December 7, 2005

National Organic Standards Board
c/o Robert Pooler, Agricultural Marketing Specialist
USDA/AMS/TM/NOP
Room 2510-So., Ag Stop 0268,
P.O. Box 96456
Washington, D.C. 20090-6456

Dear Mr. Pooler,

Attached to this letter are duplicate copies of a petition requesting addition of the non-synthetic non-agricultural substance NATAMYCIN to the National List of substances allowed in or on processed food products labeled as "organic" or "made with organic (specified ingredients)."

The petition has been compiled to comply with the requirements set forth in the Federal Register notice of 13 July 2000 [65FR43260].

Please direct all communications regarding this petition to the undersigned.

Sincerely,

John McDonnell
Director of Bread Products

Weston
George Weston Bakeries Inc.

10 Hamilton Avenue
P.O. Box 3000 • Greenwich, CT 06830
Tel: 203-531-2000

This petition seeks evaluation of the substance **NATAMYCIN** for inclusion on the National List as a **non-synthetic nonagricultural (nonorganic) substance** allowed in or on processed products labeled as "organic" or "made with organic (specified ingredients)."

1. The substance's common name.

Natamycin is an allowed and commercially available antimycotic (yeast and mold inhibitor) that has been used to prevent the growth of yeasts and molds in or on various food products, such cheese, sausage, juices, etc. (1-3). Natamycin is referred to often by other names, such as pimaricin, antibiotic A 5283, tennecetin, CL-12625, Mycophyt, Myprozine, Natacyn, Pimafucin, and Synogil (4).

2. The manufacturer's name, address and telephone number.

Natamycin for food use is available from a number of companies under various trademarks. There are two major suppliers in the world:

- DSM Food Specialties markets natamycin products under the trademark DELVOCID®. Their U.S. office is located at 2675 Eisenhower Ave, Norristown, PA 19403 (telephone: 610-650-8480).
- Danisco USA Inc. markets natamycin products under the trademark NATAMAX®. Their U.S. office is located at 440 Saw Mill River Road, Ardsley, NY 10502 (telephone 914-674-6300).

Lactose is typically the carrier in natamycin products.

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3. The intended use of the substance.

The intended use of natamycin is as a processing aid and non-agricultural non-synthetic ingredient in packaged baked goods. More specifically, natamycin is intended for use as a post-baking surface treatment of yeast-leavened and non-yeast-leavened baked goods, to prevent or delay the growth of mold and to maintain the wholesomeness of the packaged baked goods.

The intended method of application is as a spray of a water suspension of natamycin onto the surface of the baked item. Natamycin is extremely insoluble and thus remains on the surface of the baked item.

The intended commercial application level is a level of not more than 40 ppm in the sprayed water suspension, yielding an extremely small amount of natamycin in the baked item.

4. A list of the handling activities for which the substance will be used and the substance's mode of action.

"Baking" is the relevant handling activity included in the definition of "processing" in the organic food regulations at 7CFR205.2. The intended use of natamycin is as a post-baking surface treatment of yeast-leavened and non-yeast-leavened baked goods to delay the growth of mold for a sufficient period of time to permit normal commercial distribution of the packaged baked goods and their sale as wholesome foods to consumers. Molds that grow on the surface of baked goods are commonly Aspergillus and Penicillium species.

The antifungal activity of natamycin is dependent on its binding to cell membrane sterols, primarily ergosterol, the principal sterol in fungal membranes. The binding of natamycin with ergosterol makes the fungal membranes leaky. See below for a more detailed discussion.

5. The source of the substance and a detailed description of its manufacturing or processing procedures from the basic component(s) to the final product.

Natamycin (also described in the literature as pimaricin or tennecetin or pimafucin) is a water-insoluble polyene macrolide antimycotic produced by submerged aerobic fermentation of the following non-genetically modified organisms:

- *Streptomyces natalensis* (isolated from soil in Natal, South Africa)(5)
- *Streptomyces chattanoogensis* (isolated from soil in Tennessee)(6)
- *Streptomyces gilvosporeus,* and
- related *Streptomyces* species.

Streptomyces natalensis is the specific species used for most commercial production of natamycin.

The fermentation medium comprises normal bacterial fermentation substrates. See, for example, Appendix 1 for U.S. Patent No. 3,892,850, issued 1 July 1975 to Struyk et al.; U.S. Patent No. 5,231,014, issued 27 July 1993 to Eisenschink et al.; and U.S. Patent No.5,686,273, issued 11 November 1997 to Eisenschink et al. The fermentation proceeds under controlled conditions of temperature, pH, and calcium concentration to optimize the rate of natamycin production.

Natamycin is practically insoluble in water. It is present in the fermentation broth as small crystals.

Natamycin is soluble in aqueous alcohol solutions, especially under mildly alkaline and acidic conditions and also in the presence of calcium salts. These properties facilitate the isolation of natamycin from the fermentation broth.

Natamycin originally was extracted from fermentation broths or mycelia using an organic solvent, such as an alcohol (methanol (wood alcohol) or isopropanol (isopropyl alcohol, "rubbing alcohol")). Partial evaporation of the solvent in these extracts followed by addition of water yielded a crystalline precipitate of natamycin (5).

Efforts to improve the extraction process have focused on reducing the costs, hazards, and environmental impact of handling large volumes of solvent. Borden et al. (US Patent No. 5,942,611; 1999)(7) disclose a typical commercial process for recovering high purity natamycin, said process comprising the steps of adjusting the pH of the fermentation broth to above about pH 10 and adding an amount of a water-miscible solvent, such as isopropanol (isopropyl alcohol), sufficient to dissolve the natamycin in the broth, followed by removing insoluble solids from the pH-adjusted broth, followed by lowering the pH of the broth to a level sufficient to precipitate the natamycin, and then removing the precipitated natamycin from the broth. Isopropanol (isopropyl alcohol) is the specific alcohol used to extract natamycin, for health, safety, and environmental reasons. Isopropanol (isopropyl alcohol) is much less volatile than methanol. Isopropanol (isopropyl alcohol) is much less toxic than the lower molecular weight alcohols methanol and ethanol. The Scientific Committee on Food of the European Commission evaluated isopropanol

(isopropyl alcohol) as an extraction solvent for food in 1981 and 1991 and considered it acceptable for this use.

The country of origin for the natamycin in the petitioned use is Denmark, a country with very strict environmental regulations. The factory is visited regularly by government inspectors. The extracted fermentation broth is disposed of in a waste water treatment facility at the factory or for gas production by a local utility to generate electricity. No waste is dispersed into the environment.

Only food grade isopropanol (isopropyl alcohol) is used. Greater than 99% of the isopropanol (isopropyl alcohol) is recovered via distillation and re-used in the process. Essentially no isopropanol (isopropyl alcohol) is lost to the atmosphere. A very limited amount of isopropanol (isopropyl alcohol) is lost to the waste water treatment facility. Isopropanol (isopropyl alcohol) has a low potential to affect aquatic organisms and waste treatment microorganisms. It is readily biodegradable.

6. A summary of any available previous reviews by State or private certification programs or other organizations of the petitioned substance.

The non-synthetic yeast and mold inhibitor natamycin has not been previously petitioned to the National Organic Program for use in organic foods or foods labeled "made with organic (specified ingredients)."

A non-governmental organization, Demeter International, does not permit the surface treatment of cheese with the synthetic preservatives potassium sorbate or calcium sorbate or with the non-synthetic antimycotic substance natamycin (8).

Note that in the U.S. the National Organic Program Standards differentiate between synthetic and non-synthetic non-agricultural substances used as food ingredients. The prohibitions of §205.600(b) apply to a synthetic substance. Thus paragraph (b)(4) of this section, which prohibits the use of a synthetic substance as a preservative, would not be applicable to the non-synthetic substance natamycin.

Natamycin is not included in Annex VI to the European Organic Food Regulation (EEC) No. 2092/91 (9).

7. Information regarding EPA, FDA, and State regulatory authority registrations, including registration numbers.

Natamycin is a GRAS (Generally Recognized As Safe) food additive accepted as a mold and yeast inhibitor by FDA; see 21 CFR 172.155 (10). Natamycin is used to control the growth of yeasts and molds on the surface of cheese. Natamycin is allowed for use in soft tortillas in the U.S., according to a document authored by Food Standards of Australia and New Zealand (11).

Natamycin also is used for other non-sterile products, such as meat, sausages, and baked goods. See Appendix 2 for the letter of 7 January 2005 from Angela Lim, Danisco USA Inc., to Mr. Leonard Heflich, George Weston Bakeries Inc., describing the self-affirmation process of the supplier. The self-affirmation process is authorized by the FDA Modernization Act of 1997.

One producer of natamycin has petitioned the FSANZ (Food Standards Australia and New Zealand) to approve the extended use of natamycin as a food additive in bread and bakery products as well as in three other conventional food categories, since explicit approval is required for this use in New Zealand and Australia. FSANZ has issued an Initial Assessment Report (11).

8. The Chemical Abstract Service (CAS) number or other product numbers of the substance and labels of products that contain the petitioned substance.

Natamycin has the CAS Registration No. 7681-93-8. The INS (International Numbering System) Number adopted by the Codex Alimentarius Commission) for natamycin is INS 235.

Appendix 3 contains:

- the label of NATAMAX®, the natamycin product produced by Danisco A/S, Grindsted, Denmark, that is used in currently marketed conventional packaged baked goods and is intended to be used if natamycin is allowed for use in or on processed products labeled as "organic" or "made with organic (specified ingredients)";
- a copy of the label of the currently marketed natamycin-containing product THOMAS' ENGLISH MUFFINS produced by the petitioner; and
- an example of the ingredient declaration of an English muffins product (labeled "organic") which would contain natamycin.

9. The substance's physical properties and chemical mode of action including (a) chemical interactions with other substances, especially substances used in organic production; (b) toxicity and environmental persistence; (c) environmental impacts from its use or manufacture; (d) effects on human health; and, (e) effects on soil organisms, crops, or livestock.

Physical and Chemical Properties

The physical and chemical properties of natamycin are described in the Merck Index (Twelfth Edition 1996) (4,12), JECFA 2003 (13), and Food Chemicals Codex (14).

Natamycin, with an anhydrous formula weight of 665.74, is a white to creamy-white, almost odorless, crystalline powder practically insoluble in water, in lipid, and in mineral oils; slightly soluble in methanol; soluble in glacial acetic acid and dimethylformamide. The material of commerce is a trihydrate (contains three moles of water).

Chemical Interactions and Toxicity

Natamycin is extremely insoluble in water. The only significant interaction of natamycin is its formation of a complex with ergosterol, the principal sterol in fungal membranes. This complex interferes with fungal cell membrane function, causing the fungal cell to become leaky. Natamycin forms a very weak complex with the mammalian sterol cholesterol, which accounts for its safety in humans and other mammals. Oomycete fungi (water molds) and bacteria are insensitive to natamycin. The JECFA safety evaluation of natamycin (12) extensively reports on the biological effects of natamycin, including its toxicology.

The antifungal activity of natamycin is dependent on its binding to cell membrane sterols, primarily ergosterol, the principal sterol in fungal membranes, thereby making them leaky (15),(16). Natamycin has a much greater affinity for ergosterol than for cholesterol, the mammalian membrane sterol, so it is selectively antifungal. Natamycin and other polyenes form complexes with sterols and apparently disrupt membrane function by this mechanism. At low concentrations, selective changes in membrane permeability may occur. Leakage of potassium ions is the first detectable event, and, at high concentrations, leakage of amino acids and

other metabolites occurs. Susceptible fungi do not develop resistance to natamycin (15).

Natamycin is not toxic to bacteria and has low toxicity for humans and other mammalian species. See the JECFA 2002 report (12).

Environmental Persistence

The JECFA 2002 safety evaluation (12) discloses that natamycin is incompletely absorbed or degraded in the human gastrointestinal tract. Several studies in animals indicate a lack of antibiotic activity in the colon, suggesting that natamycin was degraded into microbiologically inactive compounds by bacterial flora. Significantly, susceptible fungi do not appear to develop resistance to natamycin.

Natamycin is extremely sensitive to ultraviolet (UV) light. Light exposure causes natamycin to decompose rapidly (17).

Environmental Impacts from its Use or Manufacture

The extracted fermentation broth is disposed of in a waste water treatment facility at the factory or for gas production by a local utility to generate electricity. No waste is dispersed into the environment.

Only food grade isopropanol (isopropyl alcohol) is used for natamycin extraction. Greater than 99% of the isopropanol (isopropyl alcohol) is recovered via distillation and re-used in the process. Essentially no isopropanol (isopropyl alcohol) is lost to the atmosphere. A very limited amount of isopropanol (isopropyl alcohol) is lost to the waste water treatment facility. Isopropanol (isopropyl alcohol) has a low potential to affect aquatic organisms and waste treatment microorganisms; it is readily biodegradable.

Effects on Human Health

Natamycin has FDA-approved human medicinal uses (18). It is the treatment of choice for fungal infections of the eye. Natamycin has been used for vaginal fungal and yeast infections. See Appendix 4 for a sampling of relevant research report titles, some with abstracts, identified by a search of the National Library of Medicine collection via PubMed.

The JECFA 2002 safety evaluation (12) is an extensive review of the human biology of natamycin.

Effects on Soil Organisms, Crops, or Livestock

Natamycin is a non-synthetic metabolite of soil bacteria. Natamycin has a slight effect on other soil microorganisms, principally certain fungi. This activity has been advantageously employed in methods for the enumeration of the bacterial community in soil samples, where natamycin is used to inhibit molds and yeasts. High concentrations of natamycin can bias the results of soil bacteria research by selective inhibition of some bacteria (19).

Natamycin has been used to advantage in veterinary treatment of fungal infections of the skin (ringworm), ear, and eye. See Appendix 5 for relevant research report titles, some with abstracts, identified by a search of the National Library of Medicine collection via PubMed.

10. Safety information about the substance including a Material Safety Data Sheet (MSDS) and a substance report from the National Institute of Environmental Health Sciences.

The FAO/WHO Joint Expert Committee for Food Additives ("JECFA")[1] has published a 30-page safety evaluation of natamycin (12). The MSDS for natamycin is included as Appendix 6.

The National Institute of Environmental Health Sciences does not evaluate food additives; FDA evaluates food additives. FDA concluded that natamycin is GRAS (Generally Recognized As Safe: 21 CFR 172.155).

11. Research information about the substance which includes comprehensive substance research reviews and research bibliographies, including reviews and bibliographies which present contrasting positions to those presented by the petitioner in supporting the substance's inclusion on or removal from the National List.

Natamycin has been extensively and recently reviewed for its safety by the FAO/WHO Joint Expert Committee on Food Additives (12) and for its food uses by industrial scientists (1-3,18,20).

[1] http://www.inchem.org/documents/jecfa/jecmono/v48je06.htm

12. Petition Justification Statement

The petitioner seeks inclusion of natamycin, a non-synthetic non-agricultural substance, on the National List for the following reason. Baked goods, such as English muffins, have non-sterile surfaces and are perishable foods. The amount of time required for distribution of packaged baked goods from the bakery through the distribution channel (bakery to warehouse to delivery truck) to achieve store-door delivery is a minimum of 7 days. In order to provide consumers with wholesome bakery products, packaged baked goods must remain mold-free for not less than 12 days. Untreated baked goods will normally become moldy within 5 to 9 days, depending on relative humidity, which varies with the season and the weather. Spraying a suspension containing 20 ppm natamycin delays mold development to 14 or 15 days, depending on relative humidity. Consequently, the use of this non-synthetic mold inhibitor makes it possible for the American consumer to have access to "organic" or "made with organic (specified ingredient)" packaged baked goods.

Luther (20) indicates that "pimaricin (natamycin) has been studied in various baking operations. Rye and white breads were well protected when their surfaces were sprayed with a solution of 100-500 ppm pimaricin. Uncooked doughs were also improved when their surfaces were protected by surface treatment with pimaricin. The keeping time of fillings for cakes and pies was more than doubled by the addition of 25-50 ppm of pimaricin." English muffins, similar to rye bread and white bread, are yeast-leavened baked goods.

Williams et al. (U.S. Patent Application 20050163895) performed research trials on regular muffins. Regular muffins are flour-based non-yeast-leavened fine bakery goods similarly prone to surface spoilage due to growth of molds and yeasts. Regular muffins were prepared according to a standard recipe with no added preservative in the dough. Shortly after baking, individual muffins were sprayed while still warm with a choice of four different spray treatments:

1--water only (control).

2--water containing 8% added salt

3--water containing 4 g/L of a 1:1 mixture of lactose and natamycin (2000 ppm)

4--water containing 8 g/L of a 1:1 mixture of lactose and natamycin (4000 ppm)

Each muffin was sprayed evenly over all surfaces with a minimum volume of finely adjusted spray. After cooling, the sprayed muffins were packed into heat sealed clear polythene bags with 8 to 10 muffins per treatment per bag. Initial samples were selected and tested for water activity, pH and surface natamycin concentration. Unopened bags of muffins were put for shelf life evaluation at 25 degree C and examined daily for signs of surface mold or yeast growth.

Packs of control muffins sprayed with water only or with the 8% salt in water were regarded as spoiled after only 5 and 11 days, respectively, due to the growth of clearly visible surface mold growth. In contrast, all of the packs of muffins sprayed with the two treatment levels of natamycin suspension were found to be completely free of any surface mold growth for a total incubation period of 70 days, after which time the incubation was stopped.

The amount of natamycin per muffin treated with the lower level of natamycin (2000 ppm) decreased from 400 mcg to about 135 mcg over the 70 days. The ADI (Acceptable Daily Intake) for natamycin is 0 to 0.3 mg/kg body weight, or up to 2.1 mg for a 70-kg adult.

Clearly, the intended commercial application level of not more than 40 ppm is very low, just one fiftieth of the lower level tested by Williams et al. (U.S. Patent Application 20050163895). A copy of this patent application and a copy of another recently published U.S. patent application filed by Williams et al. relating to the use of natamycin for packaged baked good are included in Appendix 7.

Bibliography (a copy of each cited publication is included)

1. Stark, J. (2003) Natamycin: an effective fungicide for food and beverages. In: Natural antimicrobials for the minimal processing of foods (Roller, S., ed.), pp. 82-97. CRC Press LCC, Boca Raton.

2. Stark, J. & Tan, H. S. (2003) Natamycin. In: Food Preservatives, Second Edition ed. (Russell, N. J. & Gould, G. W., eds.), pp. 179-195. Kluwer Academic / Plenum Publishers, New York.

3. Thomas, L. V. & Delves-Broughton, J. (2003) Natamycin. In: Encyclopedia of Food Sciences and Nutrition, Second Edition ed. (Caballero, B., Trugo, L. C. & Finglas, P. M., eds.), pp. 4109-4115. Academic Press, Amsterdam.

4. Budavari, S. (1996) Natamycin. In: Merck Index, 12th Edition ed. (Budavari, S., ed.). Merck & Co., Whitehouse Station, NJ.

5. Struyk, A. P., Hoette, I., Drost, G., Waisvisz, J. M., van Eek, T. & Hoogerheide, J. C. (1958) Pimaricin, a new antifungal antibiotic. In: Antibiotics Annual 1957-1958 (Welch, H. & Marti-Ibanez, F., eds.), pp. 878-885. Medical Encyclopedia, Inc., New York.

6. Burns, J. & Holtman, D. F. (1959) Tennecetin: A new antifungal antibiotic. Antibiotics and Chemotherapy 9: 398-405.

7. Borden, G. W., Maher, J. M. & Sklavounos, C. (1999) Process for natamycin recovery. United States Patent No. 5,942,611.

8. Demeter International e.V. (2004) Standards for the certification of DEMETER milk and dairy products. In: Processing Standards, pp. 42-47.

9. European Communities (2000) Regulation (EEC) No 207/93, defining content of Annex VI to Regulation (EEC) No. 2092/91. Office for Official Publications of the European Communities.

10. Food and Drug Administration (2005) 21 CFR 172.155 Natamycin (pimaricin). In: Title 21 Code of Federal Regulations. Government Printing Office.

11. Food Standards Australia New Zealand (2004) Initial Assessment Report. Application A542. Natamycin - extension of use as a food additive, p. 14.

12. JECFA (2002) Evaluation of certain food additives and contaminants: Natamycin (Pimaricin). In: World Health Organisation Joint Expert Committee on Food Additives Food Additive Series:48, pp. i-x, 1-171, back cover.

13. Anonymous (2003) JECFA Compendium of food additive specifications: Natamycin. In: World Health Organisation Joint Expert Committee on Food Additives Food Additive Series:52, pp. 49-52.

14. Committee on Food Chemicals Codex (2004) Natamycin. In: Food Chemicals Codex, Fifth Edition ed. (Committee on Food Chemicals Codex, Food and Nutrition Board, Institute of Medicine, ed.), pp. 298-299. National Academies Press, Washington, DC.

15. Hamilton-Miller, J. M. (1974) Fungal sterols and the mode of action of the polyene antibiotics. Adv Appl Microbiol 17: 109-134.

16. Norman, A. W., Spielvogel, A. M. & Wong, R. G. (1976) Polyene antibiotic - sterol interaction. Adv Lipid Res 14: 127-170.

17. (2003) Natamycin (Pimaricin). In: Ullmann's Encyclopedia of Industrial Chemistry, Sixth Edition ed., pp. 602-603. Wiley-VCH, Weinheim.

18. Cauwenburgh, G. (1992) Antimycotic Antibiotics. In: Kirk-Othmer Encyclopedia of Chemical Technology, pp. 475-477. John Wiley & Sons, New York.

19. Mohamed, M. A., Ranjard, L., Catroux, C., Catroux, G. & Hartmann, A. (2005) Effect of natamycin on the enumeration, genetic structure and composition of bacterial community isolated from soils and soybean rhizosphere. J Microbiol Methods 60: 31-40.

20. Luther, H. G. (1974) Antibiotics in Foods. In: Encyclopedia of Food Technology (Johnson, A. H. & Peterson., M. S., eds.), pp. 31-43. Avi Publishing Co., Inc., Westport, Conn.

Natamycin Petition

Appendix 1

Fermentation of Natamycin

U.S. Patent No. 3,892,850, issued 1 July 1975 to Struyk et al.

U.S. Patent No. 5,231,014, issued 27 July 1993 to Eisenschink et al.

U.S. Patent No. 5,686,273, issued 11 November 1997 to Eisenschink et al.

United States Patent [19]

Struyk et al.

[11] **3,892,850**

[45] **July 1, 1975**

[54] **PIMARICIN AND PROCESS OF PRODUCING SAME**

[75] Inventors: **Adrianus Petrus Struyk; Jacques Maurits Waisvisz,** both of Delft, Netherlands

[73] Assignee: **Gist-Brocades N.V.,** Delft, Netherlands

[22] Filed: **Mar. 7, 1957**

[21] Appl. No.: **644,609**

[30] **Foreign Application Priority Data**

Mar. 13, 1956 Netherlands 5620534

[52] **U.S. Cl.** **424/119;** 195/80

[51] **Int. Cl.** **A61k 21/00**

[58] **Field of Search** 424/119; 195/80

[56] **References Cited**

UNITED STATES PATENTS

2,649,401 8/1953 Haines et al. 195/51

FOREIGN PATENTS OR APPLICATIONS

712,547 7/1954 United Kingdom

OTHER PUBLICATIONS

Waksman, The Actinomyces, 1950, page 30.

Symposium Actinomycetes, 6th Cong. Intern. Microb., Rome, 1953, page 31.

Pridham, Applied Microbiology, Jan. 1958, pp. 52–79.

Sneath, J. Gen. Microbiology, Aug. 1957, pp. 184–200.

Raubitschek et al., Antibiotics and Chemotherapy, April 1952, pp. 179–183.

Schaffner et al., Antibiotics Annual, 1957–1958, pp. 869–877.

Baldacci, Archiv fur Mikrobiologie, Bd. 20, S. 347/357, 1954.

Wakaki et al., J. of Antibiotics, 1953, pp. 145–146.

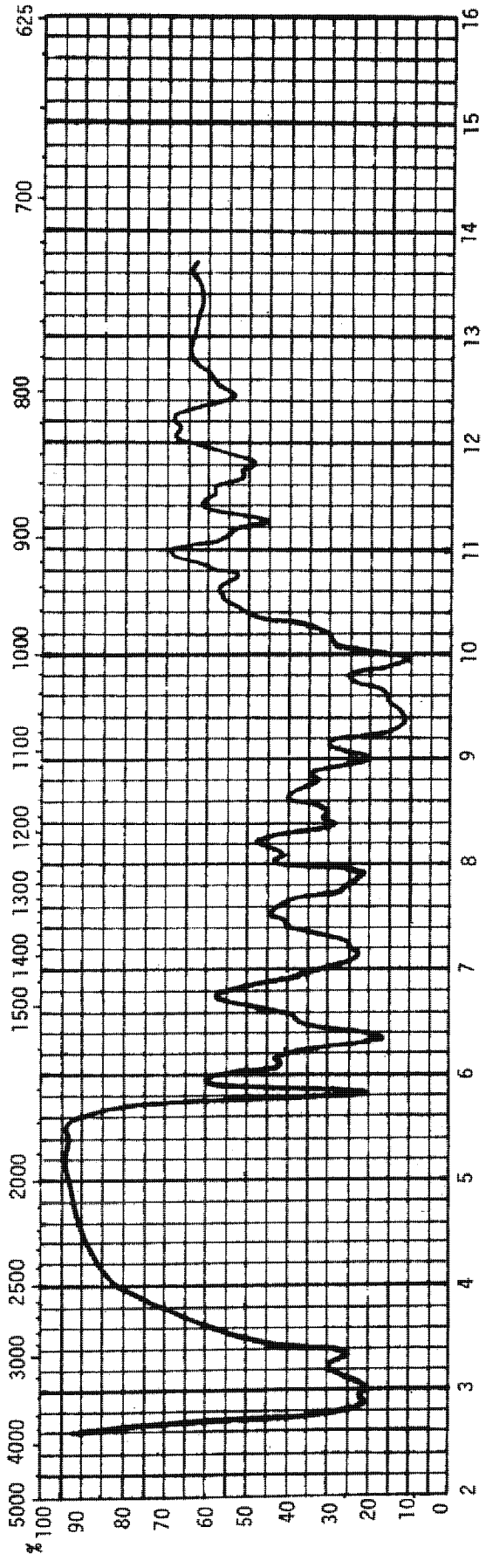
J. Waksman, "Actinomycetes and their Antibiotics", 1953, pp. 176, 177, 183, & 184.

Primary Examiner—Jerome D. Goldberg
Attorney, Agent, or Firm—Hammond & Littell

[57] **ABSTRACT**

This invention relates to an antifungal antibiotic pimaricin produced by culturing Streptomyces natalensis and the process of preparing said antibiotic.

6 Claims, 1 Drawing Figure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PIMARICIN AND PROCESS OF PRODUCING SAME

This invention relates to a new and useful antibiotic and to a process for the biosynthesis of the new antibiotic, called pimaricin, by means of a hitherto unknown Streptomyces species. For this purpose a culture is made of Streptomyces natalensis nov. spec., whose properties are described below, by the aerobic method and under appropriate conditions, and the product generically named pimaricin thus formed, whose properties are likewise given below, is obtained from the fermentation medium. The preparation of the antibiotic from the culture fluid and/or the mycelium can be advantageously effected by extraction with alcohols miscible with water to a limited extent, e.g., butanol or by a treatment with methanol (a methanol solution of calcium chloride), followed by concentrating the extract and subsequent purification with the aid of solvents.

The micro-organism which produces the antibiotic in question was isolated from a soil sample obtained from Pieter Maritzburg, in the province of Natal, South Africa. It is an actinomycete of the Streptomyces genus, now called Streptomyces natalensis.

A culture of Streptomyces natalensis is on deposit with the Centraalbureau voor Schimmelcultures, Baarn, the Netherlands, under the culture number CBS 700.57 and with the Culture Collection Unit of the Northern Utilization Research and Development Division of the United States Department of Agriculture under the culture number NRRL 2651.

The morphological and physiological characteristics of the micro-organism are as follows:

Morphological characteristics: Hyphae branched, irregularly twisted, in the growth zone often practically straight and parallel to one another, 0.3 – 0.7 μ thick, generally of uniform thickness, but also sometimes with local thickenings. Sporulating hyphae as monopodial lateral branches at the aerial mycelium. Spores in irregularly undulated chains, seldom in chains wound in loose spirals. Spores separated from one another by short intermediate pieces without protoplasm, oval, round or tangerine shaped, 0.7 – 1.2 × 0.7 – 0.8 μ.

Culture characteristics when grown on the substrates listed (after 7 days at 25°C., unless otherwise indicated) are:

TABLE I

(The colors mentioned have been taken from Ridgway, Color Standards and Nomenclature [1912]).

Oatmeal agar	Good growth. Vegetative mycelium and back colorless. After 28 days a few colonies at the bottom and the top of the tube. Chamois at the back, after 57 days slightly darker (warm buff). Colony practically flat, separate colonies slightly elevated at the center. Aerial mycelium pale mouse gray to light mouse gray, powdery and felty, after 28 days one-third gray, after 57 days drab gray. Smell earthy with sourish additional smell. No soluble pigment.
Potato agar	Moderate growth. Vegetative mycelium light mineral gray to pale olive buff. Back: cream color, after 57 days cream buff to chamois. Colonies moist, slightly elevated aerial mycelium partly covering the colony. Aerial mycelium after 7 days white and felty, after 28 days drab gray and covering the colony for 30 – 70%. No soluble pigment. Smell unpleasantly earthy.

TABLE I-Continued

(The colors mentioned have been taken from Ridgway, Color Standards and Nomenclature [1912]).

Potato	Good growth. Vegetative mycelium moist, elevated lumpy, pale pinkish buff to pinkish buff, after 28 days deep olive buff. Back on glass wall after 28 days chamois. After 7 days no aerial mycelium. After 28 days a good deal of aerial mycelium, at first white, afterwards pale mouse gray, after 57 days drab gray. Potato after 7 days not discolored, after 57 days, like the fluid at the bottom of the tube, discolored to buffy brown. Smell earthy.
Sabouraud-glucose agar	Moderate growth. Vegetative mycelium, lumpy, elevated after 7 days olive buff like back, after 28 days darker (deep olive buff to dark olive buff). Back now clay color. Aerial mycelium at first white, short and felty, afterwards pale mouse gray. No soluble pigment visible after 7 days, but after 57 days medium auburn.
Emerson agar	Very good growth. Vegetative mycelium olive buff. Back appearing darker (cream buff to chamois). Aerial mycelium short and felty, white. No soluble pigment. Smell strongly earthy.
Starch agar	Moderate growth. Vegetative mycelium and back pale olive buff. Colony slightly elevated, covered for about one half by felty white aerial mycelium. No soluble pigment. Smell strongly earthy.
Glucose nitrate agar	Good growth. Round, moist, gray colonies, slightly elevated in the center, about 2 to 2.5 mm in diameter (lighter than pale olive buff). Aerial mycelium scanty, in dots or concentric rings, felty, white. No soluble pigment. Smell earthy.
Sodium citrate agar	Very poor growth. Round, moist, gray colonies, slightly elevated in the center, about 1 mm in diameter (lighter than pale olive buff). Back pale olive buff. No soluble pigment. No smell.
Nutrient agar	Good growth. Colony moist, slightly elevated, very small, wet tufts, rough and dull, with shallow and indistinct cerebroid ridges, cream color to cream buff. Smell unpleasant. No soluble pigment.
Czapek agar (13 days)	Growth very scanty. Vegetative mycelium hyaline. No aerial mycelium. No soluble pigment.
Bacto agar 2%	Growth very scanty, not visible until after 13 days. Vegetative mycelium hyaline. No aerial mycelium. No soluble pigment.

The organism which is used according to the invention for the preparation of pimaricin belongs to the Streptomyces genus and is not identical with any Streptomyces species heretofore described. In view of the characteristics mentioned in detail above it belongs in Group I of those distinguished by Waksman in Bergey's Manual (see also S. A. Waksman, "The Actinomycetes", 1950, p. 30).

Streptomyces natalensis nov. spec. can also be classed in the series "Neo-Ingri" of Baldacci (cf. Symposium Antinomycetales, VIth Congr. Intern. Microb. Roma, 1953, page 31). It is to be noted that the same Streptomyces natalensis nov. spec. is not exclusively confined to actinomycetes which answer in a stereotyped and rigorous manner to the description given herein, but that the term also includes all related strains which broadly have the same specific properties and produce the antibiotic pimaricin, and which might be regarded as sub-species, varieties, races, forms, groups of serological or other types, variants, phases, spontaneous mutants, modifications and the like of this species. It also covers the mutants of Streptomyces natalensis which can be obtained from it with the aid of means or substances causing mutation, such as irradiation or treatment with substances having a toxic effect.

The new antibiotic pimaricin is mainly active in relation to saprophytic and parasitic fungi and yeasts.

Table II gives a survey of the antibiotic properties of pimaricin in relation to yeasts and fungi not pathogenic to the human body. For each speices the quantity of the antibiotic in micrograms/ml of nutrient medium is given which just causes checking of the growth.

Table III lists the similar checking of a number of yeasts and fungi pathogenic to the human body.

TABLE II

Test Organism	Concentration of Pimaricin in Micrograms/ml Which Inhibits Growth
Saccharomyces cerevisiae-H	0.15
Saccharomyces cerevisiae-KLL	0.9
Pichia membranifaciens	2.5
Schwanniomyces occidentalis	2.5
Aspergillus niger	1.8
Aspergillus fumigatus	1.2
Cladosporium cucumerinum	0.9
Verticillium dahliae	1.2
Fusarium spec.	1.2
Penicillium chrysogenum	0.6
Trichoderma spec.	1.2

TABLE III

Test Organism	Concentration of Pimaricin in Micrograms/ml (on Sabouraud Agar) Which Inhibits Growth
Candida albicans (3 strains)	6 – 12
Candida tropicalis[1] (2 strains)	3 – 12
Trichosporon cutaneum[1]	12
Pityrosporum spec.[1]	12
Candida parapsilosis[1]	12
Histoplasma capsulatum	3
Sporotrichum Schenckii	6
Trichophyton sulfuricum	3
Trichophyton mentagrophytes (3 strains)	50
Trichophyton violaceum	6
Trichophyton rosaceum	12.5
Trichophyton Schonleinii	6
Trichophyton rubrum	12.5
Trichophyton interdigitale	25
Microsporum lanosum	12.5
Epidermophyton floccosum	12.5
Hormodendrum compactum	6

[1]This yeast is not called pathogenic, but it was isolated from medical material.

From the above tables, among other things a clear and striking action against phytopathogenic fungi, such as Verticillium, Cladosporium, and Fusarium, becomes apparent. The checking of Candida albicans, too, is very strong.

A further important feature of the new antibiotic is the very slight phytotoxic effect of pimaricin in contrast to most of the fungicidal antibiotics hitherto found. In view of this, the new antibiotic is very suitable, among other things, in agriculture and horticulture, as a means for combating plant diseases, the more so as it has been found to diffuse easily and to act systematically.

Thus pimaricin will penetrate e.g., into pea seeds (Pisum sativum) after steeping in a dilute aqueous solution. This appears from the fact that pimaricin can be extracted from the cotyledons and the germs of these treated seeds.

Tables IV a and b show the internal disinfectant action. The fungus mycelium (among other things, Ascochyta pisi) is killed in the seed.

TABLE IV a

Concentration Pimaricin in p.p.m.[1]	% of Seeds With Mycelium[2]
150	0
75	3
37.5	11
18.7	25
blank (untreated)	80

TABLE IV b

Concentration Pimaricin in p.p.m.[1]	% of Diseased Plants Test I	Test II
150	0	0
75	0	0.7
37.5	1	2
18.7	2	3.6
blank	15	24

[1]aqueous solution in which the seeds were steeped for 24 hours at 20°C.
[2]development of mycelium on filter paper

Detailed tests indicated that pimaricin in a concentration of 75 p.p.m., which is effectively fungicidal, is not prejudicial to the germination of pea seeds and the growth of the plant (see Tables IV c and d).

TABLE IV c

Concentration Pimaricin in p.p.m. [1]	% of Germinated Seeds [2]	Length of Roots in mm after 3 Days [2]
blank	100	23
25	100	24
50	100	22
75	100	22
100	100	23
125	100	22
150	96	29
200	98	14

[1] aqueous solution in which the seeds were steeped for 24 hours at 20°C.
[2] seeds arranged on moist filter paper

TABLE IV d

Pea Seeds Steeped in Water		in concentration Pimaricin 75 p.p.m. [1]
sprouting (number)	126	128
length of plant in cm after 2 months	31.8	33.8
number of pods per plant	2.8	2.8

[1] aqueous solution in which the peas were steeped for 24 hours at 20°C.

All the data given in the tables refer to the Pea race Eminent, which is extremely sensitive to Ascochyta pisi.

Experiments with rats and mice indicate very low toxicity. To determine the acute albinorat toxicity of pimaricin, material assaying 985 micrograms/mg was administered to rats, which were then held 7 days and posted.

The following results were obtained with pimaricin suspension in water:

LD_{50} oral	1,500 mg/kg	average values of tests with 40 rats
LD_{50} intramuscular	>2,000 mg/kg	
LD_{50} subcutaneous	>5,000 mg/kg	
LD_{50} intraperitoneal	250 mg/kg	

Pimaricin has no irritating effect on the skin and mucous membranes.

In the pure state pimaricin is a white crystalline compound with an amphoteric character, salts of which can be made in the usual ways. With $FeCl_3$ it gives no color reaction; with concentrated phosphoric acid a pink, unstable color is produced. Concentrated hydrochloric acid and sulphuric acid produce a blue and olive-green discoloration. The antibiotic decolorizes bromine water. Because of this and also in view of the infra red and ultra violet spectra (see below) it is indicated that pimaricin is one of the socalled polyene antibiotics. The solubility in water is very slight, viz 8 mgs in 100 ml at 20°C. In organic solvents, such as alcohols, glycols and ketones, the antibiotic is better soluble, especially in alcohols with 1 to 6 carbon atoms, e.g., in methanol and n-butanol and in the so-called cellosolves. Further it is soluble in pyridine, dimethylformamide, dimethylacetamide, glacial acetic acid and alkali hydroxide. In aliphatic hydrocarbons, such as pentane, hexane, cyclohexane and the like, the substance is practically insoluble.

At room temperature pimaricin is a very stable compound. A solution in water (5 mg in 100 ml) preserves its full activity for 7 days at pH = 7.0 at 25°C. At pH = 2.0 this solution loses 50% of its activity in 3 days at the said temperature. At pH = 10 and at 25°C. the half value time is 6 days. At elevated temperatures the solution is less stable. Thus a solution of the above given concentration of pimaricin in water at pH = 2 and 90°C. loses 90% of its activity in 15 minutes, at pH = 6.5: 15% and at pH = 9: 50% of its activity. In methanol pimaricin is stable at higher (25°–60°C.) temperatures. The antibiotic activity of the pimaricin was tested microbiologically with Saccharomyces cerevisiae - Holland strain as test organism in relation to a pure standard preparation.

Pimaricin does not exhibit a melting point, but begins to decompose at about 150°C. The specific rotation α 25/D = + 250° (c = 0.083% in 100% methanol). The molecular weight of the substance is about 685. The presumed empirical formula is $C_{34}H_{49}NO_{14}$. The elementary analysis gave the following values: C 57.77% H 7.27%, N 1.95% and O 33.01% (calculated).

The ultra violet absorption spectrum of pimaricin is characterized by maxima at 290, 304 and 318 mμ with a shoulder at about 280 and a minimum at about 250 mμ (C = 3.93 micrograms/ml in methanol). A sample of pimaricin pressed into a potassium bromide plate (C = 0.5%) exhibits the following infra red absorption (in cm^{-1}): 3460, 2985, 1721, 1637, 1577, 1441, 1401, **1381, 1275, 1269, 1238, 1192, 1176, 1136, 1109, 1066, 1006, 988, 948, 887, 855, 844, 803, 794** (for the underlined wave lengths the absorption is strong to very strong).

FIG. 1 is a graph of the infra red spectrum of pimaricin in a potassium plate.

For the preparation of pimaricin the Streptomyces natalensis is cultivated aerobically in stationary cultures or submerged in a fluid nutrient medium under sterile conditions in closed vessels, which are equpped with stirring devices and to which, with a view to aeration, sterile oxygen or air can be supplied during cultivation.

The duration of the cultivation, the temperature, and the other conditions that have to be satisfied in order to obtain good yields of pimaricin are easily determined experimentally. They will be discussed more fully below.

The nutrient medium can be composed of the customary substances; it should contain a source of carbon and a source of fermentable organic and/or inorganic nitrogen, while further the presence of mineral salts, such as phosphates, potassium and sodium salts, and traces of various metals is desirable. However, the initial substances used in practice are often sufficiently contaminated with these mineral salts, so that extra additions of them are superfluous.

For the source of carbon, soluble as well as insoluble carbohydrates, such as glucose, saccharose, lactose or starch, can be used. Sugar alcohols, such as glycerol, are also suitable. The amount of the source of carbon in the medium may vary widely, dependent on the nature of the carbohydrate used and on the further composition of the medium: in general it is approximately between 0.5 and 5% of the weight of the medium.

For the source of nitrogen a great many substances can be used. Mention may be made of hydrolyzed or nonhydrolyzed casein, corn steep liquor, peptone, extract of meat, soybean meal (defatted or not), peanut meal, fish meal, nitrates. The choice of the source of nitrogen will usually depend on the further composition of the medium, which in turn will be chosen such that the antibiotic is prepared in the most economical manner. In this connection it may be mentioned that small amounts of nitrogeneous initial materials, such as yeast extract, distillers' solubles, fish solubles, etc. will considerably enhance the yield of pimaricin in particular media. Also lipoid substances, such as fatty oils, both of vegetable and animal origin (e.g., soy oil and fish oil) are capable of enhancing the yield to a substantial degree.

The duration of the fermentation is highly dependent on the composition of the nutrient medium. Usually it varies between 48 and 120 hours, but the fermentation may also be continued for a longer time if desired, e.g., up to 14 days, if the increased yield of antibiotic thus obtained justifies the greater expense of a longer fermentation cycle.

The temperature at which the fermentation is carried out may in principle vary between 15° and 30°C. Temperatures of about 26° – 28°C. are preferred.

With optimum growth of the micro-organism and yield of pimaricin the pH may vary, especially during the first phase of the fermentation, e.g., between 5.0 and 8.0. After the sterilization the nutrient medium is preferably adjusted to a pH between 6 and 7. The fermentation is preferably carried out at a pH between 6.5 and 8, since in this case the highest yields are obtained. the pH can be kept constant during the fermentation by adding alkali hydroxide or acid at regular intervals under sterile conditions. Usually, however, calcium carbonate is used as a kind of buffer, in quantities varying from 0.2 to 1.0% by weight of the medium. The quantity of air which is passed into the medium during the fermentation under sterile conditions is highly dependent on the shape of the vessel, on the velocity, and on the shape of the stirrers. In general this quantity varies between 0.1 and 4 liters per liter of nutrient medium per minute.

For the inoculating material in the main fermentation vessel 48 to 72 hour old precultures of Streptomyces natalensis are preferably used. In order to obtain good

yields and prevent fluctuating results the inoculation is preferably effected with amounts of a culture making up 1 to 7% by volume of the nutrient medium in the main fermentation vessel. It is obvious that with large fermentation vessels a few stages of percultures must be used. It is, however, also possible to store a portion of the culture, e.g., 10%, in the main fermentation vessel for the production of a new culture. The whole fermentation can also be carried out continuously.

The pimaricin can be obtained from the culture fluid in several ways. According to the invention use may be made of the solubility of the antibiotic in organic solvents that are miscible with water to a limted extent, such as butanol. The procedure may, for example, be as follows. After a sufficiently active fluid has been obtained, it is adjusted to a pH of about 10 in order to disengage the active substance from the so-called mycelium, upon which the latter is filtered off. The fluid can then be extracted, e.g., with n-butanol, at a pH lying between about 3 and 10. If the fluid is acidified, this is preferably done with phosphoric acid; any precipitate thus formed can be removed and, if necessary, also extracted. The n-butanol extract is concentrated by azeotropic distillation, the crude active substance finally crystallizing. This can be purified by selective precipitation from e.g., glacial acetic acid, pyridine, dimethylformamide and the like. In fermentations with yields higher than 700 micrograms/ml the yield of pimaricin can be increased very materially by extracting the mycelium. In this concentration it is advantageous to use as the extractive agent methanol in which, to increase its capacity as a solvent, 1–3% of calcium chloride has been dissolved.

EXAMPLE I

(Preparation of the inoculating culture)

From a tube with a culture of Streptomyces natalensis nov. spec. on e.g., oatmeal agar, on which good sporulation has taken place, small amounts of conidia are transferred under sterile conditions to shaking flasks with a capacity of about 2 liters, into which has been introduced 500 ml of a fluid nutrient medium. This medium consists of:

Peptone	0.5%
Concentrated corn steep liquor (50% of dry matter)	0.6%
Glucose	1.0%
Common salt (adjusted to pH = 7.0 with alkali hydroxide)	1.0%

After incubation with constant shaking at 26° C. for 48 hours the culture is suitable to be inoculated in the main fermentation medium.

EXAMPLE II

(Preparation of the culture)

One liter of the inoculating culture prepared according to Example I is transferred under sterile conditions to a fermentation vessel equipped with a stirrer and a device for blowing in sterile air and containing 15 liters of a culture medium of the following composition:

Glucose	3.0%
Concentrated corn steep liquor (50% of dry matter)	0.2%
Ammonium sulphate	0.5%
Potassium chloride	0.4%
Primary potassium phosphate	0.02%
Calcium carbonate	0.8%

The above culture medium is adjusted to a pH of 6 to 9 with alkali hydroxide. After 48 hours incubation at 27 °C. with constant aeration and stirring the culture is found to contain 610 micrograms of pimaricin per ml.

Other culture media, with which yields of the same order of magnitude can be obtained, may have the following compositions:

A.	Peanut meal	2 %
	Potato starch	1 %
	Concentrated corn steep liquor (50% of dry matter)	2 %
	Common salt	0.5%
	Magnesium sulphate	0.1%
	Primary potassium phosphate	0.05%
	Calcium carbonate	0.6%
	Potassium hydroxide to pH 6.5	

With this medium, after inoculation with 4% of inoculating culture and 72 hours incubation and aeration at 26°C., 590 micrograms of pimaricin per ml of fermentation fluid was obtained.

B.	Beet root molasses (sugar content about 50%)	4 %
	Lactose	1 %
	Concentrated corn steep liquor	2 %
	Sodium sulphate 10 aq.	0.1%
	Calcium carbonate	0.5%
	Potassium hydroxide to pH 7.1	

With this medium, after inoculation with 5% of inoculating culture and 120 hours incubation and aeration at 27°C., 640 micrograms of pimaricin per ml of fermentation fluid was obtained.

C.	Peptone ("Difco")	0.5%
	Extract of meat ("Difco")	0.5%
	Glucose	1 %
	Common salt	0.5%

In this medium, after inoculation with 3% of inoculating culture and 148 hours incubation and aeration at 26°C., 535 micrograms of pimaricin per ml of fermentation fluid was formed.

D.	Soy meal (coarse)	5%
	Soy oil	0.5%
	Concentrated corn steep liquor (50% solids)	0.2%
	Glucose	1%
	Primary potassium phosphate	0.02%
	Ammonium sulphate	0.5%
	Calcium carbonate	1%

With this medium, after inoculation with 3% of inoculating culture in a 15 liter fermentation flask and incubation and aeration during 168 hours at 28°C., 690 micrograms of pimaricin per ml of fermentation fluid was formed. After treatment of the mycelium which had been pressed off and stirred in a good deal of water

with dilute alkali hydroxide, to pH = 10, 20.4 g. of pimaricin could be found in the mycelium filtrate.

EXAMPLE III

(Isolation of crude pimaricin)

Four liters of a culture fluid, the fermentation of which was completed, which contained 590 micrograms/ml of pimaricin, was adjusted to pH = 10.0 with 10% sodium hydroxide and subsequently freed of the mycelium by filtration with the aid of Kieselguhr as filtering agent (2%). The culture filtrate, which had a volume of 3.7 liters with an activity of 570 micrograms/ml, was acidified with phosphoric acid to pH 3.0. This acidified filtrate was successively extracted with 750, 350 and 350 ml of n-butanol, respectively. The butanol extract was then washed with three times 120 ml of a 4% borax solution and then washed again with twice 120 ml of water. The butanol extract then contained 1,400 micrograms/ml. When this extract was evaporated azeotropically to 100 ml, 0.64 g. of not entirely pure pimaricin spearated in crystalline condition (act. 900 micrograms/ml). From the remainder of the extract a total quantity of 1.42 g. of impure pimaricin was obtained by further evaporation (act. 395 micrograms/mg). The yield referred to the total activity of the completely fermented fluid was 48.3%.

EXAMPLE IV

(Isolation of crude pimaricin)

Fifteen liters of a culture fluid, the fermentation of which was completed, which contained 610 micrograms/ml of pimaricin, was adjusted to pH = 10.3 with 35% potassium hydroxide, and subsequently freed of the mycelium with the aid to 50 g. of Hyflo as filtering agent. The filtrate was then acidified to pH = 2.8 with phosphoric acid. The precipitate thus formed with filtered off, this time with the aid of 10 g. of Hyflo. The precipitate was then stirred for half an hour with 100 ml of n-butanol. After settling, the butanol extract was drawn off and the sediment was washed with 100 ml of water. The total butanol extract was washed three times with 10 ml of a 4% borax solution and subsequently three times with 10 ml of water. It was then evaporated at 44°C. in vacuo to 40 ml. After cooling to 0°C., suction, and drying, 0.51 g. of pale yellow crystalline pimaricin with an activity of 850 micrograms/mg was obtained. The filtered culture filtrate was subsequently processed in the manner described in Example I. The following two fractions were obtained: 3.21 g (act. 890 micrograms/mg) and 6.8 g (act. 223 micrograms/mg). The total yield amounted to 51.5%.

EXAMPLE V

(Isolation of crude pimaricin)

Ten liters of completely fermented culture medium (act. 640 micrograms/ml) was adjusted to pH = 10 with 15% potassium hydroxide. Upon this the mycelium was centrifuged and the clear centrifugate was adjusted to pH = 6.9 with 10 N hydrochloric acid. It was subsequently extracted with successively 2,000, 1,000 and 1,000 ml of isoamyl alcohol. The combined isoamyl alcohol extracts were successively washed with three times 300 ml of 4% sodium carbonate solution, and then twice with 300 ml of water. The extract thus treated was evaporated azeotropically to 100 ml. In this process 2.62 g of pale yellow crystalline pimaricin with an activity of 900 micrograms/mg separated off. Yield, 36.8%.

EXAMPLE VI

(Isolation of crude pimaricin)

Twenty liters of a completely fermented culture of Streptomyces natalensis (act. 700 micrograns/ml) was adjusted to pH = 10 with 20% sodium hydroxide. The mycelium was subsequently filtered off with the acid of Hyflo (200 g). The clear culture filtrate was extracted successively with 4,000 2,000 and 1,000 ml of n-butanol. The combined butanol extracts were washed twice with 500 ml of 4% borax solution and then twice with 500 ml of water. After the pH of the butanol extract thus treated had been adjusted to 6.8 with 10 N hydrochloric acid, the extract was evaporated azeotropically in vacuo to a volume of 250 ml. In this process 9.85 g of pale yellow crystalline pimaricin (act. 913 micrograms/mg) separated off. Yield: 60.8%.

EXAMPLE VII

(Isolation of crude pimaricin from mycelium)

Fifteen liters of a completely fermented culture of Streptomyces natalensis nov. spec. was adjusted to pH = 4.1 with the aid of glacial acid. Subsequently 200 g of Hyflo was added as filtering agent, with which the solution was stirred. The mycelium was then pressed off to as dry as possible to a total weight of 1,700 g. (One gram of this mycelium in this condition was extracted for one hour with 40 ml of methanol in order to determine the number of micrograms of pimaricin. The methanolic extract was supplemented to 100 ml and subsequently measured spectrophotometrically. The content of pimaricin in this methanolic solution was 120 micrograms/ml.)

The pimaricin content of the total mycelium was 20.4 g. The pressed off mycelium was extracted for half an hour at room temperature with 8 liters of methanol in which 2% of calcium chloride had been dissolved. The extract thus obtained was diluted with 1 liter of water and freed of methanol in vacuo. In this process a crystalline precipitate of pimaricin was formed which, after suction, washing with water and drying in vacuo, weighed 14.73 g and had an activity of 900 micrograms/mg = 65% of the theoretical yield.

EXAMPLE VIII

(Purification of pimaricin)

Ten grams of impure pimaricin (act. 890 micrograms/ mg) was dissolved with gentle heating in 80 ml of glacial acetic acid and then freed as quickly as possible of undissolved impurities by filtration. The clear filtrate, yellow-brown in color, was diluted with 1,500 ml water and the pH of the solution thus obtained was adjusted to 6.3 with the aid of 33% sodium hydroxide. After cooling, the crystalline precipitate formed was centrifuged and washed twice with a total quantity of 500 ml of water, and sucked off. On the filter the crystalline mass was washed again with 200 ml of water, upon which it was dired in vacuo over phosphorus pentoxide. The weight of the very pale yellow product thus obtained was 7.07 g and its activity was 960 micrograms/mg.

The above treatment was repeated with 60 ml of glacial acetic acid and 1,000 ml of water. The substance was washed with three times 200 ml of water. The

product thus obtained weighed 5.35 g and had an activity of 995 micrograms/mg. Total yield: 59.8%.

EXAMPLE IX

(Purification of pimaricin)

Ten grams of impure pimaricin (act. 890 micrograms/mg) was dissolved with gentle heating in 100 ml of dimethylformamide. This solution was filtered off with a view to removing undissolved impurities, the filter being washed again with 30 ml of dimethylformamide. To the brown-colored clear filtrate was added 250 ml of water, upon which the antibiotic precipitated in the crystalline state. The product was sucked off, the filter being washed again with 100 ml of water. The crystalline mass was subsequently dried in vacuo. The product thus obtained weighed 9.24 g. After the above treatment had been repeated, a pale yellow crystalline product was finally obtained which weighed 7.9 g and had an activity of 985 micrograms/mg. Total yield: 87.5%.

EXAMPLE X

(Preparation of sodium salt)

A quantity of 2.5 g of pimaricin (act. 985 micrograms/mg) was suspended with mechanical stirring in 20 ml of methanol. Subsequently 0.145 g of sodium hydroxide dissolved in 0.3 ml of water was added to this suspension. The pimaricin, which at first dissolves, after a few minutes crystallizes as the sodium salt. After another 30 minutes stirring and 24 hours storage at 0° C. the crystalline mass was sucked off and washed with 5 ml of ethanol and 10 ml of diethyl ether. After drying in vacuo, the white needle-shaped crystalline sodium salt weighed 2.16 g (content 995 micrograms/mg = 87.3% of the calculated activity).

Other salts may be prepared in a similar manner.

The examples given above are for the purpose of illustration and it will be understood that various changes and modifications therefrom may be used without departing from the principles of the invention or the scope of the following claims.

What we claim is:

1. A substance having antibiotic properties in relation to saprophytic and parasitic fungi and yeasts which in the pure state is a white crystalline compound which begins to decompose at about 150°C., gives no color reaction with ferric chloride, which gives the concentrated phosphoric acid a pink, unstable color, which decolorizes bromine water, which is slightly soluble in water (about 8 mgs. in 100 ml at 20°C.) and more soluble in alcohols and is soluble in pyridine, dimethylformamide, dimethylacetamide, glacial acetic acid, and alkali hydroxides, which is practically insoluble in aliphatic hydrocarbons, has a specific roatation $\alpha_D^{25} = +250°$ (in a concentration of 0.083% in 100% methanol), which has a molecular weight of about 685 and an empirical formula of $C_{35}H_{50}NO_{14}$, and which displays maximum absorption of ultra violet light at 290, 304 and 318 mμ with a shoulder at about 280 and a minimum at about 250 mμ and in a potassium bromide plate exhibits the following infra red absorption (in cm^{-1}): 3460, 2985, 1721, 1637, 1577, 1441, 1401, **1381, 1275, 1269, 1238, 1192, 1176, 1136, 1109, 1066, 1006, 988, 948, 887, 855, 844, 803, 794.**

2. A process for producing pimaricin, said pimaricin being the substance of claim **2**, which comprises cultivating Streptomyces natalensis NRRL 2651 in an aqueous nutrient medium under submerged aerobic conditions at a temperature between about 15°C and about 30°C for a period of from 2 to 14 days, and recovering the so produced pimaricin from the resulting fermentation liquor.

3. A process for producing pimaricin, said pimaricin being the substance of claim **2**, which comprises cultivating Streptomyces natalensis NNRL 2651 in an aqueous nutrient-containing, carbohydrate solution having a pH from 6.5 to 8, under submerged aerobic conditions, at a temperature between about 15°C. and about 30°C. for from two to five days and recovering the pimaricin from the resulting fermentation liquor.

4. A process for the production of pimaricin, said pimaricin being the substance of claim **2**, which comprises cultivating Streptomyces natalensis NRRL 2651 under submerged aerobic conditions in an aqueous nutrient medium having a pH between 6.5 and 8 and containing a soluble carbohydrate and a source of assimilable nitrogen at temperatures within the range from about 15°C. and about 30°C. for a period of time of about two to five days, whereby the aqueous nutrient medium is fermented and pimaricin is produced, separating the active fluid from insolubles and mycelium, extracting the active fluid with an organic solvent miscible with water to a limited extent and recovering said pimaricin from said organic solvent.

5. The process of claim **4** wherein said insolubles and mycelium are extracted with methanol to recover residual pimaricin values.

6. The process of claim **4** wherein the recovery of pimaricin includes the step of extracting the active fluid into butyl alcohol at a pH of about 3.

* * * * *

UNITED STATES PATENT OFFICE

CERTIFICATE OF CORRECTION

Patent No. 3,892,850 Dated July 1, 1975

Inventor(s) ADRIANUS PETRUS STRUYK and JACQUES MAURITS WAISVISZ

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Col.	Page	Line	Page	
[73]				Assignee "Gist-Brocades N.V., Delft, The Netherlands" should be --Koninklijke Nederlandsche Gist-En Spiritusfabriek, N.V. Delft, The Netherlands--
[30]				Priority No. "5620534" should be --205,345--
3	5	5	21	speices" should be --species--
5	13	10	17 & 18	"socalled" should be --so called--
5	43	11	15	"$C_{34}H_{49}NO_{14}$" should be --$C_{33}H_{50}NO_{14}$--
5	52to54	11	23to25	Do not correspond with the original underlined numbers should be --3460, 2985, 1721, 1637, 1577, 1441, 1401, 1381, 1275, 1269, 1238, 1192, 1176, 1136, 1109, 1066, 1006, 988, 948, 887, 855, 844, 803, 794 --

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Col.	Page	Line	Page	
7	13	14	15	"limted" should be --limited--
9	21	18	7	"spearated" should be --separated--
10	7	19	21	"microgans/ml" should be --microgams/ml--
Claim 2 line 15				"Claim 2" should be --Claim 1--
Claim 3 line 23				"Claim 2" should be --Claim 1--
Claim 4 line 31				"Claim 2" should be --Claim 1--

Signed and Sealed this

thirteenth Day of *January 1976*

[SEAL]

Attest:

RUTH C. MASON
Attesting Officer

C. MARSHALL DANN
Commissioner of Patents and Trademarks

US005231014A

United States Patent [19]

Eisenschink et al.

[11] Patent Number: 5,231,014

[45] Date of Patent: Jul. 27, 1993

[54] **FERMENTATION PROCESS FOR PRODUCING NATAMYCIN**

[75] Inventors: **Michael A. Eisenschink; Phillip T. Olson,** both of Manitowoc

[73] Assignee: **Bio-Technical Resources,** Manitowoc, Wis.

[21] Appl. No.: **740,370**

[22] Filed: **Aug. 5, 1991**

[51] **Int. Cl.**[5] **C12P 1/465;** C12P 19/62; C12P 17/18

[52] **U.S. Cl.** **435/76;** 435/71.3; 435/118; 435/119; 435/242; 435/253.5; 435/886; 514/31; 536/6.5

[58] **Field of Search** 435/76, 118, 119, 253.5, 435/71.3, 886, 242; 514/31; 536/6.5

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,892,850 7/1975 Struyk et al. .

FOREIGN PATENT DOCUMENTS

218265	3/1957	Australia .
669761	3/1957	Canada .
677040	7/1957	Canada .
844289	3/1957	United Kingdom .
846933	7/1957	United Kingdom .
2106498A	7/1982	United Kingdom .

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[57] **ABSTRACT**

A natamycin producing Streptomyces species ferments to produce natamycin. A spore suspension of the Streptomyces species is propagated in a predetermined medium to obtain a quantity of Streptomyces cells. The Streptomyces cells ferment in a predetermined production medium having a controlled pH to produce a recoverable amount of natamycin.

28 Claims, 3 Drawing Sheets

FIG. 1

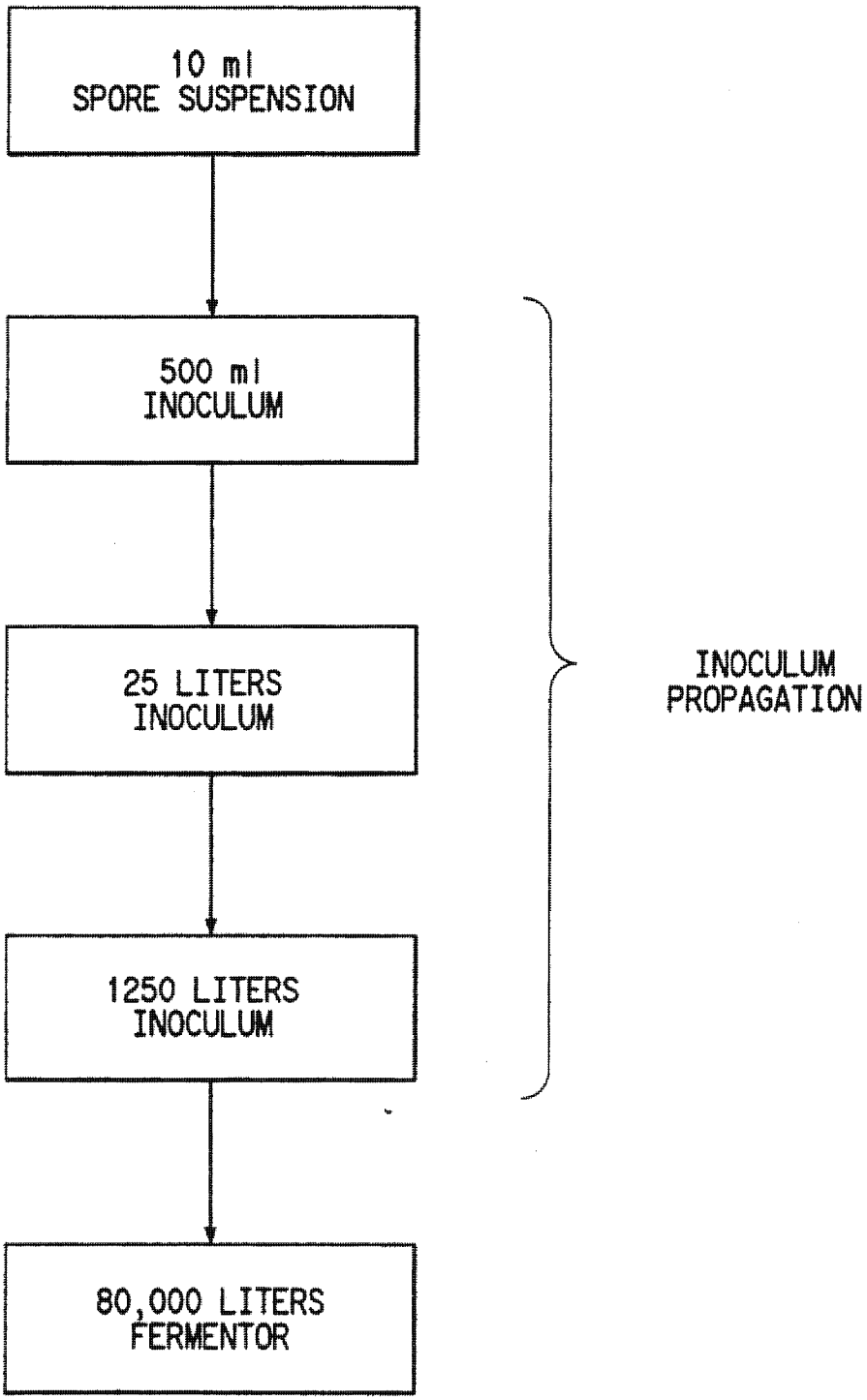

10 ml
SPORE SUSPENSION
500 ml
INOCULUM
25 LITERS
INOCULUM
1250 LITERS
INOCULUM
80,000 LITERS
FERMENTOR
INOCULUM
PROPAGATION

FIG.2

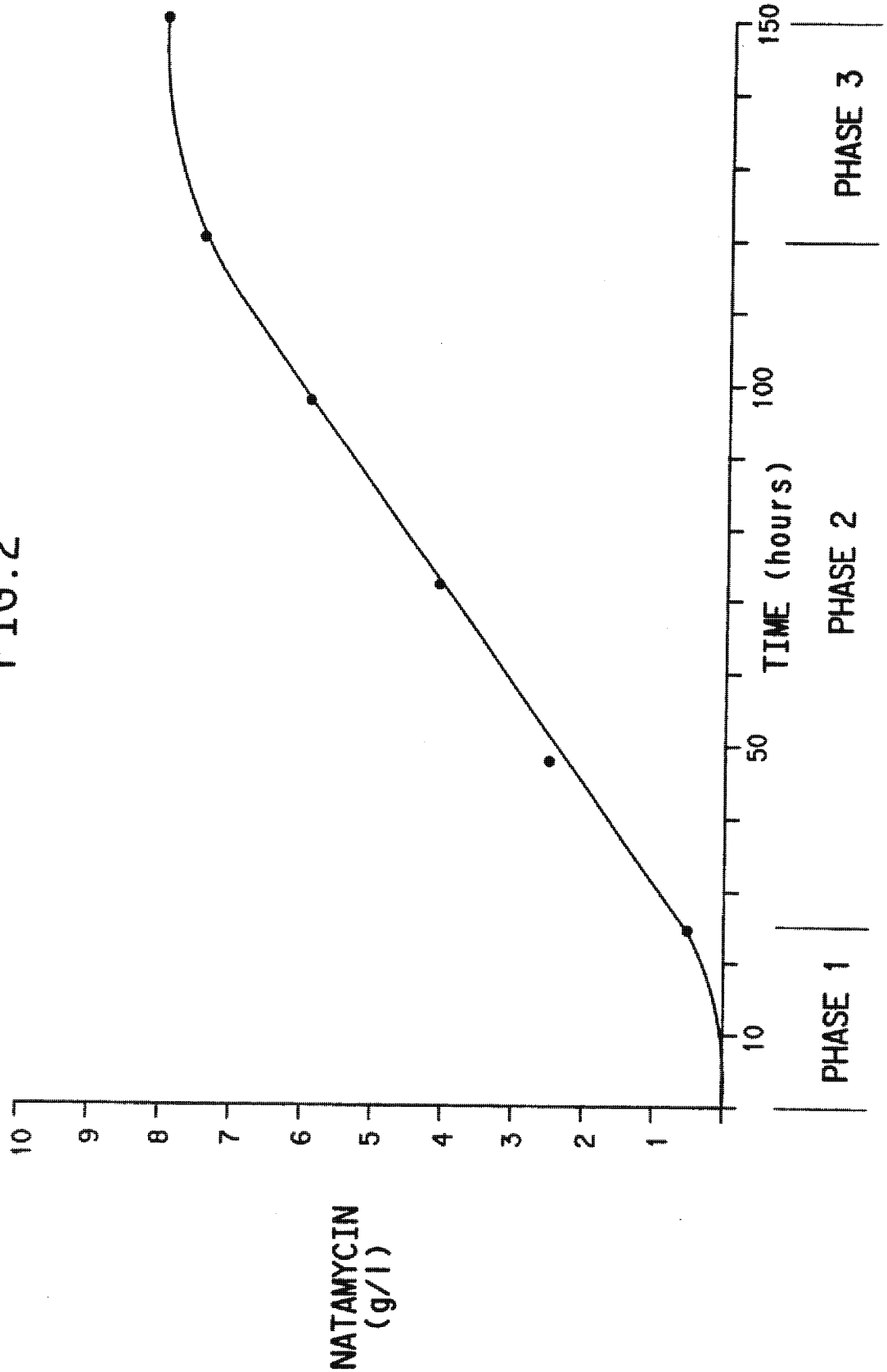
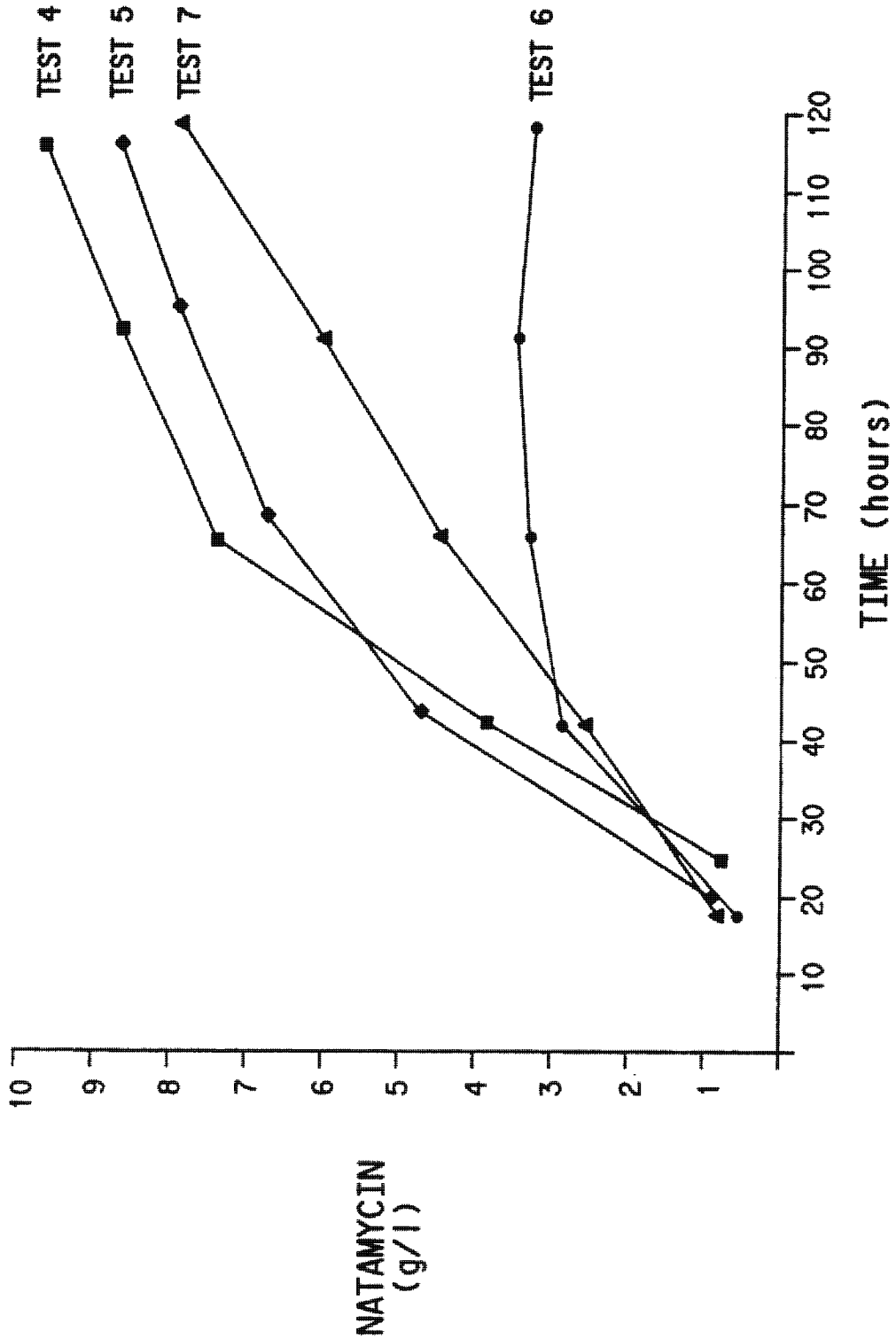

NATAMYCIN (g/l)
10
9
8
7
6
5
4
3
2
1
10
50
100
150
TIME (hours)
PHASE 1
PHASE 2
PHASE 3

FIG. 3


TEST 4
TEST 5
TEST 7
TEST 6
NATAMYCIN (g/l)
TIME (hours)
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100
110
120

FERMENTATION PROCESS FOR PRODUCING NATAMYCIN

BACKGROUND OF THE INVENTION

The present invention relates to a process for producing natamycin characterized by inoculum preparation, inoculum propagation and fermentation in a broth having a pH from about 5.0 through about 6.5.

Natamycin is a member of the polyene family of antimycotics. The compound natamycin is a tetraene with a molecular weight of about 666, empirical formula corresponding generally to $C_{33}H_{47}NO_{13}$, and it contains a glycosidically-linked carbohydrate moiety, mycosamine. Natamycin has an isoelectric point of about pH 6.5. The structure of natamycin exist typically in two configurations: the enol-structure and the keto-structure.

The production of natamycin has been known for years. A conventional fermentation process for producing natamycin is disclosed in American Cyanamid's British Patent No. 846,933 (1960). The disclosure of United Kingdom Patent No. 846,933 is hereby incorporated by reference.

Despite the antibiotic and anti-fungal value of natamycin, very little commercial use has been made of this product. One major reason for the limited use is the prohibitively high manufacturing cost of natamycin.

It is an object of the present invention to overcome the inefficiencies of conventional processes and provide a process for producing natamycin in a cost-effective manner by propagating and fermenting an inoculum in predetermined media having a controlled pH.

SUMMARY OF THE INVENTION

The present invention relates to fermentation by an organism capable of producing natamycin. Particularly the present invention is directed to preparing (e.g., sporulation) and propagating an inoculum comprising a Streptomyces species that, during fermentation, produces natamycin. The Streptomyces species is exposed to a series of predetermined environments and/or mediums which improve the rate at which natamycin is produced. It has been found that an enhanced rate of natamycin fermentation and improved yields can be achieved by adding sufficient amounts of basic pH control agents to the fermentation medium which maintain the pH between about 5.0 and 6.5.

A suitable aqueous medium for inoculum propagation comprises:

a) a protein nitrogen source in an amount of from about 2–16 g/l, normally about 8 g/l; and

b) a metabolizable carbon source present in an amount which is sufficient to avoid total carbon depletion, usually 5–30 g/l of medium, and normally about 15 g/l.

A suitable aqueous medium used during fermentation to induce the inoculum to produce natamycin comprises:

a) about 80–250 g/l of a metabolizable carbon source; and

b) a protein nitrogen source containing a high level of protein and trace ingredients. The protein nitrogen source typically comprises a non-yeast protein nitrogen component and a yeast protein nitrogen component. These two protein nitrogen components are desirably present in a ratio ranging, respectively, from about 5:1 to 11:1 based on protein contents, and for best results generally about 8:1.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic block diagram of the process which may be used in the invention for inoculum propagation.

FIG. 2 is a graphical representation of the three phases which typically occur during fermentation.

FIG. 3 is a graph of time vs. natamycin production for the natamycin produced in accordance with Tests 4 through 6 of the Example.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, an organism capable of producing natamycin is placed into contact with a predetermined medium to produce an inoculum, and then into a predetermined fermentation production medium that will support maximum metabolic activity of the organism during further propagation and natamycin producing fermentation. During fermentation the organism transforms at least a portion of the predetermined production medium into natamycin. It has been found that an enhanced rate of natamycin production and improved yields of natamycin can be achieved by adding sufficient amounts of basic pH control agents to the fermentation medium which maintain the pH between about 5.0 and about 6.5.

Any organism which comprises a natamycin producing Streptomyces species can be used in accordance with the invention. A preferred Streptomyces species comprises *Streptomyces gilvosporeus* which has been deposited previously with the American Type Culture Collection (ATCC) in Rockville, Md., United States of America, and is registered as ATCC No. 13326.

An inoculum is prepared from a spore suspension of the appropriate spores. The inoculum of the appropriate Streptomyces species is subsequently fermented which produces high yields of natamycin when placed into the fermentation medium of the invention. The inoculum is typically exposed to a series of propagation steps wherein each step increases the quantity of the natamycin producing Streptomyces cells. After the quantity of Streptomyces cells is adequate, the Streptomyces is exposed to an environment and/or a medium which is designed to enhance natamycin production when the Streptomyces species ferments.

Spore Suspension

The inoculum is started by collecting the spores of a natamycin producing Streptomyces species which was obtained from the American Type Culture Collection. The spores are germinated to produce an actively growing culture of the Streptomyces species. A sterilized (e.g., autoclaved), agar slant is inoculated heavily with the actively growing culture of the Streptomyces species (e.g., *Streptomyces gilvosporeus* or any other natamycin producing species), and incubated until the slant is covered substantially entirely with spores. The spores on the agar slant are scraped into a small amount of a liquid, such as water (e.g., distilled water), nutrient medium, etc., to produce an aqueous spore suspension. The resulting spore suspension is propagated to produce the inoculum for the fermentation operation (i.e., natamycin production). For achieving the best results, the spore suspension used to begin inoculum propagation should contain a spore concentration of about

10^5–10^{10} CFU/ml, and, normally, at least about 10^8 CFU/ml.

A number of agar slant media can be used to promote sporulation of the culture of the Streptomyces species (e.g., *S. gilvosporeus*), which will be used to form the spore suspension. Appropriate agar slant mediums typically comprise at least one member of the following group: yeast malt agar, Hickey-Turner agar, GYA agar, Pridham agar, potato dextrose, Bennett's agar, etc.

A high concentration (e.g., 10^8 CFU/ml), of viable spores within the spore suspension is a key aspect of the present invention. First, if the concentration of spores is too low, it takes much longer to obtain, through inoculum propagation, the quantity of Streptomyces cells sufficient for cost-effective natamycin fermentation production. Second, a reduced quantity of spores within the suspension lengthens the total inoculum propagation time and increases the likelihood of contamination (e.g., by an unwanted organism). Further, a low spore concentration within the suspension may tend to promote the formation of large, tightly packed mycelial pellets. These pellets are unsuitable for obtaining high yields of natamycin due to problems associated with oxygen transfer, mass transfer of nutrients into the pellets, etc. Should the size of mycelial pellets become undesirable, the pellets can be broken apart physically, such as by using a shear force (e.g., blending).

Inoculum Propagation

The aqueous spore suspension (e.g., *S. gilvosporeus*) , discussed above, is germinated and cell multiplication continued until the number of organisms is adequate to be used as an aqueous inoculum for fermentation production of natamycin. A suitable inoculum cell density comprises a dry cell weight of about 1–5 g/l and is used at a volume of about 0.1–10% of the natamycin production medium volume.

The aqueous medium used for inoculum propagation determines the cell density and the metabolic state of the inoculum (e.g., an adequate density of healthy cells is desirable). A sufficient amount of protein nitrogen, which contains complex growth factors (e.g., vitamins), and inorganic elements (e.g.,potassium, sodium, calcium, etc.), and trace elements (e.g., boron, cobalt, iron, copper, zinc, etc.), that are commonly present in the protein nitrogen source, are needed to achieve an inoculum possessing the desired cell density and metabolic state. The protein nitrogen source may be any source that will propagate the spore suspension into an inoculum that will produce the desired high yields of natamycin.

A metabolizable source of carbon must also be supplied to the aqueous inoculum medium in an amount which is sufficient to achieve the desired inoculum cell density. For best results, the carbon source should not be depleted completely during the inoculum propagation. Depletion of the carbon source tends to alter adversely the metabolic state of the inoculum, which may lead to reduced yields of natamycin during fermentation.

Although a variety of aqueous inoculum media can be used effectively in accordance with the present invention, to obtain high yields of natamycin it is advantageous to use predetermined amounts of medium ingredients.

A suitable medium for inoculum propagation may be prepared in water (e.g., low mineral content water, distilled water, etc.), and comprises:

a) a protein nitrogen source in an amount from about 2–16 g/l, normally about 8 g/l; and

b) a metabolizable carbon source present in an amount which is sufficient to avoid total carbon depletion, usually 5–30 g/l of medium, and normally about 15 g/l.

Two specific compositions of a medium appropriate for inoculum propagation are given below.

	Quantity
Composition 1	
Difco "Bacto" peptone	5 g/l
Corn steep liquor	3 g/l
Sodium chloride	10 g/l
Glucose	15 g/l
Composition 2	
Hormel peptone PSR 5	8 g/l
Sodium chloride	10 g/l
Glucose	15 g/l

The inoculum medium which provides nutrients that enhances the production rate of Streptomyces cells may be prepared by conventional techniques (e.g., separate or simultaneous sterilization of the carbon and nitrogen sources at temperatures of about 120°–140° C.). The inoculum medium after sterilization, desirably has a pH of about 7. The spore suspension is introduced to the inoculum medium and the inoculum medium is heated to a temperature of about 25°–40° C. and, normally, about 28°–35° C.

In order to achieve the large volumes of aqueous inoculum which are desirable for fermentation production of natamycin, several inoculum propagation steps are required, each carried out in a volume greater than the previous step. For example, the inoculum propagation may be conducted in a manner which achieves an exponential increase in the quantity of Streptomyces cells. Particularly, it is advantageous to keep the culture in an exponential growth mode during propagation by effectively increasing the volume of the inoculum during each step of the propagation. This can be done by either minimizing the duration of each step or by minimizing the number of steps. For example, once a predetermined cell density of inoculum has been achieved, the inoculum is transferred to a larger environment (e.g., vessel), for further propagation. By effectively controlling the inoculum propagation a minimum of time and expense is devoted to inoculum propagation and, accordingly, cost-effective natamycin yields during fermentation are increased.

The length of time an individual step in the series of inoculum propagation steps is permitted to continue depends upon the composition of the medium, quantity of Streptomyces cells desired, temperature, etc. Typically, an individual propagation step is conducted for about 6 through at least about 24 hours.

The inoculum propagation process requires aeration of the inoculum. For example, the flask or vessel housing the inoculum may be agitated on a rotary shaker at about 200 rpm. In one aspect of the invention, the inoculum may be agitated by an impeller which is located within the vessel that houses the inoculum, while sterile air is forced into the bottom of the vessel.

Now referring to FIG. 1, this figure is a schematic of the process which may be used to produce the inoculum that is fermented to produce nataymcin. FIG. 1 illustrates the volumetric increases in the inoculum which are typically achieved by propagation that are neces-

sary to obtain a quantity of aqueous inoculum that is adequate to produce natamycin in a cost-effective manner. For example, the volume of inoculum is increased from 25 liters to 1250 liters by adding the 25 liters of inoculum to a vessel containing 1225 liters of an aqueous inoculum medium.

Natamycin Production

Natamycin production is conducted in a fermentation vessel which is capable of housing the fermentation process. It has been found that an enhanced rate of natamycin fermenation and improved yields can be achieved by adding sufficient amounts of basic pH control agents to the fermentation medium which maintain the pH between about 5.0 and about 6.5. One aspect of the invention which is also important for achieving maximum yields of natamycin is the composition of the aqueous fermentation medium. The fermentation medium must contain the proper amounts of metabolizable carbon and protein nitrogen. Also, it is desirable that the medium include complex growth factors (e.g., vitamins), inorganic elements (e.g., potassium, sodium, calcium, etc.), and trace elements (e.g., boron, cobalt, iron, copper, zinc, etc.)

A suitable medium for fermentation may be prepared in water (e.g., low mineral content water, distilled water, etc.), and comprises:

a) about 80–250 g/l of a metabolizable carbon source; and

b) at least about 15 g/l and, normally about 20 g/l through 80 g/l, of a protein nitrogen source containing a high level of protein and trace ingredients. The protein nitrogen source may comprise a non-yeast protein nitrogen component and a yeast protein nitrogen component. These two protein nitrogen components are usually present in the ratio ranging, respectively, from about 5:1 to 11:1 based on protein contents, and for best results generally about 8:1.

The protein nitrogen source may be supplied from a wide range of sources. For example, soy protein products may comprise the non-yeast protein nitrogen source (e.g., desirable natamycin yields are obtained with a soy protein source comprising 80–95% protein). The protein nitrogen may also comprise beef extract and/or protein hydrolysates (e.g., peptones).

As discussed above, the production medium must also include a source of carbon which is metabolizable by the Streptomyces species. The carbon source may be supplied in any expedient form such as glucose, polysaccharide, corn and potato starches, etc.

Moreover, in one aspect of the invention, it is not necessary to initially introduce the entire quantity of the carbon source which is required to produce natamycin, as a starting component of the natamycin production medium (e.g., the initial quantity of the carbon source is not adequate for complete fermentation). In this aspect of the present invention, carbon source addition may be performed during the natamycin production so as to maintain a quantity of carbon source of about 5–30 g/l, and usually 20 g/l. Thus, an appropriate quantity of a suitable carbon source is added to the fermentation medium either initially and/or after the fermentation has begun. For example, the carbon source may be present in the fermentation medium in an amount of about 40–100 g/l. Thereafter, during the major period of fermentation, carbon source is continually added to the fermentor at a rate which is at least equivalent to the rate at which the carbon source is consumed enzymatically by the Streptomyces species during the fermentation process(e.g., to maintain the carbon source concentration at or above a minimum level). Toward the end of the fermentation process and after the major fermentation period, the carbon source addition is discontinued so that little or no carbon source is left at the end of the fermentation cycle (e.g., the quantity of the carbon source substantially equates to the particular quantity of carbon source within the fermentation medium which is necessary to complete the fermentation process).

The natamycin production medium, which provides nutrients for the Streptomyces fermentation and natamycin production may be prepared by conventional techniques (e.g., separate or simultaneous sterilization of the carbon and nitrogen sources at temperatures of about 120°–140° C.). The production medium, after sterilization, desirably has a pH of about 7.

The inoculum is introduced until a concentration of about 0.1–10%, usually about 2%, by volume is achieved in the production medium (e.g., the quantity of inoculum may be sufficient to inoculate a plurality of fermentors). The remainder of the volume of the fermentor comprises the fermentation medium discussed above. Any technique is acceptable for introducing the inoculum to the production medium within the fermentor which delivers the inoculum in an active metabolic state.

The fermentation or production medium is brought to a temperature of about 25°–40° C., and normally about 28°–35° C. The length of time which the fermentation process is allowed to continue depends upon the composition of the fermentation medium, temperature, quantity of Streptomyces cells in the inoculum, quantity of natamycin desired, etc. Typically, the fermentation process is conducted for about 70 through at least about 168 hours.

Oxygen is supplied to the natamycin production medium during fermentation. It is advantageous to maintain a dissolved oxygen level in the production medium of about 20%–80% of air saturation during the major portion of the fermentation. The ability to achieve a suitable dissolved oxygen level may be enhanced by proper adjustment of the aeration and/or agitation rate. For example, the fermentation or production medium must be aerated by forcing air (e.g., sterile air), through the fermentation medium, usually at a rate of about 0.3 through at least about 1.0 volumes of air per volume of fermentation medium. In one aspect of the invention it is desirable to agitate the fermentation medium while being aerated. Further, the rate of aeration may be sufficient to cause agitation of the fermentation medium.

Referring now to FIG. 2, the relationship between time and nataymcin production rates is shown for each of the three phases of the process. The first phase includes addition of the carbon source to the fermentation medium and growth or multiplication of the Streptomyces species. The first phase is also accompanied by natamycin production. The concentration of natamycin in the fermentation broth increases as the propagation of the cells of the Streptomyces species increases. The increase in the concentration of natamycin increases generally exponentially with time during the first phase. Eventually, the concentration of natamycin will increase constantly with time, which indicates that the second phase (i.e., the major phase) of natamycin production as been achieved. The third phase is characterized by a plateau in the concentration of natamycin (e.g., which may be due to a slowing of the metabolic

activities of the Streptomyces species). The concentration of natamycin within the fermenter may be analyzed with respect to time in order to ascertain the current phase of fermentation. It is desirable to use a medium and/or an environment which induces the second phase of fermentation to be reached rapidly and maintained in order to maximize the overall quantity of natamycin that is produced.

In one aspect of the invention, it may be desirable to add an anti-foaming agent (e.g. silicone defoamer), to the fermentation medium in an amount of from about 0.01%–1% by volume of the fermentation or natamycin production medium when it is desirable to control foaming.

The natamycin production medium after inoculum addition has a pH of about 7. As discussed above, during the relatively short first phase of fermentation, rapid culture propagation is the major activity. Thereafter natamycin production becomes the predominant activity, and the pH drops. Controlling or maintaining the pH during fermentation is a key aspect of the invention because, without pH control, the pH will drop to about pH 4.0 during natamycin production.

The major or second phase of natamycin production corresponds to the period beginning when the pH has first dropped below about pH 6.5 until near the end of the natamycin production. Toward the end of the fermentation the pH may be allowed to drop below about pH 5.0. In accordance with the invention, the major phase does not include this low pH period when the pH is below about pH 5.0.

The pH of the fermentation broth which comprises the fermentation medium may be controlled by adding pH control agents to the broth. The pH control agents used in the process of the invention comprise hydroxides and basic salts that will control the pH without adversely affecting the natamycin production and recovery. Suitable pH control agents comprise at least one of sodium potassium and calcium hydroxides, and mono-, di- and trisodium and potassium citrates, etc.

In accordance with the invention, the pH of the fermentation cycle is allowed to drop initially, (i.e., during culture propagation), to about pH 6. By this time effective natamycin production is underway. After the pH has dropped into the range of from about pH 5.0 to about 6.5, addition of the basic pH control agent is commenced and continued at rates sufficient to maintain the fermentation broth thereafter at a pH of about 5.0 through about 6.5. Normally, the pH is maintained by automatic pH controlled titration with an aqueous solution including the pH control agent.

A variety of pH control agent compositions, blends, mixtures, etc., can be used simultaneously and/or sequentially. For example, it may be desirable to introduce both a citrate salt and a hydroxide into the fermentation broth (e.g., a hydroxide could be added simultaneously to more easily maintain a pH of about 5.0–6.5). However, in some aspects of the invention, an acidic citrate can be added in conjunction with a basic pH control agent.

A key aspect of the invention comprises using a pH control agent comprising an inorganic base to maintain a pH of about 5.9–6.1. As aforementioned, the pH control of the present invention enhances the rate of natamycin production and improves the yield of natamycin. For example, the pH control of the invention may permit fermentation production of natamycin in far less time in comparison to an equivalent yield of natamycin produced without pH control. Typically, the production time is decreased 20–60%, with a reduction of about 35% being common.

When the present invention is practiced appropriately (e.g., effective handling of the Streptomyces inoculum, selection of media, etc.), the resultant fermentation broth will normally include at least about 5 g/l of natamycin. In certain cases, the level of natamycin production may range from about 7 g/l through at least about 12 g/l.

The natamycin can be separated from the production medium. In certain cases, the natamycin may be extracted from the fermentation broth and crystallized. Examples of acceptable techniques for obtaining crystalline natamycin can be found in U.K. Patent No. 846,933.

The invention is demonstrated by the following Example which is intended to illustrate, not limit, the scope of contemplated equivalents. Unless specified otherwise, commercially available reagent grade materials were used to conduct the following Example.

EXAMPLE

In the following tests, agar slants of the following compositions are prepared using distilled water.

3 g/l	yeast extract (Difco "Bacto" Yeast Extract)
3 g/l	malt extract (Difco Malt Extract)
5 g/l	peptone (Difco "Bacto" peptone)
10 g/l	glucose
15 g/l	agar.

The agar was sterilized at about 121°C for about 15 minutes.

An inoculum medium of the following composition was prepared in distilled water, and the pH was adjusted to about 7.0 with potassium hydroxide.

20 g/l	glucose
10 g/l	sodium chloride
6 g/l	corn steep liquor (PPM (brand), Corn Steep Liquid)
6 g/l	peptone (Difco "Bacto" peptone)

The inoculum medium was sterilized at about 121°C for about 15 minutes.

Streptomyces gilvosporeus, American Type Culture Collection Registration No. 13326, was obtained from the American Type Culture Collection as a freeze-dried spore suspension and used as the culture source. The culture was held on the agar slants at about 25°C until the culture sporulated.

The agar slants sporulated heavily within about 10 days and were used after 10–20 days. Spores were scraped off these agar slants into the inoculum medium to achieve a spore suspension concentration of about 10^8 CFU/ml. About 2 ml of the spore suspension was added to about 100 ml of the inoculum medium in a 500 ml baffled flask. The inoculum in the baffled flask was incubated for about 48 hours at about 29° C. and agitated at about 200 rpm on a rotary shaker. After about 48 hours about 4 ml of this culture was added to about 200 ml of inoculum medium in a 1000 ml baffled flask, to propagate the inoculum. This inoculum was then incubated for about an additional 24 hours at about 29° C. and agitated at about 200 rpm on a rotary shaker. The inoculum thus produced was used to inoculate 8 l of production medium.

The natamycin production medium used in this Example was of the following initial composition:

19.5 g/l	soy protein isolate (ADM, "Profam" S970)
4.5 g/l	yeast extract (Stauffer, Type KAT)
0.2 ml	defoamer (Mazu, DF 289)

The production medium was prepared in distilled water in a 14.0 l fermenter and the pH was adjusted to about 7.6 with potassium hydroxide. The fermenter was then sterilized for about 15 minutes at about 121° C. Glucose was sterilized separately as a 50% solution in distilled water.

Before inoculation, the production medium was heated to about 29° C. and the glucose was added to achieve an initial concentration of glucose of about 40 g/l. An aeration rate of about 0.3 v/v-min. (volumes of air per volume of medium per minute) and an agitation rate of about 300 rpm was established for the fermentor.

The inoculum discussed above containing *Streptomyces gilvosporeus*, (ATCC Registration No. 13326), was added to the fermentation vessel until the fermentation vessel had an inoculum content of about 2% by volume. Glucose was added to the inoculum after about 40 hours of fermentation in order to maintain a glucose concentration of about 20 g/l glucose in the fermentation vessel. This was done by feeding glucose to the fermenting vessel at a rate of about 1 g/l-hr. The agitation rate of the fermentation vessel was increased as necessary to maintain a dissolved oxygen level of about 50% of air saturation.

Test #1—This test shows the typical practice of the high yield fermentation process, but without the pH control of the present invention. Proceeding as described above, starting with an initial volume of about 8.0 l production medium (pH of about 7) and continuing the fermentation cycle time for about 117 hours (pH of about 4.5), with a total glucose addition of about 110 g/l, a yield of about 7.3 g/l natamycin in about 8.7 l of fermentation broth was obtained (64 g natamycin, total).

Test #2—Proceeding substantially as in Test #1, but after 18 hours when pH had dropped to about 6.0 adding about 20% KOH to maintain the pH at about 6.0, and adding about 155 g/l glucose over the cycle of about 92 hours there is obtained a natamycin yield of about 7.4 g/l in about 10.1 l (75 g natamycin, total). Thus, as compared to Test #1, this test of the present invention provides an equivalent yield of natamycin in a shorter production period.

By following substantially the same procedure but using NaOH instead of KOH, similar rapid high yield natamycin production was achieved.

Test #3—Continuing production of Test #2 and adding about 250 g/l glucose over about a 282 hour total fermentation cycle time there was obtained a natamycin yield of about 12.4 g/l in about 10.4 l of fermentation broth (129 g natamycin, total). Thus, prolonged natamycin production is possible to give high natamycin yields.

Test #4—Proceeding as in Test #2, but when the pH decreased to about pH 6, about 20% potassium hydroxide was added by automatic titration to maintain a pH of about 6.1–5.9. After about 66 hours fermentation time, about 6.6 g/l of natamycin was produced in about 9.2 l fermentation broth (61 g natamycin, total).

Test #5—Proceeding as in Test #2, but at about 24 hours when the fermentation broth was at a pH of about 5.1, about 7 g/l of trisodium citrate was added to the fermentation vessel. The pH remained in a pH range from about 5.0 to 6.5. Fermentation was continued for about 69 hours. Production of natamycin was about 6.6 g/l in about 8.7 l (57 g natamycin, total).

By following substantially the same procedure but using an equimolar amount of tripotassium citrate, similar rapid high yield natamycin production was achieved.

Test #6—By proceeding substantially in accordance with Test #5 except that in place of the trisodium citrate pH control agent, there was added at about 24 hours after the start of fermentation trisodium citrate adjusted to a pH of about 5.0 with citric acid (this addition contained about 5 g/l of citrate ion); then after about 45 hours citric acid was added to maintain pH below about 5.0. Only about 25.6 g of natamycin was produced after about 117 hours of fermentation.

Test #7—Proceeding substantially in accordance with Test #1 but without the addition of any pH control agent, after about 66 hours of fermentation the natamycin yield was only 4.3 g/l in about 8.3 l broth (36 g natamycin, total). A comparison of Test #4 and Test #7 illustrates that pH control will enhance the natamycin production rate (i.e., the 66 hour fermentation process of Test #4 produced 61 g of natamycin whereas Test #7 produced only 36 g of natamycin).

The following Table summarizes the results of Tests 1–7 in terms of relative speed of natamycin production. A review of the following Table demonstrates that the pH control of the invention improves the yield of natamycin obtained via a fermentation process.

TABLE

Test #	pH Control Agent	Production Time (hrs)	Quantity Natamycin Produced (Total/Concentration)
1	None	117	64 g/7.3 g/l
2	KOH	92	75 g/7.4 g/l
3	KOH	282	129 g/12.4 g/l
4	KOH	66	61 g/6.6 g/l
5	Trisodium citrate	69	57 g/6.6 g/l
6	Trisodium citrate/ Citric Acid	117	25.6 g/3.2 g
7	None	66	36 g/4.3 g/l

Now, refer to FIG. 3, which is a graph illustrating the natamycin production (g/l in the fermentation broth) over about 115 hours of the fermentation process which was performed in accordance with Tests 4, 5, 6 and 7. A review of FIG. 3 illustrates graphically that the pH control of the invention enhances the rate at which natamycin is produced.

Although a few embodiments of the invention have been described above in detail, those skilled in this art will readily appreciate that the present invention embraces many combinations and variations.

What is claimed:

1. In a process for preparing natamycin including the steps of (a) obtaining an inoculum by propogating a spore suspension containing a natamycin-producing Streptomyces species in an inoculum medium; (b) introducing the inoculum to a fermentation medium and providing a fermentation broth comprising said fermentation medium and inoculum; (c) producing natamycin by a fermentation in said fermentation broth; and (d)

recovering natamycin produced by said fermentation, an improvement comprising:

in (c) using a fermentation with a cell propogation stage followed by major natamycin production stage, adding a basic pH control agent at a rate sufficient to maintain the fermentation broth at a pH of from 5.0 to 6.5 during said major natamycin production stage, and continuing the fermentation to provide a fermentation broth containing at least about 5 g/l natamycin.

2. The process of claim **1** wherein said natamycin producing species comprises *Streptomyces gilvosporeus*, ATTCC 13326.

3. The process of claim **1** wherein said spore suspension contains a spore suspension of at least 10^5–10^{10} CFU/ml.

4. The process of any one of claims **1** or **2** wherein said fermentation medium contains non-yeast and yeast protein nitrogen components, said non-yeast and yeast components being present in the ratio ranging, respectively, from about 5:1 to 11:1 based on protein contents.

5. The process of any one of claims **1** or **2** wherein the inoculum medium comprises from about 2 through about 16 g/l of a protein nitrogen source and about 5 through 30 g/l of a carbon source.

6. The process of any one of claims **1** or **2** wherein the fermentation medium comprises from about 80 through about 250 g/l of a carbon source and nitrogen source; wherein the nitrogen source further comprises a non-yeast component and a yeast component.

7. The process of claim **6** wherein the ratio of non-yeast and yeast components of said protein nitrogen source ranges from about 5:1 through about 11:1, based on a protein content.

8. The process of claim **7** wherein said non-yeast component of said protein nitrogen source comprises soy protein.

9. The process of any one of claims **1** or **2** wherein the concentration of carbon source in the fermentation medium is maintained at about 5–30 g/l during the major natamycin production period.

10. The process of any one of claims **1** or **2** wherein at least 7 g of natamycin is produced per liter of fermentation medium.

11. The process of any one of claims **1** or **2** wherein the propagation and fermentation are conducted at temperature of from about 25 to about 40 degrees C.

12. The process of any one of claims **1** or **2** wherein the fermentation is conducted for a period of time from about 70 through at least about 168 hours.

13. The process of any one of claims **1** or **2** further comprising aerating the inoculum during propagation and fermentation.

14. The process of any one of claims **1** or **2** further comprising aerating the inoculum during natamycin production.

15. The process of any one of claims **1** or **2** wherein the inoculum and fermentation media include a carbon source which comprises at least one member from the group consisting of glucose, saccharide and starch.

16. The process of any one of claims **1** or **2** wherein the inoculum and fermentation media include a protein nitrogen source which comprises at least one member from the group consisting of soy protein, yeast and protein hydrolysate.

17. The process of claim **15** wherein the protein nitrogen source comprises a soy protein comprising at least one member from the group consisting of isolates, flours and meals.

18. The process of claim **15** wherein the protein nitrogen source comprises a yeast comprising at least one member from the group consisting of whole yeast extracts and autolysates.

19. The process of any one of claims **1** or **2** further comprising using a pH control agent comprising at least one member of the group consisting of hydroxides and citrates.

20. The process of claim **1** wherein carbon source is provided in the fermentation medium at from about 80 g/l to 250 g/l; wherein the carbon source addition is performed during the natamycin production stage so as to maintain a quantity of carbon source of from about 5 g/l to 30 g/l; wherein at least about 15 g/l at a protein nitrogen source is provided in the fermentation medium; wherein the protein nitrogen source comprises a non-yeast protein nitrogen component and a yeast protein nitrogen component; and wherein the ratio of non-yeast protein nitrogen component to yeast protein nitrogen component is from about 5:1 to 11:1, based upon protein content.

21. The process of claim **1** or claim **20** wherein the dissolved oxygen level is maintained at from about 20% to 80% of air saturation during said major natamycin production stage.

22. The process of claim **21** wherein the fermentation is continued until the fermentation broth includes from about 5 g/l to 12 g/l natamycin.

23. The process of claim **1** wherein the inoculum is introduced in (b) in a concentration of from about 0.1 to 10 volume percent of the fermentation broth.

24. The improved process of claim **23** wherein the inoculum introduced in (b) has a cell density based upon a dry cell weight, of from about 1 to 5 g/l.

25. The process of claim **1** wherein (a), a spore suspension containing a spore concentration of at least about 10^8 CFU/ml is propogated to obtain said inoculum.

26. The process of claim **1** or claim **25** wherein in (a) the inoculum is obtained using a series of propogation steps comprising propagating cells in one inoculum medium to achieve one inoculum having a selected range of cell density, then transferring said one inoculum to another inoculum medium of greater volume and further propagating cells to achieve larger inoculum having said selected range of cell density, and then transferring the said larger inoculum to yet another inoculum medium at yet greater volume and further propogating cells to achieve a yet larger inoculum having said selected range of cell density.

27. The process of claim **26** wherein said selected range of cell density is from about 1 g/l to 5 g/l, based upon dry cell weight.

28. The improved process of claim **1** wherein the fermentation has a natamycin plateau stage following said major natamycin production stage; and wherein the pH is allowed to drop below 5 during the natamycin plateau stage.

* * * * *

US005686273A

United States Patent [19]

Eisenschink et al.

[11] Patent Number: 5,686,273

[45] Date of Patent: *Nov. 11, 1997

[54] **FERMENTATION PROCESS FOR PRODUCING NATAMYCIN WITH ADDITIONAL CARBON AND NITROGEN**

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[73] Assignee: **Cultor Food Science, Inc.**, New York, N.Y.

[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,231,014.

[21] Appl. No.: **262,804**

[22] Filed: **Jun. 20, 1994**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 997,614, Dec. 23, 1992, abandoned, which is a continuation-in-part of Ser. No. 740,545, Aug. 5, 1991, abandoned, and a continuation-in-part of Ser. No. 997,613, Dec. 23, 1992, abandoned, which is a continuation-in-part of Ser. No. 740,536, Aug. 5, 1991, abandoned.

[51] **Int. Cl.**[6] **C12P 17/18**

[52] **U.S. Cl.** **435/119**; 435/118; 435/76; 435/244

[58] **Field of Search** 435/76, 118, 119, 435/244

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,015,612	1/1962	Pirt et al. .	
3,062,724	11/1962	Reusser	435/76
3,378,441	4/1968	Bridger et al.	435/76
4,167,450	9/1979	Chesbro et al.	435/244
4,536,494	8/1985	Carter	514/31
4,600,706	7/1986	Carter	514/31
5,231,014	7/1993	Eisenschink et al.	435/76

FOREIGN PATENT DOCUMENTS

0218265	9/1957	Australia .
669761	9/1963	Canada .
677040	12/1963	Canada .
684259	4/1964	Canada .
359517	3/1957	Sweden .
846933	7/1957	United Kingdom .
0844289	8/1960	United Kingdom .
2106498	7/1982	United Kingdom .
8400777	3/1984	WIPO .

OTHER PUBLICATIONS

Creuger, et al., On: Biotechnology, A Textbook of Industrial Microbiology, *Sinauer Associates, Inc.*, 258–261, 1990.

Omura, et al., Macrolide Antibiotics, *Biotechnology*, 4, 386–387, 1986.

Onken, et al., Control and Optimization, *Biotechnology*, 2, 792, 1985.

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Attorney, Agent, or Firm—Ronald S. Courtney, Esq.

[57] **ABSTRACT**

An improved method for producing natamycin by fermentation is disclosed. In the method, which comprises, in order, the steps of:

(a) introducing an inoculum of a natamycin producing *Streptomyces species* to a fermentation medium to produce a fermentation broth comprising the fermentation medium and inoculum; and

(b) producing natamycin by fermentation in the fermentation broth;

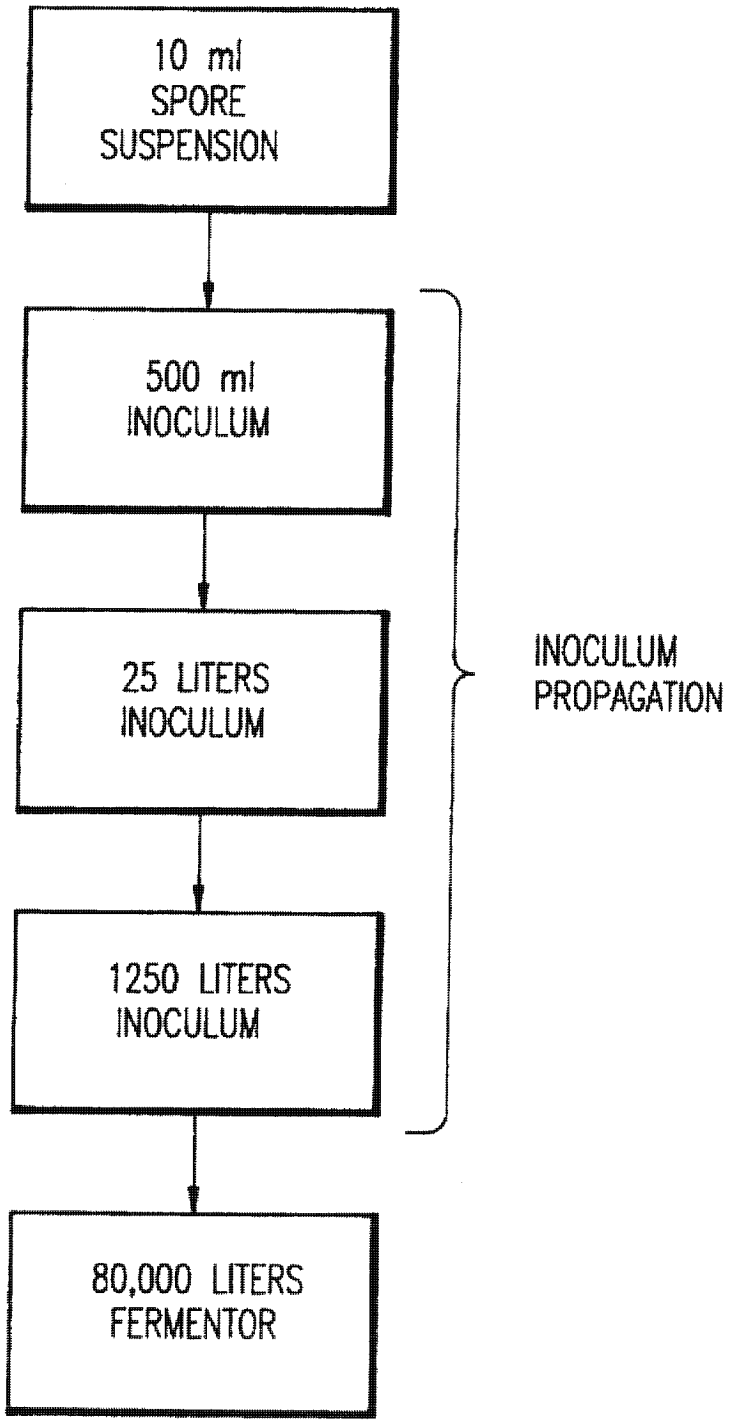
the improvement comprising:

(1) providing at least 15 g/L of a protein nitrogen source to the fermentation medium; wherein the protein nitrogen source comprises a non-yeast protein component and a yeast protein component, and the ratio of non-yeast nitrogen component to yeast protein component being from about 3:1 to 9:1, based on the protein content of the source;

(2) providing a carbon source to the fermentation medium at from about 80 g/L to 250 g/L; wherein the carbon source addition is carried out during step (b) so as to maintain a concentration of carbon source of from about 5 g/L to 30 g/L; and

(3) continuing the fermentation until the fermentation broth comprises at least 5 g/L natamycin.

16 Claims, 3 Drawing Sheets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SPORE
SUSPENSION
500 ml
INOCULUM
25 LITERS
INOCULUM
1250 LITERS
INOCULUM
80,000 LITERS
FERMENTOR
INOCULUM
PROPAGATION

FIG. 1

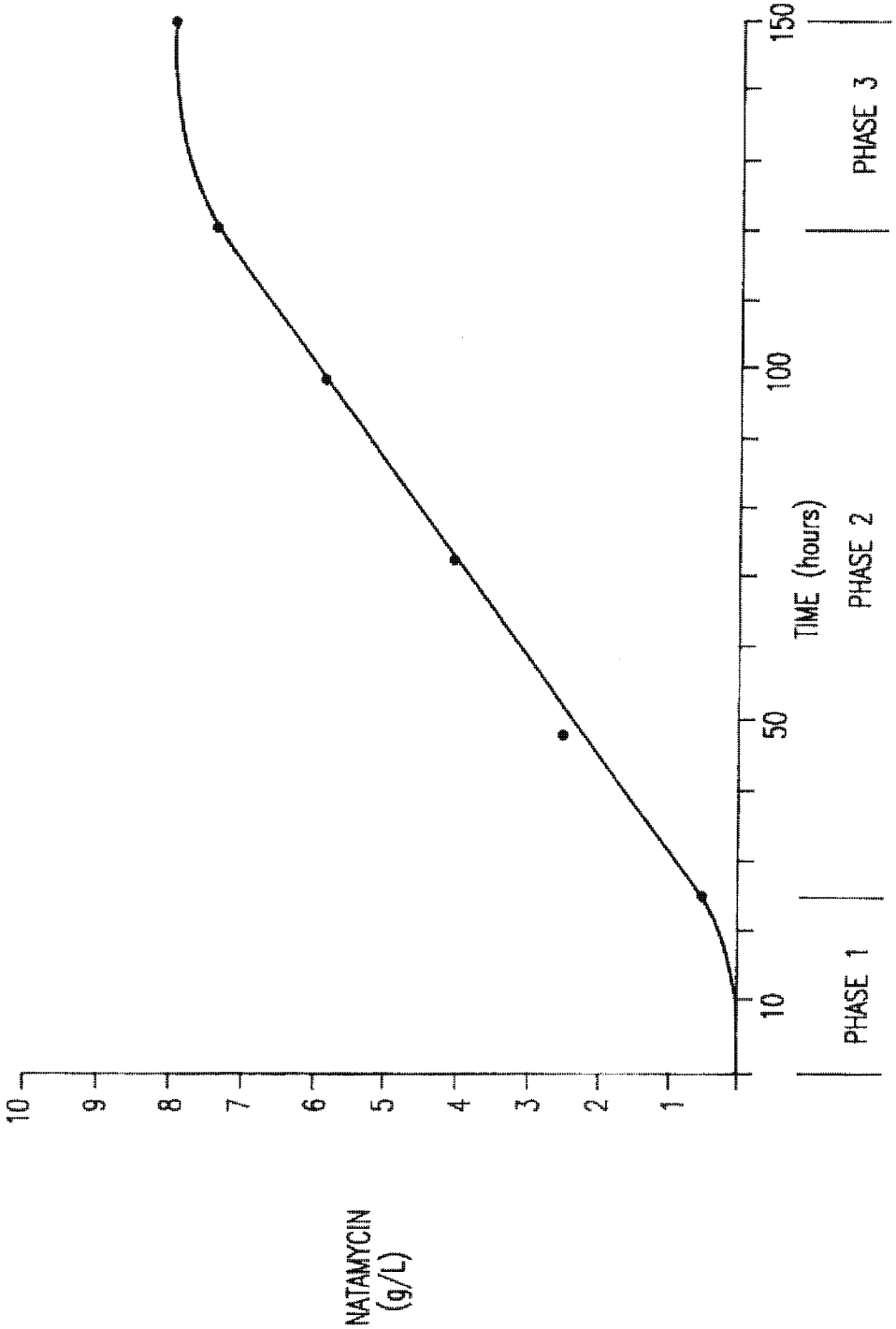
NATAMYCIN (g/L)
10
9
8
7
6
5
4
3
2
1
10
50
100
150
TIME (hours)
PHASE 1
PHASE 2
PHASE 3

FIG.2

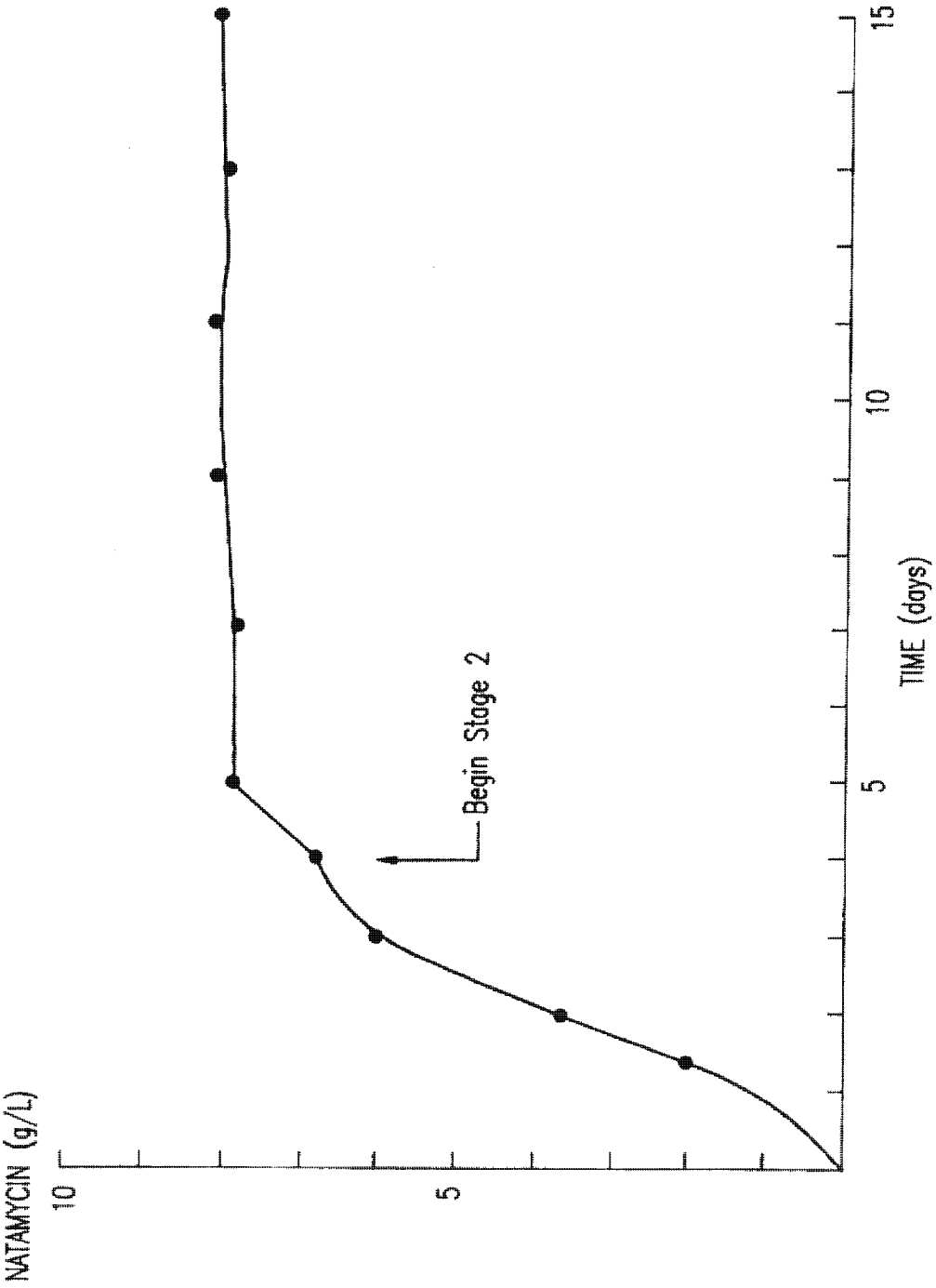

FIG.3

FERMENTATION PROCESS FOR PRODUCING NATAMYCIN WITH ADDITIONAL CARBON AND NITROGEN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. patent application 07/997,614, filed Dec. 23, 1992, now abandoned, which was a continuation-in-part of U.S. application 07/740,545, filed Aug. 5, 1991, now abandoned, and a continuation-in-part of U.S. patent application 07/997,613, filed Dec. 23, 1992, now abandoned, which was a continuation-in-part of U.S. application 07/740,536, filed Aug. 5, 1991, now abandoned, all of which are incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates to a method for producing natamycin. In particular, this invention is a process for producing natamycin by fermentation in a broth that comprises a non-yeast protein component and a yeast protein component.

BACKGROUND OF THE INVENTION

Natamycin (also known as pimaricin or tenecetin) is a member of the polyene family of antimycotics (Florey, "Analytical Profiles of Drug Substances". Vol. 10, 1981; Merck Index, 8th ed., "Pimaricin", p. 834). The compound is a tetraene with a molecular weight of about 666, empirical formula corresponding generally to $C_{33}H_{47}NO_{13}$, and it contains a glycosidically-linked carbohydrate moiety, mycosamine. Natamycin has an isoelectric point of about pH 6.5.

Fermentation processes for producing natamycin are described in: Koninklijke Netherlandsche Gist- & Spiritusfabriek, U.K. Patent 844,289; American Cyanamid, U.K. Patent 846,933; Backus, Canadian Patent 677,040; and Struyk, Australian Patent 218,265 and Canadian Patent 669,761. Although its valuable antibiotic and antifungal properties have been recognized, there has been little research or commercialization of natamycin because of the extremely high cost of its manufacture. A need exists for method for producing useful quantities of natamycin in a cost effective manner.

SUMMARY OF THE INVENTION

The invention is an improved method for producing natamycin by fermentation. In the method, comprising, in order, the steps of:

(a) introducing an inoculum of a natamycin producing *Streptomyces species* to a fermentation medium to produce a fermentation broth comprising the fermentation medium and inoculum; and

(b) producing natamycin by fermentation in the fermentation broth;

the improvement comprising:

(1) providing at least 15 g/L of a protein nitrogen source to the fermentation medium; wherein the protein nitrogen source comprises a non-yeast protein component and a yeast protein component, and the ratio of non-yeast nitrogen component to yeast protein component being from about 3:1 to 9:1, based on the protein content of the source;

(2) providing a carbon source to the fermentation medium at from about 80 g/L to 250 g/L; wherein the carbon source addition is carried out during step (b) so as to maintain a concentration of carbon source of from about 5 g/L to 30 g/L; and

(3) continuing the fermentation until the fermentation broth comprises at least 5 g/L natamycin.

In a preferred embodiment the fermentation is continued until the fermentation broth comprises at least 10 g/L natamycin. In another preferred embodiment production medium is added and broth withdrawn during fermentation so that natamycin is produced in a continuous process.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic of the method for inoculum propagation.

FIG. 2 shows the three phases that occur during batch fermentation.

FIG. 3 shows the stages of the continuous process.

DETAILED DESCRIPTION OF THE INVENTION

Spore Suspension

Any natamycin producing *Streptomyces species* can be used in the method. A preferred *Streptomyces species* is *Streptomyces gilvosporeus*. A preferred strain of *Streptomyces gilvosporeus* has been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852, USA, and assigned registration No. 13326.

Spores of the natamycin producing organism are germinated to produce an actively growing culture. A sterilized (e.g., autoclaved), agar slant is heavily inoculated with the actively growing culture and incubated until the slant is substantially covered with spores. The spores are scraped into a small amount of a liquid, such as distilled water, nutrient medium, etc., to produce an aqueous spore suspension.

A number of agar slant media can be used to promote sporulation of the culture. Appropriate agar slant media typically comprise at least one of: yeast malt agar, Hickey-Tuner agar, GYA agar, Pridham agar, potato dextrose, Bennett's agar, etc. Suitable media are given in the Examples.

The spore suspension typically contains about 10^5–10^{10} CFU/mL. A high concentration (e.g., 10^8 CFU/mL or higher), of viable spores within the spore suspension is preferred. If the concentration of spores is too low, it takes much longer to produce the cell concentration required for cost-effective natamycin production. A lower spore concentration lengthens the propagation time and increases the likelihood of contamination by an unwanted organism. In addition, a low spore concentration may tend to promote the formation of large, tightly packed mycelial pellets. These pellets are unsuitable for obtaining high yields of natamycin due to problems associated with oxygen transfer, mass transfer of nutrients into the pellets, etc. Should the size of mycelial pellets become undesirable, the pellets can be broken apart physically, such as by using a shear force (e.g., blending).

Inoculum Propagation

The aqueous spore suspension is germinated and cell growth continued until the cell density is adequate to be used as an inoculum. A suitable inoculum cell density is about 1–5 g/L (dry weight). The inoculum is used at a volume of about 0.1–10% of the natamycin production medium volume.

The medium used for propagation determines the cell density and the metabolic state of the inoculum. A sufficient amount of protein nitrogen that contains complex growth factors (e.g., vitamins), inorganic elements (e.g., potassium, sodium, calcium, etc.), and trace elements (e.g., boron, cobalt, iron, copper, zinc, etc.) that are commonly present in the protein nitrogen source is needed to produce an inoculum possessing the desired cell density and metabolic state. The protein nitrogen source may be any source that will propagate the spore suspension into an inoculum that will produce the desired high yields of natamycin.

A source of metabolizable carbon must also be supplied to the medium in an amount sufficient to achieve the desired cell density. For best results, the carbon source should not be completely depleted during propagation. Depletion of the carbon source tends to adversely alter the metabolic state of the inoculum and reduce the yield of natamycin during fermentation.

A suitable medium for inoculum propagation may be prepared in water (e.g., low mineral content water, distilled water, etc.), and comprises: (a) about 2–16 g/L, typically about 8 g/L, of a protein nitrogen source; and (b) a sufficient metabolizable carbon source to avoid total carbon depletion, usually about 5–30 g/L, typically about 15 g/L. Suitable media are given in the Examples.

The medium may be prepared by conventional techniques (e.g., separate or simultaneous sterilization of the carbon and nitrogen sources at temperatures of about 120°–140° C.). After sterilization, the medium desirably has a pH of about 7. The spore suspension is introduced to the medium and the medium is heated to about 25°–40° C., typically, about 28°–35° C.

To achieve the large volumes of aqueous inoculum which are desirable for fermentation production of natamycin, several propagation steps are required, each carried out in a volume greater than the previous step. It is advantageous to keep the culture in an exponential growth mode during propagation by increasing the volume of the inoculum during each step of the propagation. This can be done by either minimizing the duration of each step or by minimizing the number of steps. Once the desired cell density has been achieved, the inoculum is transferred to a larger vessel for further growth. This process is illustrated by FIG. 1. The length of time an individual step is permitted to continue depends upon the composition of the medium, quantity of Streptomyces cells desired, temperature, etc. Typically, a propagation step continues for about 6 to 24 hours.

Propagation requires aeration of the inoculum. The vessel or flask housing the inoculum, may be agitated on a rotary shaker at about 200 rpm, or by impeller located within the vessel that houses the inoculum while sterile air is forced into the bottom of the vessel.

Natamycin Production

The fermentation medium must contain the proper amounts of metabolizable carbon and protein nitrogen. Also, it is desirable that the medium contain complex growth factors (e.g., vitamins), and inorganic elements (e.g., potassium, sodium, calcium, etc.), and trace elements (e.g., boron, cobalt, iron, copper, zinc, etc.), that are commonly present in the protein nitrogen source.

A suitable medium for fermentation may be prepared in water (e.g., low mineral content tap water, distilled water, etc.), and comprises: (a) about 80–250 g/L of a metabolizable carbon source; and (b) at least 15 g/L and, normally about 20–80 g/L, of a protein nitrogen source containing a high level of protein and trace ingredients. The protein nitrogen source comprises a non-yeast protein nitrogen component and a yeast protein nitrogen component. These two protein nitrogen components are present in a ratio ranging, respectively, from about 3:1 to 9:1 based on protein content of the components, preferably about 4:1 to 8:1 and more preferably about 5:1 to 7:1.

The non-yeast protein nitrogen component may be supplied from a wide range of sources, such as soy protein products (e.g., isolates, flours, meals, etc.). Desirable natamycin yields are obtained with a soy protein source comprising 80–95% protein. The non-yeast protein nitrogen component may also comprise beef extract, protein hydrolysates (e.g., peptones). The yeast nitrogen component is supplied by yeast protein (e.g., extracts, autolysates, etc.).

The production medium must also include a source of metabolizable carbon. The carbon source may be supplied in any expedient form such as glucose, polysaccharide, corn and potato starches, etc.

It is not necessary to initially add the entire mount of carbon source. An appropriate amount of carbon source may be initially added to the fermentation medium and addition continued after the fermentation has begun. For example, the initial concentration of carbon source may be about 40 g/L. Thereafter, carbon source is added to the fermentor at a rate that will maintain the concentration at or above the minimum level required for fermentation. Typically the concentration is maintained at about 5–30 g/L, more typically about 20 g/L. Toward the end of the fermentation, and after the major fermentation period, carbon source addition is discontinued so that little or no carbon source is left at the end of the fermentation.

The fermentation is carried out in an appropriately sized fermentation vessel. About 0.1–10%, usually about 2%, by volume of inoculum is added to the production medium. The remainder of the volume of the fermentor comprises the fermentation medium. Any technique for introducing the inoculum that delivers the inoculum in an active metabolic state and does not cause contamination of the culture is acceptable. To control foaming it may be desirable to add to the medium 0.01–1% by volume of an anti-foaming agent (e.g., a silicone defoamer).

The fermentation medium is brought to a temperature of about 25°–40° C., and normally 28°–35° C. The length of time which the fermentation process is allowed to continue depends upon the composition of the fermentation medium, temperature, quantity of cells in the inoculum, quantity of natamycin desired, etc. Typically, the fermentation process is conducted for about 70 to 168 hours.

Oxygen is supplied to the medium during fermentation. It is advantageous to maintain a dissolved oxygen level in the medium of about 20–80% of air saturation during the major portion of the fermentation. The ability to achieve a suitable dissolved oxygen level may be enhanced by proper coordination of the aeration and/or agitation rate. For example, the medium is aerated by forcing sterile air through the medium, usually at a rate of about 0.3–1.0 volumes of air per volume of medium. Although it is generally desirable to agitate the medium during aeration, aeration may produce the desired agitation.

Referring to FIG. 2, the relationship between time and natamycin concentration is shown for each of the three phases of the process. To ascertain the phase of fermentation the concentration of natamycin may be analyzed as a function of time. During the first phase the concentration of natamycin increases generally exponentially. The first phase

includes the major portion of cell growth. During the second phase the concentration of natamycin increases linearly with time. Carbon source is added at a rate that will maintain carbon source concentration above the minimum level required for fermentation, typically at or near the rate at which carbon source is being consumed. The third phase is characterized by a plateau in natamycin concentration. In order to maximize the overall quantity of natamycin that is produced, it is desirable to use a medium and/or an environment that induces the second phase to be rapidly reached and maintained.

Initially the pH of the production medium is about 7.0. During fermentation the pH slowly decreases to about 4.5. Depending upon the end-use of the fermentation broth, a lowered pH may be desirable since the fermentation broth is more readily processed. At a pH of about 7 the fermentation broth may become relatively viscous, and recovery of natamycin may be more difficult. For natamycin recovery using the process described in U.S. patent application 08/237,437, filed May 3, 1994, a pH of about 4.5, is desirable. If a higher pH is desired, the pH by be controlled by addition of a pH control agent during fermentation. This process is described by Eisenschink, U.S. Pat. No. 5,231,014.

Continuous Process

The continuous process comprises two stages. Referring to FIG. 3, in the first stage an inoculum is added to the production medium and grown to form a broth. As described above and shown in FIG. 2, the natamycin production rate increases from zero to a steady state and then begins to decline. During the second, or continuous production, stage, natamycin production occurs at a steady rate. Natamycin production is maintained by the continuous addition of production medium and removal of fermentation broth so that both the volume of fermentation broth in the fermentor and the concentration of natamycin in the fermentation broth remain essentially constant. Only production medium is added; no new inoculum is added during this stage.

The production rate may be adjusted by control of the medium composition and addition rate. Addition and removal may be carried out by any convenient technique, such as using a pump, pressure, gravity, etc. Depending on the medium and the equipment costs, it may be desirable to add medium and remove broth at a somewhat faster rate than that which will produce maximum possible medium utilization and maximum natamycin concentration. A natamycin concentration of 7.0–9.0 g/L may be appropriate. Also, depending on the type of equipment being used, in may be desirable to add medium and/or remove broth intermittently as long as the average broth volume remains essentially constant. If desired, during the second stage the composition of the medium can be adjusted or even significantly changed to optimize natamycin production.

While, in theory, the continuous phase can continue indefinitely, in practice, this phase is limited by the contamination of the production medium with other cultures and by equipment malfunction. However, continuous production may be continued for at least 40 days or until natamycin production decreases to an uneconomical level.

Natamycin Recovery

The natamycin is recovered from the broth. In the process disclosed in U.K. Patent 846,933, natamycin is recovered by methanol extraction followed by adsorption and elution. Penick, U.S. Pat. No. 3,378,441, discloses recovery of natamycin by salting it out of the fermentation broth, extracting with methanol, removing the solids, and then evaporating the liquid. Struyk, U.S. Pat. No. 3,892,850, discloses recovery of natamycin by extraction with acidified butanol followed by distillation and precipitation. Struyk also discloses calcium chloride dissolved in methanol to improve natamycin solubility. U.K. Patent No. 844,289 shows the precipitation of natamycin from acetic acid by the addition of water. U.S. patent application 08/237,437, filed May 3, 1994, incorporated herein by reference, discloses improved methods of natamycin recovery. In one process methanol is added, the pH adjusted to 1.0 to 4.5, solids removed, the pH raised to 6.0–9.0 and the precipitated natamycin recovered.

INDUSTRIAL APPLICABILITY

The invention produces fermentation broth containing at least 5 g/L, typically 7–12 g/L, of natamycin. This high concentration improves recovery from the broth and makes the production process more economically attractive. Natamycin can be used as an anti-fungal additive for animal feed (see Carter, U.S. Pat. Nos. 4,536,494 and 4,600,706).

The invention is illustrated by reference to the following examples which illustrate, but not limit, the invention. Unless specified otherwise, commercially available reagent grade materials were used.

EXAMPLES GLOSSARY

GLOSSARY

Profam ® S970	Isolated soy protein, contains a minimum of 90% protein; Grain Processing Corp., Muscatine, IA
Flav-R-Base ™ Type KAT	Primary autolyzed yeast extract, contains about 70% protein; Stauffer Chemical, Westport, CT

MEDIA

Media were prepared in distilled water and sterilized at 121° C. for about 0.25 hr before use. The following media were prepared:

Sporulation Medium 1

4 g/L yeast extract (Difco "Bacto" Yeast Extract); 10 g/L malt extract (Difco Malt Extract); 4 g/L glucose; and 20 g/L agar.

Sporulation Medium 2

3 g/L yeast extract (Difco "Bacto" Yeast Extract); 3 g/L malt extract (Difco Malt Extract); 5 g/L peptone (Difco "Bacto" peptone); 10 g/L glucose; and 15 g/L agar.

Inoculum Medium 1

15 g/L glucose; 10 g/L sodium chloride; and 10 g/L peptone (Hormel PSR5 peptone).

Inoculum Medium 2

20 g/L glucose; 10 g/L sodium chloride; 6 g/L corn steep liquor (PPM (brand), Corn Steep Liquid); and 6 g/L peptone (Difco "Bacto" peptone).

EXAMPLES 1–4

These examples illustrate production of natamycin by *Streptomyces gilvosporeus* on a medium containing a non-yeast nitrogen component and a yeast nitrogen component in the ratio of about 5.6:1, based on protein content.

Sporulation

Streptomyces gilvosporeus, ATCC 13326, was obtained from the American Type Culture Collection as a freeze-dried spore suspension and used as the culture source. The culture was held on the agar slants (Sporulation Medium 1) at about 25° C. until the culture sporulated. The culture sporulated heavily within about 10 days and was used after 14 days.

Spores were scraped off these agar slants into a small amount of inoculum medium (Inoculum Medium 1) so that the spore concentration in the resulting spore suspension was about 10^8 CFU/mL. Glycerol was added to make the suspension 10% glycerol (volume/volume). The resulting suspension was stored at −80° C. until needed.

Inoculum Propagation

About 1.5 mL of the spore suspension was added to 100 mL of inoculum medium (Inoculum Medium 1) in a 500 mL baffled flask. The inoculum in the baffled flask was incubated for 12 hr at 29° C. and agitated at about 200 rpm on a rotary shaker. About 2 mL of the resulting culture was transferred to 100 mL of inoculum medium and incubation repeated for another 12 hr. About 2 mL of this culture was transferred to 200 mL of medium in a 1 L baffled flask and the incubation repeated for another 24 hr. This culture was used to inoculate 8 L of production medium.

Fermentation

The production medium was prepared in distilled water in a 14 L fermentor and the pH was adjusted to about 7.6 with potassium hydroxide. The fermentor was then sterilized for about 15 min at about 121° C. Glucose was sterilized separately as a 60% solution in distilled water. The composition of the production medium is given in Table 1. The medium also contained 0.05 mL/L defoamer (Mazu. DF 289).

Before inoculation, the production medium was heated to about 29° C. and glucose solution was added to produce an initial glucose concentration of about 40 g/L. An aeration rate of about 0.3 v/v-min. (volumes of air per volume of medium per minute) and an agitation rate of about 300 rpm was established for the fermentor.

Inoculum was added to the fermentation vessel until the medium in the fermentation vessel was about 2% by volume inoculum. After about 40 hr of fermentation, glucose was added at about 1–2 g/L-hr to maintain a glucose concentration of about 20 g/L in the fermentation vessel. The agitation rate was increased as necessary to maintain a dissolved oxygen level of about 50% of air saturation.

An initial volume of about 8.0 L production medium was fermented for about 120 hr. Glucose was added as necessary to maintain natamycin production. Up to 230 g/L of glucose was added (Example 4). Natamycin production is indicated in Table 1.

TABLE 1

Example	Soy Protein[a] (g/L)	Yeast Extract[b] (g/L)	Ratio[c]	Natamycin[d] (g/L)	Natamycin[e] (g/L)
1	19.5	4.5	5.6	8.1	9.3
2	26.0	6.0	5.6	10.0	11.4
3	32.5	7.5	5.6	12.9	15.3
4	39.0	9.0	5.6	15.2	18.8

[a]Profam ® S970 (minimum of 90% protein)
[b]Flav-R-Base ™ Type KAT (about 70% protein)
[c]Ratio of non-yeast protein to yeast protein corrected for protein content of the extracts.
[d]Measured concentration.
[e]Corrected to the original volume to compensate for varying amounts of glucose solution added during fermentation.

EXAMPLES 5–8

These examples illustrate natamycin production with different ratios non-yeast protein to yeast protein.

Sporulation

Streptomyces gilvosporeus, ATCC 13326, was held on the agar slants (Sporulation Medium 2) at about 25° C. until the culture sporulated. The culture sporulated heavily within about 10 days and was used after 15 days.

Spores were scraped off these agar slants into a small amount of inoculum medium (Inoculum Medium 2) so that the spore concentration in the resulting spore suspension was about 10^8 CFU/mL. The resulting suspension was used immediately after preparation.

Inoculum Propagation

About 2 mL of the spore suspension was added to 100 mL of inoculum medium (Inoculum Medium 2) in a 500 mL baffled flask. The culture was incubated for 48 hr at 29° C. and agitated at about 200 rpm on a rotary shaker. This culture (20 mL) was used to inoculate 700 mL of production medium.

Fermentation

Fermentation was carried out as described in Examples 1–4, except that fermentation was carded out in 700 mL of production medium in a 1 L fermentor using the production media given in Table 2. Natamycin production is indicated in Table 2.

TABLE 2

Example	Soy Protein[a] (g/L)	Yeast Extract[b] (g/L)	Ratio[c]	Natamycin[d] (g/L)
5	13.0	4.5	3.7	5.9
6	18.0	4.5	5.1	9.0
7	13.0	3.0	5.6	7.6
8	18.0	3.0	7.7	4.5

[a]Profam ® S970 (minimum of 90% protein)
[b]Flav-R-Base ™ Type KAT (about 70% protein)
[c]Ratio of non-yeast protein to yeast protein corrected for protein content of the extracts.
[d]Measured concentration.

EXAMPLE 9

This example illustrates natamycin production without addition of additional glucose and on a medium containing a non-yeast nitrogen component and a yeast nitrogen component in the ratio of about 1:1, based on protein content. The procedure of Example 1 of American Cyanamid, U.K. Patent 846,933, was followed.

The procedure of Examples 1–4 was followed with the following exceptions: (1) the fermentation medium contained about 10 g/L glucose, 2 g/L beef extract, 2 g/L Batco yeast extract, 0.5 g/L asparagine and 0.5 g/L dibasic potassium phosphate and (2) no glucose was added subsequently. After about 72 hr of fermentation, the concentration of natamycin was about 0.75 g/L.

EXAMPLE 10

This example illustrates continuous production of natamycin on a medium containing a non-yeast nitrogen component and a yeast nitrogen component in the ratio of about 5.6:1, based on protein content of the components.

Sporulation and Inoculum Propagation

The procedure of Examples 5–8 was followed.

First Stage

The first stage natamycin production medium had the following initial composition: 19.5 g/L soy protein isolate (ADM, "Profam" S970); 4.5 g/L yeast extract (Stauffer, Type KAT); and 0.05 mL/L defoamer (Mazu, DF 289)

About 600 mL of medium was prepared in distilled water in a 1 L fermenter. The pH was adjusted to about 7.6 with potassium hydroxide. The fermenter was sterilized for about 0.25 hr at about 121° C. A 50% glucose solution was sterilized separately and about 40 g/L added to the medium. The medium was heated to about 29° C. The aeration rate was set at about 250 mL/min and the agitation rate at 500 rpm.

The inoculum was added to the fermentation vessel until the fermentation vessel had an inoculum content of about 2% by volume. Glucose was added to the medium in the fermentor after about 40 hr of fermentation to maintain a glucose concentration of about 20 g/L glucose in the fermentation vessel. Glucose was fed to the vessel at about 1 g/L-hr. The agitation rate of the fermentation vessel was increased as necessary to maintain a dissolved oxygen level of about 20% of air saturation.

Second Stage

About 72 hr after inoculation, the natamycin production rate began to decline. The natamycin concentration in the broth was about 3.9 g/L. Second stage medium was added and the fermentation broth was withdrawn from the fermenter at a rate of about 12 mL/hr to maintain a constant broth volume of about 600 mL. The second stage medium contained about 19.5 g/L soy protein isolate, 4.5 g/L yeast extract, 50 g/L glucose and 0.2 mL/L of defoamer.

Addition and withdrawal was continued at about 12 mL/hr, and the fermentation continued until about 200 hr after the start of the first fermentation stage. The natamycin concentration was 4.5 g/L. The product was recovered from the withdrawn broth.

The medium was changed to: 26 g/L soy protein isolate, 6 g/L yeast extract, 80 g/L glucose and 0.3 mL/L of defoamer. Addition of nutrient medium and withdrawal of broth were continued at the same rate. At about 250 hr from the start, the concentration of natamycin in the broth had increased to 8.5 g/L. The concentration remained approximately constant for the rest of the fermentation. Fermentation was discontinued at about 450 hr.

EXAMPLE 11

This example illustrates continuous production of natamycin on a medium containing a non-yeast nitrogen component and a yeast nitrogen component in the ratio of about 5.6:1, based on protein content.

The general procedure of Example 10 was followed except that the production medium contained 26 g/L soy protein isolate, 6 g/L yeast extract, and 0.3 mL/L of defoamer and the volume of fermentation medium was about 3 L. After about 70 hr, the natamycin concentration was 6.0 g/L. Addition of medium and withdrawal of broth was begun. The added medium contained about 26 g/L soy protein isolate, 6 g/L yeast extract, 80 g/L glucose and 0.3 mL/L of defoamer. Second stage medium was added and the fermentation broth was withdrawn from the fermenter at a rate of about 1.5 L/day to maintain a constant broth volume of about 3 L. At about 117 hr a steady state natamycin concentration of about 8.0 g/L was attained. Fermentation was continued for about 250 hr.

What is claimed is:

1. In a method for producing natamycin, comprising, in order, the steps of:

(a) introducing an inoculum of a natamycin producing Streptomyces strain to a fermentation medium to produce a fermentation broth comprising the fermentation medium and inoculum; and

(b) producing natamycin by culturing the Streptomyces strain in the fermentation broth; the improvement comprising:

(1) providing at least about 15 g/L of a protein nitrogen source to the fermentation medium; wherein the protein nitrogen source comprises a non-yeast protein component and a yeast protein component, and the ratio of non-yeast protein component to yeast protein component being from about 3:1 to 9:1, based on the protein content of the source;

(2) providing a carbon source to the fermentation medium at from about 80 g/L to 250 g/L; wherein the carbon source addition is carried out during step (b) so as to maintain a concentration of carbon source of from about 5 g/L to 30 g/L;

(3) continuing the fermentation until the fermentation broth comprises at least 5 g/L natamycin; and

(4) recovering the natamycin from the fermentation broth.

2. The method of claim 1, wherein said natamycin producing Streptomyces strain has all the identifying characteristics of *Streptomyces gilvosporeus* ATCC 13326, and in which the fermentation is continued until the fermentation broth comprises at least 10 g/L natamycin.

3. The method of claim 1 in which the natamycin producing Streptomyces strain is *Streptomyces gilvosporeus*.

4. The method of claim 1 in which the ratio of non-yeast protein component to yeast protein component is 4:1 to 8:1.

5. The method of claim 1 in which, in step (4), methanol is added, the pH adjusted to 1.0 to 4.5, solids removed, the pH raised to 6.0–9.0 and precipitated natamycin recovered.

6. The method of claim 5 in which the natamycin producing Streptomyces strain has all the identifying characteristics of *Streptomyces gilvosporeus* ATCC 13326, the fermentation is continued until the fermentation broth comprises at least 10 g/L natamycin, and the ratio of non-yeast protein component to yeast protein component is 4:1 to 8:1.

7. The method of claim 5 in which the natamycin producing Streptomyces strain is *Streptomyces gilvosporeus*, the fermentation is continued until the fermentation broth comprises at least 10 g/L natamycin, and the ratio of non-yeast protein component to yeast protein component is 5:1 to 7:1.

8. In a method for producing natamycin, comprising, in order, the steps of:

(a) introducing an inoculum of a natamycin producing Streptomyces strain to a fermentation medium to produce a fermentation broth comprising the fermentation medium and inoculum; and

(b) producing natamycin by culturing the Streptomyces strain in the fermentation broth; the improvement comprising:

(1) providing at least about 15 g/L of a protein nitrogen source to the fermentation medium, wherein the protein nitrogen source comprises a non-yeast protein component and a yeast protein component, and the ratio of non-yeast protein component to yeast protein component being from about 3:1 to 9:1, based on the protein content of the source;

(2) providing a carbon source to the fermentation medium at from about 80 g/L to 250 g/L, wherein the carbon source addition is carried out during step (b) so as to maintain a concentration of carbon source of from about 5 g/L to 30 g/L;

(3) continuing the fermentation until the fermentation broth comprises at least 5 g/L natamycin; and

(4) removing a portion of said fermentation broth and replacing said removed portion by adding a substantially equivalent amount of fresh fermentation medium.

9. The method of claim **8** in which said removing and said replacing of step (4) are continuous.

10. The method of claim **8** in which said removing and said replacing of step (4) are intermittent.

11. The method of claim **8**, wherein said natamycin producing Streptomyces strain has all the identifying characteristics of *Streptomyces gilvosporeus* ATCC 13326, and in which step (3) is continued until the fermentation broth comprises at least 7 g/L natamycin.

12. The method of claim **8** in which the natamycin producing Streptomyces strain is a strain of *Streptomyces gilvosporeus*.

13. The method of claim **8** in which the ratio of non-yeast protein component to yeast protein component is 4:1 to 8:1.

14. The method of claim **8**, in which the ratio of non-yeast protein component to yeast protein component is 5:1 to 7:1.

15. The method of claim **8** in which, in step (4), methanol is added, the pH adjusted to 1.0 to 4.5, solids removed, the pH raised to 6.0–9.0 and the precipitated natamycin recovered.

16. The method for producing natamycin according to claim **8**, further comprising the step of recovering natamycin from the portion of said fermentation broth removed in step (4) of claim **8**.

* * * * *

Natamycin Petition

Appendix 2

U.S. Regulatory Aspects

Letter of 7 January 2005 from Angela Lim, Danisco USA Inc., to Mr. Leonard Heflich, George Weston Bakeries Inc., describing the GRAS self-affirmation process of Danisco USA.

DANISCO

First you add knowledge...

Danisco USA Inc.
440 Saw Mill River Road
Ardsley
New York 10502-2605
USA
Tel: +1 914 674 6300
Tel: +1 800 255 6837
Fax: 914-674-6513
www.danisco.com

January 7, 2005

Mr Leonard Heflich
Director of Research & Development
George Weston Bakeries Inc.
30 Inez Drive
Bay Shore, NY 11706

Dear Mr Leonard Heflich:

Re: Manufacture of Natamycin (brand name Natamax®)

In response to your recent inquiry, please be informed that natamycin in produced via fermentation. The natamycin producing organism is started on culture media and later transferred to the fermenter. Raw materials for both the inoculum media and the fermentation media include derivatives of corn and soybean. Natamycin is recovered from the fermentation broth via a proprietary process, crystallized, dried and milled.

There is no Code of Federal Regulations (CFR) definition of 'natural' apart from that used to define flavor materials. However, the Food and Drug Administration (FDA) recognizes that 'natural' additives are "manufactured from natural sources" while 'artificial' additives are "not found in nature and must be man-made." *(Reference: Food and Drug Administration/ International Food Information Council Brochure on Food Additives: January 1992).* Based on the distinction FDA makes between 'natural' and 'artificial' additives and the assessment of our manufacturing process, Danisco has determined that natamycin produced by Danisco meets the definition of 'natural' and markets it as such.

If you need further assistance regarding this inquiry, please do not hesitate to contact me at (914) 674-6300 x 2507 Thank you for your interest in our product.

Sincerely,

Angela Lim

Angela Lim
Manager, Regulatory Affairs

Natamycin Petition

Appendix 3

Label of Natamycin Product NATAMAX®, produced by Danisco A/S, Grindsted, Denmark

Label of the currently marketed product THOMAS ENGLISH MUFFINS, that contains natamycin

Ingredient Declaration Comparison of conventional English muffins and an organic English muffin product containing natamycin

DANISCO

Natamax

Natural Antimicrobial

KOSHER DAIRY (U)

Net content	Material number	Batch No.	Best before date
1.0 KG 2.2 LBS	104414	4010036007	14 Oct. 2004

INT – 3277: For use in food. E235 Natamycin. Carrier: Lactose.#

Store in unbroken packaging under conditions not exceeding 20°C/68°F and 80% relative humidity. Keep away from sunlight and odorous products.

5 700590 028921

L – 920 G5KVT 11.25:46 07052003 EN 0004 of 0100

Manufactured in Denmark: Danisco A/S, DK – 7200 Grindsted, Denmark

CONTAINS 6 MUFFINS

THOMAS' ORIGINAL

NET WT 12 OZ (340g)

Nutrition Facts

Serving Size 1 muffin (57g)
Servings Per Container 6

Amount Per Serving	
Calories 120	Calories from Fat 10
	% Daily Value*
Total Fat 1g	**2%**
Saturated Fat 0g	**0%**
Trans Fat 0g	
Polyunsaturated Fat 0g	
Monounsaturated Fat 0g	
Cholesterol 0mg	**0%**
Sodium 200mg	**8%**
Total Carbohydrate 25g	**8%**
Dietary Fiber 1g	**4%**
Sugars 1g	
Protein 4g	
Vitamin A 0% • Vitamin C 0%	
Calcium 8% • Iron 8%	

* Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs:

	Calories:	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

INGREDIENTS: **UNBLEACHED ENRICHED WHEAT FLOUR** [FLOUR, MALTED BARLEY FLOUR, REDUCED IRON, NIACIN, THIAMIN MONONITRATE (VITAMIN B1), RIBOFLAVIN (VITAMIN B2), FOLIC ACID], WATER, FARINA, YEAST, **NONFAT MILK**, HIGH FRUCTOSE CORN SYRUP, SALT, SOYBEAN OIL, PRESERVATIVES (CALCIUM PROPIONATE, SORBIC ACID), **SOY FLOUR**, GRAIN VINEGAR, MONOGLYCERIDES, **WHEY**, NATAMYCIN (A NATURAL MOLD INHIBITOR).

S.B. THOMAS, A UNIT OF
GEORGE WESTON BAKERIES INC.,
P.O. BOX 535 TOTOWA, NJ 07511-0535
CT #4734 ©2005

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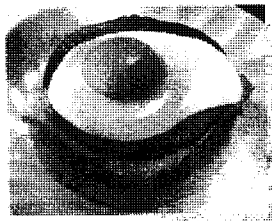

Sir Thomas

2 THOMAS'® Original or Honey Wheat English Muffins, split, toasted and buttered
2 slices American cheese
2 slices Canadian Style Bacon, cooked
2 eggs, fried or poached

On bottom half of each muffin, arrange cheese, Canadian bacon and 1 egg. Top with remaining muffin halves. Makes 2 servings

*To melt cheese, place on fried egg in skillet 1 minute before removing from skillet.

Try all of our low-fat, cholesterol-free food varieties...each with their own great recipes!
THOMAS', the Coach Logo and Nooks & Crannies are registered trademarks. Used with permission.

How To Prepare

1. Thomas'® English Muffins are fork split. Gently pull apart.
2. Toast or broil until golden brown.
3. Serve hot with your favorite toppings.

ORIGINAL MUFFINS

0 48121 20208 1

SEPARATE AT PERFORATIONS

ORIGINAL

THOMAS'

NET WT 12 OZ (340g)

CONTAINS 6 MUFFINS

Ingredient Declaration Comparison

CONVENTIONAL ENGLISH MUFFINS

INGREDIENTS: UNBLEACHED ENRICHED WHEAT FLOUR [FLOUR, MALTED BARLEY FLOUR, REDUCED IRON, NIACIN, THIAMIN MONONITRATE (VITAMIN B1), RIBOFLAVIN (VITAMIN B2), FOLIC ACID], WATER, FARINA, YEAST, NONFAT MILK, HIGH FRUCTOSE CORN SYRUP, SALT, SOYBEAN OIL, PRESERVATIVES (CALCIUM PROPIONATE, SORBIC ACID), SOY FLOUR, GRAIN VINEGAR, MONOGLYCERIDES, WHEY, NATAMYCIN (A NATURAL MOLD INHIBITOR).

ORGANIC ENGLISH MUFFINS

INGREDIENTS: ORGANIC WHOLE WHEAT FLOUR, WATER, YEAST, ORGANIC CANE SUGAR, ORGANIC WHEAT GLUTEN, CONTAINS LESS THAN 2% OF EACH OF THE FOLLOWING: ORGANIC DISTILLED VINEGAR, ORGANIC RAISIN PASTE, ORGANIC WHEAT STARCH, ORGANIC HONEY, SALT, ORGANIC SUNFLOWER OIL, DEACTIVATED YEAST, CITRIC ACID, ORGANIC PRE-GELATINIZED CORN STARCH, ORGANIC SOY LECITHIN, NATAMYCIN (A NATURAL MOLD INHIBITOR).

Appendix 4

Natamycin – Human Medicinal Use Citations (not a complete listing)

(1970). "Natamycin (pimaricin)." Drug Ther Bull **8**(13): 52.

(1978). "Natamycin approved--first U.S. drug for fungal keratitis." FDA Drug Bull **8**(6): 37-8.

Bauer, U., F. Staib, et al. (1975). "[Aspergillus infection in skin transplantation and its therapy]." Chirurg **46**(6): 279-82.

In a 10 years old girl sustaining a corrosive injury of the lower leg from sulphuric acid, in the region of a skin transplantation colonization with Aspergillus fumigatus (Fresenium) and Aspergillus niger (van Tieghem) took place. This infection endangered the attempt of transplantation and the saving of the foot. Treatment by medication with nystatin (moronal) and canesten (clotrimazol) were ineffective. Pimaricin (pimafucin, natamycin) quickly erradicated the mycotic infection and secured an undisturbed progress for the transplantation. Additionally the epidemiology of infections by Aspergillus is briefly discussed.

Chin, G. N., R. A. Hyndiuk, et al. (1975). "Keratomycosis in Wisconsin." Am J Ophthalmol **79**(1): 121-5.

Candida albicans was the most common fungus responsible for mycotic keratitis in our series from a northern climate, as opposed to southern climates where other fungi were more common. Pimaricin was effective in our patients with Candida infections and in one patient with Aspergillus infection that had been unresponsive to previous amphotericin B.

Corkill, B. M. and N. J. McCarthy (1972). "Comparative trial of fungilin (amphotericin B) and pimafucin (natamycin) pessaries in the treatment of vaginal candidiasis." Med J Aust **2**(1): 33-4.

Jones, D. B. (1981). "Decision-making in the management of microbial keratitis." Ophthalmology **88**(8): 814-20.

The successful management of suppurative microbial keratitis requires five steps: (1) make the clinical diagnosis, (2) perform the proper laboratory procedures, (3) initiate antimicrobial therapy, (4) modify the initial therapy, and (5) terminate therapy. The most helpful guidelines to decision-making in these steps are: (1) the clinical impression, (2) severity of keratitis, (3) results of laboratory studies, (4) disease potential of the responsible organism, and (5) effectiveness and toxicity of various antimicrobial agents. Selection of initial antibiotics ideally should be directed by interpretation of the corneal smears. The preferred initial antibiotic for keratitis caused by a Gram-positive coccus is cefazolin; for a Gram-negative rod, gentamicin; and for a filamentous fungi or yeast, natamycin. Broad, antibacterial therapy should be

reserved for suspected bacterial keratitis with negative smears or for severe infections with antecedent treatment. Miconazole may be an effective, alternate agent in fungal keratitis. The safety and efficacy of corticosteroids in microbial keratitis have not been established.

Jones, D. B., F. K. Forster, et al. (1972). "Fusarium solani keratitis treated with natamycin (pimaricin): eighteen consecutive cases." Arch Ophthalmol **88**(2): 147-54.

Laskownicka, Z., K. Pasyk, et al. (1971). "Pimaricin (natamycin) in the treatment of superficial fungal infections in children." Acta Paediatr Scand **60**(4): 456-60.

Masterton, G., S. M. Sengupta, et al. (1975). "Natamycin in genital candidosis in men." Br J Vener Dis **51**(3): 210-12.
In a trial of natamycin, an antifungal antibiotic in a vanishing cream base, assessment was possible in 66 men with genital or anal candidosis. The overall cure rate was 82 percent, In 43 patients with culturally proven candidosis it was 98 percent. but in 23 patients treated solely on clinical impression it was only 52 percent. Symptoms were rapidly relieved in those who responded and there were no side-effects. In our hands, natamycin 2 per cent cream has proved to be a valuable preparation in the treatment of candidal balanitis.

Newmark, E., H. E. Kaufman, et al. (1971). "Clinical experience with pimaricin therapy in fungal keratitis." South Med J **64**(8): 935-41.

Patel, V. R. (1973). "Natamycin in the treatment of vaginal candidiasis in pregnancy." Practitioner **210**(259): 701-3.

Rokicka-Milewska, R., D. Derulska, et al. (1989). "The use of 2.5% natamycin, as orally administered drops, in the treatment of fungal infections of the oral cavity in children with chronic blood diseases." J Int Med Res **17**(1): 82-6.
A total of 34 children with oral candidiasis were treated with 2.5% natamycin in the form of orally administered drops; 6-20 drops applied to oral lesions four times daily for up to 8 weeks. A total cure was achieved in 28 (82.3%) cases. No side-effects were observed. This preparation was an effective treatment for Candida albicans infections in children with blood diseases, and was well tolerated.

Zapater, R. C. and A. Arrechea (1975). "Mycotic keratitis by Fusarium. A review and report of two cases." Ophthalmologica **170**(1): 1-12.
World literature on the subject reports 112 cases of keratomycosis by Fusarium, including the two cases described in this paper and some personal communications. In most of these cases (60--70%) there is a history of trauma inflicted by vegetable matter or by foreign bodies introduced into the eye (metal, sand, stone, etc.). The different species of Fusarium isolated are F. episphaeria, F. dimerum, F. moniliforme, F. nivale, F. oxysporum, and F.

solani. The treatment recommended in a 5-percent suspension or unguent of pimaricin (natamycin).

Appendix 5

Natamycin – Veterinary Medicine Applications (not a complete listing)

Aho, R., M. Tala, et al. (1991). "Mycotic keratitis in a horse caused by Aspergillus fumigatus. The first reported case in Finland." Acta Vet Scand **32**(3): 373-6.

The first identified Finnish case of equine mycotic keratitis caused by Aspergillus fumigatus is described. The clinical picture, the sampling method, the macro- and micromorphology of the causative fungus and the therapy applied are reported in detail. Therapy with natamycin (Pimafucin 2.5% eyedrops. Gist-Brocades) was successful.

Brooks, D. E., S. E. Andrew, et al. (1998). "Antimicrobial susceptibility patterns of fungi isolated from horses with ulcerative keratomycosis." Am J Vet Res **59**(2): 138-42.

OBJECTIVES: To evaluate in vitro susceptibility to topical antifungal medications, as measured by minimum inhibitory concentration (MIC) and 50% inhibitory concentration (IC50%), of fungal isolates from horses with ulcerative keratomycosis in Florida; to compare results with those of other studies to identify differences in susceptibility patterns among fungi isolated from horses in different geographic regions; and to note indications of fungal resistance to drugs tested in other studies. SAMPLE POPULATION: Corneal fungal cultures from client-owned horses from Florida with ulcerative keratomycosis (n = 22). PROCEDURE: Fungal cultures were plated on Emmons modified Sabouraud dextrose agar and mycobiotic agar, examined weekly for growth, and kept for a total of 30 days. In vitro MIC and IC50% of fluconazole, itraconazole, ketoconazole, miconazole, and natamycin were measured for each fungal isolate. RESULTS: Aspergillus (n = 9; 41%), Fusarium (7; 32%), Penicillium (2; 9%), Cylindrocarpon (1; 4%), Scytalidium (1; 4%), and Torulopsis (1; 4%) spp and an unidentified yeast (1; 4%) were isolated. Fungi were most susceptible to antifungal drugs in the following order: natamycin and miconazole equally, itraconazole, and ketoconazole, although no significant difference was found among drugs. Fungi were significantly less susceptible to fluconazole ($P < 0.0001$) than to the other 4 drugs. CONCLUSIONS: Initial antifungal therapy with topically applied natamycin, miconazole, itraconazole, or ketoconazole is recommended for ulcerative keratomycosis in horses in the subtropical environment of Florida. CLINICAL RELEVANCE: Specific antifungal treatment of horses with ulcerative keratomycosis should be based on history, results of ophthalmic examination, cytologic findings, isolation of the pathogenic fungus, and known prevalence of unique ocular fungi in specific geographic areas. In vitro antifungal susceptibility testing may be most beneficial in aiding documentation of pharmacologic susceptibility patterns of fungi in specific geographic regions.

Brothers, A. M. and R. D. Wyatt (2000). "The antifungal activity of natamycin toward molds isolated from commercially manufactured poultry feed." Avian Dis **44**(3): 490-7.

The antifungal activity of natamycin, a polyene antifungal compound, was evaluated on molds isolated from commercial poultry feed. The antifungal activity was measured by determination of the minimal inhibitory concentration (MIC) for natamycin on molds growing on semisolid microbiological medium (potato dextrose agar) containing pure natamycin at concentrations ranging from 0 to 200 mg/liter. Natamycin exhibited a high degree of antifungal activity against the 191 isolates of aspergilli used in this study, with average MIC values ranging from 5.08 to 40.1 mg/liter for Aspergillus fumigatus and Aspergillus parasiticus, respectively. Natamycin was also equally effective in inhibiting the growth of nonaflatoxigenic compared with aflatoxigenic isolates of Aspergillus flavus and A. parasiticus. Natamycin was also efficacious against molds other than aspergilli, with MIC values ranging from 2.15 to 5.80 mg/liter for Paecilomyces and Rhizopus spp., respectively. Natamycin exhibited apparent sporicidal activity against spores of toxigenic strains of Fusarium moniliforme and A. parasiticus but not Penicillium rubrum. This sporicidal activity was evident only when spores were exposed to an in vitro concentration of natamycin of 25 mg/liter or higher for a period of time of at least 12 hr. The growth inhibiting activity of natamycin was more pronounced compared with the sporicidal activity.

Ellison, A. C. (1976). "Intravitreal effects of pimaricin in experimental fungal endophthalmitis." Am J Ophthalmol **81**(2): 157-61.

Evaluation of 25, 50, and 100 mug of pimaricin given intravitreally to albino rabbits with Aspergillus endophthalmitis revealed that 25 mug of pimaricin, while nontoxic to the retina, was ineffective in inhibiting the fungal process. A dose of 50 mug was significantly more effective in inhibiting fungal disease but also caused significant retinal damage with loss of retinal function and iridoplegia. Dosages above this level resulted in vitreous retraction and degeneration, iridoplegia, and retinal detachment. Twenty-five micrograms of pimaricin given in three dosages spaced three days apart may be effective in inhibiting fungal endophthalmitis.

Ellison, A. C. and E. Newmark (1976). "Intraocular effects of pimaricin." Ann Ophthalmol **8**(8): 987-95.

Pigmented rabbits weighing 3 to 6 lbs were given bilateral intraocular injections of 250 to 1,000 mug pimaricin. Following their injection, blood, aqueous and vitreous levels were determined at various time intervals during the first 24 hours and at 24 hour intervals thereafter for one week. In subsequent studies, pigmented rabbits were given bilateral intraocular injections of 5,000 spores of A. fumigatus and 30 hours later received intraocular injections of 250 to 1,000 mug pimaricin. These studies show that 250 mug of intraocular pimaricin is well tolerated in the infected and normal animal eye with therapeutic ocular levels maintained for over 24 hours. Drug

levels above 250 mug, although relatively notoxic in the normal eye resulted in irreversible damage to ocular structures in the infected eye that could not be resolved. Thus in the case of fungal endophthalmitis involving the anterior segment which will lead to the ultimate loss of the eye, an injection of 250 mug of pimaricin might preserve useful vision.

Greet, T. R. (1981). "Nasal aspergillosis in three horses." Vet Rec **109**(22): 487-9.
Three horses were referred for investigation of a unilateral foul smelling scanty nasal discharge, complicated in one case by intermittent epistaxis. Thick purulent material or a mycotic plaque was identified by an endoscopic examination of the middle meatus but in two horses this had to be repeated under general anaesthesia before the abnormalities were detected. Aspergillus fumigatus was cultured from all three cases and septate hyphae were identified on smears from lesions. Histological examination of the lesion in one case revealed a fungal mycelium. Topical treatment with natamycin solution in all cases plus nystatin in two of the horses resulted in complete recovery from the condition in two cases but in one case the problem recurred. The aetiology of nasal aspergillosis remains uncertain.

Grunder, H. D. and U. Muller (1979). "[Treatment with natamycin a fungicidal antimycotic in enzootic trichophyton infections of cattle (author's transl)]." Dtsch Tierarztl Wochenschr **86**(12): 457-61.

McKellar, Q., G. Fishwick, et al. (1987). "Ringworm in housed sheep." Vet Rec **121**(8): 168-9.

Oldenkamp, E. P. (1979). "Natamycin treatment of ringworm in cattle in the United Kingdom." Vet Rec **105**(24): 5554-6.
A total of 258 cattle clinically affected with Trichophyton verrucosum (ringworm) were treated twice by spraying with a suspension containing the fungicidal antibiotic natamycin. One-hundred-and-forty-seven in-contact, but unaffected cattle were also similarly treated. Forty-one of the affected animals were examined mycologically and clinically as they were taken to be representative of the whole group. At eight weeks after the last treatment 93 per cent of the affected animals had completely recovered and the remaining 7 per cent had improved markedly; 70 per cent of the mycologically examined animals proved negative at the same time. No reinfection of the recovered animals or spread of the disease to the treated in-contact animals was seen up to six months after treatment.

Oldenkamp, E. P. (1979). "Treatment of ringworm in horses with natamycin." Equine Vet J **11**(1): 36-8.
A suspension based on the antibiotic, natamycin, was applied by sponging to 83 horses of various breeds and ages with signs of clinical ringworm. A number of different causative agents were involved of which Trichophyton equinum was the most common. Treatment successfully eliminated the

disease within 4 weeks. After treatment the recovered animals did not show any evidence of re-infection for up to 6 months. The mycological clearance rate was 97 per cent and apart from the efficacy against ringworm, the preparation had the advantage of being non-irritant and odourless. It was also useful for treating the surroundings of the animals.

Oldenkamp, E. P. and B. K. Elzinga (1979). "The therapy of otitis externa." Tijdschr Diergeneeskd **104**(8): suppl 115-7.

The clinical findings following the instillation of an aqueous solution containing natamycin, neomycin and hydrocortisone into the ears of dogs exhibiting the signs of otitis externa are described. Out of a total of 143 cases, 50 were also examined microbiologically both before and after treatment in order to evaluate the antibacterial and antifungal properties of the solution. The severity of the complaint appeared to be of little significance as far as the respons is concerned. A favourable response was noted clinically in 95% and microbiologically in 86% of the cases treated.

Oldenkamp, E. P. and R. Kommerij (1976). "[A new therapeutic agent in a natamycin base in mass treatment of ringworm in cattle (author's transl)]." Tijdschr Diergeneeskd **101**(4): 178-86.

Oldenkamp, E. P. and L. Spanoghe (1976). "[Natamycin-S treatment of ringworm in cattle. Epidemiological aspects (author's transl)]." Tijdschr Diergeneeskd **101**(22): 1242-9.

At a test centre for bulls the progress of a ringworm infection was studied. By treating only the affected animals with natamycin-S the progress of the disease as well as the spreading of the infection among untreated animals could be studied. By treating only the affected animals with natamycin-S the progress of the disease as well as information could be gathered on the difference of progress of the infection in both groups and also of the role of possible immunity.

Oldenkamp, E. P. and L. Spanoghe (1977). "Natamycin-S treatment of ringworm in cattle. Epidemiological aspects." Tijdschr Diergeneeskd **102**(2): 124-5.

Panda, A., R. Ahuja, et al. (2003). "Role of 0.02% polyhexamethylene biguanide and 1% povidone iodine in experimental Aspergillus keratitis." Cornea **22**(2): 138-41.

PURPOSE: To determine the efficacy of 0.02% polyhexamethylene biguanide and 1% povidone iodine in experimental keratitis. METHODS: Aspergillus fumigatus keratitis was induced by corneal intrastromal injection of spores in 24 healthy rabbits that were randomly divided into four groups of six rabbits each. Drugs used were 5% natamycin (standard antifungal), 0.02% polyhexamethylene biguanide (PHMB) (test drug), 1% povidone iodine (test drug), and 0.5% hydroxypropylmethyl cellulose (HPMC) (control). RESULTS: The average healing times of the ulcer were 21.5 +/- 3.08 days with 5% natamycin, 27.8 +/- 2.28 days with 0.02% PHMB, 36.4 +/- 2.57 days with 1%

povidone iodine, and 38.2 +/- 4.74 days with 0.5% HPMC. While no corneal perforations occurred with natamycin treatment, one perforation was noted with PHMB, three perforations were noted with povidone iodine, and five perforations were noted with controls. CONCLUSION: Polyhexamethylene biguanide (0.02%) is a moderately effective drug for experimental Aspergillus keratitis, but 1% povidone iodine is not effective.

Sargison, N. D., J. R. Thomson, et al. (2002). "Ringworm caused by Trichophyton verrucosum--an emerging problem in sheep flocks." Vet Rec **150**(24): 755-6.

Schulz, W. (1984). "[Comparative treatment trials in enzootic cattle trichophytosis using Mycophyt-Gruntex and a new antimycotic preparation (Bay-Ve 7294)]." Dtsch Tierarztl Wochenschr **91**(10): 364-6.

Spanoghe, L. and E. P. Oldenkamp (1977). "Mycological and clinical observations on ringworm in cattle after treatment with natamycin." Vet Rec **101**(7): 135-6.

A total of 41 calves which were naturally infected with Trichophyton verrucosum were treated with natamycin used as a total body spray. Ten other infected animals were not treated and considered as control animals. Clinical observation and mycological examination show partial improvement to complete recovery with simultaneous sterilisation of the infected skin areas. Five to six weeks after treatment, 88 per cent of the treated animals had recovered or showed a distinct improvement, 65 per cent had a negative culture. After 11 to 12 weeks these percentages were 95 and 91, respectively. All controls yielded a positive culture during the whole observation period. The method used for the detection of the presence of Trich verrucosum in skin scrapings and hairs permitted accurate diagnosis in as little as two to five days.

Stack, J. A., M. Harrison, et al. (2002). "Evaluation of a selective medium for Brucella isolation using natamycin." J Appl Microbiol **92**(4): 724-8.

AIMS: To select an anti-fungal agent to replace cycloheximide in the media used for isolation of Brucella. METHODS AND RESULTS: One potential agent, natamycin, was evaluated using 28 Brucella isolates, 18 yeasts and 14 fungi. The material for the evaluation included 37 bovine milk samples, six bovine vaginal swabs and 45 milk samples artificially infected with Brucella. The recovery of Brucella only from the artificially-inoculated milk samples increased with the use of the medium containing natamycin instead of cycloheximide, at the same time significantly inhibiting the growth of yeasts, fungi and other bacteria. The inclusion of either anti-fungal agent allowed growth of the 28 Brucella isolates and totally prevented the growth of all 18 yeasts and 13 of the 14 fungi. CONCLUSIONS: Based on the results it was concluded that natamycin would be a suitable alternative to cycloheximide. SIGNIFICANCE AND IMPACT OF THE STUDY: Cycloheximide has become unavailable worldwide and is currently an anti-fungal constituent of the medium often used for isolation of Brucella organisms. The use of natamycin

as a replacement in the formulation did not inhibit growth of Brucella and was effective at eliminating most contaminants.

Sutton, D. J. and J. M. Evans (1986). "Skin lesions in horses." Vet Rec **118**(1): 27-8.

Uchida, Y., M. Mizutani, et al. (1992). "Otitis externa induced with Malassezia pachydermatis in dogs and the efficacy of pimaricin." J Vet Med Sci **54**(4): 611-4.
Eight beagles were experimentally inoculated intraotally with Malassezia pachydermatis to induce acute otitis externa. Three or 4 days after the inoculation, the animals showed the symptoms of otitis externa. All ear canals were erythematous and the dogs were shaking their heads. A large number of M. pachydermatis was noticed in exudate taken from every ear canal. Clinical signs of otitis externa were reduced after treatment with 0.1 ml (per canal) of 1% pimaricin suspension twice a day for 3 days. The amount of exudate decreased gradually and 12 of the 16 ear swabs examined, thereafter, were found to be negative for M. pachydermatis within 10 days. No side effects were observed in all the treated cases. These results suggested that M. pachydermatis could induce the canine otitis externa, and that pimaricin is effective agent for M. pachydermatis infection in ear canals.

Vanden Bossche, H., M. Engelen, et al. (2003). "Antifungal agents of use in animal health--chemical, biochemical and pharmacological aspects." J Vet Pharmacol Ther **26**(1): 5-29.
A limited number of antifungal agents is licensed for use in animals, however, many of those available for the treatment of mycoses in humans are used by veterinary practitioners. This review includes chemical aspects, spectra of activity, mechanisms of action and resistance, adverse reactions and drug interactions of the antifungals in current use.

Natamycin Petition

Appendix 6

Natamycin Material Safety Data Sheet

MATERIAL SAFETY DATA SHEET

1. CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

Trade name:	**NATAMAX®**
Product code:	104414; 104417; 104419
Supplier:	Danisco A/S Edwin Rahrs Vej 38, DK-8220 Brabrand, Denmark. Tel.: +45 89 43 50 00 Fax.: +45 86 25 10 77
Emergency telephone:	+45 79 72 56 00

2. COMPOSITION/INFORMATION ON INGREDIENTS

Description: NATAMAX® is a natural antimicrobial agent for use in the food industry.

Ingredients:	**CAS-No.**	**EINECS-No.**	**Classification**
Natamycin	7681-93-8	231-683-5	None.

3. HAZARDS IDENTIFICATION

No hazards are known under normal conditions of use.

4. FIRST-AID MEASURES

Eye contact:	Rinse with plenty of water.
Skin contact:	Wash with soap and water if necessary.
Inhalation:	Fresh air if discomfort is felt.
Ingestion:	Rinse the mouth. Drink water, milk or juice. If symptoms occur seek medical advice.
First aid facilities:	Not applicable.
Advice to doctor:	None.

5. FIRE-FIGHTING MEASURES

Extinguish with dry powder, carbon dioxide, foam or water.
Avoid inhalation of smoke and fumes.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions: Wear personal protective equipment as described under section 8.

Environmental precautions: Not applicable.

Methods for cleaning up: Wipe up spillage. Dilute remainder with water and clean up with a towel. Avoid raising dust. Wash the soiled area with soap and water or detergent.

7. HANDLING AND STORAGE

Handling:	Excessive dusting should be avoided The working area should be kept free of accumulated dust and ignition sources.
Storage:	Store NATAMAX® in original container. Avoid excessive light.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Exposure standards: Not available.

Engineering controls: Any equipment used to handle this product, should be designed to minimize the escape of aerosols, dust and vapours. Maintain adequate local exhaust ventilation which removes contaminated air, before the air reaches the respiratory system.

Protective equipment: Wear protective clothing, protective gloves and safety goggles, when there is a risk of getting in contact with the substance. In case of insufficient ventilation, wear suitable respiratory equipment to protect against dust or aerosols.

Educational demands: Instruction in the use of this product and knowledge of this Material Safety Data Sheet.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance: An off-white to cream coloured powder.
Solubility: Soluble in water.

LD_{50} (oral, rat):	2730 mg/kg.	**Melting point:**	Not available.
Boiling point:	Not applicable.	**Flammability limits:**	Not applicable.
Flash point:	Not applicable.	**Density:**	1,02 g/cm^3.
Vapour pressure:	Not applicable.		

10. STABILITY AND REACTIVITY

NATAMAX® is stable under normal conditions of use.

Conditions to avoid: Sunlight.

Materials to avoid: None.

Hazardous decomposition products: None.

11. TOXICOLOGICAL INFORMATION

In rare cases allergic reactions or other sorts of eczema may develop on prolonged or repeated contact with NATAMAX®

Eye or skin contact may cause mild transient irritation.

Ingestion of large doses may cause gastro-intestinal disturbances.

No toxic effects are known under normal conditions from inhalation of dust from this product

12. ECOLOGICAL INFORMATION

NATAMAX® is readily biodegradable and biodegrades relative fast in the environment. The primary degradation is fast followed by a slower secondary degradation.

LC_{50} (daphnia magna): 2,8 mg/l

ThOD: 60,85 ± 0,96% (10 days window).

13. DISPOSAL CONSIDERATIONS

Small quantities of waste are disposed of as domestic refuse.
Greater quantities are disposed of in accordance with the local regulations.

14. TRANSPORT INFORMATION

NATAMAX® is not considered dangerous according to ADR, RID, IMCO and IATA.

15. REGULATORY INFORMATION

NATAMAX® is not classified as dangerous according to EU-directive 91/155.

16. OTHER INFORMATION

This data sheet complies with EU Directive 91/155 as amended.
A Product Description is available on request.

Natamycin Petition

Appendix 7

Natamycin Use for Packaged Baked Goods

Published U.S. Patent Applications

U.S. Patent Application 20050163895 (Williams et al.) published July 28, 2005

U.S. Patent Application 20050191397 (Williams et al.) published Sept. 1, 2005

US 20050163895A1

(19) United States

(12) Patent Application Publication (10) Pub. No.: US 2005/0163895 A1

Williams et al. (43) Pub. Date: Jul. 28, 2005

(54) BAKED PRODUCT WITH INCREASED SHELF LIFE AND PROCESS FOR INCREASING THE SHELF LIFE OF BAKED PRODUCTS

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(21) Appl. No.: **10/765,210**

(22) Filed: **Jan. 28, 2004**

Publication Classification

(51) **Int. Cl.**[7] **A23K 1/00**

(52) **U.S. Cl.** **426/326**

(57) ABSTRACT

The present invention relates to a non yeast-leavened fine bakery product with increased shelf life and to a process for increasing the shelf life of intermediate and high moisture fine bakery products. The fine bakery product comprises an intermediate or high moisture baked product having a water activity a_w>0.8. Its surface has deposited thereon an effective amount of natamycin, which is sufficient to keep the product mould free when packaged for a storage time of 2 weeks or more at ambient temperature. In the process the outer surface of a baked product is sprayed with natamycin to deposit an effective amount of natamycin thereon, whereafter the sprayed product is packaged in a protective envelope.

BAKED PRODUCT WITH INCREASED SHELF LIFE AND PROCESS FOR INCREASING THE SHELF LIFE OF BAKED PRODUCTS

TECHNICAL FIELD

[0001] The present invention relates to a non yeast-leavened fine bakery product with increased shelf life and to a process for increasing the shelf life of intermediate and high moisture fine bakery products.

BACKGROUND

[0002] Many industrially produced baked goods emerge from the baking process with a surface that is essentially sterile but post bake handling can quickly lead to fungal surface contamination as a result of exposure to air borne contaminants as well as equipment contact. Following surface contamination, many baked goods are then very vulnerable to surface mould spoilage, the severity of which is linked to factors such as the degree of contamination, the moisture content of the product and the storage conditions. Baked products with a relatively neutral pH, high moisture content and high water activity such as cakes, muffins, waffles, and tortillas are particularly prone to rapid spoilage from a variety of moulds, principally *Penicillium* and *Aspergillus* species. Manufacturing good tasting, high moisture products with a long mould free shelf life presents a constant and ongoing technical challenge to the baking industry.

[0003] Various methods have been adopted in an attempt to achieve the required shelf life. These include addition of humectants to reduce the water activity, addition of chemical mould inhibiting preservatives such as propionates or sorbates limiting the availability of oxygen via modified atmosphere packaging and active packaging containing oxygen scavengers or providing a saturated ethanol headspace in the pack using sachet or strip inserts containing ethanol. The chemical preservatives such as sorbate and propionate are most effective at low pH so acids are often added in combination with these preservatives to reduce the pH of the baked product and hence improve the effectiveness of the added preservative.

[0004] Addition of acids, chemical preservatives and humectants can affect the taste and quality of the product and their use is often a compromise between achieving the best tasting product and the longest possible shelf life. Preservation based on packaging systems rely very much on pack integrity and even the best systems can suffer shelf life failures due to pack damage or seal failures and hence loss of pack integrity. Thus, there remains the technical problem of providing an efficient preservation system, which will not adversely affect the taste of baked goods.

[0005] Natamycin is a polyene macrolide natural antifungal agent produced by fermentation of the bacterium *Streptomyces natalensis*. Natamycin (previously known as pimaricin) has an extremely effective and selective mode of action against a very broad spectrum of common food spoilage yeasts and moulds with most strains being inhibited by concentrations of 1-15 ppm of natamycin.

[0006] Natamycin has been used for many years in a large number of countries throughout the world as an authorized preservation treatment for cheeses and certain meat products such as dried sausages. Despite this long-term use, the development of resistant strains has not been reported to date unlike the chemical organic acid sorbate and propionate preservatives for which a number of resistant yeasts and moulds have been detected and reported. Some species of *Penicillium* mould are even able to degrade and metabolise sorbate.

[0007] Natamycin is much less soluble in water than the chemical organic acid preservatives with its maximum solubility being around 40 ppm. In practice this means that when applied to the surface of the cheese or sausage, natamycin shows very limited diffusion and tends to stay on the surface of the food. Natamycin is active over a wide pH range and unlike the organic acid preservatives it is not dependant on a low pH acidic environment to show good anti-fungal activity. The effectiveness of natamycin at very low application levels on cheese and sausage has not been reported to have any adverse quality or flavour impact on the products.

[0008] Although natamycin has been used for a long time on cheese and on sausages, there is very little reported on the use of natamycin for other types of food. According to a review article in the 1974 edition of the Encyclopaedia of Food Technology by A. H. Johnson and M. S. Peters, natamycin (pimaricin) is permitted in several countries as a food additive and it is either added into the food (e.g. orange juice, wine) or the food is dipped, soaked or sprayed with aqueous natamycin (e.g. cheese, sausage, fruit).

[0009] In the U.S. the direct addition of natamycin into tortilla dough before baking is allowed. Tortilla dough is leavened without yeast and therefore adding natamycin into the tortilla dough is possible. In yeast leavened doughs, natamycin cannot be used since the natamycin would kill the yeast. Because of this limitation of using natamycin in yeast leavened bakery, natamycin seems to have been tested on the surface of yeast leavened bread. Thus, the review in the above mentioned 1974 Encyclopaedia also mentions that "rye and white bread were well protected when their surfaces were sprayed with a solution of 100-500 ppm pimaricin". No specific results are quoted to support the "well protected" comment and no reference is quoted for this work. No target levels for natamycin/pimaricin on the surface of the bread are given, no method of spraying and no shelf life targets are mentioned. In a later update of this review article for the same Encyclopaedia there was no mention of this bakery work. The review also makes similar vague references to treating the surface of uncooked doughs and to direct addition at 25-50 ppm in fillings for cakes and pies.

[0010] The direct addition of natamycin into icings and fillings of cakes is described also by J. Tichá in Mlynsko-pekarensky promysl, 7/1975, pp 225-228, as being effective in preventing the growth of moulds and yeasts for about 14 days. The article concludes that natamycin in admixture with lactose is useful to preserve curd fillings, icings and butter creams.

[0011] The addition of natamycin to protect the fillings of Cantonese mooncakes and pastry is allowed according to Chinese legislation. However, the mooncakes themselves often have a fairly low water activity and are thus not as prone to spoil as the fillings, which are often made of easily perishable foodstuffs.

[0012] Many fine bakery products are required to have a very long shelf life. Intermediate and high moisture fine

bakery products such as muffins, tortillas, waffles, sponge cakes and the like are usually packaged and kept at ambient temperatures on the shelf for 2 to 10 weeks and sometimes longer. In contrast to this, yeast-leavened baked goods, such as bread, tend to go stale in a much shorter period and the shelf life of most bread is normally not above two weeks.

[0013] The high water content of many fine bakery products makes them very sensitive to spoilage due to mould and yeast growth. This is especially true when the water activity of the product a_w is 0.8 or more, especially 0.85 or more. In an attempt to protect soft intermediate moisture and high moisture baked goods from mould growth, the present inventors added natamycin to tortilla dough, as allowed by the U.S. regulations, and to the pastry dough and egg glaze of Chinese mooncakes pre-baking. However, surprisingly, the attempts failed and did not result in any significant improvement of the shelf life of the baked goods. It was found that incorporating natamycin within the baked good was unsuccessful despite the fact that natamycin levels, which would normally be considered effective against yeasts and moulds, could still be detected in the goods after the baking process.

[0014] In two separate trials where natamycin (as Natamax) was incorporated into tortillas, the control of surface mould growth during shelf life was not achieved. Analysis of the mouldy tortillas from both trials showed that natamycin was still present within the tortillas at levels between 14.0 and 28.0 ppm. These relatively high levels of residual natamycin would normally be expected to show good control of mould growth indicating that the natamycin present within these tortillas was not biologically available at the surface of the product where it is required to be effective.

[0015] Consequently, natamycin could not be used in the non yeast-leavened baked products in the way the person skilled would normally and preferably have applied it, i.e. by direct addition of natamycin to the ingredients prior to baking. There thus remained the problem of how to increase the shelf life of fine bakery products having a high water activity and tendency to mould growth whilst optimising on desirable product characteristics such as pH and taste.

[0016] Documents cited in this text ("herein cited documents"), as well as each document or reference cited in each of the herein-cited documents, and all regulations, manufacturer's literature, specifications, instructions, product data sheets, material data sheet, and the like, as to each product mentioned in this text, are hereby expressly incorporated herein by reference.

SUMMARY

[0017] The present invention is based on the realization that natamycin can still help to overcome preservation problems of intermediate and high moisture fine bakery goods provided that it is applied to the surface of the baked products after baking. The relatively high moisture content in the baked products ascertains that an effective amount of dissolved natamycin will continuously be present to combat yeast and mould growth on the surface of the baked products.

[0018] Thus, the present invention provides an intermediate or high moisture non yeast-leavened baked product having a water activity a_w>0.8, the surface of said bakery product having deposited thereon an effective amount of natamycin which is sufficient to keep said product mould free when packaged for a storage time of 2 weeks or more at ambient temperature.

[0019] The present invention also provides a process for increasing the shelf life of fine bakery products, comprising providing a baked fine bakery product having a water activity a_w>0.8; spraying the outer surface of said baked product with natamycin to deposit an effective amount of natamycin thereon; packaging said sprayed baked product into a protective envelope; and storing said packaged product at ambient temperature. The natamycin deposited on the surface of said bakery product is provided in an amount which is effective in keeping said product mould free even when stored for 2 weeks or more.

[0020] The fine bakery product of the invention is preferably a non yeast-leavened fine bakery product having a water activity a_w between 0.8 and 0.95, preferably between 0.80 and 0.90, most preferably between 0.85 and 0.90. The preferred long shelf life fine bakery products protected by the invention are selected from muffins, waffles, tortillas, sponge cakes and the like baked fine bakery products.

[0021] The increase of mould-free shelf life achieved by the present invention is of immense significance to the baking industry and will allow the replacement of chemical preservatives such as propionate, benzoate and sorbate with natamycin, a less toxic, tasteless and naturally occurring alternative. The belief that natamycin is not effective in baked goods because it is not effective when incorporated into the food prior to baking, has been shown to be false and the problem has been overcome by the present invention by spraying the natamycin onto products post baking. Even though this requires prospective users to invest in suitable spraying equipment that can deliver an even natamycin application to all surfaces of bakery products, the advantages of natamycin are seen to outweigh the disadvantages of its use.

[0022] Without wishing to be bound by any theory, it is believed that the lack of natamycin efficacy noted when the natamycin was included directly into the ingredients of the fine bakery products was caused by a combination of 1) heat degradation of surface natamycin and 2) formation of a surface crust during baking that prevented the natamycin within the baked good from reaching the surface.

DETAILED DESCRIPTION

[0023] The lack of effectiveness that was seen when natamycin was incorporated into non yeast-leavened high water activity bakery products prior to baking led to the idea for the novel alternative solution of applying natamycin as a water based suspension precisely sprayed direct onto the exposed surfaces of the high water activity fine bakery product after the baking process. This novel approach targets a sufficient concentration of available natamycin directly at the surface of the baked product where the fungal contamination occurs. Any problem of losses during the baking process and availability on the surface has been overcome.

[0024] Consequently, the invention provides a fine bakery product the shelf life of which has been increased by the use of natamycin on the surface thereof. The preferred fine bakery products are non yeast-leavened products intended

for a long shelf life and having a moisture content which makes them susceptible to surface spoilage by moulds and yeasts. Such fine bakery products are especially intermediate or high moisture baked products having a water activity a_w>0.8, preferably 0.85 or more. A moisture content of 0.8 to 0.85 is regarded herein as an intermediate moisture content, while a moisture content above 0.85 is regarded as a high moisture content. Typical examples of such fine bakery products are cakes, muffins, waffles, tortillas and similar high water activity baked products.

[0025] The surface of the fine bakery product of the invention has deposited thereon an effective amount of natamycin which is sufficient to keep the product free of mould and yeast growth even though the product is packaged and stored for a time of 2 weeks or more. The effective amount of natamycin on the surface of the finished product is between 1-10 μg per cm^2 and it is sufficient for keeping the baked product mould free for 3 to 10 weeks, preferably for 6 to 10 weeks, or even longer, when the product is stored at ambient temperature, which usually ranges between 15 and 30° C. and which more often is from 18 to 25° C.

[0026] When the process of the present invention is operated, the outer surface of a baked fine bakery product having a water activity a_w>0.8 is sprayed with natamycin. The spraying should be performed so that a preservatively effective amount of natamycin is deposited on the exposed surface of the product. Typically, the natamycin is sprayed onto the exposed surface in the form of an aqueous suspension containing natamycin as dissolved natamycin and also in solid, crystalline, non-dissolved form. Crystalline natamycin is sparingly soluble in water and the finely divided solid natamycin crystals will be deposited on the surface together with the water and the dissolved natamycin. A preferred aqueous natamycin suspension for spraying purposes contains natamycin in a concentration of 250 to 7000 mg, more preferably 1000 to 4000 mg natamycin per litre of water.

[0027] A very small amount of natamycin is required to provide the desired protection against spoilage by moulds and yeasts. A deposited amount of from 1 to 10 μg natamycin per cm^2 of the surface of the baked product has been found to comprise an effective amount. It is of course possible to add more than the required amount of natamycin to the product. A higher amount than the above mentioned will certainly also be effective against mould and yeast growth and as natamycin has no bad taste, the product so protected would still be perfectly edible.

[0028] The natamycin should be sprayed homogeneously on all outer surfaces of the baked product so as to protect the product all over. Natamycin has a very low tendency for migration in the product and will not spread far from the point of deposition. In order to provide a homogeneous deposition the spraying equipment should be carefully selected. The natamycin based spray suspension is preferably delivered by a spinning disc, pneumatically operated spray gun or any other suitable spraying system that is capable of delivering a small but consistent and accurate spray volume over a given surface area. The volume of the water based natamycin suspension sprayed onto the product should preferably be kept to the minimum level that will allow an even surface coverage. The natamycin deposited on the surface of the bakery product should, however, be effective in keeping the fine bakery product mould free even when stored for 2 weeks or more.

[0029] After spraying, the baked product is packaged into a protective envelope, which is preferably made of a transparent material such as a plastic film or box to allow the presumptive buyer to view the product and be tempted by it. The films are generally of a moisture proof material to prevent the moist baked product from drying and loosing its softness during the several weeks of storing.

[0030] The following examples illustrate the invention.

REFERENCE EXAMPLE

Natamycin in Mooncakes

[0031] Mooncakes are traditional bakery products that are baked and eaten in large numbers once a year in China to celebrate a mid-Autumn festival. The cakes consist of a thin outer layer of pastry covering a variety of paste type fillings that are moulded into intricate shapes prior to baking. The outside of the cake is covered with an egg glaze and part cooked at 200° C.-210° C. for 15 minutes before a second coating of egg glaze is applied ready for the final bake of 10 minutes at the same temperature. Large-scale production, sale and storage of mooncakes begin in the period leading up to the festival and mould problems can occur on the surface of these products prior to consumption.

[0032] Two trial production runs of mooncakes containing lotus bean paste were made to test the efficacy of direct natamycin addition prior to baking for preserving these bakery products. The cakes were packed into individual clear plastic bags with no other preservative.

[0033] For the first production a range of four increasing natamycin levels (20, 25, 30 and 35 ppm) were mixed into the raw pastry dough for four small separate batches prior to glazing and baking. For the second production, nothing was added to the dough but an increasing range of the same four 20-35 ppm natamycin levels were added to the egg glaze that was applied to the cake surface after the first bake but before the second and final baking stage. Control cakes with no natamycin addition were also prepared.

[0034] Representative samples from each batch were assayed for residual natamycin in 10 g samples of the surface pastry layer. Good levels of residual natamycin activity were detected in the surface pastry of all samples from both production runs but despite this the growth of spoilage moulds still appeared on the surface of all of the natamycin treated cakes within 20-24 days of manufacture.

Example 1

Natamycin on the Surface of Fine Bakery

[0035] Muffins are flour-based non yeast-leavened fine bakery goods prone to surface spoilage due to growth of moulds and yeasts. Their water activity a_w is typically about 0.85.

[0036] Muffins were prepared according to a standard recipe with no added preservative in the dough. Shortly after baking, individual muffins were sprayed whilst still warm with a choice of four different spray treatments:

[0037] 1—water only (control).

[0038] 2—water containing 8% added salt.

[0039] 3—water containing 4 g per litre of Natamax™ lactose (containing 50% natamycin) obtainable from Danisco A/S.

[0040] 4—water containing 8 g per litre of Natamax™ lactose.

[0041] Spraying was done using a pneumatic hand-held spray gun with integral reservoir. The reservoir was regularly shaken during spraying to ensure that the small, undissolved crystals of natamycin stayed in suspension. Each muffin was sprayed evenly over all surfaces with a minimum volume of finely adjusted spray.

[0042] After cooling, the sprayed muffins were packed into heat sealed clear polythene bags with 8 to 10 muffins per treatment per bag. Initial samples were selected and tested for water activity, pH and surface natamycin concentration. Unopened bags of muffins were put for shelf life evaluation at 25° C. and examined daily for signs of surface mould or yeast growth.

[0043] The surface natamycin determination was performed as follows: The surface area of the muffins was calculated to be 150 cm^2. Individual muffins were shaved and the total surface material added to 100 ml high purity methanol and shaken for 1 hour. 50 ml of high purity water was added and the solution then filtered through a 0.2 μm membrane filter. The natamycin content was determined by HPLC analysis for the whole surface and then divided by 150 to give a result in μg of natamycin per cm^2 of muffin surface.

[0044] Results from analysis of the initial samples are shown in Table 1. As expected, the highest level of residual natamycin was detected on the muffins sprayed with the highest concentration of Natamax™ suspension (treatment 4).

TABLE 1

Initial analysis results

Treatment	Residual natamycin $\mu g/cm^2$	Water activity (Aw)	pH
1) Water only	<1	0.839	9.39
2) 8% salt	<1	0.854	9.45
3) 4 g/l Natamax ™	2.7	0.864	9.18
4) 8 g/l Natamax ™	4.5	0.851	9.25

[0045] Results from the incubation study of the packs of muffins at 25° C. are given in table 2. Packs of control muffins sprayed with water only or with the 8% salt in water were regarded as spoiled after only 5 and 11 days respectively due to the growth of clearly visible surface mould growth. In contrast to this, all of the packs of muffins sprayed with the two treatment levels of Natamax™ suspension were found to be completely free of any surface mould growth for a total incubation period of 70 days, after which time the incubation was stopped.

TABLE 2

Results after incubation at 25° C.

Treatment	Number of days in incubation at 25° C.	Spoilage due to mould growth
1) Water only	5 days	All spoilt
2) 8% salt	11 days	All spoilt
3) 4 g/l Natamax ™	70 days	None spoilt
4) 8 g/l Natamax ™	70 days	None spoilt

[0046] At the end of the 70 day incubation period, triplicate samples of the unspoiled muffins from Natamax™ spray treatments 3 and 4 were assayed for surface natamycin levels, water activity (Aw) and pH. The results from these final analyses are given in Table 3. Residual natamycin was still detectable at similar levels to those of the initial sample for the higher concentration spray treatment 4 and at reduced levels for the lower concentration spray treatment 3.

TABLE 3

Final analysis results

Treatment	Residual natamycin $\mu g/cm^2$	Aw (muffin outside)	pH (muffin outside)	Aw (muffin inside)	pH (muffin inside)
3) 4 g/l Natamax ™	0.7	0.85	6.97	0.85	8.15
	0.7	NT	NT	NT	NT
	0.7	NT	NT	NT	NT
4) 8 g/l Natamax ™	4.2	0.83	6.21	0.84	7.91
	3.6	NT	NT	NT	NT
	4.8	NT	NT	NT	NT

NT = Not tested

[0047] The above results clearly demonstrate the excellent preservative efficacy of natamycin, when sprayed onto the surface of this relatively high moisture flour based baked product, in preventing or delaying spoilage due to surface growth of yeast and moulds.

Example 2

Natamycin on the Surface of Fine Bakery

[0048] Muffins were prepared according to the same standard recipe as in Example 1. Individual muffins were subjected to one of three treatments with a minimum of 50 muffins per treatment.

[0049] 1—No spray (control)

[0050] 2—Water only spray (control)

[0051] 3—Water containing 4.2 g per litre of Natamax™ SF (sugar free containing 87% natamycin) obtainable from Danisco A/S.

[0052] The muffins were sprayed shortly after baking whilst still warm. Spraying was done with a pneumatic hand-held spray gun with integral reservoir. The reservoir was regularly shaken during spraying to ensure that the small, undissolved crystals of natamycin stayed in suspension. The approximate minimum volume of spray required to evenly cover the whole surface of a muffin was measured and a concentration of 4.2 g per litre of Natamax™ SF

calculated to target a spray delivery of 5 μg of natamycin per cm^2 of muffin in treatment 3. Each muffin was sprayed evenly over all surfaces.

[0053] After cooling, the sprayed muffins were packed into individual, heat sealed, clear plastic bags. One sample was selected from each of the two control treatments and five samples from the Natamax™ SF spray treatment and tested for water activity, pH, yeast and mould count at 25° C. and residual surface natamycin levels by HPLC analysis. Forty unopened muffins per treatment were put for shelf life evaluation at 25° C. and examined daily for signs of surface mould or yeast growth.

[0054] Results from analysis of the initial samples are shown in Table 4. The residual natamycin levels detected on the muffins sprayed with the Natamax™ SF suspension were found to be close to the target level of 5 μg per cm^2.

TABLE 4

Initial analysis results

Treatment	Residual natamycin μg/cm^2	Water activity (muffin outside)	Water activity (muffin inside)	Mould at 25° C./g	pH
1) No spray (control)	<0.2	0.855	0.867	<50	8.55
2) Water only (control)	<0.2	0.863	0.877	<50	8.65
3) 4.2 g/l Natamax ™ SF	5.1	0.863	0.879	<50	8.27
	4.3	NT	NT	<50	NT
	3.4	NT	NT	<50	NT
	3.8	NT	NT	<50	NT
	5.8	NT	NT	<50	NT

NT = Not Tested

[0055] Results from the incubation study of the 40 muffins per treatment are given in Table 5. The first non-spray control sample (treatment 1) developed surface mould growth after only 7 days giving a mould free shelf life of only 6 days. The first water sprayed control sample (treatment 2) developed surface mould growth after only 10 days. In contrast to this, all of the 40 muffins sprayed with Natamax™ SF suspension remained completely free of any surface mould growth for a total incubation period of 68 days, after which time the incubation was stopped. Thus, in this trial experiment, a natamycin surface spray treatment of approximately 5 μg/cm^2 increased the mould-free shelf life of muffins at 25° C. from 6 days to at least 68 days.

TABLE 5

Results after incubation at 25° C.

Days incubated at 25° C.	Number showing mould growth/Number under incubation: Treatment 1 No. spray control	Treatment 2 Water only control	Treatment 3 4.2 g/l Natamax ™ SF
6	0/40	0/40	0/40
7	1/40	0/40	0/40
9	2/40	0/40	0/40
10	3/40	1/40	0/40
14	5/40	1/40	0/40
17	6/40	2/40	0/40

TABLE 5-continued

Results after incubation at 25° C.

Days incubated at 25° C.	Number showing mould growth/Number under incubation: Treatment 1 No. spray control	Treatment 2 Water only control	Treatment 3 4.2 g/l Natamax ™ SF
23	6/40	3/40	0/40
24	7/40	4/40	0/40
63	8/40	4/40	0/40
68	8/40	4/40	0/40

[0056] The above examples 1 and 2 clearly demonstrate the preservative efficacy of natamycin when sprayed on the outer surface of fine bakery products having a water activity above 0.8, which are susceptible to surface spoilage by moulds and yeasts during storage. Based on the description and examples a person skilled in the art is able to apply the invention to a wide variety of fine bakery goods.

What is claimed is:

1. A non-yeast leavened fine bakery product with increased shelf life comprising an intermediate or high moisture baked product having a water activity a_w>0.8, the surface of said bakery product having deposited thereon an effective amount of natamycin which is sufficient to keep said product mould free when packaged for a storage time of 2 weeks or more at ambient temperature.

2. The fine bakery product of claim 1, wherein said fine bakery product is selected from muffins, waffles, tortillas, sponge cakes and the like baked products.

3. The fine bakery product of claim 1, wherein the water activity a_w of said baked product is between 0.8 and 0.95.

4. The fine bakery product of claim 3, wherein the water activity a_w of said baked product is between 0.8 and 0.90.

5. The fine bakery product of claim 3, wherein the water activity a_w of said baked product is between 0.85 and 0.90.

6. The fine bakery product of claim 1, wherein the water activity of said baked product is sufficient to keep at least a part of said deposited natamycin in dissolved form.

7. The fine bakery product of claim 1, wherein said effective amount of natamycin comprises from 1 to 10 μg per cm^2 of the surface of said baked product.

8. The fine bakery product of claim 1, wherein said natamycin is deposited on said surface in an amount sufficient to keep said baked product mould free for a storage time of 3 to 10 weeks.

9. The fine bakery product of claim 8, wherein said natamycin is deposited on said surface in an amount sufficient to keep said baked product mould free for a storage time of 6 to 10 weeks.

10. The fine bakery product of claim 1, wherein said ambient temperature comprises a temperature of 15 to 30° C.

11. The fine bakery product of claim 10, wherein said ambient temperature is from 18 to 25° C.

12. The fine bakery product of claim 1, wherein said product is packaged in a protective envelope.

13. The fine bakery product of claim 12, wherein said envelope is of a moisture proof and/or transparent material.

14. A process for increasing the shelf life of fine bakery products, comprising

providing a baked fine bakery product having a water activity a_w>0.8;

spraying the outer surface of said baked product with natamycin to deposit an effective amount of natamycin thereon;

packaging said sprayed baked product into a protective envelope; and

storing said packaged product at ambient temperature;

the natamycin deposited on the surface of said bakery product being effective in keeping said product mould free even when stored for 3 weeks or more.

15. The process of claim 14, wherein said natamycin is sprayed onto said surface in the form of an aqueous suspension.

16. The process of claim 15, wherein said suspension contains natamycin in a concentration of 250 to 7000 mg natamycin per litre of water.

17. The process of claim 16, wherein said suspension contains natamycin in a concentration of 1000 to 4000 mg natamycin per litre of water.

18. The process of claim 15, wherein said natamycin suspension contains dissolved natamycin and crystalline natamycin.

19. The process of claim 14, wherein said effective amount of natamycin comprises from 1 to 10 μg per cm^2 of the surface of said baked product.

20. The process of claim 14, wherein said natamycin is sprayed homogeneously on all outer surfaces of said baked product.

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US 20050191397A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2005/0191397 A1**

Williams et al. (43) **Pub. Date: Sep. 1, 2005**

(54) **BAKED PRODUCT WITH INCREASED SHELF LIFE AND PROCESS FOR INCREASING THE SHELF LIFE OF BAKED PRODUCTS**

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(21) Appl. No.: **11/041,943**

(22) Filed: **Jan. 26, 2005**

Related U.S. Application Data

(63) Continuation-in-part of application No. 10/765,210, filed on Jan. 28, 2004.

Publication Classification

(51) **Int. Cl.**[7] **A21D 10/00**

(52) **U.S. Cl.** **426/549**

(57) **ABSTRACT**

The present invention relates to a non yeast-leavened fine bakery product with increased shelf life and to a process for increasing the shelf life of intermediate and high moisture fine bakery products. The fine bakery product comprises an intermediate or high moisture baked product having a water activity a_w>0.8. Its surface has deposited thereon an effective amount of natamycin, which is sufficient to keep the product mould free when packaged for a storage time of 2 weeks or more at ambient temperature. In the process the outer surface of a baked product is sprayed with natamycin to deposit an effective amount of natamycin thereon, whereafter the sprayed product is packaged in a protective envelope.

BAKED PRODUCT WITH INCREASED SHELF LIFE AND PROCESS FOR INCREASING THE SHELF LIFE OF BAKED PRODUCTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in part of application Ser. No. 10/765,210, filed Jan. 28, 2004, the contents of which are incorporated herein by reference to the extend they are consistent with this application and inventions described herein.

BACKGROUND OF THE INVENTION

[0002] The present invention relates to a non yeast-leavened fine bakery product with increased shelf life and to a process for increasing the shelf life of intermediate and high moisture fine bakery products.

[0003] Many industrially produced baked goods emerge from the baking process with a surface that is essentially sterile but post bake handling can quickly lead to fungal surface contamination as a result of exposure to airborne contaminants as well as equipment contact. Following surface contamination, many baked goods are then very vulnerable to surface mould spoilage, the severity of which is linked to factors such as the degree of contamination, the moisture content of the product and the storage conditions. Baked products with a relatively neutral pH, high moisture content and high water activity such as cakes, muffins, waffles, and tortillas are particularly prone to rapid spoilage from a variety of moulds, principally *Penicillium* and *Aspergillus* species. Manufacturing good tasting, high moisture products with a long mould free shelf life presents a constant and ongoing technical challenge to the baking industry.

[0004] Various methods have been adopted in an attempt to achieve the required shelf life. These include addition of humectants to reduce the water activity, addition of chemical mould inhibiting preservatives such as propionates or sorbates, limiting the availability of oxygen via modified atmosphere packaging and active packaging containing oxygen scavengers or providing a saturated ethanol headspace in the pack using sachet or strip inserts containing ethanol. The chemical preservatives such as sorbate and propionate are most effective at low pH so acids are often added in combination with these preservatives to reduce the pH of the baked product and hence improve the effectiveness of the added preservative.

[0005] Addition of acids, chemical preservatives and humectants can affect the taste and quality of the product and their use is often a compromise between achieving the best tasting product and the longest possible shelf life. Preservation based on packaging systems rely very much on pack integrity and even the best systems can suffer shelf life failures due to pack damage or seal failures and hence loss of pack integrity. Thus, there remains the technical problem of providing an efficient preservation system, which will not adversely affect the taste of baked goods.

[0006] Natamycin is a polyene macrolide natural antifungal agent produced by fermentation of the bacterium *Streptomyces natalensis*. Natamycin (previously known as pimaricin) has an extremely effective and selective mode of action against a very broad spectrum of common food spoilage yeasts and moulds with most strains being inhibited by concentrations of 1-15 ppm of natamycin.

[0007] Natamycin has been used for many years in a large number of countries throughout the world as an authorized preservation treatment for cheeses and certain meat products such as dried sausages. Despite this long-term use, the development of resistant strains has not been reported to date unlike the chemical organic acid sorbate and propionate preservatives for which a number of resistant yeasts and moulds have been detected and reported. Some species of *Penicillium* mould are even able to degrade and metabolise sorbate.

[0008] Natamycin is much less soluble in water than the chemical organic acid preservatives with its maximum solubility being around 40 ppm. In practice this means that when applied to the surface of the cheese or sausage, natamycin shows very limited diffusion and tends to stay on the surface of the food. Natamycin is active over a wide pH range and unlike the organic acid preservatives it is not dependant on a low pH acidic environment to show good anti-fungal activity. The effectiveness of natamycin at very low application levels on cheese and sausage has not been reported to have any adverse quality or flavour impact on the products.

[0009] Although natamycin has been used for a long time on cheese and on sausages, there is very little reported on the use of natamycin for other types of food. According to a review article "Antibiotics in Food: Primarcin" in the Encyclopaedia of Food Technology Volume 2. 1974, The Avi Publishing Co. Inc. Westport, Conn. USA, pp 36-37, Eds. A. H. Johnson and M. S. Peters, natamycin (pimaricin) is permitted in several countries as a food additive and it is either added into the food (e.g. orange juice, wine) or the food is dipped, soaked or sprayed with aqueous natamycin (e.g. cheese, sausage, fruit).

[0010] US 2004/0013781 discloses a fully baked bread product which remains soft for an extended shelf life. The bread may be protected by a microbial inhibitor which may be natamycin. In the described embodiments the inhibitor is included in the dough at baking. However, there is also a suggestion that a potassium sorbate inhibitor may be sprayed in an aqueous solution on the bread after baking.

[0011] In the U.S. the direct addition of natamycin into tortilla dough before baking is allowed. Tortilla dough is leavened without yeast and therefore adding natamycin into the tortilla dough is possible. In yeast leavened doughs, natamycin cannot be used since the natamycin would kill the yeast. Because of this limitation of using natamycin in yeast leavened bakery, natamycin seems to have been tested on the surface of yeast leavened bread. Thus, the review in the above mentioned 1974 Encyclopaedia also mentions that "rye and white bread were well protected when their surfaces were sprayed with a solution of 100-500 ppm pimaricin". No specific results are quoted to support the "well protected" comment and no reference is quoted for this work. No target levels for natamycin/pimaricin on the surface of the bread are given, no method of spraying and no shelf life targets are mentioned. In a later update of this review article for the same Encyclopaedia there was no mention of this bakery work. The review also makes similar vague references to treating the surface of uncooked doughs and to direct addition at 25-50 ppm in fillings for cakes and pies.

[0012] The direct addition of natamycin into icings and fillings of cakes is described also by J. Tichá in Mlynsko-pekarensky promysl, July 1975, pp 225-228, as being effective in preventing the growth of moulds and yeasts for about 14 days. The article concludes that natamycin in admixture with lactose is useful to preserve curd fillings, icings and butter creams.

[0013] The use of natamycin to protect the surface of the fillings of Cantonese mooncakes and pastry is allowed according to Chinese legislation. However, the mooncakes themselves often have a fairly low water activity and are thus not as prone to spoil as the fillings, which are often made of easily perishable foodstuffs.

[0014] Many fine bakery products are required to have a very long shelf life. Intermediate and high moisture fine bakery products such as muffins, tortillas, waffles, pancakes, pizzas, pastry, sponge cakes and the like are usually packaged and kept at ambient temperatures on the shelf for 2 to 10 weeks and sometimes longer. In contrast to this, yeast-leavened baked goods, such as bread, tend to go stale in a much shorter period and the shelf life of most bread is normally not above two weeks.

[0015] The high water content of many fine bakery products makes them very sensitive to spoilage due to mould and yeast growth. This is especially true when the water activity of the product a_w is 0.8 or more, especially 0.85 or more. In an attempt to protect intermediate moisture and high moisture baked goods from mould growth, the present inventors added natamycin to tortilla dough, as allowed by the U.S. regulations, and to the pastry dough and egg glaze of Chinese mooncakes pre-baking. However, surprisingly, the attempts failed and did not result in any significant improvement of the shelf life of the baked goods. It was found that incorporating natamycin within the baked good was unsuccessful despite the fact that natamycin levels, which would normally be considered effective against yeasts and moulds, could still be detected in the goods after the baking process.

[0016] In two separate trials where natamycin (as Natamax) was incorporated into tortillas, the control of surface mould growth during shelf life was not achieved. Analysis of the mouldy tortillas from both trials showed that natamycin was still present within the tortillas at levels between 14.0 and 28.0 ppm. These relatively high levels of residual natamycin would normally be expected to show good control of mould growth indicating that the natamycin present within these tortillas was not biologically available at the surface of the product where it is required to be effective.

[0017] Consequently, natamycin could not be used in the non yeast-leavened baked products in the way the person skilled would normally and preferably have applied it, i.e. by direct addition of natamycin to the ingredients prior to baking. There thus remained the problem of how to increase the shelf life of fine bakery products having a high water activity and tendency to mould growth whilst optimising on desirable product characteristics such as pH and taste.

[0018] Documents cited in this text ("herein cited documents"), as well as each document or reference cited in each of the herein-cited documents, and all regulations, manufacturer's literature, specifications, instructions, product data sheets, material data sheet, and the like, as to each product mentioned in this text, are hereby expressly incorporated herein by reference.

BRIEF SUMMARY OF THE INVENTION

[0019] The present invention is based on the realization that natamycin can still help to overcome preservation problems of intermediate and high moisture fine bakery goods provided that it is applied to the surface of the baked products after baking. The relatively high moisture content in the baked products ascertains that an effective amount of dissolved natamycin will continuously be present to combat yeast and mould growth on the surface of the baked products.

[0020] Thus, the present invention provides an intermediate or high moisture non yeast-leavened baked product having a water activity $a_w>0.8$, the surface of said bakery product having deposited thereon an effective amount of natamycin which is sufficient to keep said product mould free when packaged for a storage time of 2 weeks or more at ambient temperature.

[0021] The present invention also provides a process for increasing the shelf life of fine bakery products, comprising providing a baked fine bakery product having a water activity $a_w>0.8$; spraying the outer surface of said baked product with natamycin to deposit an effective amount of natamycin thereon; packaging said sprayed baked product into a protective envelope; and storing said packaged product at ambient temperature. The natamycin deposited on the surface of said bakery product is provided in an amount which is effective in keeping said product mould free even when stored for 2 weeks or more.

[0022] The fine bakery product of the invention is preferably a non yeast-leavened fine bakery product having a water activity a_w between 0.8 and 0.95, preferably between 0.80 and 0.90, most preferably between 0.85 and 0.90. The preferred long shelf life fine bakery products protected by the invention are selected from muffins, waffles, tortillas, pizzas, pizza bases, pancakes, pastry, sponge cakes and the like baked fine bakery products. The term fine bakery should be considered as including also part-baked products of fine bakery.

[0023] The increase of mould-free shelf life achieved by the present invention is of immense significance to the baking industry and will allow the replacement of chemical preservatives such as propionate, benzoate and sorbate with natamycin, a less toxic, tasteless and naturally occurring alternative. The belief that natamycin is not an effective preservative for baked goods (based on evidence of lack of efficacy when incorporated into the food prior to baking), has been shown to be false and has been overcome by the present invention by spraying the natamycin onto products post baking. Even though this requires prospective users to invest in suitable spraying equipment that can deliver an even natamycin application to all surfaces of bakery products, the advantages of natamycin are seen to outweigh the disadvantages of its use.

[0024] Without wishing to be bound by any theory, it is believed that the lack of natamycin efficacy noted when the natamycin was included directly into the ingredients of the fine bakery products was caused by a combination of 1) heat degradation of surface natamycin and 2) formation of a surface crust during baking that prevented the natamycin within the baked good from reaching the surface.

DETAILED DESCRIPTION OF THE INVENTION

[0025] The lack of effectiveness that was seen when natamycin was incorporated into non yeast-leavened high water activity bakery products prior to baking led to the idea for the novel alternative solution of applying natamycin as a water based suspension precisely sprayed direct onto the exposed surfaces of the high water activity fine bakery product after the baking process. This novel approach targets a sufficient concentration of available natamycin directly at the surface of the baked product where the fungal contamination occurs. Any problem of losses during the baking process and availability on the surface has been overcome.

[0026] Consequently, the invention provides a fine bakery product the shelf life of which has been increased by the use of natamycin on the surface thereof. The preferred fine bakery products are non yeast-leavened products intended for a long shelf life and having a moisture content which makes them susceptible to surface spoilage by moulds and yeasts. Such fine bakery products are especially intermediate or high moisture baked products having a water activity a_w>0.8, preferably 0.85 or more. A moisture content of 0.8 to 0.85 is regarded herein as an intermediate moisture content, while a moisture content above 0.85 is regarded as a high moisture content. Typical examples of such fine bakery products are cakes, muffins, waffles, tortillas, pizzas, pancakes, pastry, sponge cakes and similar high water activity baked products as well as similar part-baked fine bakery products. Pizzas are for example ready made pizzas or pizza bases.

[0027] The surface of the fine bakery product of the invention has deposited thereon an effective amount of natamycin which is sufficient to keep the product free of mould and yeast growth even though the product is packaged and stored for a time of 2 weeks or more. The effective amount of natamycin on the surface of the finished product is between 1-10 μg per cm^2 and it is sufficient for keeping the baked product mould free for 3 to 10 weeks, preferably for 6 to 10 weeks, or even longer, when the product is stored at ambient temperature, which usually ranges between 15 and 30° C. and which more often is from 18 to 25° C.

[0028] When the process of the present invention is operated, the outer surface of a baked fine bakery product having a water activity a_w>0.8 is sprayed with natamycin. The spraying should be performed so that a preservatively effective amount of natamycin is deposited on the exposed surface of the product. Typically, the natamycin is sprayed onto the exposed surface in the form of an aqueous suspension containing natamycin as dissolved natamycin and also in solid, crystalline, non-dissolved form. Crystalline natamycin is sparingly soluble in water and the finely divided solid natamycin crystals will be deposited on the surface together with the water and the dissolved natamycin. A preferred aqueous natamycin suspension for spraying purposes contains natamycin in a concentration of 250 to 7000 mg, more preferably 1000 to 4000 mg natamycin per litre of water.

[0029] In a preferred embodiment of the invention the natamycin is deposited on said surface in a suspension which includes a thickener. The thickener is preferably selected from the group consisting of agar, alginates, carrageenan, cellulose and derivatives, gums, gelatin, pectins and derivatives thereof, polyvinyl acetate, starches and modified starches, and suspending agents. Useful derivatives of cellulose are such as microcrystalline cellulose sodium, hydroxypropylmethyl cellulose, carboxymethyl cellulose and methyl cellulose. The gums used are e.g. xanthan gum, gellan gum, locust bean gum, gum arabic, gum tragacanth, gum karaya, guar gum, rhamxam gum, conjac gum and seed gum. Suspending agents used are e.g. sodium dodecyl sulphate, polyethylene glycol, fumed silica, glycol and glycerol.

[0030] The thickener in the suspension ensures that the sprayed natamycin remains evenly distributed at the point of deposition and does not collect in pools in the crevices of the baked goods.

[0031] A very small amount of natamycin is required to provide the desired protection against spoilage by moulds and yeasts. A deposited amount of from 1 to 10 μg natamycin per cm^2 of the surface of the baked product has been found to comprise an effective amount. It is of course possible to add more than the required amount of natamycin to the product. A higher amount than the above mentioned will certainly also be effective against mould and yeast growth and as natamycin has no bad taste, the product so protected would still be perfectly edible.

[0032] The natamycin should be sprayed homogeneously on all outer surfaces of the baked product so as to protect the product all over. Natamycin has a very low tendency for migration in the product and will not spread far from the point of deposition. In order to provide a homogeneous deposition the spraying equipment should be carefully selected. The natamycin based spray suspension is preferably delivered by a spinning disc, pneumatically operated spray gun or any other suitable spraying system that is capable of delivering a small but consistent and accurate spray volume over a given surface area. The volume of the water based natamycin suspension sprayed onto the product should preferably be kept to the minimum level that will allow an even surface coverage. The natamycin deposited on the surface of the bakery product should, however, be effective in keeping the fine bakery product mould free even when stored for 2 weeks or more.

[0033] After spraying, the baked product is packaged into a protective envelope, which is preferably made of a transparent material such as a plastic film or box to allow the presumptive buyer to view the product and be tempted by it. The films are generally of a moisture proof material to prevent the moist baked product from drying and loosing its softness during the several weeks of storing.

[0034] The following examples illustrate the invention.

REFERENCE EXAMPLE

Natamycin in Mooncakes

[0035] Mooncakes are traditional bakery products that are baked and eaten in large numbers once a year in China to celebrate a mid-Autumn festival. The cakes consist of a thin outer layer of pastry covering a variety of paste type fillings that are moulded into intricate shapes prior to baking. The outside of the cake is covered with an egg glaze and part cooked at 200° C.-210° C. for 15 minutes before a second coating of egg glaze is applied ready for the final bake of 10

minutes at the same temperature. Large-scale production, sale and storage of mooncakes begin in the period leading up to the festival and mould problems can occur on the surface of these products prior to consumption.

[0036] Two trial production runs of mooncakes containing lotus bean paste were made to test the efficacy of direct natamycin addition prior to baking for preserving these bakery products. The cakes were packed into individual clear plastic bags with no other preservative.

[0037] For the first production a range of four increasing natamycin levels (20, 25, 30 and 35 ppm) were mixed into the raw pastry dough for four small separate batches prior to glazing and baking. For the second production, nothing was added to the dough but an increasing range of the same four 20-35 ppm natamycin levels were added to the egg glaze that was applied to the cake surface after the first bake but before the second and final baking stage. Control cakes with no natamycin addition were also prepared.

[0038] Representative samples from each batch were assayed for residual natamycin in 10 g samples of the surface pastry layer. Good levels of residual natamycin activity were detected in the surface pastry of all samples from both production runs but despite this the growth of spoilage moulds still appeared on the surface of all of the natamycin treated cakes within 20-24 days of manufacture.

EXAMPLE 1

Natamycin on the Surface of Fine Bakery

[0039] Muffins are flour-based non yeast-leavened fine bakery goods prone to surface spoilage due to growth of moulds and yeasts. Their water activity a_w is typically about 0.85.

[0040] Muffins were prepared according to a standard recipe with no added preservative in the dough. Shortly after baking, individual muffins were sprayed whilst still warm with a choice of four different spray treatments:

[0041] 1—water only (control).

[0042] 2—water containing 8% added salt.

[0043] 3—water containing 4 g per litre of Natamax™ lactose (containing 50% natamycin) obtainable from Danisco A/S.

[0044] 4—water containing 8 g per litre of Natamax™ lactose.

[0045] Spraying was done using a pneumatic hand-held spray gun with integral reservoir. The reservoir was regularly shaken during spraying to ensure that the small, undissolved crystals of natamycin stayed in suspension. Each muffin was sprayed evenly over all surfaces with a minimum volume of finely adjusted spray.

[0046] After cooling, the sprayed muffins were packed into heat sealed clear polythene bags with 8 to 10 muffins per treatment per bag. Initial samples were selected and tested for water activity, pH and surface natamycin concentration. Unopened bags of muffins were put for shelf life evaluation at 25° C. and examined daily for signs of surface mould or yeast growth.

[0047] The surface natamycin determination was performed as follows: The surface area of the muffins was calculated to be 150 cm^2. Individual muffins were shaved and the total surface material added to 100 ml high purity methanol and shaken for 1 hour. 50 ml of high purity water was added and the solution then filtered through a 0.2 μm membrane filter. The natamycin content was determined by HPLC analysis for the whole surface and then divided by 150 to give a result in μg of natamycin per cm^2 of muffin surface.

[0048] Results from analysis of the initial samples are shown in Table 1. As expected, the highest level of residual natamycin was detected on the muffins sprayed with the highest concentration of Natamax™ suspension (treatment 4).

TABLE 1

Initial analysis results

Treatment	Residual natamycin $\mu g/cm^2$	Water activity (Aw)	pH
1) Water only	<1	0.839	9.39
2) 8% salt	<1	0.854	9.45
3) 4 g/l Natamax ™	2.7	0.864	9.18
4) 8 g/l Natamax ™	4.5	0.851	9.25

[0049] Results from the incubation study of the packs of muffins at 25° C. are given in table 2. Packs of control muffins sprayed with water only or with the 8% salt in water were regarded as spoiled after only 5 and 11 days respectively due to the growth of clearly visible surface mould growth. In contrast to this, all of the packs of muffins sprayed with the two treatment levels of Natamax™ suspension were found to be completely free of any surface mould growth for a total incubation period of 70 days, after which time the incubation was stopped.

TABLE 2

Results after incubation at 25° C.

Treatment	Number of days in incubation at 25° C.	Spoilage due to mould growth
1) Water only	5 days	All spoilt
2) 8% salt	11 days	All spoilt
3) 4 g/l Natamax ™	70 days	None spoilt
4) 8 g/l Natamax ™	70 days	None spoilt

[0050] At the end of the 70 day incubation period, triplicate samples of the unspoiled muffins from Natamax™ spray treatments 3 and 4 were assayed for surface natamycin levels, water activity (Aw) and pH. The results from these final analyses are given in Table 3. Residual natamycin was still detectable at similar levels to those of the initial sample for the higher concentration spray treatment 4 and at reduced levels for the lower concentration spray treatment 3.

TABLE 3

Final analysis results

Treatment	Residual natamycin $\mu g/cm^2$	Aw (muffin outside)	pH (muffin outside)	Aw (muffin inside)	pH (muffin inside)
3) 4 g/l Natamax ™	0.7	0.85	6.97	0.85	8.15
	0.7	NT	NT	NT	NT
	0.7	NT	NT	NT	NT
4) 8 g/l Natamax ™	4.2	0.83	6.21	0.84	7.91
	3.6	NT	NT	NT	NT
	4.8	NT	NT	NT	NT

NT = Not tested

[0051] The above results clearly demonstrate the excellent preservative efficacy of natamycin, when sprayed onto the surface of this relatively high moisture flour based baked product, in preventing or delaying spoilage due to surface growth of yeast and moulds

EXAMPLE 2

Natamycin on the Surface of Fine Bakery

[0052] Muffins were prepared according to the same standard recipe as in Example 1. Individual muffins were subjected to one of three treatments with a minimum of 50 muffins per treatment.

[0053] 1—No spray (control)

[0054] 2—Water only spray (control)

[0055] 3—Water containing 4.2 g per litre of Natamax™ SF (sugar free containing 87% natamycin) obtainable from Danisco A/S.

[0056] The muffins were sprayed shortly after baking whilst still warm. Spraying was done with a pneumatic hand-held spray gun with integral reservoir. The reservoir was regularly shaken during spraying to ensure that the small, undissolved crystals of natamycin stayed in suspension. The approximate minimum volume of spray required to evenly cover the whole surface of a muffin was measured and a concentration of 4.2 g per litre of Natamax™ SF calculated to target a spray delivery of 5 μg of natamycin per cm^2 of muffin in treatment 3. Each muffin was sprayed evenly over all surfaces.

[0057] After cooling, the sprayed muffins were packed into individual, heat sealed, clear plastic bags. One sample was selected from each of the two control treatments and five samples from the Natamax™ SF spray treatment and tested for water activity, pH, yeast and mould count at 25° C. and residual surface natamycin levels by HPLC analysis. Forty unopened muffins per treatment were put for shelf life evaluation at 25° C. and examined daily for signs of surface mould or yeast growth.

[0058] Results from analysis of the initial samples are shown in Table 4. The residual natamycin levels detected on the muffins sprayed with the Natamax™ SF suspension were found to be close to the target level of 5 μg per cm^2.

TABLE 4

Initial analysis results

Treatment	Residual natamycin $\mu g/cm^2$	Water activity (muffin outside)	Water activity (muffin inside)	Mould at 25° C./g	pH
1) No spray (control)	<0.2	0.855	0.867	<50	8.55
2) Water only (control)	<0.2	0.863	0.877	<50	8.65
3) 4.2 g/l Natamax ™ SF	5.1	0.863	0.879	<50	8.27
	4.3	NT	NT	<50	NT
	3.4	NT	NT	<50	NT
	3.8	NT	NT	<50	NT
	5.8	NT	NT	<50	NT

NT = Not Tested

[0059] Results from the incubation study of the 40 muffins per treatment are given in Table 5. The first non-spray control sample (treatment 1) developed surface mould growth after only 7 days giving a mould free shelf life of only 6 days. The first water sprayed control sample (treatment 2) developed surface mould growth after only 10 days. In contrast to this, all of the 40 muffins sprayed with Natamax™ SF suspension remained completely free of any surface mould growth for a total incubation period of 68 days, after which time the incubation was stopped. Thus, in this trial experiment, a natamycin surface spray treatment of approximately 5 $\mu g/cm^2$ increased the mould-free shelf life of muffins at 25° C. from 6 days to at least 68 days.

TABLE 5

Results after incubation at 25° C.

Number showing mould growth/Number under incubation

Days incubated at 25° C.	Treatment 1 No spray control	Treatment 2 Water only control	Treatment 3 4.2 g/l Natamax ™ SF
6	0/40	0/40	0/40
7	1/40	0/40	0/40
9	2/40	0/40	0/40
10	3/40	1/40	0/40
14	5/40	1/40	0/40
17	6/40	2/40	0/40
23	6/40	3/40	0/40
24	7/40	4/40	0/40
63	8/40	4/40	0/40
68	8/40	4/40	0/40

EXAMPLE 3

Natamycin Suspension on the Surface of Muffins

[0060] Natamycin suspension as such, as well as natamycin suspension containing a thickener was tested on the surface of muffins. The natamycin suspension contained 2000 ppm natamycin or 2000 ppm natamycin and 0.25% of thickener HPMC, respectively.

[0061] Muffins were prepared according to a standard recipe with no added preservative in the dough. Shortly after baking, one third of the muffins were sprayed whilst still warm with the natamycin suspension without a thickener and one third with the natamycin suspension including the thickener. Spraying was done using a pneumatic hand-held spray gun with integral reservoir. Each muffin was sprayed evenly over all surfaces with a minimum volume of finely adjusted spray.

[0062] After cooling, the sprayed muffins were packaged into heat sealed clear polythene bags. Unopened bags of muffins were put for shelf life evaluation at 25° C. and examined daily for signs of surface mould or yeast growth.

[0063] Natamycin treated muffin samples as well as untreated control muffin samples (which had no natamycin sprayed on the surface) were stored at ambient temperature (25° C.). Natamycin treated samples showed no mould after 16 days of storage, whereas the control samples displayed mould growth very quickly—after 5 days, as can be seen on the mould observation Table 6 below.

TABLE 6

Days until mould observed	Control/untreated muffin	Natamycin treated muffin	Natamycin + thickener treated muffin
1	0	0	0
2	0	0	0
5	0	0	0
6	1	0	0
7	2	0	0
8	3	0	0
9	4	0	0
10	4	0	0
11	4	0	0
12	4	0	0
13	5	0	0
14	5	0	0
15	5	0	0
16	5	0	0

Scale:
0 = no mould observed
5 = extensive mould spoilage

[0064] The Table shows quite clearly that the treatment with a natamycin suspension both with or without a thickener prevents mould growth on muffin.

[0065] The above examples clearly demonstrate the preservative efficacy of natamycin when sprayed on the outer surface of fine bakery products having a water activity above 0.8, which are susceptible to surface spoilage by moulds and yeasts during storage. Based on the description and examples a person skilled in the art is able to apply the invention to a wide variety of fine bakery goods.

1. A non-yeast leavened fine bakery product with increased shelf life comprising an intermediate or high moisture baked product having a water activity a_w>0.8, the surface of said bakery product having deposited thereon an effective amount of natamycin which is sufficient to keep said product mould free when packaged for a storage time of 2 weeks or more at ambient temperature.

2. The fine bakery product of claim 1, wherein said fine bakery product is selected from muffins, waffles, tortillas, sponge cakes, pizzas, pastry, pancakes, and the like baked or part-baked products.

3. The fine bakery product of claim 1, wherein the water activity a_w of said baked product is between 0.8 and 0.95.

4. The fine bakery product of claim 3, wherein the water activity a_w of said baked product is between 0.8 and 0.90.

5. The fine bakery product of claim 3, wherein the water activity a_w of said baked product is between 0.85 and 0.90.

6. The fine bakery product of claim 1, wherein the water activity of said baked product is sufficient to keep at least a part of said deposited natamycin in dissolved form.

7. The fine bakery product of claim 1, wherein said effective amount of natamycin comprises from 1 to 10 μg per cm^2 of the surface of said baked product.

8. The fine bakery product of claim 1, wherein said natamycin is deposited on said surface in a suspension which includes a thickener selected from the group consisting of agar, alginates, carrageenan, cellulose and derivatives, gums, gelatin, pectins and derivatives thereof, polyvinyl acetate, starches and modified starches, and suspending agents.

9. The fine bakery product of claim 1, wherein said natamycin is deposited on said surface in an amount sufficient to keep said baked product mould free for a storage time of 3 to 10 weeks.

10. The fine bakery product of claim 9, wherein said natamycin is deposited on said surface in an amount sufficient to keep said baked product mould free for a storage time of 6 to 10 weeks.

11. The fine bakery product of claim 1, wherein said ambient temperature comprises a temperature of 15 to 30° C.

12. The fine bakery product of claim 11, wherein said ambient temperature is from 18 to 25° C.

13. The fine bakery product of claim 1, wherein said product is packaged in a protective envelope.

14. The fine bakery product of claim 13, wherein said envelope is of a moisture proof and/or transparent material.

15. A process for increasing the shelf life of fine bakery products, comprising

providing a baked fine bakery product having a water activity a_w>0.8;

spraying the outer surface of said baked product with natamycin to deposit an effective amount of natamycin thereon;

packaging said sprayed baked product into a protective envelope; and

storing said packaged product at ambient temperature;

the natamycin deposited on the surface of said bakery product being effective in keeping said product mould free even when stored for 3 weeks or more.

16. The process of claim 15, wherein said natamycin is sprayed onto said surface in the form of an aqueous suspension.

17. The process of claim 16, wherein said suspension includes a thickener selected from the group consisting of agar, alginates, carrageenan, cellulose and derivatives, gums, gelatin, pectins and derivatives thereof, polyvinyl acetate, starches and modified starches, and suspending agents.

18. The process of claim 16, wherein said suspension contains natamycin in a concentration of 250 to 7000 mg natamycin per litre of water.

19. The process of claim 18, wherein said suspension contains natamycin in a concentration of 1000 to 4000 mg natamycin per litre of water.

20. The process of claim 16, wherein said natamycin suspension contains dissolved natamycin and crystalline natamycin.

21. The process of claim 15, wherein said effective amount of natamycin comprises from 1 to 10 μg per cm^2 of the surface of said baked product.

22. The process of claim 15, wherein said natamycin is sprayed homogeneously on all outer surfaces of said baked product.

* * * * *

Bibliography (a copy of all cited literature is included)

1. Stark, J. (2003) Natamycin: an effective fungicide for food and beverages. In: Natural antimicrobials for the minimal processing of foods (Roller, S., ed.), pp. 82-97. CRC Press LCC, Boca Raton.

2. Stark, J. & Tan, H. S. (2003) Natamycin. In: Food Preservatives, Second Edition ed. (Russell, N. J. & Gould, G. W., eds.), pp. 179-195. Kluwer Academic / Plenum Publishers, New York.

3. Thomas, L. V. & Delves-Broughton, J. (2003) Natamycin. In: Encyclopedia of Food Sciences and Nutrition, Second Edition ed. (Caballero, B., Trugo, L. C. & Finglas, P. M., eds.), pp. 4109-4115. Academic Press, Amsterdam.

4. Budavari, S. (1996) Natamycin. In: Merck Index, 12th Edition ed. (Budavari, S., ed.). Merck & Co., Whitehouse Station, NJ.

5. Struyk, A. P., Hoette, I., Drost, G., Waisvisz, J. M., van Eek, T. & Hoogerheide, J. C. (1958) Pimaricin, a new antifungal antibiotic. In: Antibiotics Annual 1957-1958 (Welch, H. & Marti-Ibanez, F., eds.), pp. 878-885. Medical Encylopedia, Inc., New York.

6. Burns, J. & Holtman, D. F. (1959) Tennecetin: A new antifungal antibiotic. Antibiotics and Chemotherapy 9: 398-405.

7. Demeter International e.V. (2004) Standards for the certification of DEMETER milk and dairy products. In: Processing Standards, pp. 42-47.

8. European Communities (2000) Regulation (EEC) No 207/93, defining content of Annex VI to Regulation (EEC) No. 2092/91. Office for Official Publications of the European Communities.

9. Food and Drug Administration (2005) 21 CFR 172.155 Natamycin (pimaricin). In: Title 21 Code of Federal Regulations. Government Printing Office.

10. Food Standards Australia New Zealand (2004) Initial Assessment Report. Application A542. Natamycin - extension of use as a food additive, p. 14.

11. JECFA (2002) Evaluation of certain food additives and contaminants: Natamycin (Pimaricin). In: World Health Organisation Joint Expert Committee on Food Additives Food Additive Series:48, pp. i-x, 1-171, back cover.

12. Anonymous (2003) JECFA Compendium of food additive specifications: Natamycin. In: World Health Organisation Joint Expert Committee on Food Additives Food Additive Series:52, pp. 49-52.

13. Committee on Food Chemicals Codex (2004) Natamycin. In: Food Chemicals Codex, Fifth Edition ed. (Committee on Food Chemicals Codex, Food and Nutrition

Board, Institute of Medicine, ed.), pp. 298-299. National Academies Press, Washington, DC.

14. Hamilton-Miller, J. M. (1974) Fungal sterols and the mode of action of the polyene antibiotics. Adv Appl Microbiol 17: 109-134.

15. Norman, A. W., Spielvogel, A. M. & Wong, R. G. (1976) Polyene antibiotic - sterol interaction. Adv Lipid Res 14: 127-170.

16. (2003) Natamycin (Pimaricin). In: Ullmann's Encyclopedia of Industrial Chemistry, Sixth Edition ed., pp. 602-603. Wiley-VCH, Weinheim.

17. Cauwenburgh, G. (1992) Antimycotic Antibiotics. In: Kirk-Othmer Encyclopedia of Chemical Technology, pp. 475-477. John Wiley & Sons, New York.

18. Mohamed, M. A., Ranjard, L., Catroux, C., Catroux, G. & Hartmann, A. (2005) Effect of natamycin on the enumeration, genetic structure and composition of bacterial community isolated from soils and soybean rhizosphere. J Microbiol Methods 60: 31-40.

19. Luther, H. G. (1974) Antibiotics in Foods. In: Encyclopedia of Food Technology (Johnson, A. H. & Peterson., M. S., eds.), pp. 31-43. Avi Publishing Co., Inc., Westport, Conn.

5

Natamycin: an effective fungicide for food and beverages

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5.1 Introduction

Natamycin was discovered in the 1950s and was described for the first time by Struyck as follows:

> A new crystalline antibiotic, pimaricin, has been isolated from the fermentation broth of a culture of a *Streptomyces* species, isolated from a soil sample obtained near Pietermaritzburg, State of Natal, Union of South Africa. This organism has been named *Streptomyces natalensis* (Struyck *et al.*, 1957–58).

Today, almost half a century later, the antimycotic natamycin (the name used in earlier literature was pimaricin) is still widely used for the prevention of mould and yeast growth on food products.

Natamycin is produced by fermentation using *Strep. natalensis*. It can be recovered using extraction, filtration and drying. The dry powder is sufficiently stable to be stored for years without substantial loss of activity. Besides its broad-spectrum activity against moulds and yeasts, the biopreservative natamycin has some unique characteristics, which make it particularly suitable for preventing fungal growth on the surface of food products and in beverages. It is safe for the consumer, is effective at low concentrations, has no negative effects on the quality of food products, remains on the surface of the cheese or sausage and has a prolonged working time. Although natamycin has been used for almost 40 years in the food industry, the development of resistant strains has not been reported to date. As natamycin has no antibacterial activity, the bacterial ripening processes of food products such as cheese and sausages are not influenced in a negative way.

5.2 Chemical and physical properties

Natamycin belongs to the group of polyene macrolide antimycotics. This group is characterised by its macrocyclic lactone-ring with a number of conjugated carbon–carbon double bonds (Fig. 5.1). The molecular weight of natamycin is 665.75. Its empirical formula is $C_{33}H_{47}NO_{13}$. Its CAS registry number is 7681-93-8. The correct gross structure was determined using proton- and ^{13}C-NMR (nuclear magnetic resonance) spectroscopy (Ceder *et al.*, 1977). The complete three-dimensional structure was determined later (Lancelin and Beau, 1990, 1995; Duplantier and Masamune, 1990). The isoelectric point of natamycin is 6.5 (Raab, 1974).

Natamycin is a white or creamy-white powder with almost no odour or taste. Its most common form, the trihydrate, can be stored for many years at room temperature, provided the powder is protected from moisture and light. Even after several years of storage under these conditions only a few per cent loss of activity is observed. Neutral aqueous suspensions of natamycin are also very stable. A suspension containing 0.5% natamycin at pH 6.5 lost hardly any activity at room temperature after two years of storage in the dark (Clark *et al.*, 1964). Under these conditions, most of the natamycin is in the stable crystalline form. Only a minor fraction (about 40 ppm) is in solution. Although dissolved natamycin is relatively unstable, these results demonstrate that under optimal storage conditions it can be quite stable. The amphoteric character of natamycin is responsible for its low solubility in most solvents. The solubility is increased at high or low pH values (Brik, 1981).

At extreme pH values, natamycin is completely soluble. Since only the dissolved fraction has antifungal activity, extremes of pH can be used to

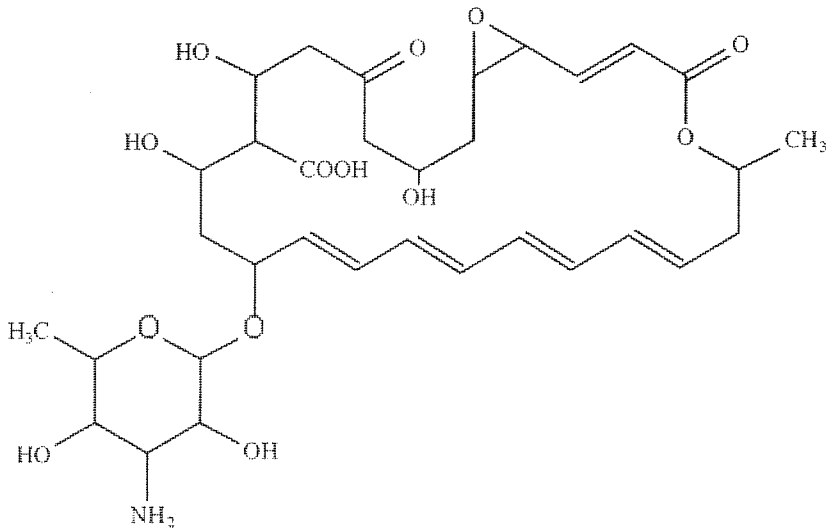

Fig. 5.1 Structure of natamycin.

optimise/control the availability of active natamycin. However, natamycin is rapidly decomposed under these conditions. Therefore such solutions are effective only when used immediately after preparation. In practice, this way of applying natamycin might be beneficial in cases of extreme contamination, but under normal hygienic conditions there is no added value in this approach. Therefore, such treatment is not normally recommended.

Solid natamycin and natamycin suspensions are quite stable to heat and it has been reported that natamycin suspensions could withstand a temperature of 50 °C for several days without losing much activity. Even heating for several hours at 100 °C causes only a slight decrease of its activity.

Natamycin is decomposed by ultraviolet light with loss of the tetraene structure so it is best stored in the dark. Gamma irradiation decomposes natamycin as well. Natamycin is inactivated by chlorine, peroxides, antioxidants, sulphites and sodium formaldehyde sulphoxidate (Brik, 1981). Some of these compounds are applied as cleaning agents in the food industry. Uncontrolled use of chlorine in a factory may indirectly induce mould growth on cheese. In the presence of deactivating compounds or less optimal conditions, solid natamycin is the most stable form. Dissolved natamycin is much more susceptible to inactivation. Under neutral aqueous conditions most of the natamycin on the surface of a food product or in a stock suspension is present as the more stable crystal. Under extremely unfavourable conditions, rapid elimination of the dissolved fraction will ultimately lead to complete decomposition of natamycin through enhanced dissolution.

Natamycin trihydrate, crystallised from aqueous solvents, contains approximately 7.5% water. Methanol extracts can be obtained by crystallisation from a saturated methanolic solution. As soon as the methanol extract is in contact with water, it rapidly converts to the trihydrate form. The anhydrous form of natamycin can be prepared by drying the trihydrate in vacuum at room temperature over phosphorus pentoxide. The anhydrate is unstable; it loses 15% of its activity when stored for 48 hours under optimal conditions (Brik, 1981). However, when stored under nitrogen or at low temperatures it is more stable (van Rijn *et al.*, 1995). The barium and calcium salts of natamycin and a new crystalline form, also a trihydrate, have been described by van Rijn *et al.* (1995). The stability of the new trihydrate is somewhat less than the stability of the regular type. The salts in their solid form are stable but, once in contact with water, they dissolve rapidly and are converted into the more stable regular trihydrate. In principle, this phenomenon could be used to obtain a highly active preparation of dissolved natamycin for a short period of time.

5.3 Mechanism of action

The eukaryotic cell membrane contains lipids, phospholipids, proteins and sterols. The sterols play an important role in the selective action of polyene antimycotics. Ergosterol is the major sterol in the cell membrane of moulds and

yeasts, while cholesterol is the major sterol in the mammalian cell membrane. Natamycin has a high affinity for ergosterol and binds irreversibly to the ergosterol in the fungal cell membrane. This perturbs the permeability of the cell membrane, which leads to rapid leakage of essential ions and small peptides and so causes cell lysis.

The detailed mechanism of action of natamycin is thought to resemble that of another polyene antimycotic, amphotericin B. The latter forms chiral aggregates at the bilayer–water interface. In the absence of ergosterol, the aggregates remain at the surface of the bilayer (Milhaud *et al.*, 2002). In the presence of ergosterol, the aggregates are embedded in the phospholipid bilayers. The embedding in the hydrophobic bilayer requires a rearrangement of the aggregates that is, in turn, responsible for the hollowing of the aggregates. These structures are thought to be the precursors of pore formation.

Vesicles of *Acholeplasma laidlawii* have been prepared with either ergosterol or cholesterol as the membrane sterol. When the vesicles contained 20–40 mol% of ergosterol, which is equivalent to the ergosterol content of a fungal cell membrane, natamycin induced leakage of potassium ions. Natamycin caused no damage when only cholesterol was present in the vesicles (De Kruijff *et al.*, 1974; Teerlink *et al.*, 1980). It can be concluded that ergosterol is the molecular target for natamycin on the fungal cell membrane. Since bacteria do not contain sterols in their cell membrane, they are not susceptible to lysis by natamycin.

Studies with amphotericin B suggest that the cell wall forms a natural barrier to the fungicide. This protective effect increases as the culture ages (Malewicz and Borowski, 1979). It is possible that a similar protective effect occurs in the case of natamycin. This is supported by observations made in practice, which indicate that natamycin is very effective in preventing fungal growth, but ineffective in killing mature fungal tissue. Therefore natamycin cannot be used as a disinfectant. Most moulds grow by extension of the hyphal tip and the cell wall is formed just behind the new tissue of the hyphal tip. If the cell wall indeed prevents interaction of natamycin with the cell membrane, this should mean that natamycin stops the growth of the fungal mycelium only at the tip of the growing hypha by interacting with the fungal cell membrane. Thus the cell wall prevents direct interaction of natamycin with the older tissue in the lower part of the hyphae. The older cells are not killed directly but are also unlikely to survive. Natamycin also inhibits the germination of fungal spores by an unknown mechanism.

Mycotoxins produced by fungi can cause health problems and some are carcinogenic. Removal or elimination of moulds after growth on a food does not affect mycotoxins, which can migrate deep into the foodstuff. Therefore, it is extremely important to prevent growth of toxigenic fungi on food products. The growth-inhibiting mode of action of natamycin lends itself well to this application.

5.4 Sensitivity of moulds and yeasts to natamycin

Natamycin is active against most fungi at low concentrations. The minimum inhibitory concentration (MIC) for natamycin against almost all foodborne fungi is less than 20 ppm, while the solubility of natamycin in aqueous food systems is around 40 ppm. It has been demonstrated in practice that under acceptable hygienic conditions, this concentration of dissolved natamycin is sufficient to prevent fungal growth.

Struyk *et al.* (1957–58) determined the MICs of natamycin against 66 moulds and yeasts, including food spoilage strains and those that were pathogenic to people and plants. The majority of yeasts and moulds were inhibited at concentrations of 1–15 ppm of natamycin. Some species of *Trichophyton* and *Phythium* were less sensitive but these were irrelevant for the food industry. Struyk concluded that natamycin has a broad spectrum of activity against fungi, is fungicidal and is not effective against bacteria. Over the past 50 years, the original conclusions of Struyk have been confirmed in many studies using a wide range of species of relevance to the food industry (Tables 5.1–5.3).

Several well-known methods for determining the MICs of antimicrobials against bacteria and yeasts are recognised and used widely. By contrast, there are no standardised methods for the determination of MICs for filamentous fungi. The quantities of mould spores used by different investigators can vary or are not

Table 5.1 Sensitivity to natamycin of fungi isolated in Dutch cheese factories and warehouses

Microorganism	MIC* (ppm)
Aspergillus flavus & parasiticus	10–20
Aspergillus fumigatus, penicillioides & versicolor *Cladosporium candidum* *Debaryomyces hansenii* *Eurotium herbariorum* *Geotrrichum candidum* *Mucor racemosus* *Penicillium brevicompactum, camembertii, commune, corylophilum, glabrum, nalgiovense, roquefortii & solitum* *Scopulariopsis brevicaulis & fusca* *Syncephalastrum racemosum* *Wallemia sebi*	< 10
Penicillium discolor[+]	20–30

* 5μl of a suspension of 10^5 spores/ml was spotted on agar plates containing 0, 10, 20, 30 or 40 ppm of natamycin and incubated at 24 °C for 6 days. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of natamycin at which no growth was observed. The MIC of natamycin for most moulds and yeasts is most likely < 5 ppm (see Tables 5.2 and 5.3).
[+] At a concentration of 10^4 spores/ml the MIC for *Pen. discolor* is 10–20 ppm.
Source: from DSM Food Specialties Research Laboratorium Delft, The Netherlands; Dr R.A. Samson, Centraal Bureau voor Schimmelcultures, Utrecht, The Netherlands.

Table 5.2 Sensitivity to natamycin of fungi occurring on sausages*

Microorganism	MIC (ppm)*	Source of microorganism
Moulds		
Alternaria alternata	< 5	Blood
Aspergillus flavus	10–20	Air
Aspergillus niger	< 5	Fruit
Aspergillus versicolor	< 5	Soil
Cladosporium cladosporioides	< 5	Meat stamp
Eurotium appendiculatum	< 5	Smoked sausage
Eurotium herbariorum	< 5	Board
Geotrichum candidum	5–10	Soil
Mucor racemosus	< 5	Sausage
Penicillium chrysogenum	< 5	Meat
Penicillium glabrum	5–10	Soil
Penicillium nalgiovense	< 5	Sausage
Penicillium verrucosum	< 5	Meat
Rhizopus stolonifer	5–10	Bread
Wallemia sebi	< 5	Sea salt
Yeasts		
Candida zeylandoides	< 5	Sausage
Cryptococcus laurentii	< 5	Air
Debaryomyces hansenii	< 5	Sausage
Rhodutorula mucilaginosa	< 5	Air
Trichosporon pullulans	< 5	Frozen beef

* The selection of strains was based on their occurrence on sausages and meat products. When not available from meat sources, representative isolates were selected.
* 0.1 ml of a suspension of 10^4 spores/ml (moulds) or CFU/ml (yeasts) was spread on agar plates containing natamycin and incubated at 24 °C for 7 days. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of natamycin at which no growth was observed.
Source: from DSM Food Specialties Research Laboratorium Delft, The Netherlands; Dr R.A. Samson, Centraal Bureau voor Schimmelcultures, Utrecht, The Netherlands.

reported at all, although this is just as important as the concentration of fungicide added to the medium. Nevertheless, in most cases, the MIC of natamycin is independent of spore concentration. The manufacturer DSM Food Specialties recommends spreading 0.1 ml of a suspension of at least 10^4 spores/ml on an agar plate. This concentration of spores is high but reflects the levels of contamination that can be expected in practice. The use of fungal mycelia for determining MICs is not advised, as it is impossible to obtain reliable cell counts.

It is recommended that freshly prepared spores are used in MIC determinations. Moulds are grown on appropriate media such as malt extract agar (MEA) and incubated in darkness at 24 °C for 7–10 days. The spores are gently scraped from the agar surface and suspended in sterile water with 0.05% Tween 80. Stock suspensions are diluted using sterile water. A spore suspension of 0.1 ml is spread on suitable agar plates containing different concentrations of natamycin. Alternatively, a droplet of 5 μl of the concentrated spore suspension may be spotted onto the agar. After incubation for 4–6 days in darkness at 24 °C, the

Table 5.3 Sensitivity to natamycin of fungi isolated from beverages and fruit products

Microorganism	MIC (ppm)*	Source of microorganism
Yeasts		
Saccharomyces cerevisiae	1.5	Grape juice
Saccharomyces cerevisiae	1.5	Apple juice
Saccharomyces exiguus	< 1.0	Soft drink
Saccharomyces carlsbergensis	< 1.0	Brewers yeast
Zygosaccharomyces microellipsoides	1.0	Apple juice
Zygosaccharomyces microellipsoides	2.5	Orange juice
Zygosaccharomyces bailii	1.0	Apple juice
Zygosaccharomyces bailii	1.0	Pear juice
Zygosaccharomyces rouxii	1.0	Strawberry juice
Schizosaccharomyces pombe	< 1.0	Grape juice
Brettanomyces bruxellensii	2.5	Wine
Brettanomyces intermedius	< 1.0	Wine
Dekkara anomala	2.5	Stout
Dekkara anomala	5.0	Soft drink
Candida etchellsii	< 1.0	Concentrated lemon juice
Candida glabrata	4.0	Concentrated orange juice
Debaromyces hansenii	1.0	White wine
Pichia membranaefaciens	1.0	Lemonade
Pichia kluyverii	1.0	Fruit
Pichia angusta	< 1.0	Orange juice
Lodderomyces elongisporus	2.0	Concentrated orange juice
Torulaspora dellbreuckii	< 1.0	Lemonade
Hanseniaspora osmophila	< 1.0	Cider
Williopsis saturnus	1.0	Fruit juice
Rhodutorula rubra	< 1.0	Yoghurt
Moulds		
Byssochlamys nivea	2.0	Fruit juice
Trichosporonoides nigrescens	5.0	Melon jam
Mucor circinelloides	2.0	Strawberries
Stemphiliomma valparadisiacum	4.0	Apple juice
Talaromyces macrosporus	2.0	Pineapple juice
Cladosporium tenuissimum	2.0	Fruit
Zygosporium mycophilum	2.0	Apple pulp
Gilbertella persiacaria	10.0	Fruit

* MICs were determined as described in Table 5.2.
Source: from DSM Food Specialties Research Laboratorium Delft. The Netherlands.

MIC is determined as the lowest concentration of natamycin at which no growth is observed.

During storage of the agar plates, the fully dissolved natamycin is partly inactivated. After 20 days at 24 °C, the concentration of natamycin in MEA plates can be reduced by up to 50% (unpublished results, DSM Food Specialties). Therefore, it is recommended that only freshly prepared agar plates are used and that the concentration of natamycin in control agar plates is checked analytically at the end of prolonged incubation times.

Natamycin is poorly soluble in water and its crystals have no antifungal activity. Therefore, experiments to determine the MIC using agar plates containing natamycin suspensions higher than its maximum solubility in neutral aqueous environments are scientifically meaningless. However, such experiments might be useful to examine the prolonged working time of natamycin, e.g. in a cheese model.

Several surveys on the mycoflora in the cheese industry have been reported (De Boer and Stolk-Horsthuis, 1977; Fente-Sampayo, 1995; Hoekstra *et al.*, 1998). Natamycin sensitivity of fungi from Dutch cheese warehouses was determined by De Boer and Stolk-Horsthuis (1977). The main fungi detected were *Penicillium* and *Aspergillus* species although *Cladosporium*, *Scopulariopsis* and *Acremomium* species were also isolated. MICs of less than 10 ppm were reported for 26 species. More recently, Hoekstra *et al.* (1998) reported that a stable 'home flora' was present in all cheese factories. Environmental conditions, especially humidity, determine which species becomes predominant. In the more humid areas of a cheese production site, the composition of the mycoflora differed from that in the dryer areas of the factory. The predominant fungi detected in cheese factories were *Penicillium brevicompactum*, *Pen. corylophilum*, *Aspergillus penicillioides*, *Cladosporium* species and yeast species. The yeast *Debaryomyces hansenii* was predominant in the brines of several factories. The predominant fungi in cheese warehouses were *A. penicillioides* and *A. versicolor*. The sensitivity to natamycin of the most important fungi isolated in this study is shown in Table 5.1. Only three organisms, *A. flavus*, *A. parasiticus* and *Pen. discolor*, had a MIC greater than 10 ppm. In practice, the amount of active natamycin of about 40 ppm present on cheese surfaces is sufficient to inhibit all these species.

Fente-Sampayo *et al.* (1995) determined the composition of the fungal flora on soft cheeses and the production environment in 10 farm-level cheese production plants in Spain. In total, 35 species were isolated. Species of the genera *Penicillium*, *Aspergillus*, *Cladosporium*, *Rhizopus* and *Geotrichum* were most frequently isolated. The MIC for natamycin of all isolates was 10 ppm or less, except for a *Geotrichum* species that had a MIC of 12.5 ppm. A survey of 16 Dutch factories producing dry sausages was carried out to isolate less sensitive moulds and yeast using agar plates containing 0, 1, 2, 3 or 4 ppm of natamycin (De Boer *et al.*, 1979). Airborne yeasts and moulds were isolated using uncovered Petri dishes. For each natamycin concentration, 20 plates were used per factory. Yeasts and moulds from various objects in manufacturing and storage areas were isolated using Rodac plates (30 per factory) or swabs (25 per factory). Growth was observed on very few plates containing 2 ppm natamycin. More recently, the sensitivity to natamycin of fungi isolated from sausages was determined, as shown in Table 5.2. Again, a concentration of 10 ppm of natamycin was sufficient to inhibit most species.

Yeasts are predominant in the spoilage of acidic beverages such as fruit juices, lemonades, wine and beer. Some moulds, especially species producing heat-resistant spores or with thicker heat-resistant hyphae, are also associated

with the spoilage of beverages and pasteurised fruit products. Important heat-resistant spoilage moulds are species belonging to the genera *Byssochlamys* and *Talaromyces*. Some strains are also a health risk, since the mycotoxin patulin is produced. Ascospores of *Byssochlamys* can survive several minutes at 90 °C. This mould is also able to grow at low O_2 or elevated CO_2 concentrations. Its unique characteristics give it a selective advantage in products such as fruit juices and canned fruits.

In a German study the sensitivity of 83 yeasts to natamycin was examined (Henninger, 1977). None of the yeasts isolated from food products were able to grow at a concentration of 3 ppm of natamycin. More recently, the MICs for natamycin of yeasts associated with spoilage of beverages and fruit products were determined, as shown in Table 5.3. The MICs of most species was less than 2.5 ppm. Notably, several sorbate-resistant yeast species, which can cause severe spoilage problems in the food industry, were also inhibited at these low concentrations of natamycin. *Zygosaccharomyces bailii*, a well-known sorbate-resistant yeast was inhibited by 1 ppm of natamycin. In the same study, many heat-resistant moulds of relevance to the food industry were also inhibited by as little as 2.5 ppm of natamycin.

5.5 Resistance

After decades of continuous use of natamycin, no resistant moulds and yeasts have been reported. This is quite remarkable, since most preservatives induce resistance. For example, several moulds and yeasts resistant to sorbate have been isolated. However, it is unlikely that fungi will develop resistance to natamycin because it interacts with ergosterol, a major constituent of the fungal cell membrane. Although low-ergosterol or ergosterol-free mutants that are also resistant to natamycin have been produced in the laboratory, they have slower growth rates and are unlikely to survive in nature (Ziogas *et al.*, 1983; Hamilton-Miller, 1974).

Furthermore, there is no separation between fungistatic and fungicidal concentrations of natamycin. Natamycin appears to have an all-or-none effect, which destroys the cell membrane without noticeable prior damage (Kotler-Brajtburg *et al.*, 1979). An interesting additional explanation is the single-hit theory. It is suggested that in aqueous solutions natamycin always occurs as micelles. If such a micelle comes into contact with a fungal cell, the local concentration of natamycin is always high enough to kill the cell immediately. Therefore selection of resistant mutants is unlikely.

Attempts have been made to induce natamycin tolerance in moulds from cheese warehouses by serial transfer onto media with increasing concentrations of natamycin (De Boer and Stolk-Horsthuis, 1977). After 25–30 transfers, none of the 26 strains studied became obviously less sensitive to natamycin. A study with *Penicillium discolor* (Frisvad *et al.*, 1997), a relatively natamycin-tolerant mould (see Table 5.1), showed that on agar plates inoculated with 10^2 spores/ml,

the MIC was 10–20 ppm. However, when the agar plate was inoculated with 10^3–10^4 spores/ml, the MIC was 20–30 ppm. These results illustrated the importance of control of inoculum size in MIC determinations. Moreover, attempts to induce resistance in *Pen. discolor* have not been successful (unpublished results, DSM Food Specialties). It is concluded that *Pen. discolor* is a more tolerant mould species due to natural variation.

5.6 Applications

5.6.1 Surface treatments

One of the advantages of natamycin over other preservatives such as sorbate is that natamycin crystals remain on the surface of the product. Also, the dissolved fraction of natamycin hardly penetrates the product. In a study with Gouda cheese, no migration of natamycin was observed further than 3–4 mm into the rind (Daamen and van den Berg, 1985). In another study, a maximal penetration depth of 2.6 mm in Tilsiter cheese was reported (Kiermeier and Zierer, 1975). As another example, natamycin can be used to ensure a clean surface on blue cheese without affecting the internal mould growth in the punch holes of the cheese (Morris and Castberg, 1980). Growth of spoilage moulds on the surface of blue cheese must be avoided as it closes the punch holes and inhibits the development of *Penicillium roquefortii*, which give the cheese its characteristic appearance and flavour. Dipping the blue cheese in a suspension of 1 g/l of natamycin was sufficient to prevent fungal growth on the surface for three weeks, while the development of the blue mould in the punch holes was not affected.

Another advantage of natamycin is its prolonged working time. In practice, cheeses or sausages are treated with coatings, casings or suspensions containing 100–2000 ppm of natamycin. The initial concentration of natamycin on the surface of the product depends on the treatment. Usually this concentration is 1–2 mg/dm^2. At these concentrations, only a fraction of 30–40 ppm is in solution. Most of the natamycin is present in the form of stable crystals. Disappearance of dissolved natamycin, e.g. due to interaction with fungal cell membranes, diffusion or chemical decomposition, is compensated for by dissolution from the crystals and by diffusion over the surface of the product. This protects the product for a longer period of time against fungal growth.

Many scientific studies have been published about the successful application of natamycin on almost every type of cheese: Gouda (Lück and Cheesman, 1978; Engel *et al.*, 1983; De Ruig and van den Berg, 1985; Daamen and van den Berg, 1985), Edam (Engel *et al.*, 1983), Cheddar (Lück and Cheesman, 1978; Sachdeva *et al.*, 1994); Tilsiter (Engel *et al.*, 1983), Italian cheeses such as Caciotta (Neviani *et al.*, 1981) and Fontina, Tallegio, Montasio, Asiago, Provolone, Pecorino and Romano (Lodi *et al.*, 1989); Swedish hard cheeses (Mattson, 1977); blue cheese (Morris and Castberg, 1980); Indian cheeses (Verma *et al.*, 1988; Pugazhenthe *et al.*, 1999). For the surface treatment of

cheese, natamycin is usually added to the aqueous polymer dispersion, the so-called plastic emulsion that is applied to the cheese rind as a coating. On cheese types that are not treated with a coating, natamycin can be applied by dipping or spraying. Natamycin can also be added to the brine as an alternative treatment for round cheeses such as Edam (Zuthof and Isidorus, 1981).

The antifungal effect of natamycin on sausages has also been extensively studied. Natamycin prevents fungal growth during normal ageing and storage without affecting the quality of Dutch raw sausages (Moerman, 1972), German raw sausages (Hechelman and Leistner, 1969; Stiebing *et al.*, 2001) and Italian sausages such as dry salami (Cattaneo *et al.*, 1978; Baldini *et al.*, 1979; Holley, 1981, 1986). Sausages are treated with natamycin by dipping (e.g. at 500–1000 ppm) or spraying (e.g. at 2000 ppm). Natamycin can also be applied by treatment of sausage casings before stuffing by soaking for 1–2 h in a suspension containing 500–1000 ppm of natamycin.

The efficacy of natamycin and sorbate in the prevention of mould growth on raw German sausages has been compared (Stiebing *et al.*, 2001). Sausages were dipped in suspensions containing 0.2% natamycin or a solution containing 10% sorbate. It was demonstrated that natamycin treatment was superior to that afforded by sorbate. Sorbate gave less protection against mould growth, inhibited the bacterial starter cultures, causing a delay of the ripening process, which led to defects in the colour development of the product and penetrated into the sausages. In the case of natamycin no negative effects on the quality of the product were observed.

Natamycin can also be used to prevent fungal growth on fruit. Strawberries, cranberries and raspberries can be sprayed in the field. A more effective protection is obtained when the berries are dipped in a suspension of 10–100 ppm natamycin after harvest. The shelf-life of the berries was prolonged by several days (Ayres and Denisen, 1958). Natamycin was shown to be effective in preventing fungal growth on the surface of apples and pears. Treatment with a lecithin emulsion containing 200 ppm of natamycin was the most effective way to prevent fungal growth (Staden and Witmondt, 1967).

5.6.2 Beverages

Owing to its optimal availability, low concentrations of natamycin (1–5 ppm) are sufficient to prevent fungal spoilage of juices, lemonades, beer, wine, iced tea and fruit yoghurts. Natamycin reduces the initial mould and yeast population rapidly. This treatment is sufficient since after production the packaging of most beverages is usually well sealed and contamination before the consumer opens the packaging is unlikely. It has also been demonstrated that low concentrations of natamycin (1–20 ppm) are quite stable in orange juice. After 12 weeks of refrigerated storage, 70% of the initial natamycin remained active (Shirk and Clark, 1963). This means that natamycin is also suitable to protect 'fresh' beverages with a short shelf-life, such as chilled orange juice, against fungal spoilage. Natamycin is also effective in protecting iced tea, a beverage with

higher pH values. Sorbic acid is currently used in iced tea but the pH of the product has to be reduced for the sorbic acid to be effective. Both the low pH and the taste of the sorbic acid itself can have adverse effects on the organoleptic properties of iced tea.

5.7 Toxicology

The mutagenicity, absorption, distribution, excretion and metabolism of natamycin have been investigated in several studies. Mutagenicity has been examined with the Ames test using two *Salmonella* Typhimurium strains and two *Escherichia coli* strains. Another mutagenicity test was executed using a wild type *Bacillus subtilis* and a mutant deficient in its DNA repair system. If the mutant was more sensitive to natamycin than its wild-type parent, it could be concluded that natamycin acted on DNA. The degradation products aponatamycin, dinatamycinolidediol and mycosamine and the commercial product Delvocid® were included in these studies. All the mutagenicity tests were negative.

In several studies it was demonstrated that natamycin and its degradation products are hardly absorbed by the body. After oral administration of ^{14}C-labelled natamycin to rats, none of the label was detected outside the gastrointestinal tract. After a very long exposure time of 150 days, small amounts were detected in the liver, kidney and fatty tissue. Similar results were found with dogs. Acute intraperitoneal toxicity of natamycin and its degradation products was determined in mice. Neither natamycin nor its degradation products presented a toxic risk. Finally, the very long history of safe use of natamycin as food additive confirms that natamycin is a safe fungicide for preventing growth of moulds and yeasts in foods and beverages.

5.8 Regulatory status for use in foods

Natamycin is allowed for use in many countries as a food additive in a variety of foods to control the growth of yeasts and moulds. The primary food uses are in the surface treatment of cheese and sausages. These uses are covered in the Food and Agriculture Organization/World Health Organization (FAO/WHO) Codex Alimentarius (see Chapter 15 for details of international food regulations). Several Codex standards for cheese permit the presence of natamycin on the cheese surface/rind at a level of 2 mg/dm^2 to a maximum depth of 5 mm. Recently, the use of natamycin was incorporated in a new Codex group standard for unripened cheese and fresh cheese. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed the safety of natamycin in 1968, 1976 and 2001. JECFA assigned an Acceptable Daily Intake value (ADI) of 0.3 mg/kg body weight per day. This ADI was reaffirmed by JECFA during the latest review of natamycin in 2001.

In the European Union, natamycin is permitted as a food additive for the surface treatment of hard, semi-hard and semi-soft cheese and dried, cured sausages. The maximum permitted level is 1 mg/dm^2 surface and it must not be present at a depth greater than 5 mm (EU Directive 95/2/EC). In the USA, natamycin is an approved food additive. It may be applied on cheese in amounts not to exceed 20 mg/kg in the finished product as determined by International Dairy Federation Standard 140A: 1992 (21 CFR 172.155). In Canada, natamycin is permitted on 47 named cheeses up to 20 ppm and in shredded/grated cheese up to 10 ppm residual level (*Canada Gazette* Part II, Vol. 116, no. 7, 14 April 1982).

In Australia, natamycin is approved in cheese and cheese products at a level of 15 mg/kg on cheese surfaces based on individual cheese weight. It is also allowed on fermented, uncooked processed comminuted meat products when determined in a surface sample taken to a depth of not less than 3 mm and not more than 5 mm including the casing, applied to the surface of food (ANZA Food Standard 1.3.1). In South Africa, natamycin is allowed in a broad range of food products and beverages (*Government Gazette* no. 8436, 1982, and no. 5729, 1977). Elsewhere, the permitted uses differ from country to country. In most non-EU European countries, natamycin is allowed for the surface treatment of cheeses and/or sausages or other meat products. In Brazil, Argentina and Venezuela, it is allowed for the application on cheese and sausages. In Colombia it is only allowed for the treatment of meat products. In countries such as Mexico, Chile, Costa Rica and some Arab countries, it is permitted as a general food additive. Since regulations may change, one always has to check the regulation in a country before applying natamycin in food products.

5.9 Future prospects

Natamycin has a long history of safe use in the prevention of fungal growth on the surface of cheese and sausages. In the future, economic losses due to spoilage and health risks associated with the growth of pathogenic or mycotoxin-producing moulds will remain important issues. Therefore, it is expected that natamycin will remain the most important antifungal agent for the surface treatment of cheese and sausages. Although natamycin has also been shown to inhibit moulds and yeasts in other foods and beverages at very low concentrations (1–5 ppm), its use in other food products is still not permitted in most countries. The recent inclusion of natamycin in a new Codex group standard for unripened cheese and fresh cheese can be considered as a first step towards a more general approval in the future.

The increasing occurrence of sorbate-resistant yeasts and heat-resistant moulds will require improved preservation systems. Also, the trend towards more fresh products such as fruit juices represents a severe risk of increasing mould problems. Sterilization processes will be replaced by milder heat

treatments or by new mild preservation techniques such as ultra high pressure or pulsed electric field processing. These techniques will improve the organoleptic and nutritional properties of the products; however, they will also introduce new spoilage problems by selecting resistant strains. Therefore, it is to be expected that in the future natamycin will be accepted in more countries for application in products containing fresh fruit, beverages and other products sensitive to fungal spoilage.

5.10 References

AYRES, J.C. and DENISEN, E.L. (1958), 'Maintaining freshness of berries using selected packaging materials and antifungal agents', *Food Technology*, **12** (10), 562–567.

BALDINI, P., PALMIA, F., RACZYNSKI, G. *et al.* (1979), 'Impiego della pimaricina nella prevenzione della crescita della muffe sui prodotti di salumeria italiani', *Industria Conserve*, **54** (4), 305–307.

BRIK, H. (1981), 'Natamycin', in K. Florey, *Analytical Profiles of Drug Substances* Vol. 10, Academic Press, New York, 513–561.

CATTANEO, P., D'AUBERT, S. and RIGHETTI, A. (1978), 'Attivita antifungina della pimaricina in salami crudi stagionati. *Industrie Alimentari*', **17** (9), 658–664.

CEDER, O., HANSSON, B. and RAPP, U. (1977), 'Pimaricin. VIII. Structural and configurational studies by electron impact and field desorption mass spectrometry, carbon-13 (25.2 Mhz) and proton (270 Mhz)-NMR spectroscopy', *Tetrahedron*, **33** (20), 2703–2714.

CLARK, W.L., SHIRK, R.J. and KLINE, E.F. (1964), 'Pimaricin, a new food fungistat', in Molin, N., *Microbial Inhibitors in Food*, Almquist & Wiksell, Gotenborg, 167–184.

DAAMEN, C.B.G. and VAN DEN BERG, G. (1985), 'Prevention of mould growth on cheese by means of natamycin', *Voedingsmiddelentechnologie*, **18** (2), 26–29.

DE BOER, E. and STOLK-HORSTHUIS, M. (1977), 'Sensitivity to natamycin (pimaricin) of fungi isolated in cheese warehouses', *Journal of Food Protection*, **40** (8), 533–536.

DE BOER, E., LABOTS, M., STOLK-HORSTHUIS, M. *et al.* (1979), 'Sensitivity to natamycin of fungi in factories producing dry sausage', *Fleischwirtschaft*, **59** (12), 1868–1869.

DE KRUIJFF, B., GERRITSEN, W.J., OERLEMANS, F. *et al.* (1974), 'Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. II. Temperature dependence of the polyene antibiotic-sterol complex formation', *Biochimica et Biophysica Acta*, **339** (1), 44–56.

DE RUIG, W.G. and VAN DEN BERG, G. (1985), 'Influence of the fungicides sorbate and natamycin in cheese coatings on the quality of the cheese', *Netherlands Milk and Dairy Journal*, **39** (3), 165–172.

DUPLANTIER, A.J. and MASAMUNE, S. (1990), 'Pimaricin. Stereochemistry and synthesis of its aglycon (pimarolide) methyl ester', *Journal of the American Chemical Society* **112** (19), 7079–7081.

ENGEL, G., HERTEL, K. and TEUBER M. (1983), 'Nachweis und Abbau von Natamycin (Pimaricin) auf der Käseoberfläche', *Milchwissenschaft*, **38** (3), 145–147.

FENTE-SAMPAYO, C.A., VAZQUEZ-BELDA, B., FRANCO-ABUIN, C. *et al.* (1995), 'Distribution of fungal genera in cheese and dairies. Sensitivity to potassium sorbate and natamycin', *Archiv für Lebensmittelhygiene*, **46** (3), 62–65.

FRISVAD, J.C., SAMSON, R.A., RASSING, B.R. *et al.* (1997), '*Penicillium discolor*, a new species

from cheese, nuts and vegetables', *Antonie van Leeuwenhoek*, **72**, 119–126.

HAMILTON-MILLER, J.M.T. (1974), 'Fungal sterols and the mode of action of the polyene antibiotics', *Advances in Applied Microbiology*, **17**, 109–134.

HECHELMANN, H. and LEISTNER, L. (1969), 'Hemmung von unerwünschtem Schimmelpilzwachstum auf Rohwürsten durch Delvocid (Pimaricin)', *Fleischwirtschaft*, **49** (12), 1639–1641.

HENNINGER, W. (1977), 'Die Abtötung von getränkeverderbenden Hefen durch Pimaricin', *Das Erfrischungsgetränk Mineralwasser-Zeitung*, **30** (13), 1–6.

HOEKSTRA, E.S., VAN DER HORST, M.L., SAMSON, R.A. *et al.* (1998), 'Survey of the fungal flora in Dutch cheese factories and warehouses', *Journal of Food Mycology*, **1** (1), 13–22

HOLLEY, R.A. (1981), 'Prevention of surface mould growth on Italian dry sausage by natamycin and potassium sorbate', *Applied and Environmental Microbiology*, **41** (2), 422–429.

HOLLEY, R.A. (1986), 'Effect of sorbate and pimaricin on surface mould and ripening of Italian dry salami', *Lebensmittel Wissenschaft und Technologie*, **19** (1), 59–65.

KIERMEIER, F. and ZIERER, E. (1975), 'Zur Wirkung von Pimaricin auf Schimmelpilze und deren Aflatoxinbildung bei Käsen', *Zeitschrift für Lebensmitteluntersuchung und Forschung*, **157** (5), 253–262.

KOTLER-BRAJTBURG, J., MEDOFF, G., KOBAYASHI, G.S. *et al.* (1979), 'Classification of polyene antibiotics according to chemical structure and biological effects', *Antimicrobial Agents and Chemotherapy*, **15** (5), 716–722.

LANCELIN, J.M. and BEAU, J.M. (1990), 'Stereostructure of pimaricin', *Journal of the American Chemical Society* **112** (10), 4060–4061.

LANCELIN, J.M. and BEAU, J.M. (1995), 'Stereostructure of glycosylated polyene macrolides: the example natamycin', *Bulletin Societé Chimie de France* **132**, 215–223.

LODI, R., TODESCO, R. and BOZZETTI, V. (1989), 'Nouvelles applications de la natamycine sur des fromages typiques Italiens', *Microbiologie-Aliments-Nutrition*, **7** (1), 81–84.

LÜCK, H. and CHEESMAN, C.E. (1978) 'Mould growth on cheese as influenced by pimaricin or sorbate treatments', *South African Journal of Dairy Technol.*, **10** (3), 143–146

MALEWICZ, B. and BOROWSKI, E. (1979), 'Energy dependence and reversibility of membrane alterations induced by polyene macrolide antibiotics in *Chlorella vulgaris*', *Nature* **281**, 80–82.

MATTSSON, N. (1977), 'Mould control on cheese. Experience with potassium sorbate and pimaricin for surface treatment of hard cheese', *Svenska Mejeritidningen*, **69** (3), 14–15.

MILHAUD, J., PONSINET, V., TAKASASHI, M. *et al.* (2002) 'Interactions of the drug amphotericin B with phospholipid membranes containing or not ergosterol: new insight into the role of ergosterol', *Biochimica et Biophysica Acta*, **1558**, 95–108.

MOERMAN, P.C. (1972), 'Schimmelwering op vleeswaren door Pimaricine', *Voedingsmiddelentechnologie*. **3** (51/52), 261–264.

MORRIS, H.A. and CASTBERG, H.B. (1980), 'Control of surface growth on blue cheese using pimaricin', *Cultures Dairy Products Journal*, **15** (2), 21–23.

NEVIANI, E., EMALDI, G.C. and CARINI, S. (1981), 'L'impiego di pimaricina come antifungino sulle croste dei formaggi: technologia e microflora di superficie', *Latte*, **6** (5), 335–343.

PUGAZHENTHI, T.R., DHANALAKSHMI, B., NARASIMHAN, R. *et al.* (1999), 'Effect of antimycotic agents on *Penicillium citrinum* in cheese', *Indian Veterinary Journal*, **76**,

537–539.

RAAB, W.P. (1974), '*Natamycin (Pimaricin). Properties and Medical Applications*', Georg Thieme, Stuttgart.

SACHDEVA, S., SING, S., TIWARI, B.D. *et al.* (1994), 'Effect of processing variables on the quality and shelf-life of processed cheese from buffalo milk Cheddar cheese', *The Australian Journal of Dairy Technology*, **49** (2), 75–78.

SHIRK, R.J. and CLARK, W.L. (1963), 'The effect of pimaricin in retarding the spoilage of fresh orange juice', *Food Technology*, **17** (8), 1062–1066.

STADEN, O.L. and WITMONDT, M. (1967), 'Lecithine-pimaricine-perspectieven op hard fruit', *De Fruitteelt*, **57** (38), 1180–1182.

STIEBING, A., OBERHAUS, T. and BAUMGART J. (2001), 'Natamycin-Verhinderung des Schimmelpilzwachtums bei Rohwurst', *Fleischwirtschaft*, **81** (8), 97–100.

STRUYK, A.P., HOETTE, I., DROST, G. *et al.* (1957–58), 'Pimaricin, a new antifungal antibiotic', *Antibiotics Annual*, 878–885.

TEERLINK, T., DE KRUYFF, B. and DEMEL, R.A. (1980), 'The action of pimaricin, etruscomycin and amphotericin B on liposomes with varying sterol content', *Biochimica et Biophysica Acta*, **599** (2), 484–492.

VAN RIJN, F.T.J., STARK, J., TAN, H.S. *et al.* (1995), 'A novel antifungal composition', US Patent Application No. 08/446,782.

VERMA, H.S., YADIV, J.S. and NEELAKANTAN S. (1988), 'Preservative effect of selected antifungal agents on butter and cheese', *Asian Journal of Dairy Research*, **7** (1), 34–38.

ZIOGAS, B.N., SISLER, H.D. and LUSBY, W.R. (1983), 'Sterol content and other characteristics of pimaricin-resistant mutants of *Aspergillus nidulans*, *Pesticide Biochemistry and Physiology*, **20** (3), 320–329.

ZUTHOF, J.B.I. and ISIDORUS, J.B. (1981), 'Cheese manufacturing – Flowing brine over mould pressed cheese in racks', GB Patent 2072481.

Natural antimicrobials for the minimal processing of foods

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CRC Press
Boca Raton Boston New York Washington, DC

WOODHEAD PUBLISHING LIMITED
Cambridge England

Published by Woodhead Publishing Limited
Abington Hall, Abington
Cambridge CB1 6AH
England
www.woodhead-publishing.com

Published in North America by CRC Press LLC
2000 Corporate Blvd, NW
Boca Raton FL 33431
USA

First published 2003, Woodhead Publishing Limited and CRC Press LLC

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library.

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress.

Woodhead Publishing Limited ISBN 1 85573 669 1 (book); 1 85573 703 5 (e-book)
CRC Press ISBN 0-8493-1753-3
CRC Press order number: WP1753

Cover design by The ColourStudio
Project managed by Macfarlane Production Services, Markyate, Hertfordshire
(e-mail: macfarl@aol.com)
Typeset by MHL Typesetting Limited, Coventry, Warwickshire
Printed by TJ International, Padstow, Cornwall, England

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9 Natamycin

J. STARK and H.S. TAN

9.1 Introduction

Prevention of mold growth is an important issue for the food industry because economic losses due to fungal spoilage of foods can be considerable. Apart from some deliberately fermented foods, products containing visible molds or yeasts are generally not acceptable to the consumer. Also, from a health point of view it is necessary to prevent fungal growth on food products. Mycotoxins produced by spoilage fungi can cause various health problems, and some mycotoxins even have highly carcinogenic properties. Superficial removal of visible molds and yeasts from food products therefore gives no guarantee of safety to the consumer. Such treatment is not very effective and does not affect toxic fungal metabolites which could have been excreted and then diffused into the food product.

A precondition for the efficacy of a preservative is a good hygienic production process. However, in spite of optimal hygienic production and storage conditions some products remain sensitive to fungal growth. In these cases the use of an antifungal agent is usually the only way to prevent fungal spoilage. Because of its effectiveness in controlling the growth of fungi, natamycin has found its way as a preservative in the food industry.

Natamycin has been used to prevent fungal growth on foods for more than 30 years. In low concentrations this fungicide is active against nearly all molds and yeasts. It is used mainly for the surface treatment of cheeses and dry sausages. As natamycin has no antibacterial activity, the natural ripening processes in cheeses and sausages are not influenced in a negative way.

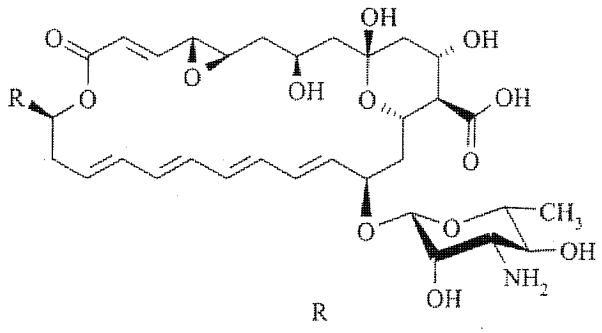
Natamycin, also known as pimaricin, belongs to the group of polyene macrolide antimycotics. It is produced on an industrial scale by fermentation using *Streptomyces natalensis*. This strain was found in 1955 in a soil sample from the province of Natal, South Africa (Struyk *et al.*, 1957–1958; Brik, 1981).

Thus far, natamycin is the only microbially derived antifungal compound that is used as a food preservative.

9.2 Physical and chemical properties

The physical and chemical properties of natamycin have been described in detail by Brik (1981, 1994), and in 1993 natamycin was the subject of a comprehensive review article (Davidson and Doan, 1993). Therefore, instead of mainly reviewing the physical and chemical properties of natamycin as such, it seems more relevant to have a closer look at the relationship between these properties and the application of natamycin as a food preservative.

Natamycin belongs to the large group of polyene antifungal antibiotics and has a molecular weight of 665.75. The correct chemical structure was documented by Ceder *et al.* (1977) and the complete stereostructure was elucidated by Lancelin and Beau (1990, 1995) and Duplantier and Masamune (1990) (Figure 9.1).



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Natamycin: CH_3
Etruscomycin: $n\text{-}C_4H_9$

Figure 9.1 Chemical structures of natamycin and etruscomycin.

Natamycin is a white-to-cream colored powder, which has little or no odor or taste. Because of these properties natamycin will have no influence on the taste and appearance when applied on food or in drinks.

Owing to its amphoteric character natamycin has a low solubility in most solvents. The isoelectric point was reported to be 6.5 (Raab, 1974). In general an aqueous suspension of natamycin will have a pH value between 5 and 7.5. There are no accurate data available of the solubility in aqueous systems. According to Brik's own measurement, the solubility of natamycin in water is about 30 ppm (mg/L) (Brik, 1981). Others have reported values of 50–100 ppm (Struyk *et al.*, 1957–1958; Clark *et al.*, 1964). In view of the fact that the minimum inhibitory concentration (MIC) of natamycin for most of the food-spoilage fungi is less than 10 ppm (De Boer and Stolk-Horsthuis, 1977; Hoekstra *et al.*, 1998) the solubility of natamycin usually will be more than sufficient to inhibit those microorganisms.

On the other hand, for the inhibition of fungi with a relatively high MIC, the low solubility of natamycin may be a problem. Therefore, when dealing with more tolerant species it is important to know what the real solubility will be under the conditions of the application. Otherwise the limitations of the application will be unclear.

Enhanced solubility in aqueous systems can be obtained at either low or high pH, which can be used to inhibit more tolerant species. An example of such an application is reported in a Dutch patent application for a veterinary preparation consisting of a solution of 100–200 ppm natamycin in aqueous citric acid of pH 3 (Oldenkamp *et al.*, 1976). A disadvantage of an acidic solution, however, is the chemical instability of natamycin. Such solutions are only effective when used immediately after preparation. It seems that the low chemical stability is also a common property of dissolved natamycin in organic solvents such as dimethylformamide (Brik, 1981). This is probably one of the reasons why ready-to-use preparations based on natamycin are suspensions with a pH value in the neutral region, for example, the medical preparation Pimafucin® of Yamanouchi, which contains 25 mg of natamycin per milliliter. Natamycin as the trihydrate is a stable compound when protected from light and moisture. At room temperature the loss of activity was only a few percent after storage for several years. Neutral aqueous suspensions are nearly as stable as the dry powder. For example, a suspension containing 0.5% of natamycin with a pH of 6.5 which is stored in the dark at room temperature will keep its activity for more than 2 years

(Clark *et al.*, 1964). Clearly, this stability is due to the fact that only a small fraction is dissolved under such conditions. At lower concentrations the stability of the suspensions are limited. There are no accurate stability data available for natamycin at lower concentrations.

Besides the aqueous trihydrate, solvates of natamycin can be prepared with organic solvents such as methanol or dioxane. The stability of these solvates, however, is rather low (Brik, 1981). Moreover, for food applications these kinds of compounds are unacceptable. The stability of the anhydrate under ambient conditions is also poor. A recent patent application (Van Rijn *et al.*, 1995) claims that the anhydrate (δ-natamycin) is more stable when stored under nitrogen or at low temperatures. In addition, a new crystalline form (γ-natamycin), also a trihydrate, is described. With a stability that is somewhat less than that of the original trihydrate (α-natamycin). The same patent application described the synthesis of the barium and calcium salts of natamycin. Contrary to the anhydrate and other salts, such as the potassium salt and the sulfate, these salts possess high stability. An interesting property of these compounds is their conversion under aqueous conditions into the more stable and therefore energetically more preferable trihydrate. In principle this phenomenon may be used to inhibit species with a relatively high MIC value. At the conversion process the amount of natamycin that is in solution will be temporarily higher than the solubility of natamycin trihydrate, resulting in an improvement of the availability of the natamycin. The disadvantage, however, is that only freshly prepared solutions will be effective against the tolerant species.

In conclusion, there is much known about the properties of natamycin, but under application conditions more accurate data are still required.

9.3 Mechanism of action

The mechanism of action of polyene antibiotics has been reviewed by several authors (Hamilton-Miller, 1974; Norman *et al.*, 1976; Hammond, 1977; Gale, 1984; Bolard, 1986; Ali, 1989). Polyene antibiotics are toxic to eukaryotic but not generally to prokaryotic organisms (some Gram-positive bacteria are sensitive for reasons that are not understood). A major difference between the two types of organisms lies in the presence of sterols as a functional part of the membrane in eukaryotic and not in prokaryotic organisms. It is generally accepted that sterols play an important role in the selective action of polyene antibiotics toward eukaryotic organisms (Norman *et al.*, 1972; Thomas, 1986).

The major sterol in yeasts and fungi is ergosterol (Marriott, 1975). It is suggested that natamycin binds to ergosterol in the cell membranes, resulting in disturbance of the cell permeability. Rapid leakage of essential ions and small peptides occurs and so causes lysis of the cell (Hamilton-Miller, 1973; Kotler-Brajtburg *et al.*, 1979). The smaller polyenes, such as natamycin, bind irreversibly to sterols in the cell membrane (Bulder, 1971; Norman *et al.*, 1972; Kerridge *et al.*, 1976) while the binding of the larger molecular-size ones is reversible (De Kruijff *et al.*, 1974; Malewicz and Borowski, 1979).

Most resistant mutants have no or only a limited amount of ergosterol in the cell membrane (Hamilton-Miller, 1974; Ziogas *et al.*, 1983). Although natamycin has been used for many years, primary resistance to it has not arisen so far (Lieske *et al.*, 1983). Hamilton-Miller (1974) made some interesting speculations on the remarkable fact that resistance to polyene antibiotics in general seemed to be non-existent. The suggestion is that contrary to other antibiotics, polyenes are virtually insoluble in water. Thus, even in very dilute aqueous solution, it is probable that polyene antibiotics exist in micelles or in aggregates.

A sub-inhibitory concentration of a polyene antibiotic may therefore be one in which there are fewer quanta than organisms. It can be visualized that any cell that comes in contact with a quantum will perish. In this way an all-or-none phenomenon will exist. Cells will either die or not be exposed at all, thus preventing the selection of mutants.

Observations of resistance of *Candida* species to polyenes have been made only by *in vitro* experiments, and resistance is always associated with diminished virulence (Athar and Winner, 1971). Moreover, such yeasts have little or no ergosterol in their cell membranes and they also have slower metabolism and growth rates (Ziogas *et al.*, 1983). If sterols are indeed an integral and functional part of the cell membrane of these organisms, then this decrease of virility is not surprising at all. Ziogas *et al.* (1983) suggested that there well may be bases other than a reduced ergosterol content for resistance to polyene antibiotics, for instance the capacity to cause the degradation of the antibiotic. However, the experiments did not make clear whether these cases concerned mutants or naturally existing variants within the species.

The molecular size and differences in amphilicity, charge, and side-chain structure of the molecule also have influence on the selectivity of the different polyene antibiotics (Gale, 1984). Etruscomycin, for instance, having a more hydrophobic side chain than natamycin (Figure 9.1), shows a quite different behavior than natamycin (Kotler-Brajtburg *et al.*, 1979; Teerlink *et al.*, 1980; Nadeau *et al.*, 1982; Gale, 1984). Physical studies of polyene–sterol–phospholipid complexes showed that the range of effectiveness of polyene interaction with cholesterol is filipin > amphotericin B > etruscomycin > natamycin (Norman *et al.*, 1972). Experiments with artificial membrane systems demonstrated that the degree of disorganization seemed to depend more on the ratio of sterol to phospholipid than on the amount of sterol itself (Demel *et al.*, 1965, 1968; Kinsky *et al.*, 1968; Gale, 1984).

It can be concluded that much is known about the mode of action of polyene antibiotics in general, and natamycin in particular, but that the mechanism is still not fully understood. Moreover, most investigations have been done with yeasts, and the question remains as to just how relevant the results are to molds.

9.4 Sensitivity of fungi to natamycin

Several methods for determining the MIC values of natamycin for molds and yeasts are known. Usually spore suspensions are prepared to final concentrations of 10^4–10^6 spores/ml. The freshly-prepared spore suspension is inoculated on suitable agar plates with different concentrations of natamycin. The spore suspension may be spread over the surface of the agar or may be inoculated in a spot. After incubation for 4–6 days at 24°C, the MIC is measured as the lowest concentration at which no growth is observed. As far as we know there is no clear statement or agreement concerning the quantity of spores that have to be inoculated on the agar plate. In our opinion, if MIC values have to be determined, then the number of spores is just as important as the concentration of preservative added to the medium. Nevertheless, in the literature different numbers of spores are described or not mentioned at all. The National Committee for Clinical Laboratory Standards conducted a study to develop standard guidelines for the antifungal susceptibility testing of filamentous fungi (Espinel-Ingroff *et al.*, 1997). MIC values of amphotericin B and itraconazole were determined in 11 laboratories against 6 different mold species. The effects of inoculum density, incubation time, and procedure of MIC determination were established. The test condition proposed as a guideline for a reference broth microdilution method is by

determination of colorimetric MIC values using Almar Blue after 48–72 hr of incubation with an inoculum density of 104 CFU/ml (colonies forming units per milliliter). We suppose that this method can also be used to determine the MIC values of natamycin for filamentous fungi.

Natamycin is active in small quantities against almost all fungi that may occur in food products. The sensitivity to natamycin of most molds is lower than 10 ppm (De Boer and Stolk-Horsthuis, 1977; Hoekstra *et al.*, 1998). The MIC values of natamycin for most yeasts is even lower than 3 ppm (Henninger, 1977). Some species are less sensitive to natamycin owing to the fact that variation in nature is inevitable. For example, a more tolerant mold species which can be found in the cheese industry is *Penicillium discolor*. This new species was first described by Frisvad *et al.* (1997), and it has an MIC value for natamycin that varies from less than 20 ppm to more than 60 ppm depending on the spore concentration used (see Table 9.1). These results illustrate that simply mentioning a MIC value as such is not sufficient. In particular, in the case of less sensitive species, the number of spores added to the agar greatly influences the results.

The determination of the MIC value of natamycin for *P. discolor* was part of a survey carried out to study the composition of the fungal flora in four cheese factories and three cheese warehouses in the Netherlands (Van Rijn *et al.*, 1997; Hoekstra *et al.*, 1998). The fungal flora detected in different sites at the cheese factories and warehouses consisted mainly of *Aspergillus* and *Penicillium* species. In most factories a fairly constant home flora was found. Fungal species occurring at a percentage of more than 30% of the total mold count in the different sampling sites were *Aspergillus penicillioides, A. versicolor, P. brevicompactum, P. corylophilum, P. solitum, P. echinulatum, Wallemia sebi, Eurotium* species, and *Cladosporium* species. The xerophilic fungi were encountered more frequently in the warehouses and the drier areas of the cheese factories. Yeasts were detected mainly in the more humid locations such as the brine bath areas.

The sensitivity to natamycin of 10 fungal species, which commonly occur in the cheese industry, was determined by the Centraal Bureau voor Schimmelcultures, Baarn. Also, the sensitivity to natamycin of 12 fungal strains from other sources was determined (unpublished results). Spore suspensions of the strains were made up to give final concentrations of 10^6, 10^5, and 10^4 spores/ml. Malt extract agar plates containing 0, 10, 20, 30, 40, 50, and 60 ppm (mg/kg) of natamycin were prepared and inoculated with 5 µl of the spore suspensions at a spot in the middle of the plate. The growth diameter of the colonies was measured after 6 days of incubation at 24°C in the dark. All molds and yeasts but one were fully inhibited by the lowest concentration of natamycin (10 ppm). Only *P. discolor* was able to grow on natamycin-containing agar plates. As can be seen in Table 9.1 the diameter of the

Table 9.1 Growth diameter (mm) of *P. discolor* on duplicate malt extract agar plates after 6 days of incubation at 24°C in the dark as a function of the spore density (spores/ml) at different concentrations of natamycin (ppm)

Spore density	Natamycin concentration (ppm)						
	0	10	20	30	40	50	60
10^4	23/24	9/0	0/0	0/0	0/0	0/0	0/0
10^5	25/25	12/15	5/12	0/0	0/0	0/0	0/0
10^6	24/25	21/21	17/17	13/12	12/11	9/9	9/9

colonies decreased as the concentration of natamycin in the medium increased. Only at the highest spore concentration can some growth be observed on medium with natamycin concentrations higher than 20 ppm.

The amount of dissolved natamycin on cheese surfaces is between 30 and 50 ppm. If *P. discolor* is present in high concentrations on the surface of the cheese, than the available amount of active natamycin could be insufficient to prevent fungal growth. Under good hygienic and processing conditions this species, which occurs commonly in the cheese industry, will not cause spoilage problems. However, it is observed that *P. discolor* will grow on natamycin-treated cheeses if both the conditions are optimal for fungal growth and the species is present in high quantities. In the case of mold problems, extra hygienic measurements have to be executed. The sensitivity of fungi, isolated in cheese warehouses where natamycin has been used for various periods, was first determined in the 1970s by De Boer and Stolk-Horsthuis (1977). Concentrated spore suspensions were inoculated on agar plates containing 0–32 ppm of natamycin. Natamycin-insensitive mold or yeast strains were not detected. Laboratory experiments were carried out with the aim of inducing tolerance to natamycin in 26 mold strains isolated in cheese warehouses. The initial MIC value of natamycin varied from 2 to 8 ppm, and the final value after 23–31 transfers varied from 1 to 12 ppm. The MIC value after multiple transfers increased in 8 out of 26 strains, but decreased in 10 out of 26 strains, whereas in 8 strains there was no change. It was concluded that none of the strains became notably less sensitive to natamycin.

De Boer *et al.* (1979) also investigated the natamycin sensitivity of fungi isolated from 16 factories producing dry sausages. Molds and yeasts were obtained from factories where natamycin had never been used and from factories where it had been used for several years. Samples were taken by using Rodac plates and by placing uncovered Petri dishes with various natamycin concentrations in areas where the sausages were being manufactured, ripened, or stored. Objects difficult to sample with Rodac plates were sampled with swabs. Only on agar plates containing 2 ppm of natamycin was a little growth observed.

The inhibition of 13 different aflatoxin-producing molds was investigated by Kiermeier and Zierer (1975). Agar plates containing 5 or 25 ppm of natamycin were prepared and inoculated with different mold strains (*A. flavus, A. parasiticus, P. aurantio virens, P. islandicum,* or an *Epatulium* sp.). The agar plates were incubated for 3 weeks at 30°C. Three strains were fully inhibited by 5 ppm of natamycin. After 3 weeks of incubation on most plates with 5 ppm of natamycin and on five agar plates containing 25 ppm of natamycin some colonies were observed. De Boer and Stolk-Horsthuis (1977) also found growth of a mold on an agar plate with 25 ppm of natamycin. It was reported that this particular mold was a slow-growing one. However, it is likely that after 3 weeks of incubation the concentration of natamycin in the agar plates was much lower than at the beginning of the experiment and a few remaining spores could germinate or slow-growing molds could develop.

The decomposition of natamycin in malt extract agar (MEA) plates is presented in Figure 9.2 (unpublished results). After 20 days at 24°C the concentration of natamycin is reduced from 11.4 to 5.5 ppm and from 12.6 to 7.3 ppm. Therefore, we recommend the use of freshly prepared agar plates and determination of the natamycin concentration in the plates, especially in the case of longer incubation periods.

The sensitivity to natamycin of 83 different yeast strains from several sources was determined by Henninger (1977). Yeast colonies were transferred using a stamper onto agar plates containing different concentrations of natamycin. None of the strains was able to grow at a concentration of 5 ppm of natamycin. The MIC values of natamycin for most of the species that can cause spoilage in beverages varied from 0.5 to 2 ppm.

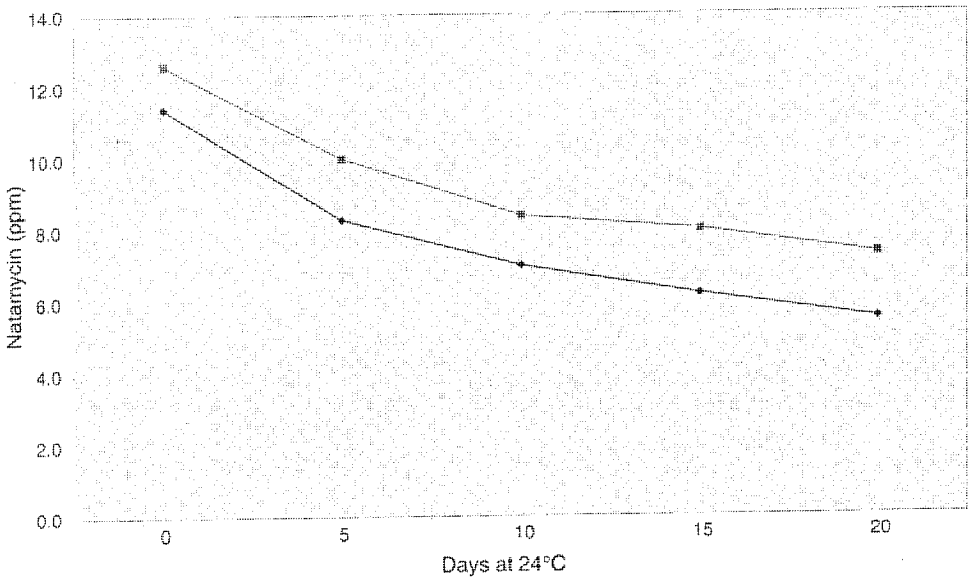

Figure 9.2 Decomposition of natamycin in malt extract agar.

The composition of the fungal flora on soft cheese and the production environment in 10 farm-level cheese-making units in NW Spain was determined by Fente-Sampayo *et al.* (1995). *Penicillium*, *Aspergillus*, *Cladosporium*, *Rhizopus*, and *Geotrichum* species were most frequently isolated. The sensitivity to natamycin and sorbate of 35 fungal strains was determined. Spore suspensions containing 10^4 spores/ml were prepared and 0.1–0.2 ml of each suspension was inoculated into agar with different concentrations of natamycin. The plates were incubated for 5 days at 4°C and 25°C. For the *Geotrichum* species a MIC value of 12.5 ppm of natamycin was reported. The MIC value of natamycin for *Fusarium culmorum* and *F. heterosporum* was 10 ppm, and for all the other strains the values at both temperatures were equal to or less than 5 ppm for total inhibition. The sensitivity to sorbate varied from 375 to 15,000 ppm.

Most fungi are very sensitive to natamycin. Some less sensitive molds are described. In most scientific literature the absolute number of spores added to the agar plates or the concentration of natamycin in the agar plates during the experiment is not mentioned. We recommend that the natamycin concentration during the experiment, the inoculum density, the incubation time, and the determination procedure should all be detailed. This prevents wrong conclusions and misinterpretation of the data, in particular for less sensitive molds, and offers the possibility to evaluate and compare data from different laboratories.

9.5 Inhibition of mycotoxin formation by natamycin

For food and feed the definition of mycotoxins can be restricted to "those fungal secondary metabolites that in small concentrations are toxic to vertebrates and other animals when introduced via a natural route" (Frisvad and Thrane, 1995). Common food-borne fungi such

as species in the genera *Aspergillus*, *Penicillium*, and *Fusarium* are able to produce mycotoxins. *Penicillium* and *Aspergillus* species are the most common molds that contaminate cheese. Some of these species can produce mycotoxins on cheese. Aflatoxins and sterigmatocystin produced by *Aspergillus* species can penetrate into cheese as far as 4 cm. Patulin and penicillic acid produced by *Penicillium* species are not very stable in cheese, in contrast to the aflatoxins and sterigmatocystin (Scott, 1989).

Lund *et al.* (1995) studied the mycoflora of hard, semi-hard, and semi-soft cheeses from several countries. Of the 371 identified isolates, 91% were *Penicillium* species, and *P. commune* was the most widespread and most frequently occurring species (42%). All *P. commune* isolates from cheese were able to produce mycotoxins, including cyclopiazonic acid, rugulovasine A and B, palitantin, cyclopaldic acid, and viridicatin. Other species that were able to produce mycotoxins on cheese are *P. verrucosum* (ochratoxin A), *A. versicolor* (sterigmatocystin), and *P. crustosum* (penitrem A and roquefortine C).

Ray and Bullerman (1982) discussed the effect of natamycin on growth and mycotoxin production by *A. flavus* (aflatoxin B_1), *A. ochraceus* (ochratoxin), *P. cyclopium* (penicillic acid), and *P. patulum* (patulin). In all cases the inhibitory effect of natamycin was greater on mycotoxin production than on fungal growth. Just 1 ppm of natamycin inhibited the production of aflatoxin B_1, ochratoxin, penicillic acid, and patulin by, respectively, 25.0%, 93.2%, 70.6%, and 97.8%, whereas the inhibitory effects on growth were respectively 0.3%, 16.0%, 16.4%, and 23.6%.

Gourama and Bullerman (1988) studied the effects of natamycin on growth and penicillic acid production by *A. ochraceus* in yeast extract sucrose medium and on olive paste. Also in this study it was observed that natamycin inhibited mycotoxin production more effectively than fungal growth. Natamycin at 20 ppm inhibited nearly all penicillic acid production. Penicillic acid production in olive paste was almost completely inhibited (96%) at 350 ppm of natamycin. It was concluded that natamycin may provide a practical solution to the economic and health problems that can be caused by mold growth on olives.

Kiermeier and Zierer (1975) studied the inhibition of fungal growth and mycotoxin formation for 13 different aflatoxin-forming molds. Aflatoxin formation was only inhibited if the growth of the molds was inhibited. Incomplete growth inhibition resulted in lowered mycotoxin production. Even in the inhibition zones of the agar plates mycotoxins could be detected. The mycotoxin produced by the mold apparently diffuses through the agar into the inhibition zone. Obviously natamycin was not effective against the mycotoxins themselves.

The effectiveness of natamycin in retarding mold growth and aflatoxin production in raw ground peanuts was studied by Gelda *et al.* (1974). Peanuts were inoculated with *A. parasiticus* spores and incubated for 11 days at 25°C. Growth and aflatoxin production were completely inhibited by 50 ppm of natamycin during 11 days. After 5 days of incubation with 5 ppm of natamycin, growth was inhibited by 88% and aflatoxin production by 95%.

In several publications natamycin inhibition of mycotoxin formation is described, sometimes as even more effective than mycelial growth. The mechanism of inhibition of mycotoxin formation by natamycin is unknown, which may be a challenge for further research.

9.6 Applications of natamycin

9.6.1 Surface treatment

Natamycin is a suitable fungicide for the surface treatment of products such as cheeses and dry sausages. Mold growth occurs on the surface of these products. Owing to its low solubility in

aqueous systems with a pH around 7, natamycin is mainly present in the form of crystals on the surface of the product. The dissolved fraction of natamycin hardly penetrates into the cheese. The fact that natamycin remains on the surface of the product is an important advantage over other preservatives such as sorbate. Daamen and Van den Berg (1985) reported that the penetration of natamycin into Dutch cheese is limited to 2–4 mm, whilst Kiermeier and Zierer (1975) found that the maximum penetration depth of natamycin in Tilsiter cheese was 2.6 mm. The standard of the European Community concerning the maximum permissible penetration depth of natamycin in cheese is 5 mm.

The crystalline form of natamycin is very stable and guarantees a prolonged working time, but only the dissolved fraction of natamycin has antifungal activity. In aqueous systems such as cheese surfaces 30–50 ppm of natamycin will be present in the active form. Natamycin has an MIC value of less than 10 ppm for most food-borne fungi (De Boer and Stolk-Horsthuis, 1977; Hoekstra *et al.*, 1998). Under normal hygienic and processing conditions 30 ppm of dissolved natamycin is sufficient to prevent fungal growth, because the solubility is much higher than the MIC value. The dissolved fraction is less stable than the natamycin crystals (Brik, 1981). Elimination of dissolved natamycin will be compensated by dissolution of natamycin from the crystals and by diffusion of the dissolved natamycin over the surface of the cheese. Elimination of dissolved natamycin occurs when natamycin interacts with the ergosterol of the fungal cell membrane. Inactivation may also occur by decomposition through the action of ultraviolet light or by hydrolysis.

9.6.2 *Cheese*

The prevention of fungal growth is an important issue in the cheese industry. Cheese can be considered as a good substrate for many mold and yeast species. The storage and ripening conditions of cheese make this product even more susceptible to fungal growth. The relative humidity (RH) of the air in cheese factories is usually high, for example, around 80–85% in modern warehouses where Gouda-type cheeses are ripened. Cheeses are often ripened in the open air, which means that contamination with fungal spores or mycelium may occur during the whole ripening period.

Natamycin has several advantages over other fungicides such as sorbate, in particular the fact that it does not penetrate far into the cheese. The cheese is protected for a longer period of time because the natamycin slowly dissolves from the crystals and remains on the surface of the cheese. Furthermore, natamycin has no antibacterial activity. Consequently, maturation of the cheese is unaffected and bacterial surface growth which is required for some cheese types is not inhibited. Natamycin has no color, odor, or taste and causes no defects in the cheese.

Natamycin can be added to the aqueous polymer dispersion (usually polyvinylacetate) that is applied to the cheese rind as a coating. It can also be applied by means of dipping or spraying. Alternatively, natamycin can be added to the brining bath. Zuthof and Isidorus (1981) describe a method in which brine containing natamycin is poured over the cheese. The cheeses are placed in mobile racks with tiers of liquid-permeable structures for brining by passing the racks under a fixed watering system. This method is particularly suitable for the treatment of round cheeses such as Edam.

Cheeses are treated with a coating by pouring the required amount on the surface. The amount should be sufficient to obtain a thin layer over the whole surface of the cheese. The concentration of natamycin in the cheese coating usually varies from 100 to 750 ppm. The required concentration depends on the type of cheese, the time of storage, and the

number of treatments. Furthermore, the effective amount in the coating may also be affected by the quality and homogeneity of the dispersion. In general, after the first treatment the protection is 3 weeks or longer. Most cheeses are treated more than once to maintain a protection for a longer period of time. For example, a 5-month-old Gouda-type cheese usually has been treated four or five times with a cheese coating containing 100–250 ppm of natamycin.

Cheese can also be dipped in an aqueous suspension of natamycin, usually of 1–3 g/L of water (1,000–3,000 ppm). The amount of natamycin applied on the cheese surface depends on the concentration of natamycin in the dipping bath and the volume of the suspension that remains attached to the cheese (which is correlated to the type of cheese, the total surface area of the cheese, and the dryness of the surface). The dipping application is carried out after brining, preferably with the dried cheese. The cheese is dipped for some seconds in the natamycin suspension and then stored for ripening. This treatment can be repeated after several days. Alternatively, the natamycin suspension can be sprayed onto the cheese surface.

De Ruig and Van den Berg (1985) treated Gouda cheeses with coatings containing either natamycin (100 and 250 ppm) or sorbate (3–10%). Both fungicides were effective in protecting the cheese against mold growth. The amount of sorbate has to be 200 times higher than the amount of natamycin. The rind of cheeses treated with potassium sorbate or calcium sorbate did not ripen well and a pink coloring of the rind could be observed. Even after several weeks the cheeses still had a more or less yellow-pink gloss. Furthermore, in the case of sorbate, so-called chemical off-flavors were noticed, mainly just below the rind. The treatment with coatings containing natamycin did not affect the quality of the cheese in a negative way. Both potassium and calcium sorbate could be detected in the inner parts of the cheese and after 10 weeks even in the center of the cheese. Natamycin could only be detected in the rind (about 1 mm thick).

Natamycin can be used to prevent fungal growth on Blue cheese (Morris and Castberg, 1980). Application of a wax coating or plastic bags often results in the growth of molds and yeast on the surface of Blue cheese. Extensive growth on the surface will close the punch holes and inhibits *P. roqueforti* which has to grow inside the holes. In addition, fungal growth on the surface may occur during ripening. This will increase the pH which may lead to a secondary infection with bacteria. Removal of the surface growth by scraping results in economic losses. Sorbate is not suitable for this application as it penetrates into the cheese and so inhibits the growth of the blue mold. In addition, off-flavors may occur and sorbate-resistant *Penicillium* species may develop. Dipping of Blue cheese in a suspension of 1 g/L of natamycin, prior to waxing, was sufficient to prevent fungal growth on the surface of the cheese. The treated surface remained whiter and cleaner than the surface of untreated cheeses. Furthermore, the treated cheeses were superior in their internal mold growth and flavor. The blue mold which grows in the relatively large holes is not inhibited since natamycin remains on the cheese surface. This implies that fungal growth inside cheese may also occur when the cheese is damaged, even with cheese where internal mold growth is not required.

The application of natamycin on the Italian cheeses Fontina, Taleggio, Montasio, Asiago, Provolone, and Pecorino Romano has been studied by Lodi *et al.* (1989). Neviani *et al.* (1981) tested the inhibitory effect of natamycin on mold growth on a Caciotta type cheese. Natamycin did not change the typical characteristics of the Italian cheeses. Fungal growth was prevented without disturbing the growth of the bacterial surface flora which is required for these cheeses.

The effect of natamycin (500 ppm), nystatin (1,000 ppm), sorbic acid (2%), and benzoic acid (1%) on the growth of four selected mold strains, one selected yeast strain, and the wild

flora on Indian green cheese and butter was investigated by Verma *et al.* (1988). The molds and yeasts (*A. terreus, Trichoderma harzianum, P. janthinellum*, and a *Saccharomyces* sp.) were isolated from butter and cheese samples. The fungal counts were determined after 15 and 30 days of storage at 7°C. Nystatin (1,000 ppm) followed by natamycin (500 ppm) decreased the fungal counts by the greatest extent. The effect of natamycin and potassium sorbate on the shelf-life of Indian processed cheese made from buffalo milk was investigated by Sachdeva *et al.* (1994). In samples treated with 500 ppm of natamycin or with 1,000 ppm of sorbate no molds or yeasts could be detected throughout the storage period of 12 months. Because of bacterial growth, addition of the antifungal agents did not improve the shelf-life of the product.

The shelf-life of cottage cheese was extended from 20 days to more than 35 days by using combinations of natamycin and propionate or natamycin and nisin (Tortorello *et al.*, 1991). Several microorganisms from spoiled cottage cheese were isolated. A *Penicillium* sp., a Gram-positive spore-forming *Bacillus* sp. a Gram-negative psychrotrophic *Pseudomonas* sp., and a yeast were identified as spoilage organisms. Samples of cottage cheese were inoculated with a mixture of the four species (100 CFU/g cottage cheese for each spoilage organism) and with combinations of natamycin (200 or 400 ppm), propionate (200 or 400 ppm), or natamycin (200 or 400 ppm) and nisin (10 or 20 IU/g). Upon storage at 7°C, the inoculated control samples showed visible spoilage after 21 days, whereas all samples to which the preservatives were added showed no spoilage even after 35 days.

Nilson *et al.* (1975) studied the effect of natamycin on the shelf-life of cottage cheese inoculated with *A. niger* or *Saccharomyces cerevisiae*. Natamycin was added to the curd wash-water (20, 50, or 100 ppm) or directly to the cheese dressing (1, 2, or 5 ppm). Samples of cottage cheese were stored at 4.4°C, 10.0°C, or 15.6°C. In all cases the shelf-life of the samples was improved. Adding natamycin to the cheese dressing was more effective than adding it to the wash-water. Addition of just 5 ppm of natamycin extended the shelf-life of the cottage cheese by 2 days when stored at 15.6°C and by up to 20 days when stored at 4.4°C.

Recently, Berry (1999) reported the use of natamycin as a dry blend with powdered cellulose for the preservation of shredded cheese. This technology was claimed to be user friendly, time saving, and beneficial to the manufacturer.

From the fungicidal effect on many different cheese types, it can be concluded that natamycin is a very effective preservative to prevent fungal spoilage of cheese without causing any negative effect on the quality of the cheese.

9.6.3 *Sausages*

Dried sausages are susceptible to the growth of undesirable molds on the surface. In particular, sausages which are not smoked, such as Italian dry salami, are most affected. Mold growth can occur during the production process, storage, and distribution. Although in some countries a certain degree of mold growth is accepted by the consumer, molds are not always part of the normal microflora of sausages.

The advantages of natamycin for this application are similar to the advantages described earlier in this chapter for the surface treatment of cheese. Natamycin can be applied by treatment of the casings. Usually casings are soaked for 20–60 min (protein fiber, cellulose, and textile casings) or for about 2 hr (natural casings) in an aqueous suspension containing 500–1,000 ppm of natamycin. Natamycin can also be applied by means of dipping or spraying. Freshly prepared sausages are usually dipped in a suspension of 500–1,000 ppm of natamycin or sprayed with a suspension containing 2,000 ppm of natamycin. Sometimes

sausages are dipped in a coating suspension of polyvinylacetate. The concentration of natamycin is usually 250–1,000 ppm.

Baldini *et al.* (1979) studied the mold-inhibiting effect of natamycin on Mortadella sausages, Italian salami, and raw hams. Aqueous suspensions of natamycin (1,000–5,000 ppm) were sprayed on the cured meat products during maturation and at the beginning of storage. Natamycin prevented mold growth during normal aging and storage times without affecting the quality of the product. The bacteria responsible for the ripening of the Italian salami were not inhibited. Residual amounts of natamycin were below the detection limit of the analytical method (5 ppm). The antifungal effect of natamycin on Italian dry salami and bresaole was investigated by Cattaneo *et al.* (1978). A spray treatment with a suspension of 2,000 ppm of natamycin prevented mold growth almost completely.

The antifungal effect of natamycin on Dutch raw sausages was studied by Moerman (1967). Three methods of applying natamycin were investigated. Casings were soaked in a suspension of natamycin before stuffing, and the stuffed sausages were dipped in a suspension of natamycin or the suspension was sprayed on their surface. Generally, a concentration of 1,000 ppm of natamycin was sufficient to prevent the growth of molds and yeasts during the whole ripening and storage period (usually 6 weeks). Although after some weeks the natamycin was decomposed, no fungal growth was observed. This was explained by the fact that during the first period of ripening no mycelium was formed in the sausages and after a few weeks the molds cannot penetrate through the thin hard outer layer that is formed during smoking and storage. Moerman (1972) also studied the fungicidal effect of natamycin on Dutch sausages. The sausages were treated with 50–1,000 ppm of natamycin either during brining, by dipping or spraying before or after smoking, or by preliminary soaking of the casings. Natural protein fiber and cellulose fiber casings were used. The type of casing seems to have an influence on the efficacy of natamycin. After 4 weeks of storage only some mold growth was observed on sausages with natural casings. Pre-soaking the protein-based and cellulose-based casings in a suspension of 500–1,000 ppm of natamycin appeared not to be effective owing to insufficient absorption of natamycin into these coatings. Spraying or dipping the sausages with a natamycin suspension (250–1,000 ppm), however, effectively inhibited mold growth on all three casings. Spraying is the method of choice because the natamycin is better distributed over the surface of the sausages. An additional study demonstrated that natamycin can be used to prevent mold growth on raw ham and smoked meat. Both dipping (1,000–2,500 ppm) and spraying (1,500–2,500 ppm) have been applied successfully to inhibit fungal growth. Hechelmann and Leistner (1969) demonstrated that treatment of German raw sausages with 1,000–2,500 ppm of natamycin was sufficient to prevent fungal growth. Here, spraying was also more effective than dipping.

The inhibition of mold growth on raw cured Italian dry salami was studied by Holley (1981). A collagen type of casing was used. An immersion treatment with natamycin (2,000 ppm) had hardly any effect. Spraying the sausages twice (days 0 and 5) gave better results, although fungal growth was still not prevented effectively. In an additional study Holley (1986) tried to improve the effectiveness of natamycin. A method was described to prevent drip-loss of natamycin from the salami surfaces by adding a viscous gum (hydroxypropyl methylcellulose) to the dipping bath. After 17 days mold counts were substantially lower on sausages dipped in a suspension with 2,000 ppm of natamycin and 1% of the viscous gum compared with the untreated sausages. After 25 days mold growth could no longer be prevented. The poor performance of natamycin in these trials was due to the high levels of mold contamination and the crowded conditions of the curing areas. In addition,

we suppose that the high rate of contamination will require replenishment of natamycin to keep the required level of effective dose. Furthermore, Holley concluded that these results confirm the earlier results of Moerman (1972), namely that immersion treatment on artificial casings does not work well.

In conclusion, natamycin can be used to prevent fungal growth on sausages. Several studies demonstrate that spraying seems to be more effective than dipping. Also the type of casing determines the effectivity of natamycin.

9.6.4 *Other applications*

The efficacy of natamycin against fungal growth on other products has been investigated. A broad spectrum fungicide such as natamycin is effective on many products. Studies have been reported where natamycin was successfully tested on fruit (Ayres and Denisen, 1958; Staden and Witmondt, 1967), peanuts (Gelda *et al.*, 1974), juices (Shirk and Clark, 1963; Bärwald, 1976), butter (Verma *et al.*, 1988), quark, cream fillings, and icings (Ticha, 1975), olive paste (Gourama and Bullerman, 1988), raw cut chicken (Ayres *et al.*, 1956), and poultry feed (Wyatt and Brothers, 1997).

Fruit can easily be spoiled by molds or yeasts. The quality of apples and pears coated with an emulsion of lecithin is affected during storage in a positive way. However, mold growth on the surface of the fruit was frequently observed. Many fungicides were tested. Dipping the fruit in an emulsion containing lecithin and natamycin (200 ppm) was the most effective way to prevent fungal growth (Staden and Witmondt, 1967). When natamycin is applied to the fruit in the lecithin emulsion it will be distributed homogeneously over the surface of the fruit. Furthermore, it was found that the lecithin coating prevented the degradation of natamycin during the storage period of several months.

Natamycin can also be used to prevent fungal growth on berries (Ayres and Denisen, 1958). Strawberries, raspberries, and cranberries that were sprayed in the field shortly before harvest with a solution of natamycin or that were dipped after harvesting in a solution of natamycin had lower yeast and mold counts. Less spoilage was observed. The shelf-life of berries dipped in a solution of 10–100 ppm of natamycin was prolonged by several days. Less spoilage was also observed in berries sprayed in the field with a solution containing 50 ppm of natamycin. However, dipping after harvesting was always more effective.

Spoilage of juices by yeasts is a well-known problem. Certain spoilage yeasts are resistant to organic acids such as sorbic acid which are used to preserve beverages. The growth of yeast causes off-flavors and gas is produced. The production of gas can cause swelling or even bursting of the container. In fluids the availability of natamycin is optimal. For that reason very low concentrations of natamycin are effective in preventing fungal growth in beverages. Furthermore, after production the packaging of the beverages is usually well sealed which excludes recontamination before opening of the packaging.

It was demonstrated that 1.25 ppm of natamycin was sufficient to prevent spoilage of both inoculated and uninoculated fresh orange juice during 8 weeks of storage (Shirk and Clark, 1963). It was also reported that natamycin is quite stable in orange juice when stored in the dark under refrigeration. After 12 weeks of storage 70% of the natamycin was still active.

Bärwald (1976) investigated the effectiveness of natamycin against spoilage yeast in apple juice. Different naturally occurring spoilage yeasts were selected. Apple juice was inoculated with 10^2–10^4 CFU/ml. The growth of yeasts in the juice was fully inhibited during 25 days by 5 ppm of natamycin.

9.7 Regulatory food status of natamycin

9.7.1 General considerations

Beside the application as a food additive, natamycin is also used in human and veterinary medicine. In practice, application is limited to the topical therapy of candidiasis of the skin and the mucous membranes. Nevertheless, authorities are reluctant to give permission for a wider food use of natamycin. In most countries the allowance for food application is limited to the surface treatment of cheeses and sausages. The Food and Agricultural Organization/World Health Organization has recommended an Acceptable Daily Intake (ADI) of 0–0.3 mg/kg body weight (Twentieth Report of the Joint FAO/WHO Expert Committee on Food Additives, 1976).

9.7.2 Regulatory food status in different countries

(a) European Union. Natamycin is permitted as a food additive for the surface treatment of hard, semi-hard, and semi-soft cheese and dried, cured sausages. The maximum level is 1 mg/dm^2 surface and not present at a depth greater than 5 mm (European Parliament and Council Directive 95/2/EC of 20 February 1995 on food additives other than colors and sweeteners).

(b) United States of America. Natamycin is allowed as a 0.02%–0.03% suspension on cuts and slices of those cheese varieties of which the cheese standards provide for the use of safe and suitable mold-inhibiting ingredients (Federal Register 21 CFR §172.155).

(c) Canada. Natamycin is permitted for the surface treatment of certain cheeses. Based on the weight, the maximum level in the finished cheese should not exceed 20 ppm (*Canada Gazette* Part II, Vol. 116, no. 7, 14 April 1982).

(d) Australia. Natamycin is approved for the surface treatment of rinded cheeses and manufactured meat. The proportion of natamycin in a sample taken from a depth not less than 3 mm and not more than 5 mm shall not exceed 2 mg/dm^2 for cheese and 1.2 mg/dm^2 for meat (National Health and Medical Research Council, Australian Food Standards Code, October 1983).

(e) Former Eastern European Countries. The details of allowance in the Czech Republic, Estonia, Hungary, Poland, Russia, Slovakia, and former Yugoslavia differ from country to country, but in general the application is permitted in the dairy industry and sometimes also in the meat industry.

(f) Latin America. Details for approval limitations in Argentina, Brazil, Chile, Colombia, Costa Rica, Mexico, and Venezuela vary in the different countries. In Brazil, natamycin is only allowed on hard cheese. Colombia allows the use on meat products only. In Argentina and Venezuela the allowance is for application on cheese and on sausages. In Chile, Costa Rica, and Mexico the use as a food additive is allowed.

(g) Middle East Countries. The details in Bahrain, Dubai, Egypt, Israel, Kuwait, Oman, Qatar, Saudi-Arabia, Turkey, and United Arab Emirates vary in the different countries.

In Bahrain, Egypt, Israel, Oman, Qatar, Saudi-Arabia, and Turkey the use for cheese is permitted. Dubai, Kuwait, and the United Arab Emirates approve the use of natamycin as a food additive.

(h) Other countries. In South Africa natamycin is allowed in a broad range of food products (Government Gazette no. 8436, 5 November 1982 and no. 5729, 2 September 1977). In Cyprus and Philippines natamycin is allowed for the surface treatment of hard cheeses.

9.7.3 Conclusion

Natamycin is approved as a food additive in many countries, but in most the approval is for use in the dairy industry only. In a number of countries a wider use is permitted, for example, in the meat industry or as a general food additive.

References

Ali, H.J. (1989) Development and application of an enzyme immunoassay for the detection of natamycin in food. Dissertation at the Veterinarian Faculty of the University of Munich, Germany.

Athar, M.A. and Winner, H.I. (1971) The development of resistance by *Candida* species to polyene antibiotics *in vitro*. *Journal of Medical Microbiology*, **4**, 505–517.

Ayres, J.C. and Denisen, E.L. (1958) Maintaining freshness of berries using selected packaging materials and antifungal agents. *Food Technology*, **12**, 562–567.

Ayres, J.C., Walker, H.W., Fanelli, M.J. *et al.* (1956) Use of antibiotics in prolonging storage life of dressed chicken. *Food Technology*, **10**, 563–568.

Baldini, P., Palmia, F., Raczynski, G. *et al.* (1979) Impiego della pimaricina nella prevenzione della crescita della muffe sui prodotti di salumeria italiani. *Industria Conserve*, **54**, 305–307.

Bärwald, G. (1976) Über die Kaltenkeimung von Apfelsaft mit dem Fungicid Pimaricin. *Die industrielle Obst- und Gemüseverwertung*, **61**, 453–458.

Berry, D. (1999) Natamycin for shredded cheese. *Dairy-Foods*, **100**, 45.

Bolard, J. (1986) How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochimica et Biophysica Acta*, **864**, 257–304.

Brik, H. (1981) Natamycin. In *Analytical Profiles of Drug Substances*, Vol. 10 (ed. K. Florey), Academic Press, New York, pp. 513–561.

Brik, H. (1994) Natamycin (Supplement). In *Analytical Profiles of Drug Substances and Excipients*, Vol. 23 (ed. H.G. Brittain), Academic Press, San Diego, pp. 399–419.

Bulder, C.J.E.A. (1971) Anaerobic growth, ergosterol content, and sensitivity to a polyene antibiotic, of the yeast *Schizosaccharomyces japonicus*. *Antonie van Leeuwenhoek*, **37**, 353–358.

Cattaneo, P., D'Aubert, S., and Righetti, A. (1978) Attivita antifungina della pimaricina in salami crudi stagionati. *Industrie Alimentari*, **17**, 658–664.

Ceder, O., Hansson, B., and Rapp, U. (1977) Pimaricin. VIII. Structural and configurational studies by electron impact and field desorption mass spectrometry, carbon-13 (25.2 MHZ) and proton (270 MHZ)-NMR spectroscopy. *Tetrahedron*, **33**, 2703–2714.

Clark, W.L., Shirk, R.J., and Kline, E.F. (1964) Pimaricin, a new food fungistat. In *Microbial Inhibitors in Food* (ed. N. Molin), Almquist & Wiksell, Uppsala, pp. 167–184.

Daamen, C.B.G. and Van den Berg, G. (1985) Prevention of mould growth on cheese by means of natamycin, *Voedingsmiddelentechnologie*, **18**, 26–29.

Davidson, P.M. and Doan, C.H. (1993) Natamycin. In *Antimicrobials in Foods* (eds P.M. Davidson and A.L. Branen), Marcel Dekker, New York, pp. 395–407.

De Boer, E., Labots, M., Stolk-Horsthuis, M. *et al.* (1979) Sensitivity to natamycin of fungi in factories producing dry sausage. *Fleischwirtschaft*, **59**, 1868–1869.

De Boer, E. and Stolk-Horsthuis, M. (1977) Sensitivity to natamycin (pimaricin) of fungi isolated in cheese warehouses. *Journal of Food Protection*, **40**, 533–536.

De Kruijff, B., Gerritsen, W.J., Oerlemans, A. *et al.* (1974) Polyene antibiotic–sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. II. Temperature dependence of the polyene antibiotic–sterol complex formation. *Biochimica et Biophysica Acta*, **339**, 44–56.

De Ruig, W.G. and Van den Berg, G. (1985) Influence of the fungicides sorbate and natamycin in cheese coatings on the quality of the cheese. *Netherlands Milk and Dairy Journal*, **39**, 165–172.

Demel, R.A., Crombag, F.J.L., Van Deenen, L.L.M., and Kinsky, S.C. (1968) Interaction of polyene antibiotics with single and mixed lipid molecular layers. *Biochimica et Biophysica Acta*, **150**, 1–14.

Demel, R.A., Van Deenen, L.L.M., and Kinsky, S.C. (1965) Penetration of lipid monolayers by polyene antibiotics. Correlation with selective toxicity and mode of action. *Journal of Biological Chemistry*, **240**, 2749–2753.

Duplantier, A.J. and Masamune, S. (1990) Pimaricin. Stereochemistry and synthesis of its aglycon (pimarolide) methyl ester. *Journal of the American Chemical Society*, **112**, 7079–7081.

Espinel-Ingroff, A., Bartlett, M., Bowden, R. *et al.* (1997) Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. *Journal of Clinical Microbiology*, **35**, 139–143.

Fente-Sampayo, C.A., Vazquez-Belda, B., Franco-Abuin, C. *et al.* (1995) Distribution of fungal genera in cheese and dairies. Sensitivity to potassium sorbate and natamycin. *Archiv für Lebensmittelhygiene*, **46**, 62–65.

Frisvad, J.C., Samson, R.A., Rassing, B.R. *et al.* (1997) *Penicillium discolor*, a new species from cheese, nuts and vegetables. *Antonie van Leeuwenhoek*, **72**, 119–126.

Frisvad, J.C. and Thrane, U. (1995) Mycotoxin production by food-borne fungi. In *Introduction to Food-borne Fungi*, 4th edn (eds R.A. Samson *et al.*), Centraal Bureau voor Schimmelcultures, Baarn, pp. 251–260.

Gale, E.F. (1984) Mode of action and resistance mechanisms of polyene macrolides. In *Macrolide Antibiotics. Chemistry, Biology and Practice* (ed. S. Omura), Academic Press, New York, pp. 425–453.

Gelda, C.S., Mathur, A.D., and Stersky, A.K. (1974) The retarding effect of the antifungal agent pimaricin on the growth of *Aspergillus parasiticus*. *Proceedings IV International Congress of Food Science and Technology*, **3**, 261–265.

Gourama, H. and Bullerman, L.B. (1988) Effects of potassium sorbate and natamycin on growth and penicillic acid production by *Aspergillus ochraceus*. *Journal of Food Protection*, **51**, 139–144.

Hamilton-Miller, J.M.T. (1973) Chemistry and biology of the polyene macrolide antibiotics. *Bacteriological Reviews*, **37**, 166–196.

Hamilton-Miller, J.M.T. (1974) Fungal sterols and the mode of action of the polyene antibiotics. *Advances in Applied Microbiology*, **17**, 109–134.

Hammond, S.M. (1977) Biological activity of polyene antibiotics. In *Progress in Medicinal Chemistry*, Vol. 14 (eds G.P. Ellis and G.B. West), North-Holland Publishing Company, Amsterdam, pp. 105–179.

Hechelmann, H. and Leistner, L. (1969) Hemmung von unerwünschtem Schimmelpilzwachstum auf Rohwürsten durch Delvocid (Pimaricin). *Fleischwirtschaft*, **49**, 1639–1641.

Henninger, W. (1977) Die Abtötung von getränkeverderbenden Hefen durch Pimaricin. *Das Erfrischungsgetränk Mineralwasser-Zeitung*, **30**, 1–6.

Hoekstra, E.S., Van der Horst, M.I., Samson, R.A. *et al.* (1998) Survey of the fungal flora in Dutch cheese factories and warehouses. *Journal of Food Mycology*, **1**, 13–22.

Holley, R.A. (1981) Prevention of surface mold growth on Italian dry sausage by natamycin and potassium sorbate. *Applied and Environmental Microbiology*, **41**, 422–429.

Holley, R.A. (1986) Effect of sorbate and pimaricin on surface mold and ripening of Italian dry salami. *Lebensmittel Wissenschaft und Technologie*, **19**, 59–65.

Kerridge, D., Koh, T.Y., and Johnson, A.M. (1976) The interaction of Amphotericin B methyl ester with protoplasts of *Candida albicans*. *Journal of General Microbiology*, **96**, 117–123.

Kiermeier, F. and Zierer, E. (1975) Zur Wirkung von Pimaricin auf Schimmelpilze und deren Aflatoxinbildung bei Käsen. *Zeitschrift für Lebensmitteluntersuchung und Forschung*, **157**, 253–262.

Kinsky, S.C., Haxby, J., Kinsky, C.B. *et al.* (1968) Effect of cholesterol incorporation on the sensitivity of liposomes to the polyene antibiotic, filipin. *Biochimica et Biophysica Acta*, **152**, 174–185.

Kotler-Brajtburg, J., Medoff, G., Kobayashi, G.S. *et al.* (1979) Classification of polyene antibiotics according to chemical structure and biological effects. *Antimicrobial Agents and Chemotherapy*, **15**, 716–722.

Lancelin, J.M. and Beau, J.M. (1990) Stereostructure of pimaricin. *Journal of the American Chemical Society*, **112**, 4060–4061.

Lancelin, J.M. and Beau, J.M. (1995) Stereostructure of glycosylated polyene macrolides – the example of pimaricin. *Bulletin Société Chimie France*, **132**, 215–223.

Lieske, K., Lieske, C., and Refai, M. (1983) Resistance determinations in mycology. *GIT Supplement*, **3**, 41–43.

Lodi, R., Todesco, R., and Bozzetti, V. (1989) Nouvelles applications de la natamycine sur des fromages typiques italiens. *Microbiologie–Aliments–Nutrition*, **7**, 81–84.

Lund, F., Filtenborg, O., and Frisvad, J.C. (1995) Associated mycoflora of cheese. *Food Microbiology*, **12**, 173–180.

Malewicz, B. and Borowski, E. (1979) Energy dependence and reversibility of membrane alterations induced by polyene macrolide antibiotics in *Chlorella vulgaris*. *Nature*, **281**, 80–82.

Marriott, M.S. (1975) Isolation and chemical characterization of plasma membranes from the yeast and the mycelial forms of *Candida albicans*. *Journal of General Microbiology*, **86**, 115–132.

Moerman, P.C. (1967) Evaluation of the fungicide pimaricin (Delvocid) for the prevention of mould growth on sausages. *Vleesdistributie en Vleestechnologie*, **2**, 243.

Moerman, P.C. (1972) Schimmelwering op vleeswaren door Pimaricine. *Voedingsmiddelentechnologie*, **3**, 261–264.

Morris, H.A. and Castberg, H.B. (1980) Control of surface growth on Blue cheese using pimaricin. *Cultures Dairy Products Journal*, **15**, 21–23.
Nadeau, P., Gruda, I., Medoff, G., and Brajtburg, J. (1982) Relative avidity of etruscomycin to cholesterol and ergosterol. *Antimicrobial Agents and Chemotherapy*, **21**, 545–550.
Neviani, E., Emaldi, G.C., and Carini, S. (1981) L'impiego di pimaricina come antifungino sulle croste dei formaggi: Technologia e microflora di superficie. *Latte*, **6**, 335–343.
Nilson, K.M., Shahani, K.M., Vakil, J.R. *et al.* (1975) Pimaricin and mycostatin for retarding cottage cheese spoilage. *Journal of Dairy Science*, **58**, 668–671.
Norman, A.W., Demel, R.A., De Kruijff, B. *et al.* (1972) Biological properties of polyene antibiotics. Comparison of other polyenes with filipin in their ability to interact specifically with sterol. *Biochimica et Biophysica Acta*, **290**, 1–14.
Norman, A.W., Spielvogel, A.M., and Wong, R.G. (1976) Polyene antibiotic–sterol interaction. In *Advances in Lipid Research*, Vol. 14 (eds. R. Paoletti and D. Kritchevsky), Academic Press, New York, pp. 127–170.
Oldenkamp, E.P., Smink, D.A., and Van Os, J.L. (1976) Antifungal compositions and method (natamycin and citric acid). U.S. Patent Application No. 76US-744513.
Raab, W.P. (1974) Natamycin (Pimaricin). In *Properties and Medical Applications*, Georg Thieme Verlag, Stuttgart.
Ray, L.L. and Bullerman, L.B. (1982) Preventing growth of potentially toxic molds using antifungal agents. *Journal of Food Protection*, **45**, 953–963.
Sachdeva, S., Sing, S., Tiwari, B.D. *et al.* (1994) Effect of processing variables on the quality and shelf-life of processed cheese from buffalo milk Cheddar cheese. *The Australian Journal of Dairy Technology*, **49**, 75–78.
Scott, P.M. (1989) Mycotoxigenic fungal contaminants of cheese and other dairy products. In *Mycotoxins in Dairy Products*, 1st edn (ed. H.P. van Egmond), Elsevier Science Publishers Ltd, Essex, pp. 193–259.
Shirk, R.J. and Clark, W.L. (1963) The effect of pimaricin in retarding the spoilage of fresh orange juice. *Food Technology*, **17**, 1062–1066.
Staden, O.L. and Witmondt, M. (1967) Lecithine-pimaricine-perspectieven op hard fruit. *De Fruitteelt*, **57**, 1180–1182.
Struyk, A.P., Hoette, I., Drost, G. *et al.* (1957–1958) Pimaricin, a new antifungal antibiotic. *Antibiotics Annual*, **1957–1958**, 878–885.
Teerlink, T., De Kruyff, B., and Demel, R.A. (1980) The action of pimaricin, etruscomycin and amphotericin B on liposomes with varying sterol content. *Biochimica et Biophysica Acta*, **599**, 484–492.
Thomas, A.H. (1986) Suggested mechanisms for the antimycotic activity of the polyene antibiotics and the N-substituted imidazoles. *Journal of Antimicrobial Chemotherapy*, **17**, 269–279.
Ticha, J. (1975) A new fungicide, pimaricin, and its application in the baking industry. *Mlynsko-Pekarensky Prumysl*, **21**, 225–228.
Tortorello, M.L., Best, S., Batt, C.A. *et al.* (1991) Extending the shelf-life of cottage cheese: Identification of spoilage flora and their control using food grade preservatives. *Cultured Dairy Products Journal*, **26**, 8–12.
Van Rijn, F.T.J., Hoekstra, E.S., Van der Horst, M.I. *et al.* (1997) The occurrence of *Penicillium discolor* in the Dutch cheese industry. *Voedingsmiddelentechnologie*, **20**, 19–23.
Van Rijn, F.T.J., Stark, J., Tan, H.S. *et al.* (1995) A novel antifungal composition. *U.S. Patent Application No. 08/446,782*.
Verma, H.S., Yadav, J.S., and Neelakantan, S. (1988) Preservative effect of selected antifungal agents on butter and cheese. *Asian Journal of Dairy Research*, **7**, 34–38.
Wyatt, R.D. and Brothers, A.M. (1997) The antifungal activity of natamycin in commercial poultry feed. *Poultry Science*, **76**, 32.
Yamanouchi. *Pimafucin® in fungal skin infections*. Technical Bulletin. Leiderdorp, The Netherlands.
Ziogas, B.N., Sisler, H.D., and Lusby, W.R. (1983) Sterol content and other characteristics of pimaricin-resistant mutants of *Aspergillus nidulans*. *Pesticide Biochemistry and Physiology*, **20**, 320–329.
Zuthof, J.B.I. and Isidorus, J.B. (1981) Cheese manufacturing – flowing brine over mould pressed cheese in racks. GB Patent 2072481.

Library of Congress Cataloging-in-Publication Data
Food preservatives / edited by N.J. Russell and G.W. Gould.—2nd ed.
p. cm.
Includes bibliographical references and index.
ISBN 0-306-47736-X
1. Food preservatives. I. Russell, Nicholas J. II. Gould, G.W. (Grahame Warwick)

TP371.2.F67 2003
664'.028—dc21

2003044723

ISBN 0-306-47736-X

233 Spring Street, New York, N.Y. 10013

http://www.wkap.nl/

10 9 8 7 6 5 4 3 2 1

A C.I.P. record for this book is available from the Library of Congress

Printed in the United States of America

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L V Thomas and J Delves-Broughton, Danisco Innovation, Beaminster, Dorset, UK

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Introduction

Natamycin (also known as pimaricin) is a polyene macrolide antimycotic produced by *Streptomyces natalensis*. It is marketed commercially as the Natamax™ family of products by Danisco, and as the Delvocid® family of products by DSM. Natamycin is used worldwide as a food preservative; its primary application is surface treatment of cheese and processed meat (e.g., dry sausages) by dipping, spraying, or in emulsion coatings. It has several advantages as a preservative, including broad activity spectrum, efficacy at low concentrations, lack of resistance, and activity over a wide pH range. Due to its low solubility, natamycin does not migrate from the surface into the food, and thus does not affect the organoleptic properties. It has no effect on bacteria, including those used as starter cultures or to promote ripening. It is chemically stable and has prolonged effectiveness. Moreover, it is easy to apply and has a proven safety record. Its general characteristics are summarized in Table 1.

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History

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Natamycin was first discovered in 1955, when it was isolated from culture filtrates of an *Actinomycetes* bacterium isolated from soil near the town of Pietermaritzburg, in the Natal province of South Africa. The antimycotic was first called pimaricin, a name derived from this place of origin. Its current name natamycin, as well as the name of its producer organism *Streptomyces natalensis*, was similarly derived from Natal. Natamycin has also been produced from closely related *Streptomyces* strains isolated from other locations and has been given various names (e.g., tennecetin, antifungal A-5283) and trade names. Its potential as a food preservative was recognized shortly after its discovery, when its efficacy against molds and yeasts was investigated in foods including fruit juice, carbonated drinks, fresh strawberries and raspberries, dressed poultry, sausages, cottage cheese, and hard cheese.

Structure

Natamycin has the empirical formula $C_{33}H_{47}NO_{13}$ and a molecular weight of 665.7. The structure was first determined in 1958, and later revised. It belongs to the group of polyene macrolide antifungals, compounds characterized by possession of a macrocyclic ring of carbon atoms closed by lactonization, and four conjugated double bonds. This classes it as a tetraene antimycotic (**Figure 1**). The structure is closely related to other antimycotics such as nystatin, rimodicin, and amphotericin. The molecule is amphoteric due to one basic and one acid group and has an isoelectric point of 6.5.

Stability and Solubility

Natamycin is a white/cream-colored crystalline powder with no taste and little odor. It is usually found as the stable trihydrate formulation. No significant loss of activity occurs for several months if the powder is stored in the dark at room temperature. Aqueous suspensions are less stable, particularly if exposed to light, certain oxidants, and heavy metals, but remain sufficiently stable during practical use. Although solutions are more unstable in acid or alkaline conditions, the pH of food products is not normally at levels that would cause problems. For instance, natamycin stored at 30 °C for 21 days reportedly retained 100% activity at pH 5–7, retained *c.* 85% at pH 3.6, and 75% at pH 9.0. In acid solutions, the molecule undergoes hydrolysis at the glycosidic linkage, forming mycosamine and aponatamycin and subsequently other compounds. Aqueous suspensions at neutral pH are stable for 24 h at 50 °C and show little reduction in activity at this temperature for several days and for shorter periods at 100 °C.

Natamycin is poorly soluble in water (30–100 p.p.m. at room temperature) and almost insoluble

in nonpolar solvents, but shows good solubility in strongly polar organic solvents (Table 1). To be active, natamycin must be in solution. However, solubility is not normally a limiting factor as natamycin is usually effective at relatively low concentrations. In solution, natamycin has an ultraviolet absorption spectrum with minima at 250, 295.5, and 311 nm, and maxima at 220, 290, 303, and 318 nm.

Table 1 General characteristics of natamycin

Characteristic	*Description*
Names	Natamycin, pimaricin (pimafucin), tennecetin, antifungal A-5283 (trade names: Natacyn, Myprozine)
Commercial products	Natamax™, Delvocid®
Producer organism	*Streptomyces natalensis*
EU number	E235
Formula	$C_{33}H_{47}NO_{13}$
Molecular weight	665.7 Da
Structure	Polyene macrolide antimycotic compound with a macrocyclic ring of carbon atoms closed by lactonization, and four conjugated double bonds
Properties	Amphoteric. Isoelectric point: 6.5
Solubility in different solvents	Water: 30–100 p.p.m. *n*-Butanol: 50–120 p.p.m. Glycerol: 15 000 p.p.m. Methylpyrrolidone: 120 000 p.p.m. Glacial acetic acid: 185 000 p.p.m.
Absorption spectrum	Maxima: 290, 303, 318 nm Minima: 250, 295.5, 311 nm
Antimicrobial spectrum	Most molds and yeasts (MIC: < 5–20 p.p.m.)
JECFA ADI	0.3 mg kg^{-1} body weight

MIC, minimum inhibitory concentration; JECFA, Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives; ADI, acceptable daily intake.

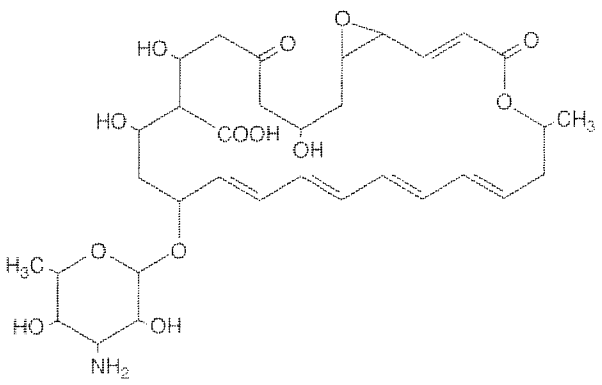

Figure 1 The structure of natamycin.

Mode of Action and Antimicrobial Effect

Mechanism of Action

Natamycin acts against yeasts and molds but is ineffective against bacteria. It combines with ergosterol and other sterols such as 24, 28-dehydroergosterol and cholesterol, compounds present in the cell membranes of yeast and molds but (with rare exceptions) not present in bacteria. The irreversible binding of natamycin to sterols, in particularl ergosterol, disrupts the cell membrane integrity and increases membrane permeability. This results in leakage of essential cellular constituents including cations, causing a rapid drop in intracellular pH and ultimately cell lysis. A study of laboratory-forced natamycin-resistant mutants of *Aspergillus flavus* has confirmed this theory. These either had reduced levels of ergosterol or none at all (and consequently much slower growth rates – critical to survival *in vivo*). A secondary mode of action is the inhibition of glycolysis and respiration.

Antimicrobial Spectrum

Natamycin is active against an extensive range of yeasts and molds (Table 2). The preservative is usually effective at concentrations between < 1 and 10 p.p.m. In general, yeasts are more sensitive than molds: the minimum inhibitory concentration (MIC) of yeasts is usually < 5 p.p.m., whereas that of molds can be at least 10 p.p.m. Examples of MICs are shown

Table 2 Antimicrobial spectrum: examples of yeast and molds sensitive to natamycin

Absidia	*Penicillium camemberti*
Alternaria	*P. chrysogenum*
Aspergillus chevalieri	*P. digitatum*
A. clavatus	*P. expansum*
A. flavus	*P. glabrum*
A. nidulans	*P. islandicum*
A. niger	*P. notatum*
A. ochraceus	*P. roqueforti* var. *punctatum*
A. oryzae	*Rhizopus oryzae*
A. penicilloides	*Rhodotorula gracilis*
A. roquefortii	*Saccharomyces bailii*
A. versicolor	*S. bayanus*
Botrytis cinerea	*S. cerevisiae*
Brettanomyces bruxellensis	*S. exiguus*
Candida albicans	*S. ludwigii*
C. guilliermondii	*S. rouxii*
C. vini	*S. sake*
Cladosporium cladosporioides	*Sclerotina fructicola*
Fusarium	*Scopulariopsis asperula*
Gloeosporium album	*Torulopsis candida*
Hansenula polymorpha	*T. lactis* var. *condensi*
Kloeckera apiculata	*Wallenis sebi*
Mucor mucedo	*Zygosaccharomyces barkeri*
M. racemosus	

Table 3 Examples of sensitivity of yeasts and molds to natamycin

Strain	Minimal inhibitory concentration (p.p.m.)
Aspergillus chevalleri 4296	0.1–2.5
Saccharomyces cerevisiae H	0.15
Penicillium chrysogenum	0.6–1.0
Aspergillus niger	1.0–1.8
Saccharomyces bailii	1.0
Candida albicans	1–2.5
Mucor mucedo	1.2–5.0
Penicillium notatum 4640	5.0
Saccharomyces rouxii 0562	5.0
Rhizopus oryzae 4758	10.0

in Table 3. Less susceptible species include *Verticillium cinnabarinum*, *Botrytis cinerea*, and *Penicillium discolour*, and also occur among the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichophyton*. The outcome of antifungal activity is usually cidal, in contrast to sorbate, which is fungistatic. Natamycin can inhibit aflatoxin synthesis, furthermore overall control of fungi leads to control of aflatoxins.

Resistance to polyene antimycotics such as natamycin does not seem to occur naturally and it has also proved difficult to generate resistant mutants in the laboratory. For example, in a survey of factories of natamycin-producers, no detectable difference in natamycin sensitivity was found for isolates compared to other factories. Similarly, in both a cheese warehouse and sausage factory where natamycin had been used for some time, no resistant strains were isolated. This lack of resistance may be partly explained by the fact that natamycin occurs as micelles in solution. Thus if a cell comes into contact with natamycin in solution, the antimycotic concentration is high and consequently lethal. Furthermore, the site of activity is an essential component of the cell membrane.

Factors Affecting Efficacy

The antimycotic activity of natamycin is affected by several factors, which may also affect its stability, such as pH, temperature, light, oxidants, and heavy metals. Natamycin is active over a wide pH range (pH 3–9); pH does not appear to affect its antimycotic activity but does affect stability. Compounds such as peroxides or chlorides, often used as cleaning/disinfecting agents, should be used with care in the proximity of natamycin.

Methods of Assay

The natamycin content of food products can be determined by microbiological, immunochemical, spectrophotometric, and liquid chromatographic (LC) procedures. The agar diffusion bioassay using *Saccharomyces cerevisiae* can also be used for quantitative assessment. Spectrophotometric and LC methods are commonly used for routine analysis. High-performance liquid chromatography (HPLC) with ultraviolet detection is considered one of the most sensitive and accurate methods, with a detection limit of 0.5 mg kg^{-1}. (*See* **Chromatography**: High-performance Liquid Chromatography.)

International Dairy Federation standard 140A: 1992 specifies the method for determining the natamycin contents of cheese rind and cheese adjacent to the rind. In this method a known quantity of sample is extracted with methanol, which is then diluted with water and cooled to between −15 and −20 °C to congeal and precipitate the majority of fat, followed by filtration. The natamycin content can then be determined in the filtrate by either a spectrometric or HPLC method. For the spectrometric measurement the spectrum of both a natamycin standard solution and the test sample is recorded in the range 300–340 nm. Absorbance is measured at the maximum of approximately 318 nm, at the minimum of approximately 311 nm, and then at 329 nm. For measurement by HPLC, ultraviolet detection should be at 308 nm and it is recommended that the mobile phase of the column should comprise methanol/water/acetic acid, 60 + 40 + 5 (v/v/v). Before analysis of test samples, a standard natamycin solution is first injected to determine retention time and for calibration.

More recently, a rapid method using derivative spectrophotometry has been described. In this method cheese is extracted with acidified aqueous acetonitrile, and natamycin content is directly quantified in filtered extracts on the basis of the depth of the trough at 322.6 nm after third-derivative processing of the normal ultraviolet spectrum. An enzyme immunoassay involving the use of a natamycin–protein conjugate has also been reported, in which natamycin coupled to horseradish peroxidase was used as the labeled ligand. Detection limits were 200–2000 pg ml^{-1}, which enabled the determination of concentrations as low as 0.005 mg dm^{-2} (0.1 mg kg^{-1}) in cheese rinds.

Toxicology and Legislation

Toxicology studies have been undertaken using mice, rats, rabbits, and guinea pigs. Natamycin was least toxic if administered orally (LD50 = 1500 mg kg^{-1} in rats and mice) or subcutaneously (LD50 = 5000 mg kg^{-1} in rats), and most toxic if administered intravenously (LD50 = 5–10 mg kg^{-1}). No natamycin was

absorbed from the human intestinal tract after 7 days' feeding of up to a maximum of 500 mg day^{-1}. Feeding studies have been conducted in rats, rabbits, and dogs. The acceptable daily intake was set at 0.3 mg kg^{-1} body weight per day in 1976.

Specification of natamycin in the USA (21 CFR 172.155) requires purity of the anhydrous compound to be 97% ± 2%, containing < 1 p.p.m. arsenic and not more than 20 p.p.m. heavy metals.

Natamycin is approved for use as an antimycotic in various cheeses and processed meats in 32 countries worldwide. A more general use as a food additive is allowed in a few countries such as South Africa (Table 4). In the European Union (EU) natamycin (designated E235) is permitted for surface treatment of hard, semihard, and semisoft cheese as well as dry sausages. The maximum surface coverage permitted in the EU is 1 mg dm^{-2}, and penetration is restricted to 5 mm from the surface. In the USA, natamycin is permitted only if the cheese standard allows the use of 'safe and suitable' antimycotics. **Table 4** is a guide to food legislation. This list is not comprehensive – one should be aware that legislation for food additives is under constant review. The reader is advised to check the current legal situation with the appropriate authorities.

Table 4 Food legislation on the use of natamycin

Country	*Food in which natamycin is permitted*	*Maximum permitted level*
Algeria	Cheese rinds	Used in suspension at 2.5 g l^{-1}
Argentina	Surface treatment of hard and semihard paste cheeses	Limit of 1 mg dm^{-2}. Penetration limit of 2 mm
Australia	Surface treatment of cheese rind	Limit of 15 mg/kg
	Uncooked fermented manufactured meat products	Penetration limit of 3–5 mm
Bahrain	Permitted food preservative	
Brazil	Surface treatment of cheese	Limit of 2 mg dm^{-2}
Bulgaria	Cheese rind	500 mg kg^{-1}
Canada	Surface treatment of 47 listed cheeses	20 mg kg^{-1} based on total weight
	Grated/shredded cheese (0.5% sodium lauryl sulfate prohibited as dispersant)	10 mg kg^{-1}
Chile	Surface treatment of hard cheese (prohibited in wine)	
China	Surface treatment of cheese, processed meat products, moon cakes, baked goods, fruit juices, and processing utensils for easily moldy foods	Application by spraying or dipping in 200–300 mg kg^{-1}, to leave a residue of < 10 mg kg^{-1}
Colombia	Cheese	12.5 mg kg^{-1}
Czech Republic	Dairy and meat products – as EU regulations (contact authorities for further information)	Limit of 1 mg dm^{-2}. Penetration limit of 5 mm
Cyprus	Surface treatment of specified cheeses	
Egypt	Surface treatment of cooked cheese (dehydrated, semidehydrated, and semisoft cheese)	Limit of 2 mg 100 cm^{-2} (1 mg dm^{-2})
European Union (EU)	Surface treatment of specified cheese and sausage	Limit of 1 mg dm^{-2}. Penetration limit of 5 mm
Hungary	Surface treatment of hard and semihard cheese, dried, cured sausage	Limit of 1 mg dm^{-2}
Iceland	Surface treatment of ripened and whey cheese	Limit of 2 mg dm^{-2}
India	Surface treatment of hard cheese	Maximum application level: 2 mg dm^{-2}. Maximum residual level in finished cheese: 1 mg dm^{-2}. Penetration limit of 2 mm
Israel	Surface treatment of specified cheese	
Kuwait	Permitted food additive	
Lithuania	Surface treatment of hard, seminard, and semisoft cheese	Limit of 1 mg dm^{-2}. Penetration limit of 5 mm
Mauritius	Surface treatment of hard, semihard, and semisoft cheese and dried cured sausage	Limit of 1 mg dm^{-2}. Penetration limit of 5 mm

Continued

Table 4 Continued

Country	*Food in which natamycin is permitted*	*Maximum permitted level*
Mercosur	Surface treatment of cheese	Limit of 1 mg dm^{-2}. Maximum application of 5 mg kg^{-1}. Penetration limit of 2 mm
Mexico	Cheese surfaces	Limit of 0.002 %
Norway		Maximum level of suspension: 2 g kg^{-1}
	Surface treatment of hard, firm, and semifirm cheese, dried, cured sausage	Limit of 1 mg dm^{-2}. Penetration limit of 5 mm
Oman	Surface treatment of specified cheese	
Philippines	Surface treatment of specified cheese	
Poland	Permitted in colored and uncolored soft wax and polyvinyl acetate for application to skin of hard cheese	No limits
	Surface treatment of smoked, dried sausage	Limit of 1 mg dm^{-2}
Saudi Arabia	Permitted as a mold inhibitor in foodstuffs but controlled by standards of composition	
Slovakia	Surface treatment of cheeses and dried, cured sausage	Limit of 1 mg dm^{-2}
South Africa	Wine, alcoholic fruit beverages, and grape-based liquors	30 mg l^{-1}
	Fresh fruit pulp	5 mg kg^{-1}
	Fruit juice (blackcurrant, pineapple, etc.)	5 mg kg^{-1}
	Fish sausages	6 mg kg^{-1} to be applied to the outer inedible casing only
	Manufactured fish products, fish pastes, fish roe and spawn, with the exception of frozen fish, salted snoek, and canned fish products	6 mg kg^{-1}
	Lobsters (quick frozen)	6 mg kg^{-1}
	Edam, Gouda, Tilsiter, Limburger, Cheddar, Cheshire	2 mg kg^{-1} in rind without plastic coating; 500 mg kg^{-1} in a plastic coating; 10 mg kg^{-1} for application to the surface of the cheese only
	Cottage cheese, cream cheese	Limit of 10 mg kg^{-1}
	Process or blended cheese, including cheese spread, process cheese preparations, and soft cheese	10 mg kg^{-1} for application to the surface of the cheese only
	Yogurt	10 mg kg^{-1}
	Canned foods	6 mg kg^{-1}
	Manufactured meat products	Limit of 500 mg kg^{-1} on casing or 6 mg kg^{-1} in contents
	Canned chopped meat, canned corned beef, cooked cured luncheon meat, cooked cured pork shoulder, Biltong, frozen cooked-meat pie fillings	Limit of 6 mg kg^{-1}
Tunisia	Surface treatment of hard, semihard and semisoft cheese; dried, cured sausage	Limit of 1 mg dm^{-2}
Turkey	Surface treatment of hard and semihard cheese, dried cured sausages, salami, and hot dogs	Limit of 1 mg dm^{-2}. Penetration limit of 5 mm
Ukraine	Surface treatment of cheese	Limit of 1 mg dm^{-2}. Penetration limit of 5 mm
United Arab Emirates	Permitted food additive	
USA	Surface treatment of cuts and slices of cheese	Limit of 20 mg kg^{-1}
	Nonstandard of identity yogurt	Limit of 7 mg kg^{-1}
	Nonstandard of identity cream cheese	Limit of 7 mg kg^{-1}
	Cottage cheese	Limit of 7 mg kg^{-1}
	Sour cream	Limit of 7 mg kg^{-1}
	Soft tortillas	Limit of 20 mg kg^{-1}
	Nonstandard of identity salad dressing	Limit of 20 mg kg^{-1}
Venezuela	Surface treatment of specified cheeses and sausages	Maximum 0.5% suspension

Preservation of Foods with Natamycin

Types of Food Suitable for Natamycin Use

The principal use of natamycin is on the surface of cheese and dry sausages. Due to its low solubility, natamycin remains effective on the surface for extended periods. When first applied, only 30–50 p.p.m. will be present in solution on the surface – the remainder is present in the more stable crystal formation. The preservative then gradually dissolves, insuring a slow release and prolonged effectiveness. An additional advantage of surface treatment is that natamycin does not penetrate far into the food; a limit of penetration of 1–4 mm has been reported in cheese rind. This is particularly useful for the preservation of blue cheese, especially in comparison to sorbate. Sorbate migrates into the cheese matrix where it inhibits the desired blue mold development inside the cheese. Natamycin remains on the surface, acting only where it is needed to prevent the growth of surface spoilage molds.

Much of the early work on natamycin investigated its ability to inhibit fungal growth on fruit. Strawberries, raspberries, and cranberries sprayed with 50 p.p.m. natamycin in the field prior to harvesting showed reduced spoiling during storage. Treatment by immersion in 10–100 p.p.m. proved more effective. A lecithin coating containing natamycin can be used to treat harder fruit, such as apples and pears. An addition level of 1–10 p.p.m. natamycin may be effective for a variety of beverages, including juices, beer, wine, and beverages containing tea or milk solids. Natamycin (at 20 p.p.m.) has proved effective against *S. cerevisiae* spoilage of orange juice. Further potential applications include ready-to-eat frostings, salad dressings, and mayonnaise susceptible to yeast spoilage. Natamycin control of *Aspergillus niger* and *S. cerevisiae* in cottage cheese has been investigated. Levels of 20–100 p.p.m. added to wash water or 1–5 p.p.m. added to the cheese dressing inhibited fungal growth. A level of 5–10 p.p.m. can be effective in controlling yeast and mold growth in yogurt. Natamycin has also been shown to be effective against molds isolated from bakery products. A level of 100 p.p.m. has been shown to inhibit the growth of yeasts and molds in fillings and icings. (*See* Yeasts.)

Natamycin can also be used to treat the surface of cured-meat products such as raw hams and investigations have been conducted into its ability to inhibit the spoilage microflora on raw cut chicken. Dipping in a solution of 10 p.p.m. inhibited yeast counts for 12–15 days at refrigeration temperature.

Mode of Application

To treat the surface of cheese and sausages, the food can be immersed, sprayed, or coated in an aqueous suspension or the suspension can be used in a plastic coating. Penetration into the food then depends on the food type, being greater in soft cheese compared to hard cheese.

Aqueous dipping solutions for cheese usually contain 1250 p.p.m. natamycin, but concentrations vary depending on cheese type. For example, dips for blue cheese may require dip concentrations as high as 2500 p.p.m. The cheese should be dipped into the natamycin slurry for a few seconds, whilst the slurry is kept constantly agitated to maintain the natamycin in suspension. The concentration of natamycin in the dipping solution becomes reduced after each dipping, at a rate dependent on the ratio of surface area to weight of each piece of cheese.

A suspension of 1250 p.p.m. is recommended for spraying cheese, applied at a rate of 6 l t^{-1} to achieve 7–15 p.p.m. natamycin on the surface. It is important that the natamycin is evently applied as it binds rapidly and tightly on contact with the cheese surface, so that subsequent mixing after initial application will not achieve better distribution of the preservative. A tumbler of at least 1 m in length is recommended with a space of at least 0.3 m between the natamycin and flow agent application.

Lower levels of natamycin (100–750 p.p.m.) can also be added to an emulsion of a polymer in water, mostly polyvinyl acetate, which can be applied as a cheese coating. Alternatively, 100–20 000 p.p.m. of natamycin can be mixed with 0.5–50 g l^{-1} of a suitable thickener and approximately 20–250 g l^{-1} salt and used on the surface of cheese or sausages. This method of application overcomes the problem of uneven distribution of the fungicide caused by the heterogeneity of the fat content of the product. Coatings can be applied by 'painting' multiple layers on to the surface with a sponge or soft cloth, or by a commercially available coating machine.

Natamycin can also be used in natural and fibrous casings of dry fermented sausage products, preventing mold growth during the ripening and storage process. Commonly, casings can be prepared by immersion for 2 h in a 1000-p.p.m. suspension. Alternatively the sausages can be dipped or sprayed in a 1000–5000-p.p.m. natamycin suspension.

Fermentation and Production

Natamycin is produced by fermentation of *Streptococcus natalensis* in an aqueous nutrient medium containing a carbon source (e.g., starch, molasses,

glycerol, glucose, lactose, maltrose, sucrose, alcohols, organic acids), a fermentable organic and/or inorganic nitrogen source (e.g., corn steep liquor, casein, zein, lactalbumin, soya bean meal). The carbon source usually comprises 0.5–5% of the medium. Inorganic cations (potassium, sodium, or calcium) and anions (sulfate, phosphate, or chloride) may be needed as well as trace elements such as boron, molybdenum, or copper. Fermentation is aerobic and mechanical agitation and use of antifoaming agents can aid the process. The temperature is usually 26–30 °C and the pH range pH 6–8. The period of fermentation, varying with the medium, can be between 48 and 120 h.

Due to its low solubility in water, natamycin will accumulate mainly as crystals in the broth. Recovery commonly involves dissolving the natamycin using polar solvents with limited water miscibility such as butanol, methanol, and acetone and adjusting the medium to approximately pH 10. The fermentation broth is filtered to remove the mycelial biomass and impurities, and adjusted to a lower pH (*c.* pH 7) in order to crystallize the natamycin, which is then recovered and finally dried. An alternative process that does not use organic solvents involves the disintegration of the fermentation biomass by homogenization, high shear mixing, or ultrasonic techniques or treatment with heat, alkali, or enzymes.

See also: **Legislation**: History; **Mycotoxins**: Classifications; **Preservatives**: Classifications and Properties; Food Uses; Analysis; **Spoilage**: Molds in Spoilage; Yeasts in Spoilage; **Yeasts**

Further Reading

Anonymous (1992) Cheese and cheese rind determination of natamycin content method by molecular absorption spectrometry and by high-performance liquid chromatography. *IDF Standard 140A*. Brussels: International Dairy Federation.

Davidson PM and Doan CH (1993) Natamycin. In: Davidson PM and Branen AL (eds) *Antimicrobials in Foods*, pp. 395–407. New York: Marcel Dekker.

Hamilton-Miller JMT (1973) Chemistry and biology of the polyene macrolide antibiotics. *Bacteriological Reviews* 37: 166–196.

Raab WP (1972) *Natamycin (Pimaricin). Its Properties and Possibilities in Medicine*. Stuttgart: Georg Thieme.

Raghoenath D and Webbers JJP (1997) *Natamycin recovery*. AU patent application no. 1999717229 B2.

Stark J (1999) Permitted preservatives – natamycin. In: Robinson RK, Batt CA and Patel PD (eds) *Encyclopedia of Food Microbiology*, pp. 1776–1781. London: Academic Press.

Struyk AP and Waisvisz JM (1975) *Pimaricin and Process of Producing Same*. US patent 3892850.

Struyk AP, Hoette I, Drost G *et al.* (1957) Pimaricin, a new antifungal antibiotic. *Antibiotics Annual* 878–885.

Nectarines *See* **Peaches and Nectarines**

Neural Tube Defects *See* **Pregnancy**: Maternal Diet, Vitamins, and Neural Tube Defects; **Folic Acid**: Properties and Determination; Physiology

ENCYCLOPEDIA OF FOOD SCIENCES AND NUTRITION

SECOND EDITION

Editor-in-Chief

BENJAMIN CABALLERO

Editors

LUIZ C TRUGO

PAUL M FINGLAS

2003

ACADEMIC PRESS

An imprint of Elsevier Science

Amsterdam Boston London New York Oxford Paris
San Diego San Francisco Singapore Sydney Tokyo

VOLUME 7

THE MERCK INDEX

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TWELFTH EDITION

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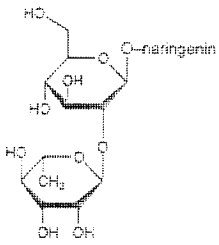
Published by
Merck Research Laboratories
Division of

MERCK & CO., INC.

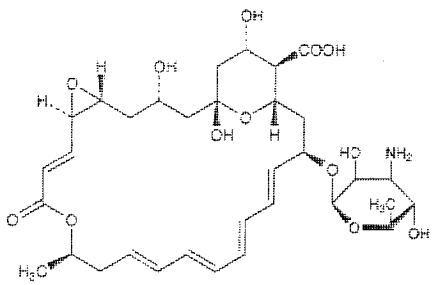
Whitehouse Station, NJ

1996



When crystallized from water, it contains 6 to 8 mols H_2O, mp ~83°. After drying at 110° to constant weight, it contains 2 mols H_2O, mp 171°. Bitter taste (1:10,000 H_2O can be tasted). $[\alpha]_D^{25}$ −82° (alcohol). One gram dissolves in 1000 ml water at 40°. At 75° one gram dissolves in 10 ml water. Sol in acetone, alcohol, warm acetic acid.

6513. Natamycin. *Pimaricin;* antibiotic A 5283; tennecetin; CL-12625; Mycophyt; Myprozine; Natacyn; Pimafucin; Synogil. $C_{33}H_{47}NO_{13}$; mol wt 665.74. C 59.54%, H 7.12%, N 2.10%, O 31.24%. Polyene antifungal antibiotic produced by *Streptomyces natalensis* from soil near Pietermaritzburg, South Africa and by *S. chattanoogensis.* Isoln: Struyk *et al., Antibiot. Ann.* **1957-1958,** 878. Prodn: **Brit.** pat. **844,289** (1960 to Koninklijke Nederlandsche Gist en Spiritus-Fabriek); **Brit.** pat. **846,933** (1960 to Am. Cyanamid). Identity with tennecetin: Divekar *et al., Antibiot. & Chemother.* **11,** 377 (1961). Structure: Golding *et al., Tetrahedron Letters* **1966,** 3551; Meyer, *Chem. Commun.* **1968,** 470. Configuration: Gaudiano *et al., Chim. Ind. (Milan)* **48,** 1327 (1966). Revised structure: R. C. Pandey, K. L. Rinehart, *J. Antibiot.* **29,** 1035 (1976). Soly data: Marsh, Weiss, *J. Assoc. Offic. Anal. Chem.* **50,** 457 (1967). Toxicology: G. J. Levinskas *et al., Toxicol. Appl. Pharmacol.* **8,** 97 (1966). Comprehensive description: H. Brik in *Analytical Profiles of Drug Substances* vol. **10,** K. Florey, Ed. (Academic Press, New York, 1981) pp 513-561.



Crystals from methanol + water, darkens ~200°, dec 280-300°. $[\alpha]_D^{20}$ −278° (c = 1 in CH_3COOH). Sensitive to light, but otherwise very stable in the dry state. uv max (methanol + 0.1% CH_3COOH): 220, 280, 290, 303, 318 nm (ε 21300, 26630, 52930, 83220, 76230). Practically insol in higher alcohols, ether, esters, aromatic or aliphatic hydrocarbons, chlorinated hydrocarbons, ketones, dioxane, cyclohexanol, oils. LD_{50} orally in male, female rats (g/kg): 2.73, 4.67 (Levinskas).

THERAP CAT: Antifungal (topical).

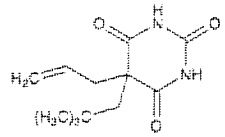
6514. Natural Gas. American natural gas consists of about 85% methane, 9% ethane, 3% propane, 2% nitrogen, 1% butane. Northern Texas gas contains more nitrogen, also sufficient helium to warrant commercial extraction.

Caution: Narcotic in high concns. Incomplete combustion can result in production of carbon monoxide.

USE: Fuel gas, in the manuf of hydrogen, methane, ammonia.

6515. Nauheim Salts (Artificial). Bad Nauheim salts (artificial). The composition used in European pharmaceutical dispensing practice is as follows: three lbs $CaCl_2 \cdot 2H_2O$, two lbs NaCl, 1½ lbs $Na_2CO_3 \cdot 10H_2O$ and ½ lb $NaHCO_3$. This mixture is dissolved in a bath of 50 gallons of water. Then one lb of $NaHSO_4 \cdot H_2O$ is slowly added.

6516. Nealbarbital. *5-(2,2-Dimethylpropyl)-5-(2-propenyl)-2,4,6(1H,3H,5H)-pyrimidinetrione; 5-allyl-5-neopentylbarbituric acid;* nealbarbitone; neallymal; Neventa; Censedal. $C_{12}H_{18}N_2O_3$; mol wt 238.29. C 60.49%, H 7.61%, N 11.76%, O 20.14%. Synthesis: Brandstrom, *Acta Chem. Scand.* **13,** 613, 615, 619 (1959); **Brit.** pat. **797,017** and **U.S.** pat. **2,899,435** (1958, 1959, both to Pharmacia). Metabolism: J. N. T. Gilbert *et al., J. Pharm. Pharmacol.* **26,** 119 (1974); J. N. T. Gilbert, J. W. Powell, *Eur. J. Drug Metab. Pharmacokinet.* **3,** 195 (1978).

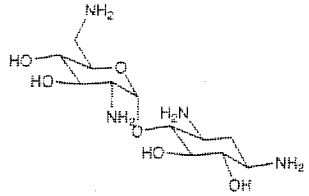


Slightly bitter crystals, mp 155-157°. Practically insol in water, petr ether. Moderately sol in chloroform; freely sol in alcohol, ether, acetone. Also sol in aq alkaline solns.

Note: This is a controlled substance listed in the U.S. Code of Federal Regulations, Title 21 Part 1308.13 (1995).

THERAP CAT: Sedative, hypnotic.

6517. Neamine. *2-Deoxy-4-O-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-D-streptamine;* neomycin A. $C_{12}H_{26}N_4O_6$; mol wt 322.36. C 44.71%, H 8.13%, N 17.38%, O 29.78%. Component of the antibiotic complex neomycin, *q.v.* Isoln: Dutcher *et al., J. Am. Chem. Soc.* **73,** 1384 (1951). Identity of neomycin A and neamine: Leach, Teeters, *ibid.* **74,** 3187 (1952). Prepn from neomycin B: Peck *et al.,* **U.S.** pat. **2,691,675** (1954 to Merck & Co.). Structure: Carter *et al., J. Am. Chem. Soc.* **83,** 3723 (1961); Hichens, Rinehart, *ibid.* **85,** 1547 (1965). Synthesis: Umezawa *et al., J. Antibiot.* **20A,** 53 (1967); H. Kohno *et al., Agr. Biol. Chem.* **39,** 1091 (1975); A. Harayama *et al., Bull. Chem. Soc. Japan* **52,** 3626 (1979).



Crystals from water or aq alc, dec 225-226°, $[\alpha]_D^{25}$ +112.8° (c = 1). IR spectrum: Leach, Teeters, *J. Am. Chem. Soc.* **73,** 2794 (1951).

Hydrochloride, $C_{12}H_{26}N_4O_6 \cdot 4HCl$, amorphous, dec 250-260°, $[\alpha]_D^{25}$ +83° (c = 1).

N-Acetyl deriv, $C_{12}H_{26}N_4O_6 \cdot (CH_3CO)_4$, crystals from methanol, mp 334-336°, $[\alpha]_D^{25}$ +87° (c = 1).

6518. Neatsfoot Oil. Fixed oil from feet of neat (bovine) cattle.

Pale yellow liquid; peculiar odor. d 0.915. Solidif 0° to −10°. n_D^{20} 1.4695-1.4708. Sapon no. 192-203. Iodine no. 44-73.2.

USE: Waterproofing and softening leather; lubricant; oiling wool, etc.

6519. Nebivolol. *α,α'-[Iminobis(methylene)]bis[6-fluoro-3,4-dihydro-2H-1-benzopyran-2-methanol];* α,α'-(iminodimethylene)bis[6-fluoro-2-chromanmethanol]; *dl*-nebivolol; narbivolol; R-65824; Nebilet. $C_{22}H_{25}F_2NO_4$; mol wt 405.44. C 65.17%, H 6.22%, F 9.37%, N 3.45%, O 15.78%. β_1-Adrenergic blocker; prepd as racemic mixture of RSSS and SRRR enantiomers. Prepn: G. R. E. Van Lommen *et al.,* **Eur. pat. Appl. 145,067;** *eidem,* **U.S.** pat. **4,654,362** (1985, 1987 both to Janssen); *eidem, J. Pharm. Belg.* **45,** 355 (1990). Pharmacology of racemate: A. Van de Water *et al., J. Cardiovasc. Pharmacol.* **11,** 552 (1988); and of enantiomers: A. Van de Water *et al., Eur. J. Pharmacol.* **156,** 95

Pimaricin, a New Antifungal Antibiotic

A. P. Struyk, I. Hoette, G. Drost, J. M. Waisvisz, T. van Eek, and J. C. Hoogerheide

Research Department of the Royal Dutch Yeast and Fermentation Industries, Delft, Holland

A new crystalline antibiotic, pimaricin, has been isolated from the fermentation broth of a culture of a *Streptomyces* species, isolated from a soil sample obtained near Pietermaritzburg, State of Natal, Union of South Africa.

This organism has been named *Streptomyces natalensis* nov. sp. and will be described elsewhere in more detail, including some of its biochemical characteristics.

When grown under submerged conditions in media containing soybean meal, glucose, and nutrient salts, an antibiotic is produced that is highly active against a large variety of yeasts and fungi but shows no activity against bacteria.

Good yields of the antibiotic may be isolated from the insoluble part of the culture, such as the centrifuged or filtered mycelial mat, by extraction with organic solvents, such as the lower alcohols or glycols or with formamide and its derivatives. Partial evaporation of these extracts followed by addition of water yields a crystalline precipitate of pimaricin.

The antibiotic may be further purified by dissolving in hot methanol, followed by filtration and precipitation with two volumes of water. Figure 1 is a photomicrograph of crystals of purified pimaricin thus obtained.

There have been no indications that more than one active component is present in culture filtrates of *S. natalensis*.

Pimaricin was found to belong to the group of polyene antibiotics and thus is related to, but not identical with, nystatin, amphotericin, trichomycin, ascosin, candicidin, and others.

PHYSICAL PROPERTIES

In pure form pimaricin is a colorless compound, decomposing at approximately 200 C. without a definite melting point. Table I gives the solubility of pimaricin in several solvents. The antibiotic is very insoluble in water. However, because of its amphotheric character, pimaricin is readily soluble in dilute alkali as well as in dilute acids. In fact, water-soluble crystalline alkali salts may be prepared. These salts, however, have a marked tendency to hydrolyze when dissolved in water. Also, crystalline salts of organic acids, such as its picrate, may be prepared.

In the dry state pimaricin is very stable. Solutions or suspensions in water retain their activity for several weeks, especially in the optimum *p*H range of 5 to 7. However, when such solutions are exposed to direct sunlight or ultraviolet light, decomposition occurs. Addition of antioxidants, e.g. ascorbic acid, markedly improves stability. Solutions or suspensions of pimaricin may be heated to the

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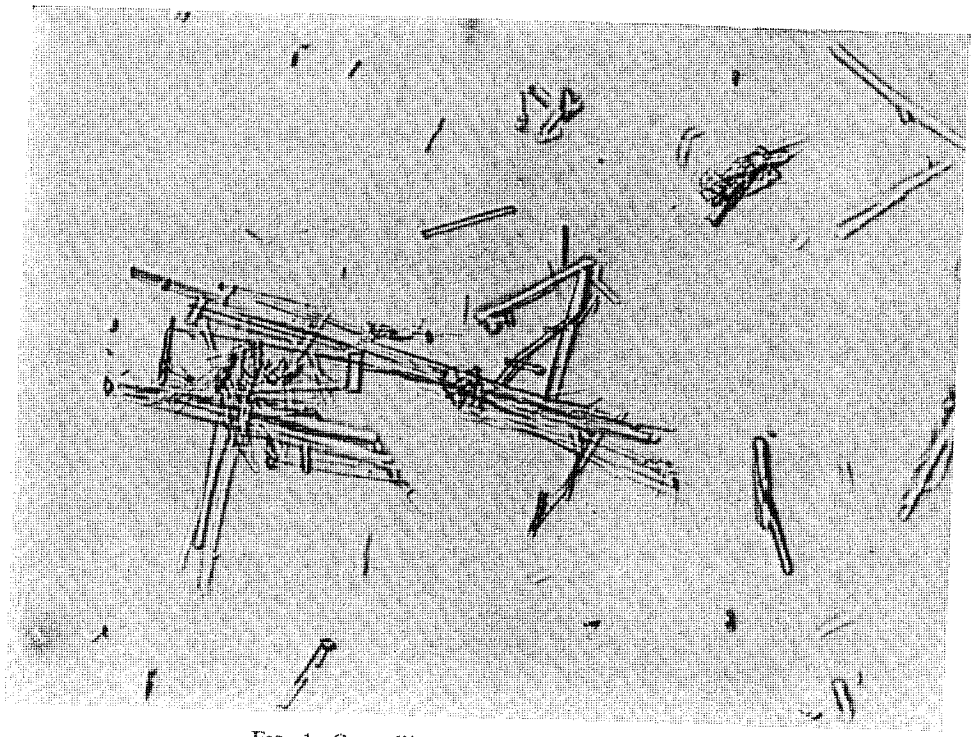

FIG. 1. Crystalline pimaricin is illustrated.

boiling point without any loss of activity; heat sterilization for 20 minutes at 110 C. gives only a moderate potency reduction.

Repeated reprecipitation from methanol with water or reisolation of pimaricin through its crystalline inorganic salts or through its salts with organic acid failed to indicate the presence of impurities (constant biological activity as well as extinction spectrum).

TABLE I

Solubility of Pimaricin

Solvent	Solubility, %*
Water	0.005–0.01
Methanol	0.2
Methanol, 2 per cent calcium chloride	1.5
Propylene glycol or diethylene glycol	2.0
Formamide	2.0
Dimethylformamide	5.0
Methylpyrrolidone	12
Methylcellosolve, 2 per cent calcium chloride	14

* Pimaricine is insoluble in higher alcohols, ethers, esters, aromatic or aliphatic hydrocarbons, chlorinated hydrocarbons, ketones, dioxane, cyclohexanol, and diverse oils.

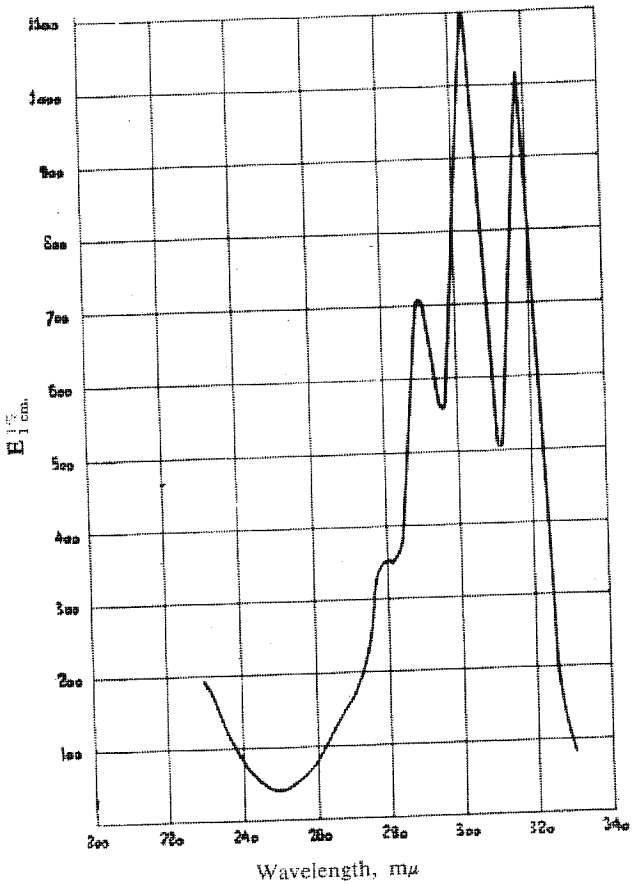

FIG. 2. The ultraviolet absorption spectrum of pimaricin is shown. Concentration: 6.75 μg./ml. methanol.

Pimaricin has the following probable structural fomula: $C_{30-32}H_{45-50}NO_{13}$. This indicates a somewhat smaller molecular size than nystatin has ($C_{46}H_{83}NO_{18}$).[1]

The ultraviolet absorption spectrum has maxima at 279, 290, 303, and 318 mμ, these being the characteristic peaks typical of a conjugated tetraene structure, and as such there is great similarity with the absorption spectra of rimocidin, nystatin, and amphotericin A (fig. 2). Pimaricin can be distinguished readily from these antibiotics by paper chromatographic technique, as is demonstrated in figure 3.

ACTIVITY

Pimaricin is active against a wide variety of fungi and yeasts, including strains pathogenic for man and plants as well as saprophytic varieties. Tables II, III, IV, and V give a brief summary of these sensitivity tests.

It may be seen from these tables that the majority of yeasts and fungi are inhibited by concentrations of 1 to 10 μg./ml. of pimaricin. Least sensitive are some *Trichophyton* and *Pythium* species.

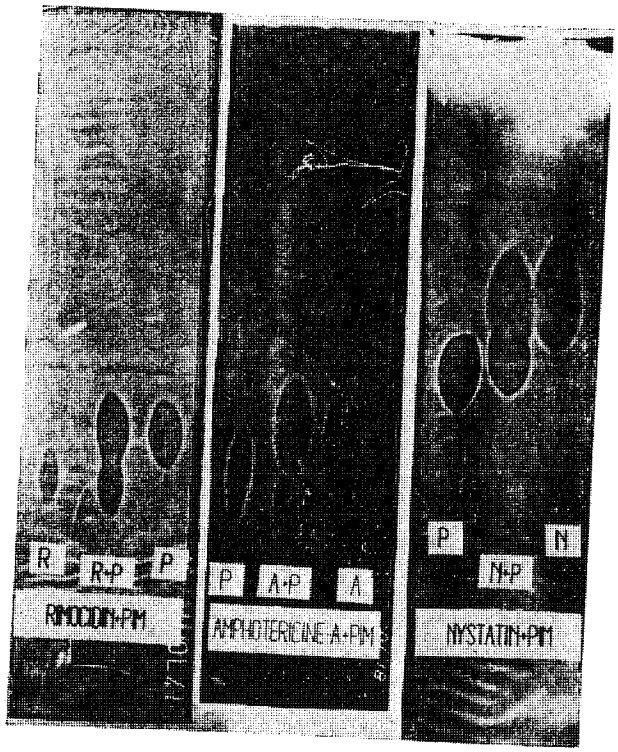

FIG. 3. The paper chromatographic separation of nystatin, rimocidin, and amphotericin from pimaricin on Whatman no. 1 paper (descending method) is illustrated. Nystatin: not buffered, 17 hour elution; system: butanol-ethanol-water (5:1:4). Rimocidin: not buffered, 25 hour elution; system: propanol-water (7:3). Amphotericin A: phosphate-buffered, *p*H 4, 41 hour elution; system: propanol-water (8:2). Test organism: *Saccharomyces cerevisiae.*

Pimaricin also shows marked activity against *Trichomonas vaginalis,* but probably not against amebas.

At no time could we observe any appearance of acquired resistance to pimaricin due to prolonged contact with inhibitory concentrations.

TABLE II

Activity of Pimaricin against Yeasts

Strains	No. of strains tested	Test medium	Minimum inhibitory concentration, μg./ml.
Saccharomyces cerevisiae	2	Malt extract	0.9 to 1.2
Pichia membranfaciens	1	Malt extract	2.5
Schwanniomyces occidentalis	1	Malt extract	2.5
Candida tropicalis	2	Sabouraud agar	3 to 12
Pityrosporum species	1	Sabouraud agar	12
Candida parapsilosis	1	Sabouraud agar	12
Trichosporon cutaneum	1	Sabouraud agar	12
Cryptococcus neoformans (*Blastomyces neoformans*)	6	Malt extract–peptone	5 to 10
Candida albicans	4	Sabouraud agar	6

TABLE III

Activity of Pimaricin against Saprophytic Fungi

Strains	No. of strains tested	Test medium	Minimum inhibitory concentration, μg./ml.
Aspergillus niger	1	Malt extract	1.8
Penicillium chrysogenum	1	Malt extract	0.6
Penicillium species	4	Malt extract	1.0 to 1.5
Mucor mucedo	1	Malt-glucose agar	1.2
Trichoderma species	1	Malt extract	1.2
Cladosporium herbarium	1	Malt-glucose agar	2.5
Paecilomyces species	1	Malt-glucose agar	2.5
Podospora setosa	1	Malt-glucose agar	25.0
Trichocladium asperum	1	Malt-glucose agar	10.0
Chaetomium globosum	1	Malt-glucose agar	25.0
Stemphylium consortiale	1	Malt-glucose agar	25.0

The effect of pimaricin is mainly fungicidal. It is equally active over a wide *p*H range.

TOXICITY

Table VI summarizes the preliminary acute toxicity data (expressed as LD_{50}) obtained with pimaricin for rats, mice, and guinea pigs. Comparison of these data with data in the literature on the toxicity of nystatin[2] shows that the acute intraperitoneal toxicity of pimaricin is only one tenth of that of nystatin. However, its oral toxicity is two to three times higher than that of nystatin. This may be due to a better resorption of pimaricin in the intestinal tract.

Oral administration of 50 to 70 mg. pimaricin/Kg. body weight to rats, daily for a period of 5 or even 10 weeks, had no untoward serious effects. Normal growth was not disturbed, no changes in the blood picture were observed during the test

TABLE IV

Activity of Pimaricin against Fungi Pathogenic for Plants

Strains	No. of strains tested	Test medium	Minimum inhibitory concentration, μg./ml.
Cladosporium cucumerinum	1	Malt extract	0.9
Verticillium dahliae	1	Malt extract	1.2
Verticillium cinnabarinum	1	Malt-glucose agar	50
Fusarium species	1	Malt extract	1.2
Stereum purpureum	1	Malt extract	1.2
Botrytis cinerea	1	Malt agar	25
Alternaria species	1	Malt agar	2.5
Gloeosporium album	1	Malt agar	2.5
Gloeosporium perennans	1	Malt agar	2.5
Phialophora cinerescens	1	CS-glucose agar	5.0
Pythium species	2	Oatmeal agar	150 to 300

TABLE V

Activity of Pimaricin against Fungi Pathogenic for Human Beings

Strains	No. of strains tested	Test medium	Minimum inhibitory concentration, μg./ml.
Histoplasma capsulatum	1	Sabouraud agar	3
Sporotrichum schenckii	1	Sabouraud agar	12
Microsporum lanosum.	1	Sabouraud agar	12
Epidermophyton floccosum	1	Sabouraud agar	12
Hormodendrum compactum	1	Sabouraud agar	6
Trichophyton sulfureum	1	Sabouraud agar	3
Trichophyton mentagrophytes	3	Sabouraud agar	50
Trichophyton violaceum	1	Sabouraud agar	12
Trichophyton rosaceum	1	Sabouraud agar	12
Trichophyton schönleini	1	Sabouraud agar	6
Trichophyton interdigitale	2	Sabouraud agar	25 to 100
Trichophyton rubrum	4	Sabouraud agar	12 to 50
Aspergillus fumigatus	3	Sabouraud agar	1.2 to 20

period, and no histological changes were noticeable in any of the organs at the end of the experimental period.

However, daily oral administration of 150 mg./Kg. for a period of nine weeks gave some growth inhibition.

Much higher doses of 500 mg./Kg. body weight administered daily caused 30 per cent mortality within a two week period with diarrhea, serious growth inhibition, and edema as major abnormalities.

EFFECT ON THE CIRCULATORY SYSTEM

The effect of intravenous injection of pimaricin (dissolved in propylene glycol) on the blood pressure of narcotized cats and dogs is, if at all present, short-lived and may consist of a temporary drop lasting less than one minute after injection of 5 and 10 mg. pimaricin/Kg. body weight. Sometimes a brief halving of the heart frequency is observed due to a negative chronotropic effect on the heart. Porst complexes always retain their normal form.

TABLE VI

Preliminary Acute Toxicity Data

Administration	LD_{50}, mg./Kg. body weight		
	Rat	Mouse	Guinea pig
Oral	1500	1500	450
Intraperitoneal	250		
Intramuscular	2000		
Subcutaneous	5000		

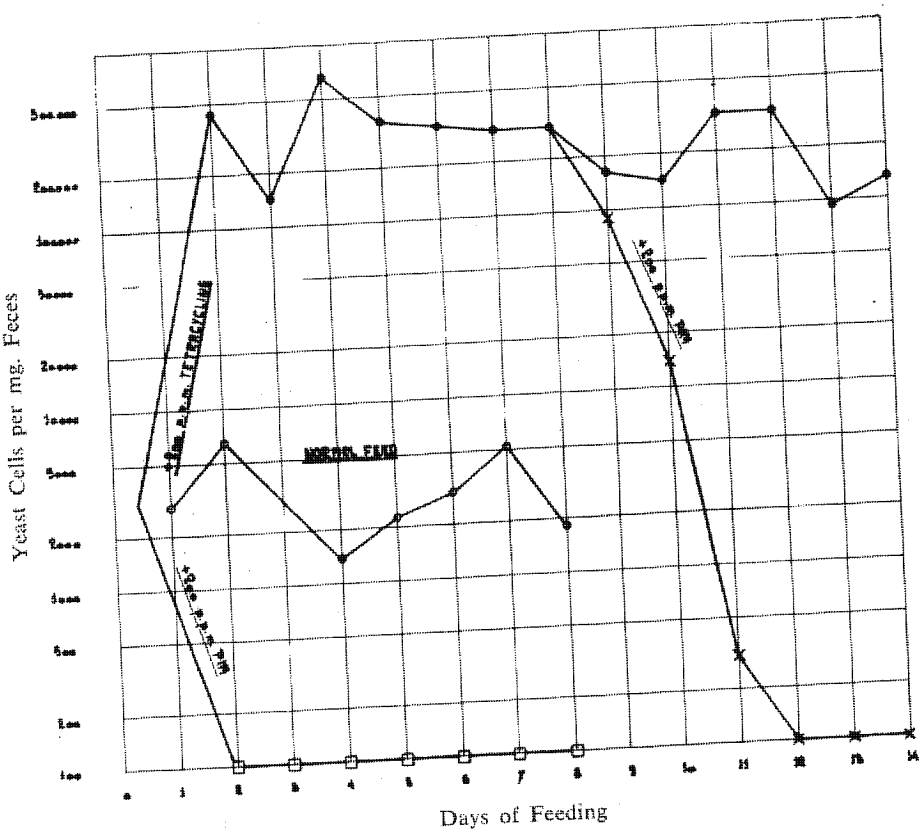

FIG. 4. The yeast cell counts in rat feces after normal feeding, addition of 200 ppm pimaricin to normal feed, and addition of 200 ppm tetracycline to normal feed. After 9 days, some of the rats were transferred to feed containing 200 ppm pimaricin in addition to tetracycline.

EFFECT OF PIMARICIN ADMINISTRATION ON FECAL FLORA

It is a well-known fact that oral administration of broad-spectrum antibiotics, especially the tetracyclines, causes a marked increase of the yeast flora of feces. This could readily be demonstrated by addition of 200 p.p.m. tetracycline to the feed of rats.

Whereas normally the yeast flora of rat feces was found to be of the order of 0 to 500 yeast cells/Gm., this value increased rapidly to 140,000 to 160,000 cells/Gm. after administration of feed medicated with 200 p.p.m. tetracycline.

Addition of pimaricin to this feed either with or without 200 p.p.m. tetracycline reduced the yeast count almost to zero. A concentration of 50 p.p.m. pimaricin or more was sufficient to suppress completely any yeast accumulation in the feces due to tetracycline present in the feed fig. 4).

Pimaricin is at present under extensive clinical investigation for the treatment

of vaginal moniliasis as well as *T. vaginalis* infections. Also, its possible use in the treatment of dermatomycosis is being studied.

Results of these clinical investigations will be published shortly.

ACKNOWLEDGMENT

We are very much indebted to Dr. E. G. Jurgens and Dr. G. A. Overbeek from Organon for conducting certain pharmacological studies and to Dr. A. Manten and Dr. G. J. van Esch from the Netherlands Institute for Public Health for bacteriological and toxicological data reported here.

BIBLIOGRAPHY

1. DUTCHER, J. D.; BOYACK, G., AND FOX, S.: The preparation and properties of crystalline fungicidin (Nystatin). *In:* Antibiotics Annual 1953–1954, New York, Medical Encyclopedia, Inc., 1954, p. 191.
2. HAZEN, E. L., AND BROWN, R.: Fungicidin, an antibiotic produced by a soil actinomycete, Proc. Soc. Exper. Biol. & Med. *76*:93, 1951.

Antibiotics annual

1957·1958

EDITED BY

HENRY WELCH, Ph.D.

and

FELIX MARTÍ-IBAÑEZ, M.D.

MEDICAL ENCYCLOPEDIA, INC.

NEW YORK, N. Y.

1958

Tennecetin: A New Antifungal Antibiotic

General Characteristics

JAMES BURNS AND D. FRANK HOLTMAN

Department of Bacteriology, University of Tennessee, Knoxville, Tenn.

A class of compounds previously unknown in biological systems has, within recent years, been intensively studied because of the antibiotic activity of individual members of the group. These are the conjugated polyenes, identified by their characteristic chromophores in the ultraviolet region[1] and by their selective antifungal antibiotic activity as opposed to antibacterial activity. Recent reports indicate that the ability to produce polyene antibiotics is widespread among streptomycetes.[2–4] This contention is supported by the fact that some 30 or more named polyene antibiotics have been reported in the literature.

Several of these polyenes, notably nystatin and amphotericin B, have found useful application as antibiotics in clinical medicine. Others are subjects of continued study with a view toward useful medical or agricultural applications. All are of interest as unique products of microbial metabolism. Little is known concerning their biological synthesis or their mode of action as antifungal agents.

An interest in these compounds and their biological implications has led us to a study of a single streptomycete and the antifungal antibiotic produced by it. The streptomycete, isolated from Tennessee soil, does not appear to be identical with any previously described organism, and its antibiotic, a tetraene, does not appear to be identical with any previously described antibiotic. We have therefore assigned the appellation *Streptomyces chattanoogensis* for the organism and tennecetin for its antifungal antibiotic. A description of the general characteristics of *S. chattanoogensis* and tennecetin forms the substance of this report.

CHARACTERISTICS OF THE ORGANISM

One of the most prominent characteristics of the organism is its ability to produce a deep yellow-orange pigment in most media. On solid media the pigment diffuses into the agar. The vegetative mycelium of the organism itself is of orange-yellow color.

Sporulation occurs only on certain media, e.g., Carvajal's oatmeal agar. The spore coat is at first white, later turning gray. On oatmeal agar the soluble pigment is at first bright yellow, later becoming darker, almost orange.

Microscopically, spores are found in chains at the ends of coiled sporophores. Spores are spherical, 1.0 to 1.3 μ in diameter.

Other characteristics are: gelatin is rapidly liquefied; starch is hydrolyzed; milk is coagulated and peptonized; hydrogen sulfide is not produced; growth without sporulation occurs at 37 C. but not at 45 C.; growth is inhibited in media containing

Received for Publication: December 30, 1958

3 per cent sodium chloride; and a yellow ring often appears at the surface of litmus milk tubes, but a true pellicle has not been observed on any liquid medium.

Comparison of our culture with authentic strains of named streptomycetes and with descriptions in the literature has not permitted identification as a previously described species. Using the system of Waksman and Lechevalier,[5] the organism may be placed in the *Streptomyces flavus* group. Or, it may be placed in group VIa of the earlier grouping of workers at the Northern Regional Research Laboratory[6] or in Section *Spira*, gray series, of the same workers' later classification.[7]

PRODUCTION AND RECOVERY OF TENNECETIN

Tennecetin has been prepared by surface culture of *S. chattanoogensis* on agar media, in shake flasks, and in laboratory fermenters. Experiments designed to determine the optimal medium for antibiotic production have established that glycerol, dextrin, galactose, and inositol are the best carbon sources. Soybean hydrolysates and yeast extract have been found to stimulate markedly the production of the antibiotic. Most of our experiments have employed a medium of the following composition: glycerol, 2 per cent; phytone (BBL), 0.5 per cent; peptone (Difco), 0.5 per cent; yeast extract (Difco), 0.3 per cent; beef extract (Difco), 0.3 per cent; and tap water. We refer to this culture liquid as "GPY medium." Additions to this medium of corn steep liquor, sodium chloride, or potassium phosphate have not improved the medium to any noticeable extent. The addition of other substances, such as polysorbate 80, oleic acid, palmitic acid, and mevalonic acid, shown by others[8–10] to favor polyene synthesis, has not resulted in higher yields of tennecetin.

The *p*H of autoclaved GPY medium is 7.0. During the first 48 hours of fermentation there is a rapid drop in *p*H, to about *p*H 4.5. This drop may be controlled if the medium is adjusted to *p*H 7.6 before sterilization and 0.25 per cent calcium carbonate is added following sterilization. Controlling the *p*H, however, does not appear to have a great effect on the amount of antibiotic produced in the medium. Omitting of any one of the ingredients from the GPY medium results in decreased yields, although the concentration of yeast extract can be reduced to 0.1 per cent without markedly affecting the fermentation.

Five hundred ml. Erlenmeyer flasks containing 100 ml. GPY medium were inoculated with 1 to 2 ml. of a 24 to 48 hour shaken culture (same medium) of the organism. Incubation on a reciprocal or a rotary shaker at 25–28 C. resulted in good growth and antibiotic production after three to four days. For production of larger quantities, five gallon carboys or fermenters, charged with 10 liters of GPY medium, were inoculated with 100 ml. of a 24 to 48 hour shaken culture of the organism. The carboys are agitated on reciprocal shakers, while the fermenters are equipped with mechanical agitators. Both may be aerated with filtered air or oxygen during the fermentation period. Growth under these conditions is very good, and antibiotic production is at its peak at about 60 to 72 hours at temperatures of 25–28 C.

Unlike many other polyenes, tennecetin occurs almost entirely in the culture

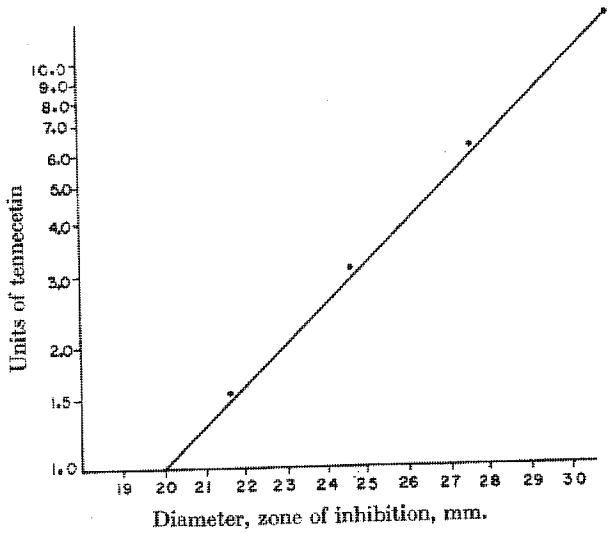

FIG. 1. Tennecetin assay dose-response curve.

liquid. For this reason, we have filtered off the mycelium before extracting the antibiotic. Extraction is accomplished with *n*-butanol, followed by concentration of the butanol extract in vacuo and precipitation of the active material by addition of four volumes of anhydrous ether.

The product resulting from this procedure is a yellow amorphous powder containing up to 100 units of tennecetin activity per mg. of dried powder.

ASSAY OF TENNECETIN

A quantitative microbiological assay method has been developed for measuring the amount of tennecetin present in crude broths, extracts, and dried powders. This is the familiar paper disc–agar diffusion method, using *Saccharomyces carlsbergensis* as the test organism. The assay method gives a straight line response with a good slope over a tenfold range in concentration when plotted on semi-logarithmic paper. Figure 1 shows this dose-response curve.

Using this method, an arbitrary unit of tennecetin has been defined as that amount of antibiotic which, when contained in 1.0 ml. broth or diluent, will give a 20 mm. zone (diameter) of inhibition against the test strain of *S. carlsbergensis* (strain K-20) when tested in accordance with the conditions prescribed for the standard assay method. (The paper discs used in this method are Schleicher and Schuell 740-E, 12.7 mm. diameter.)

The yeast is carried by daily serial transfer in a broth medium composed of glucose, 1 per cent; peptone, 0.5 per cent; and yeast extract, 0.3 per cent. Assay plates are prepared by flooding standard Petri dishes with 20 ml. of 2 per cent purified agar to form a base layer. When the base layer has hardened, 10 ml. of an overlay nutrient agar (same composition as broth medium but with 1 per cent agar added) inoculated with 1 per cent yeast culture is pipetted over the base and evenly distributed. A standard response line is prepared from various dilutions of a reference preparation. Samples are diluted to 2.0 units per ml.

TABLE I

Sensitivity of Microorganisms to Tennecetin by Cross-Streak Agar Diffusion Technique

Organisms Inhibited	
Penicillium sp.	*Corynebacterium* sp.
Aspergillus sp.	*Corynebacterium diphtheriae*
Aspergillus niger	*Pseudomonas tabaci*
Absidia spinosa	*Candida albicans*
Mucor sp.	*Candida krusei*
Syncephalastrum racemosum	*Candida parakrusei*
Cunninghamella sp.	*Candida stellatoidea*
Thamnidium elegans	*Geotrichum* sp.
Circinella tenella	*Cryptococcus neoformans*
Penicillium citrenum	*Torulopiss* sp.
Aspergillus candidus	*Rhodotorula* sp.
Aspergillus glaucus	*Saccharomyces cerevisiae*
Aspergillus fumigatus	*Saccharomyces carlsbergensis*
Aspergillus clavatus	*Saccharomyces fragii*
Aspergillus ochraceus	*Hansenula anomola*
Paecilomyces sp.	*Hansenula mrakii*
Scopulariopsis sp.	*Hansenula silvacola*
Fusarium graminum	*Pichia membranefaciens*
Fusarium sp.	*Schwanniomyces* sp.
Penicillium canescens	*Trigonopsis variabilis*
Blastomyces dermatitidis (yeast phase)	*Mycoderma* sp.
Blastomyces dermatitidis (mycelium phase)	*Debaromyces globosus*
Sporotrichum schenckii	*Sporobolomyces salmonicolor*
Trichophyton mentagrophytes	*Zygosaccharomyces lactis*
Microsporum gypseum	*Candida tropicalis*
Microsporum audouini	
Organisms Slightly or Questionably Inhibited	
Oospora lactis	*Sarcina lutea*
Staphylococcus aureus	*Corynebacterium* sp.
Staphylococcus sp.	*Mycobacterium* sp. (ATCC 607)
Organisms Not Inhibited	
Escherichia coli	*Bacillus cereus*
Serratia sp.	*Bacillus subtilis*
Salmonella typhosa	*Bacillus graveolus*
Paracolobactrum sp.	*Neisseria catarrhalis*
Pseudomonas fragii	*Neisseria perflava*
Pseudomonas fluorescens	*Mycobacterium avium*
Alcaligenes viscosus	*Streptomyces* sp.
Pseudomonas aeruginosa	*Nocardia* sp.
Salmonella enteritidis	*Nocardia asteroides*
Bacterium cadaveris	*Micromonospora* sp.

(estimated). The filter paper discs are soaked in the solutions of standards and samples and placed on the plates. A standard preparation is included on each plate along with each sample.

CHEMICAL AND PHYSICAL PROPERTIES OF TENNECETIN

The antibiotic is soluble in water, methanol, 95 per cent ethanol, propylene glycol, pyridine, formamide, and *n*-butanol. It is insoluble in chloroform, ethyl acetate, amyl acetate, ether, and petroleum ether. Aqueous solutions of tennecetin have an alkaline reaction.

Solutions of tennecetin kept in the refrigerator have retained full activity for more than a month. Likewise they are quite insensitive to visible light. At *p*H

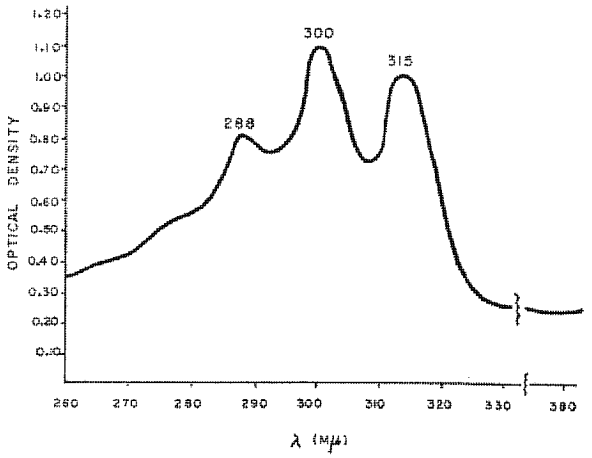

FIG. 2. Ultraviolet absorption spectrum of tennecetin.

7.0, solutions of the antibiotic have retained full activity following treatment at 100 C. for 20 minutes. Stability is markedly decreased at *p*H 4.0 and *p*H 10.0, as is shown in table I.

All active preparations of tennecetin have exhibited a characteristic ultraviolet absorption spectrum with a slight shoulder at 270–280 mμ, and peaks at 288, 300–302, and 315–318 mμ. (This latter peak is always sharp but varied between 315 and 318 mμ in different determinations.) Such an absorption spectrum is characteristic of polyene compounds in general and tetraenes in particular.[1] Figure 2 shows the ultraviolet absorption spectrum plotted from one of many similar determinations.

The infrared absorption spectrum of tennecetin in a potassium bromide pellet is shown in figure 3. A comparison of this with the published spectra of other tetraenes shows several variations in the major and minor absorption bands of all, indicating that they are not identical compounds.

Paper chromatography of tennecetin, nystatin, rimocidin sulfate, and mixtures of these was done in a water-saturated *n*-butanol solvent employing the descending, one-dimensional technique on Whatman no. 1 paper. Strips were spotted with 10 drops of the test material, and, after being subjected to the wet butanol

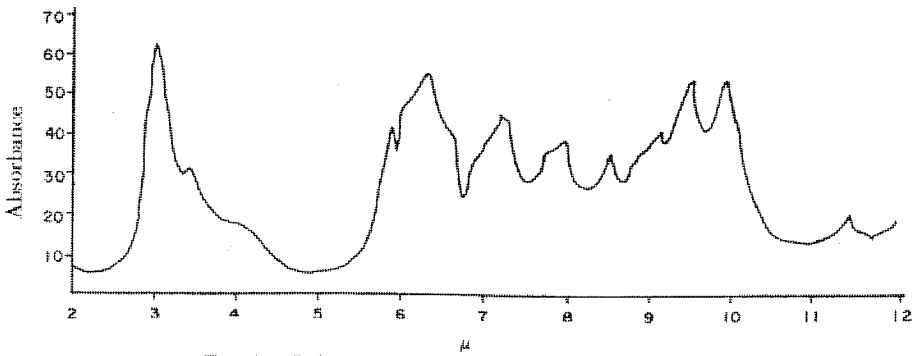

FIG. 3. Infrared absorption spectrum of tennecetin.

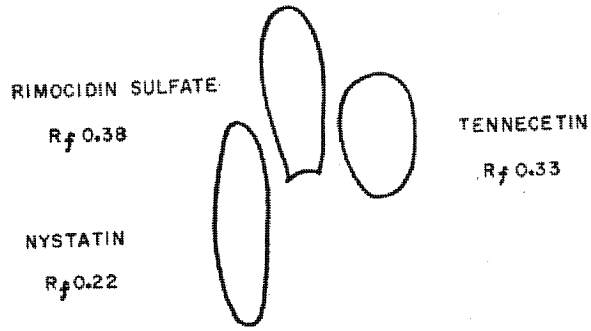

FIG. 4. Chromatograms of tennecetin, rimocidin, and nystatin, showing comparative R_f values.

solvent for 18 hours, they were dried in air and placed on trays of agar medium seeded with *S. carlsbergensis* K-20. The strips were removed after 15 minutes, incubated at room temperature overnight, and developed with 2,3,5-triphenyltetrazolium chloride in glucose. Results are shown in figure 4.

Tennecetin in concentrated sulfuric acid gives a wine-red color of indefinite stability. However, the acid rapidly destroys antibiotic activity. Potassium permanganate is rapidly decolorized. There is no reaction with ferric chloride. The ninhydrin test is negative.

BIOLOGICAL PROPERTIES OF TENNECETIN

Tennecetin has been found to be markedly fungistatic and/or fungicidal to yeasts and filamentous fungi in vitro. More than 80 different species of microorganisms were tested for their susceptibility to the action of tennecetin. No yeast or mold has yet been found that has not been inhibited by this antibiotic,

TABLE II

*Concentration of Tennecetin Required to Inhibit Growth of Certain Fungi In Vitro**

Organism	Tennecetin, u./ml. medium			
	0.5	0.25	0.05	None
Sporotrichum schenkii	0	4	4	4
Cryptococcus neoformans	0	4	4	4
Candida albicans	0	0	4	4
Candida parakrusei	0	0	4	4
Debaryomyces globosus	0	4	4	4
Saccharomyces cerevisiae	0	0	4	4
Geotrichum candidum	0	4	4	4
Saccharomyces carlsbergensis	0	2	4	4
Allescheria boydii	0	0	4	4
Trichophyton rubrum	0	0	4	4
Microsporum audouini	0	3	3	4
Blastomyces dermatitidis (yeast phase)	0	0	4	4
Blastomyces dermatitidis (mycelial phase)	0	0	2	2
Coccidioides immitis	0	0	1	2

* Tennecetin was dissolved in Sabouraud dextrose agar. Cultures were incubated at appropriate temperature for 3, 4, or 5 days, depending upon growth of control cultures containing no added antibiotic. Numerals in table refer to extent of growth judged on an arbitrary scale between 0 and 4. (0 indicates no growth; 4 indicates heavy growth.)

although certain of the human dematophytes have required somewhat higher in vitro concentrations to demonstrate the inhibitory effect. Most bacteria appear to be natively resistant to the action of tennecetin.

Among the fungi that have been found to be sensitive to the antibiotic are: human pathogens causing deep mycoses, such as *Cryplococcus neoformans, Blastomyces dermalitidis, Candida albicans;* dermatophytes, such as *Trichophyton mentagrophytes,* and *Microsporum lypseum;* and plant pathogens, such as *Fusarium* sp. and *Monascus purpurea.* Table I lists some of the species that have been tested for in vitro sensitivity to tennecetin. Table II shows the amounts required, in units, for inhibition of some of the strains tested.

Tennecetin has been tested for toxicity in laboratory animals and in human beings. The results of these tests indicate that the antibiotic is of relatively low toxicity. Toxicological and other pharmacological properties are being investigated. The results of these investigations will be the subject of a subsequent report.

DISCUSSION

Despite the apparent wide distribution of polyene-producing streptomycetes in nature, little is known of the biological significance of these compounds. All have certain characteristics in common, including characteristic ultraviolet absorption spectra, insolubility in nonpolar solvents, and antifungal activity with little or no antibacterial activity. These antibiotic substances apparently contain only carbon, hydrogen, oxygen, and sometimes nitrogen. Those already described appear to be either acidic, neutral, or amphoteric compounds.[11] Often they are quite toxic and unstable to light. Most are also insoluble in water.

In the absence of a pure, crystalline compound, we are not able to present a precise chemical characterization of tennecetin. Certain of its properties, however, indicate that this substance may possess especially desirable advantages for antibiotic use. Among these properties are its solubility, low toxicity, wide antifungal spectrum, and stability.

SUMMARY

A species of the genus *Streptomyces,* differing from previously classified members of this genus, was isolated from Tennessee soil and found to produce an antifungal antibiotic.

The antibiotic, designated as tennecetin, was identified as a tetraene unlike the known polyene compounds of that type.

The antibiotic has a wide antifungal spectrum in vitro. This, and other of its properties suggest the possibility of its use in the treatment of fungus infections.

BIBLIOGRAPHY

1. Oroshnik, W.; Vining, L. G.; Mebane, A. D., and Taber, W. A.: Polyene antibiotics, Science *121*:147, 1955.

2. Pledger, R. A., and Lechevalier, H.: Survey of the production of polyenic substances by soil streptomycetes. *In:* Antibiotics Annual 1955–1956, New York, Medical Encyclopedia, Inc., 1956, p. 249.
3. Ball, S.; Bessell, C. J., and Mortimer, A.: The production of polyenic antibiotics by soil streptomycetes, J. Gen. Microbiol. *17*:96–103, 1957.
4. Vaněk, L.; Doležilova, L., and Řeháček, Z.: Formation of a mixture of antibiotic substances including antibiotics of a polyene character, by strains of actinomycetes freshly isolated from soil samples, J. Gen. Microbiol. *18*:649–657, 1958.
5. Waksman, S. A., and Lechevalier, H. A.: Actinomycetes and Their Antibiotics, Baltimore, Williams and Wilkins Co., 1953.
6. Hesseltine, C. W.; Benedict, R. G., and Pridham, T. G.: Useful criteria for species differentiation in the genus Streptomyces, Ann. New York Acad. Sc. *60*:136–151, 1954.
7. Pridham, T. G.; Hesseltine, C. W., and Benedict, R. G.: A guide for the classification of streptomycetes according to selected groups, Appl. Microbiol. *6*:52–79, 1958.
8. McCarthy, F. J.; Fisher, W. P.; Charney, J., and Tytell, A. A.: Effects of oils and fatty acids on the production of fungichromin. *In:* Antibiotics Annual 1954–1955, New York, Medical Encyclopedia, Inc., 1955, p. 719.
9. Schaffner, C. P.; Steinman, I. D.; Safferman, R. S., and Lechevalier, H.: Role of inorganic salts and mevalonic acid in the production of a tetraenic antifungal antibiotic. *In:* Antibiotics Annual 1957–1958, New York, Medical Encyclopedia, Inc., 1958, p. 869.
10. Brock, T. D.: The effect of oils and fatty acids on the production of filipin, Applied Microbiol. *4*:131–133, 1956.
11. Koe, B. K.; Sobin, B. A., and Celmer, W. D.: PA 132, a new antibiotic. I. Isolation and chemical properties. *In:* Antibiotics Annual 1956–1957, New York, Medical Encyclopedia, Inc., 1957, pp. 672–675.

US005942611A

United States Patent [19]

Borden et al.

[11] **Patent Number: 5,942,611**

[45] **Date of Patent: Aug. 24, 1999**

[54] **PROCESS FOR NATAMYCIN RECOVERY**

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[73] Assignee: **Cultor Ltd.**, Finland

[21] Appl. No.: **08/696,841**

[22] PCT Filed: **Jan. 19, 1995**

[86] PCT No.: **PCT/IB95/00040**

§ 371 Date: **Aug. 28, 1996**

§ 102(e) Date: **Aug. 28, 1996**

[87] PCT Pub. No.: **WO95/27073**

PCT Pub. Date: **Oct. 12, 1995**

[51] **Int. Cl.**[6] **C07H 1/00**

[52] **U.S. Cl.** **536/127**; 536/6.5; 536/16.9

[58] **Field of Search** 536/6.5, 127, 16.9

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,378,441	4/1968	Bridge	536/127
3,892,850	7/1975	Struyk et al.	435/76
4,568,740	2/1986	Oppici et al.	536/7.5
5,231,014	7/1993	Eisenschink et al.	435/76

FOREIGN PATENT DOCUMENTS

846933	9/1960	United Kingdom .
2106498	4/1983	United Kingdom .
9210580	6/1992	WIPO .
9507998	3/1995	WIPO .

Primary Examiner—Elli Peselev

[57] **ABSTRACT**

A new process for recovering high purity natamycin from fermentation broth containing natamycin comprises the steps of adjusting the pH of the broth to above about 10 and adding an amount of a water-miscible solvent, such as isopropanol, sufficient to dissolve the natamycin in the broth, followed by removing insoluble solids from the pH-adjusted broth, followed by lowering the pH of the broth to a level sufficient to precipitate the natamycin, and removing the natamycin from the broth.

12 Claims, No Drawings

PROCESS FOR NATAMYCIN RECOVERY

BACKGROUND OF THE INVENTION

Natamycin, also known as pimaricin, was first isolated in 1955 from the fermentation broth of a culture of *Streptomyces natalensis* obtained from soil taken near the town of Pietermaritzburg In Natal, South Africa. (A. P. Struyk et al, *Antibiotics Annual,* 878 (1957–1958)). Pimaricin, designated natamycin by the WHO, is produced either from *Streptomyces natalensis* or *Streptomyces gilvosporeus.*

Early patents describe recovery processes requiring multiple purification steps and involving relatively expensive unit operations. British patent GB 846,933 discloses an adsorption/elution recovery of natamycin from fermentation broth using water miscible polar solvents such as methanol, butanol and acetone. U.S. Pat. No. 3,892,850 discloses recovery from fermentation broth by extraction into an organic solvent having limited water miscibility, followed by recovery from the solvent. U.S. Pat. No. 3,378,441 claims recovery of natamycin by salting it out of fermentation broth followed by solvent dissolution and evaporative precipitation. A later patent, GB 2,106,498, describes vacuum concentration or butanol extraction of filtered fermentation broth to obtain a raw antifungal complex from which natamycin can be isolated. WO 92/10580 discloses solubilization of natamycin with methanol at low pH, followed by removal of broth solids. Under these conditions, natamycin is susceptible to acid degradation, with potential for depressed recovery yield and reduced product purity. Thus, the prior art does not teach an efficient method for recovery of high quality natamycin.

SUMMARY OF THE INVENTION

The present invention concerns a new, simple and efficient process to recover high quality natamycin from fermentation broth using a substantially water-miscible solvent at alkaline pH. Specifically, natamycin is recovered from the fermentation broth via a process comprising the steps:

a) adjusting the pH of the broth to greater than about 10 and adding an amount of a substantially water-miscible solvent sufficient to dissolve the natamycin in the broth;

b) removing insoluble solids from the pH-adjusted broth;

c) lowering the pH of said broth to a level sufficient to precipitate the natamycin from the broth; and

d) removing the natamycin from the broth.

In its preferred embodiment, the pH of the broth is adjusted to between 10 and 11 in step a) and to between 5.5 and 7.5 in step c). Any of several methods may be used in steps b) and d), for example, centrifugation, depth filtration and crossflow filtration, with a preferred method being crossflow filtration. It is preferred that, prior to the recovery process, the fermentation broth is concentrated, for example, to a concentration of from 10 to 50% solids, on a weight/volume basis. For greater stability of the natamycin during the recovery process, an antioxidant such as BHA, BHT, ascorbic acid or sodium erythorbate can be added.

DETAILED DESCRIPTION OF THE INVENTION

The production of natamycin by fermentation is well known. The aforementioned British patent, GB 846,933, is representative and discloses the production of natamycin via fermentation using *Streptomyces gilvosporeus*. The fermentation method itself is not critical to the present invention.

Fermentation broth containing natamycin may be concentrated, if desired, through any suitable method such as evaporation, depth filtration, crossflow filtration or centrifugation, with a preferred method being crossflow filtration. Concentration to a range of about 10 to 50% solids on a weight/volume basis is preferred. During concentration, the broth may be heated to a range of 50 to 70° C. to improve evaporation or filtration rate.

The broth is then adjusted to a pH greater than about 10, preferably between about 10 and about 11, using an appropriate basifying agent such as, for example, Na_2CO_3, K_2CO_3, KOH, NaOH, or a combination thereof. Depending on the degree of water removal in the concentration step, dilution of the concentrate with water or a water-miscible solvent may be necessary to facilitate agitation during the pH adjustment. Further, a substantially water-miscible solvent is added to the pH-adjusted concentrate in an amount sufficient to solubilize natamycin. Suitable water-miscible solvents include, for example, ethanol, propanol, isopropanol, acetone, tetrahydrofuran and combinations of the foregoing. Of these, isopropanol is preferred. Typically, one to two volumes of isopropanol is required per volume of concentrate.

Although the solubility of natamycin is low in either water or isopropanol ("Solubility of Antibiotics in Twenty-Six Solvents," Journal of the Association of Official Analytical Chemists, vol. 50, no. 2, 1967), it is soluble at 40 to 120 g/λ or more in the solvent mixture at elevated pH. Although the literature reports that natamycin is unstable at alkaline pH (H. Brik in "Analytical Profiles of Drug Substances"), in fact it is reasonably stable under the conditions of the present invention. If desired, stability can be further improved through addition of antioxidants such as ascorbic acid, erythorbic acid, BHA and BHT, at levels up to about 0.2 weight percent of the natamycin activity. Under the conditions of low pH recovery processes such as that disclosed in WO 92/10580, natamycin is susceptible to rapid activity loss and to the formation of natamycin analogs which are not readily separated from natamycin.

Inactive broth solids are removed from the natamycin solution by any of several methods such as depth filtration, crossflow filtration, or centrifugation. If desired, the solids may be washed, for example with 40 to 60% aqueous isopropanol, to extract residual natamycin activity and improve recovery yield. Crossflow filtration is a preferred method for removal of broth solids. Crossflow filtration, as defined by J. M. Walker and M. Cox in "The Language of Biotechnology" (American Chemical Society, Washington, D.C., 1988) is "an operating regime for a filtering device in which the main fluid flow is parallel to the filter, such that the fluid passes through the filter perpendicular to the main flow. This regime minimizes the buildup of filter cake and also the consequential reduction in filtration rate. It allows rapid filtration without the need for filter aids or flocculants." In the process claimed herein, crossflow filtration gives rapid filtration rates and high final solids concentrations without addition of filter aids. The ceramic filter elements which are used allow high crossflow rates, imparting an effective sweep of the filtration surfaces. They can be used at high temperatures, allowing further flux enhancement. Their stability to harsh pH and temperature conditions facilitates frequent cleaning, and they can be backpulsed during operation to minimize plugging.

The pH of the product-rich solution is then adjusted with a suitable acid such as hydrochloric acid to effect precipitation of natamycin. The choice of acid is not critical; for economy and simplicity, hydrochloric acid is preferred. The precipitated natamycin crystals are then isolated by an appropriate method such as, for example, depth filtration,

crossflow filtration or centrifugation. This can be followed by further known purification steps, for example, washing with a water-isopropanol mixture followed by drying. Isolation of natamycin by centrifugation or crossflow filtration requires that the filtration mother liquor be displaced with a wash of similar solvent composition to avoid precipitating water-insoluble impurities. The dry product typically has a purity of at least 94% on an anhydrous basis. Recovery yields of 40–70% are typical. Crossflow filtration, as defined above, is a preferred method for isolation of natamycin, giving rapid removal of the mother liquor and wash liquid. The product in this case is a concentrated crystal slurry which can be converted to a dry solid by any of a number of methods, for example, evaporation or spray drying.

In crystalline form, natamycin is temperature stable (H. A. Morris, et al., *Cultured Dairy Products Journal*, p 23, (August 1978)). Sustained drying temperatures of 70° C. are acceptable as long as the product moisture is not driven below about 6%. Natamycin normally exists as a trihydrate. Anhydrous natamycin is less stable than the hydrate, so excessive drying adversely affects product purity.

Dry product purities of 94 to 99% (calculated on an anhydrous basis) and recovery yields of 40 to 70% are typically achieved through the inventive recovery process.

EXAMPLES

Example 1

On a rotary evaporator, 1027 ml of fermentation broth with a natamycin concentration of 10.5 g/λ was concentrated to 500 ml. The pH was adjusted to 10.5 with 10 M sodium hydroxide, and 500 ml of isopropanol was added. The mixture was stirred for about two hours, then centrifuged. The clear dark natamycin-rich solution was decanted, adjusted to pH 6.5–7.5 with 12 M hydrochloric acid, and allowed to crystallize for several hours. Natamycin crystals were isolated by centrifugation, transferred to a filter funnel with 40 ml of 1:1 water-isopropanol, filtered, washed with three 20 ml portions of 1:1 water-isopropanol, and dried to constant weight at 35° C. in a vacuum oven, yielding 8 g of natamycin solids which were 96.2% pure on an anhydrous basis. The yield from broth was 69%.

Example 2

A 10.2 liter portion of fermentation broth concentrate with a natamycin concentration of 134 g/λ was adjusted to pH 10.6 by addition of 1100 g of sodium carbonate, 140 ml of 50% sodium hydroxide solution, and sufficient water (1.3 λ) to allow the heavy concentrate to be mixed during pH adjustment. To the resulting slurry, 6 kg of isopropanol containing 6 g of BHA was added, and the mixture was stirred to dissolve natamycin. Broth solids were removed by crossflow filtration at 25–30° C. During this operation, as the retained slurry of broth solids thickened, 10 λ of 1:1 isopropanol-water was added to wash out residual natamycin. The filtrate was adjusted to pH 6.6 with 5 M hydrochloric acid, and natamycin was allowed to crystallize for 1–2 hours. Natamycin was isolated by filtration on a Buchner funnel, washed with about 500 ml of 40:60 isopropanol-water followed by about 500 ml of water, and dried in a laboratory hood to 550 g of natamycin solids which were greater than 97% pure on an anhydrous basis. The yield from broth concentrate was about 40%.

Example 3

A 9600-gallon volume of fermentation broth containing 1.06% natamycin was concentrated to a volume of 535 gallons by crossflow filtration at 55–60° C. During this operation, as the retained slurry thickened, 915 gallons of water was added as a wash. When the retained washed broth concentrate was no longer filterable, it was adjusted to pH 10.6 with sodium carbonate and sodium hydroxide, 8000 kg of isopropanol was added to dissolve natamycin, and one pound of sodium erythorbate was added to protect against oxidation. Broth solids were removed by crossflow filtration at 23–28° C. During this operation, as the retained slurry of broth solids thickened, 1625 gallons of 1:1 isopropanol-water was added to wash out residual natamycin. The filtrate containing natamycin was adjusted to pH 6.5 with hydrochloric acid. Natamycin was allowed to crystallize for 1–2 hours, then isolated by filtration, washed with water and dried, giving about 240 kg of natamycin which was 98% pure on an anhydrous basis. The yield was about 60%.

We claim:

1. A process for recovering natamycin from fermentation broth containing natamycin comprising the steps:

a) adjusting the pH of said broth to greater than about 10 and adding an amount of a substantially water-miscible solvent sufficient to dissolve the natamycin in said broth;

b) removing insoluble solids from said pH-adjusted broth;

c) lowering the pH of said broth to a level sufficient to precipitate the natamycin; and

d) removing the natamycin from said broth.

2. A process according to claim **1** wherein said water-miscible solvent is selected from the group consisting of ethanol, propanol, isopropanol, acetone, tetrahydrofuran and combinations thereof.

3. A process according to claim **2** wherein said water-miscible solvent is isopropanol.

4. A process according to claim **1** wherein, in step a), the pH of said broth is adjusted to between 10 and 11.

5. A process according to claim **3** wherein, in step a), the pH of said broth is adjusted to between 10 and 11.

6. A process according to claim **1** wherein, in step c), the pH of said broth is lowered to between 5.5 and 7.5.

7. A process according to claim **5** wherein, in step c), the pH of said broth is lowered to between 5.5 and 7.5.

8. A process according to claim **1** wherein step b) utilizes crossflow filtration.

9. A process according to claim **7** wherein step b) utilizes crossflow filtration.

10. A process according to claim **1** further comprising the preliminary step of concentrating said broth.

11. A process according to claim **10** wherein said broth is concentrated to a concentration of 10 to 50% solids on a weight/volume basis.

12. A process according to claim **10** wherein said preliminary step utilizes crossflow filtration.

* * * * *

PROCESSING

STANDARDS

Passed in Sabaudia, Italy, on June 25th 1999

Revised June 2004

to be implemented by each member country by the 1st July 2005

Demeter International e.V.

VII

Standards for the certification of DEMETER milk and dairy products

Table of contents

1. Transportation of the milk

The milk must be picked up by special milk trucks, which are used only for DEMETER milk, or have special tanks labelled for DEMETER milk. Transport is also possible in DEMETER labelled cans, or may be delivered directly from the farm to the dairy.

2. Storage of the milk

The storage of milk takes place in special tanks which are designated for DEMETER milk. Any confusion with bio or conventional milk must be avoided through the use of an appropriate labelling system.

3. Ingredients and additives

3.1 Ingredients

In principle all DEMETER raw materials may be used as ingredients.

3.1.1. Starter cultures, micro-organism cultures

3.1.1.1 Cultures using milk as a growing medium

Starter cultures (also direct starters) may be used. They are to be bred in the usual manner at the processing facility, and preferably used in production only from the third generation onward. The raising and multiplication must take place in DEMETER milk. Micro-organism cultures such as Brevibacterium Linens may be used. The use of genetically modified micro-organisms is not allowed. The manufacturer of DEMETER milk products must find out the production details of the starter cultures from the supplier of these cultures, in writing.

3.1.1.2 Starter cultures not grown on milk.

The use of cultures that have not been grown on milk (e.g. moulds) may be used for specific recipes.

3.1.2 Rennet

Rennet of calves, microbial rennet, rennet-pepsin mixtures (calf rennet) and plant extracts (Artichokes, Ladies' bedstraw – Gallium verum) may be used to curdle milk. The rennet should contain no preservatives.

Fruit vinegar and starter cultures are allowed for the souring of milk proteins.

3.1.3 Salt

See table 5.5., Part A

3.1.4 Sweetening agents

See table 5.5., Part A

3.1.5 Oil

Oil may be used to treat the surfaces of cheese.

3.1.6 Herbs and spices

Any herbs used must meet the requirements of the „Standards for the Processing of DEMETER Herbs and Spices".

3.1.7 Fruit preparations

Any fruit preparations used must have met the production requirements of the „Standards for the Certification of Processed DEMETER fruit and vegetables."

3.2 Additives

3.2.1 Calcium carbonate ($CaCO_3$) and Calcium chloride ($CaCl_2$)

Calcium carbonate (E 170) is allowed solely for the production of sour milk cheese. Sodium bicarbonate may not be used.

Calcium chloride (E 509) may be used as processing-aid in the cheese production.

3.2.2 Coatings

The following coatings can be used for hard cheeses, sliceable cheeses and for semi-hard cheeses:

- Beeswax
- Natural hard paraffin wax
- Microcrystaline waxes

These three substances can be mixed with each other. Natural hard paraffin wax and microcrystalline wax may contain no other additives such as polyethylene, short chain polyolefine, polyisobutylene, butyl or cyclic rubber. In addition the waxes may not be coloured.

Plastic film is provisionally permitted for covering the outer layer of sliceable cheese, and semi- hard cheese, as long as it is free from potassium sorbate, calcium sorbate and natamycin. (This is permitted only until a suitable replacement material or method is found).

3.2.3 Smoking of cheese

The wood is burnt either directly in the smoking chamber or outside of it in a suitable facility. Cold and warm smoking processes (< 70°C) are permitted. The individual cheese types determine the exact method required.

Permitted smoking agents:

- suitable native wood types (as wood, shavings or sawdust, preferably from beech, oak and plane trees.

- Pine cones
- Herbs
- Other types of plants such as juniper, heather, branches, conifer cones and spices

4. Processing methods

In order to maintain the inner quality of the milk right through to consumption, it should be processed whole as far as possible and also fresh from the cow.

The use of aluminium vats is not allowed for either storage or processing.

4.1 Milk (for drinking)

The legally permitted pasteurisation methods, to a maximum temperature of 80 degrees C, may be used to pasteurise milk. After treatment the milk must have a positive peroxidase index. The same applies in principle to all processed milk products. Other heat processes such as sterilisation or UHT treatment are not permitted, and the milk may not be homogenised.

The following types of milk can be made commercially available:

- Gold-top milk
- Whole milk with natural fat content
- Standardised whole milk (at least 3.5% fat)
- Low fat and skim milk

Enriching milk with milk proteins and vitamins etc is not allowed.

4.2 Butter

The following butter types can be produced:

- Full cream butter
- Sour cream butter

Brought in cream may be processed. For ease of spreading, physical methods for cream ripening may be used, such as cold-warm-cold or warm-cold-cold processing.

Salting with table salt is permitted if indicated on the label. Colouring with beta-carotene is not permitted. Indirectly acidified butter, made according to the NIZO method is not permitted. The other common methods of butter manufacture are allowed. Butter may be cold stored for up to half a year. Cold stored butter may not be mixed with fresh butter.

4.3 Fresh cheese and curd cheese (Quark)

Fresh and curd cheese may be produced with the addition solely of starter cultures and rennet. The utilisation of whey proteins using methods such as thermo-curd methods and ultrafine filtration are permitted. The use of centrifugal whey separation methods is not allowed. The adjustment of fat

content using the addition of high or low fat curd cheese, or of cream, is permitted. The other common methods of fresh cheese manufacture are allowed.

4.4 Sour milk cheese

Sour milk cheese may only be manufactured from sour milk curd cheese. The use of calcium carbonate is permitted. The addition of cooking salt to the cheese must not exceed 2.5%. The use of beta-carotine and lactoflavin is prohibited.

4.5 Sour milk products, joghurt production, kefir production, buttermilk production

A heat treatment of 85-95 degrees C, not exceeding 5-10 minutes in duration, is permitted for treating the milk products. It is desirable to work, as far as possible, at the lower limits. UHT treatment and homogenisation are prohibited.

The following options are available for increasing the dry matter

- Addition of powdered milk
- Evaporating under vacuum
- Evaporating in a downdraft, multi-stage evaporator.

The finished products may not be heat- treated.

Only pure buttermilk may be produced for sale. The other common methods of sour milk production are allowed.

4.6 Sweet milk products

The same processing standards are applied as for sour milk products. As thickening agents starch and agar agar may be used.

4.7 Cream

Cream may not be enriched with milk protein products to increase the milk solids. After pasteurisation the cream must have a positive peroxidase index. Homogenisation and the use of thickening agents (e.g. Carrageen) are not permitted.

4.8. Whey

Both sweet and sour whey can be produced.

4.9. Milk powder production

The production of dried milk products from DEMETER milk and milk products is permitted (e.g. Whole milk powder, skim milk powder, buttermilk powder, whey powder.) The process of reduction and drying should be gentle, using optimal temperatures and pressures.

Milk powder is allowed only as an ingredient for processed products.

4.10. Cheese

The milk is to be purified by separation or appropriate filtration methods. To prevent bacterial contamination, the approved pasteurisation methods may be used (see section 4.1) or the milk subjected to thermal treatment. Bacteria may also be removed by bactofuging, but the material that has been separated out may no longer be used.

The milk may be curdled with acid starters, rennet or a combination of the two. It may not however be curdled with pure acid. To renew the salt brine, the cheese is to be removed and the precipitate cleared away. The salt brine can be re-boiled and enriched with salt accordingly. Sterilisation with sodium hypochlorite, hydrogen peroxide etc. is not permitted.

Only pure herbs and spices, or extracts made from pure herbs and spices, may be added to the cheese.

The use of lactoflavin or beta carotin colourings is prohibited. Surface treatment of the cheese with potassium sorbate, calcium sorbate, or natamycin is not permitted.

The individual cheese types will be manufactured according to the method typical for each respective type. Cheese may be matured in foil, as long as the foil type used is free from substances which could reduce the quality of the DEMETER-product. Plastic film is permitted for the covering of the outer layers of sliceable cheese and semi-hard cheese, provided that it is free of the above mentioned substances. This approval will apply until such time as an appropriate replacement material or method is found.

4.11. Ice-cream

Ingredients and thickeners:

All DEMETER products including aroma-extracts, herbs and spices may be used in the production of ice-cream.

Allowable thickening agents are carob bean gum, pectin, guar gum, agar agar.

Colourings are not allowed.

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CONSLEG: 1993R0207 — 03/10/2000

Number of pages: 8

Office for Official Publications of the European Communities

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COMMISSION REGULATION (EEC) No 207/93

of 29 January 1993

defining the content of Annex VI to Regulation (EEC) No 2092/91 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs and laying down detailed rules for implementing the provisions of Article 5 (4) thereto

(OJ L 25, 2.2.1993, p. 5)

Amended by:

		Official Journal		
		No	page	date
►M1	Commission Regulation (EC) No 345/97 of 26 February 1997	L 58	38	27.2.1997
►M2	Commission Regulation (EC) No 2020/2000 of 25 September 2000	L 241	39	26.9.2000

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defining the content of Annex VI to Regulation (EEC) No 2092/91 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs and laying down detailed rules for implementing the provisions of Article 5 (4) thereto

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Regulation (EEC) No 2092/91 of 24 June 1991 on organic production of agricultural products and indications referring thereto on agricultural products and foodstuffs(1) and in particular Article 5 (7) and (8) thereof,

Whereas for the purposes of Article 5 (3) (b) and (c) and (4) of Council Regulation (EEC) No 2092/91 (hereinafter referred to as 'the Regulation'), exhaustive lists have to be established in Sections A, B and C of Annex VI thereto;

Whereas for the purposes of the three sections of Annex VI to the Regulation, certain definitions have to be developed in order to ensure coherence with other related Community regulatory provisions;

Whereas any ingredients or processing aids to be mentioned in Annex VI to the Regulation should be used only in accordance with legislative requirements concerning the preparation of foodstuffs and according to good practice of in the manufacturing of foodstuffs;

Whereas Annex VI to the Regulation should take account of consumers' expect actions that processed products from organic production will be composed essentially of ingredients as they occur in nature;

Whereas, however, other ingredients or processing aids which may be used in conventionally processed foodstuffs, and which preferably exist in nature, may be included in Annex VI to the Regulation, provided it has been shown that, without having recourse to such substances, it is impossible to produce or preserve organic foodstuffs;

Whereas, with regard to enzymes derived from micro-organisms, it has to be further examined whether such products obtained from micro-organisms genetically modified within the meaning of Council Directive 90/220/EEC(2), can be used in foodstuffs whose labelling refers to organic production methods; whereas this question will be examined in detail when such enzymes are approved for use in foodstuffs according to the relevant Community legislation;

Whereas the said Annex VI will have to be reviewed regularly in the light of experience gained and of developments with in the availability on the Community market of certain organically produced ingredients of agricultural origin;

Whereas detailed rules for the implementation of the derogation pursuant to Article 5 (4) of the Regulation are necessary to ensure coherent implementation of this derogation in the Member States, as long as products covered by this derogation have not been included in Section C of Annex VI to the Regulation;

Whereas the measures provided for in this Regulation are in accordance with the opinion of the committee referred to in Article 14 of Regulation (EEC) No 2092/91,

(1) OJ No L 198, 22. 7. 1991, p. 1.
(2) OJ No L 117, 8. 5. 1990, p. 15.

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HAS ADOPTED THIS REGULATION:

Article 1

The content of Annex VI to Regulation (EEC) No 2092/91 is defined in the Annex to the present Regulation.

Article 2

No amendments to Sections A and B of Annex VI shall be adopted unless at least the following requirements are satisfied:

(a) for food additives covered by Section A, point 1 of Annex VI: without prejudice to the requirements for acceptance of additives provided for in Council Directive 89/107/EEC [1] only substances shall be included for which it has been shown that, without having recourse to such substances, it is impossible to produce or preserve such foodstuffs;

(b) for processing aids covered by Part B of Annex VI: only substances are included which are accepted in general food processing and for which it has been shown that, without having recourse to such substances, it is impossible to produce such foodstuffs.

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Article 3:

1. As long as an ingredient of agricultural origin is not included in Section C of Annex VI to Regulation (EEC) No 2092/91, that ingredient may be used according to the derogation provided for in Article 5(3)(b) and Article 5(5a)(b) of that Regulation on the following conditions:

(a) that the operator has notified to the competent authority of the Member State all the requisite evidence showing that the ingredient concerned satisfies the requirements of Article 5(4) of Regulation (EEC) No 2092/91; and

(b) that the competent authority of the Member State has provisionally authorised, in accordance with the requirements of Article 5(4) of Regulation (EEC) No 2092/91, the use for a maximum period of three months after having verified that the operator has taken the necessary contacts with the other suppliers in the Community to ensure himself on the unavailability of the ingredients concerned with the required quality requirements; without prejudice of the provisions of paragraph 6, the Member State may prolong this authorisation maximum three times for seven months each; and

(c) no decision has been taken, in accordance with the provisions of paragraphs 4 or 6 that a granted authorisation with regard to the ingredient concerned shall be withdrawn.

2. Where an authorisation as referred to in paragraph 1 has been granted, the Member State shall immediately notify to the other Member States and to the Commission, the following information:

(a) the date of the authorisation and in case of a prolonged authorisation, the date of the first authorisation;

(b) the name, address, telephone, and where relevant, fax and e-mail of the holder of the authorisation; the name and address of the contact point of the authority which granted the authorisation;

(c) the name and, where necessary, the precise description and quality requirements of the ingredient of agricultural origin concerned;

(d) the type of products for the preparation of which the requested ingredient is necessary;

(e) the quantities that are required and the justification for those quantities;

(f) the reasons for, and expected period of, the shortage;

[1] OJ No L 40, 11. 2. 1989, p. 27.

▼M2

(g) the date on which the Member State sends this notification to the other Member States and the Commission.

The Commission and/or Member States may make this information available to the public.

3. Where a Member State submits comments to the Commission and to the Member State which granted the authorisation, which show that supplies are available during the period of the shortage, the Member State shall consider withdrawal of the authorisation or reducing the envisaged period of validity, and shall inform the Commission and the other Member States of the measures it has taken or will take, within 15 days days from the date of receipt of the information.

4. At the request of a Member State or at the Commission's initiative, the matter shall be submitted for examination to the Committee referred to in Article 14 of Regulation (EEC) No 2092/91. It may be decided, in accordance with the procedure laid down in Article 14, that a granted authorisation shall be withdrawn or its period of validity amended, or where appropriate, that the ingredient concerned be included in Section C of Annex VI.

5. In case of a prolongation as referred to in paragraph 1(b), the procedures of paragraphs 2 and 3, will apply.

6. Where a Member State wants to ensure that an ingredient from conventional production can still be used after the third prolongation of the authorisation referred to in paragraph 1(b), this Member State shall, together with the notification for the third prolongation of a granted authorisation notify a request for inclusion of the ingredient in Annex VI, Section C. As long as no decision has entered into force in accordance with the procedure referred to in Article 14, in order to include the ingredient in Annex VI, Section C or to withdraw the authorisation, the Member State may continue to prolong the authorisation for successive periods of seven months in due respect of the conditions of paragraphs 1, 2 and 3.

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Article 4

This Regulation shall enter into force 15 days from the date of publication in the *Official Journal of the European Communities*.

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ANNEX

'ANNEX VI

INTRODUCTION

For the purposes of this Annex, the following definitions will apply:

1. ingredients: substances as defined in Article 4 of this Regulation under the restrictions as referred to in Article 6 (4) of Council Directive 79/112/EEC of 18 December 1978 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs for sale to the ultimate consumer (1).

2. ingredients of agricultural origin:

   (a) single agricultural products and products derived therefrom by appropriate washing, cleaning, thermic and/or mechanical processes and/or by physical processes having the effect of reducing the moisture content of the product;

   (b) also, products derived from the products mentioned under (a) by other processes used in food processing, unless these products are considered food additives or flavourings as defined under points 5 or 7 hereunder.

3. ingredients of non-agricultural origin: ingredients other than ingredients of agricultural origin and belonging to at least one of the following categories:

3.1. food additives, including carriers for food additives, as defined under points 5 and 6 hereunder;

3.2. flavourings, as defined under point 7 hereunder;

3.3. water and salt;

3.4. micro-organism preparations

3.5. minerals (including trace elements) and vitamins.

4. processing aids: substances as defined in Article 1 (3) (a) of Council Directive 89/107/EEC (2) on the approximation of the laws of the Member States concerning food additives authorized for use in foodstuffs intended for human consumption;

5. food additives: substances as defined in Article 1 (1) and (2) of Directive 89/107/EEC and covered by that Directive or by a comprehensive Directive as referred to in Article 3 (1) of Directive 89/107/EEC;

6. carriers, including carrier solvents: food additives used to dissolve, dilute, disperse or otherwise physically modify a food additive without altering its technological function in order to facilitate its handling, application or use;

7. flavouring: substances and prducts as defined in Article 1 (2) of Council Directive 88/388/EEC of 22 June 1988 on the approximation of the laws of the Member States relating to flavourings for use in foodstuffs and to source materials for their production (3), and covered by that Directive.

GENERAL PRINCIPLES

Sections A, B and C cover the ingredients and processing aids which may be used in the preparation of foodstuffs composed essentially of one or more ingredients of plant origin, referred to in Article 1 (1) (b) of this Regulation, with the exception of wines.

Notwithstanding reference to any ingredient in Sections A and C or any processing aid in Section B, any ingredient or such processing aid shall be used only in accordance with relevant Community legislation and/or national legislation compatible with the Treaty and, in the absence thereof, in accordance with the principles of good manufacturing practice for foodstuffs. In particular additives shall be used according to the provisions of Directive 89/107/EEC and, where relevant, those of any comprehensive Directive as referred to in Article 3 (1) of Directive 89/107/EEC; flavourings shall be used according to the provisions of Directive 88/388/EEC and solvents according to the provisions of Council Directive 88/344/EEC of 13 June 1988 on the approximation of the laws of the Member States on extraction solvents used in the production of foodstuffs and food ingrediends (4).

(1) OJ No L 33, 8. 2. 1979, p. 1.
(2) OJ No L 40, 11. 2. 1989, p. 27.
(3) OJ No L 184, 15. 7. 1988, p. 61.
(4) OJ No L 157, 24. 6. 1988, p. 28.

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SECTION A — INGREDIENTS OF NON-AGRICULTURAL ORIGIN (REFERRED TO IN ARTICLE 5 (3) (B) OF REGULATION (EEC) No 2092/91)

A.1. Food additives, including carriers

	Name	Specific conditions (*)
E 170	Calciumcarbonates	—
E 270	Lactic acid	—
E 290	Carbondioxyde	—
E 296	Malic acid	—
E 300	Ascorbic acid	—
E 322	Lecithins	—
E 330	Citric acid	—
E 334	Tartaric acid [L (+) –]	—
E 335	Sodium tartrate	—
E 336	Potassium tartrate	—
E 400	Alginic acid	—
E 401	Sodium alginate	—
E 402	potassium alginate	—
E 406	Agar	—
E 410	Locust beam gum	—
E 412	Guar gum	—
E 413	Tragacanth gum	—
E 414	Arabic gum	—
E 415	Xanthan gum	—
E 416	Karaga gum	—
E 440 (i)	Pectin	—
E 500	Sodiumcarbonates	—
E 501	Potassiumcarbonates	—
E 503	Ammoniumcarbonates	—
E 504	Magnesiumcarbonates	—
E 516	Calcium sulphate	CR
E 938	Argon	—
E 941	Nitrogen	—
E 948	Oxygen	—

(*) CR-carrier

A.2. Flavourings within the meaning of Directive 88/388/EEC

Substances and products as defined in Article 1 (2) (b) (i) and 1 (2) (c) of Directive 88/388/EEC labelled as natural flavouring substances or natural flavouring preparations, according to Article 9 (1) (d) and (2) of that Directive.

A.3. Water and salt

Drinking water

Salt (with sodium chloride or potassium chloride as basic components), generally used in food processing.

A.4. Micro-organism preparations

(i) Any preparations of micro-organisms normally used in food processing, with the exception of micro-organisms genetically modified within the meaning of Article 2 (2) of Directive 90/220/EEC;

(ii) Micro-organisms genetically modified within the meaning of Article 2 (2) of Directive 90/220/EEC: if they have been included according to the decision procedure of Article 14.

A.5. Minerals (including trace elements) and vitamins

Only authorized as far as their use is legally required in the foodstuffs in which they are incorporated.

SECTION B — PROCESSING AIDS AND OTHER PRODUCTS WHICH MAY BE USED FOR PROCESSING OF ORGANICALLY PRODUCED INGREDIENTS OF AGRICULTURAL ORIGIN, REFERRED TO IN ARTICLE 5 (3) (C) OF REGULATION (EEC) No 2092/91

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Name	Specific conditions
Water	
Calcium chloride	coagulation agent
Calcium carbonate	
Calcium hydroxide	
Calcium sulphate	coagulation agent
Magnesium chloride (or nigari)	coagulation agent
Potassium carbonate	drying of grapes
Carbon dioxide	
Nitrogen	
Ethanol	solvent
Tannic acid	filtration aid
Egg white albumen	
Casein	
Gelatin	
Isinglass	
Vegetable oils	greasing or releasing agent
Silicon dioxide gel or colloidal solution	
Activated carbon	
Talc	
Bentonite	
Kaolin	
Diatomaceous earth	
Perlite	
Hazelnut shells	
Beeswax	releasing agent
Carnauba wax	releasing agent

Preparations of micro-organisms and enzymes:

(i) Any preparations of micro-organisms and enzymes normally used as processing aids in food processing, with the exception of micro-organisms genetically modified within the meaning of Article 2 (2) of Directive 90/220/EEC;

(ii) Micro-organisms genetically modified within the meaning of Article 2 (2) of Directive 90/220/EEC: If they have been included hereunder according to the decision procedure of Article 14.

SECTION C — INGREDIENTS OF AGRICULTURAL ORIGIN WHICH HAVE NOT BEEN PRODUCED ORGANICALLY, REFERRED TO IN ARTICLE 5 (4) OF REGULATION (EEC) No 2092/91

C.1. Unprocessed vegetable products, as well as products derived therefrom by processes referred to under definition 2 (a):

C.1.1. Edible fruits, nuts and seeds

Coconuts

Brazil nuts

Cashew nuts

Dates

Pineapples

Mangoes

Papayas

Sloes

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Cocoa

Maracujas (Passion fruit)

Colanuts

Peanuts

Rosehips

Sallowthorns

Bluberries

Maple syrup

Quinoa

Amaranth

Horseradish seed

Pumpkin seed

Pine kernels

Radish seeds

C.1.2. Edible spices and herbs

All products with the exception of thyme

C.1.3. Cereals

Millet

Wild rice (Zizania plauspra)

C.1.4. Oil seeds and oleaginous fruits

Sesamum seeds

C.1.5. Miscellaneous

Algae, including seaweed

C.2. Vegetable products, processed by processes as referred to under definition 2 (b):

C.2.1. Fats and oils, whether or not refined, but not chemically modified, derived from plants other than:

olive

sunflower

C.2.2. Sugars; starch; other products from cereals and tubers

Cane and beet sugar

Starches produced from cereals and tubers, not chemically modified

Rice paper

Gluten

C.2.3. Miscellaneous

Lemon juice

Vinegar from fermented beverages other than wine

C.3. Animal Products

Honey

Gelatin

Milk powder and skimmed milk powder

Edible aquatic organisms, not originating from aquaculture.'

§ 172.150 4-Hydroxymethyl-2,6-di-*tert*-butylphenol.

The food additive 4-hydroxymethyl-2,6-di-*tert*-butylphenol may be safely used in food in accordance with the following prescribed conditions:

(a) The additive has a solidification point of 140 °C–141 °C.

(b) The additive is used as an antioxidant alone or in combination with other permitted antioxidants.

(c) The total amount of all antioxidants added to such food shall not exceed 0.02 percent of the oil or fat content of the food, including the essential (volatile) oil content of the food.

§ 172.155 Natamycin (pimaricin).

(a) Natamycin (CAS Reg. No. 7681–93–8), also known as pimaricin, is a polyene macrolide antimycotic substance possessing an empirical formula of $C_{33}H_{47}NO_{13}$ and a molecular weight of 665.7.

(b) The additive shall conform to the following specifications:

Purity: 97 percent ±2 percent on an anhydrous basis.
Arsenic: Not more than 1 part per million.
Heavy metals (as Pb): Not more than 20 parts per million.

(c) The additive may be applied on cheese, as an antimycotic, in amounts not to exceed 20 milligrams per kilogram (20 parts per million) in the finished product as determined by International Dairy Federation (IDF) Standard 140A:1992, "Cheese and Cheese Rind-Determination of Natamycin Content-Method by Molecular Absorption Spectrometry and by High-Performance Liquid Chromatography," which is incorporated by reference. The Director of the Office of the Federal Register approves this incorporation by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the Division of Product Policy (HFS–206), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740, or may be examined at the Center for Food Safety and Applied Nutrition's Library, 5100 Paint Branch Pkwy., College Park, MD 20740, or at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202–741–6030, or go to: *http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html.*

[47 FR 26823, June 22, 1982, as amended at 50 FR 49536, Dec. 3, 1985; 63 FR 66015, Dec. 1, 1998; 66 FR 13847, Mar. 8, 2001]

§ 172.160 Potassium nitrate.

The food additive potassium nitrate may be safely used as a curing agent in the processing of cod roe, in an amount not to exceed 200 parts per million of the finished roe.

§ 172.165 Quaternary ammonium chloride combination.

The food additive, quaternary ammonium chloride combination, may be safely used in food in accordance with the following conditions:

(a) The additive contains the following compounds: *n*-dodecyl dimethyl benzyl ammonium chloride (CAS Reg. No. 139–07–1); *n*-dodecyl dimethyl ethylbenzyl ammonium chloride (CAS Reg. No. 27479–28–3); *n*-hexadecyl dimethyl benzyl ammonium chloride (CAS Reg. No. 122–18–9); *n*-octadecyl dimethyl benzyl ammonium chloride (CAS Reg. No. 122–19–0); *n*-tetradecyl dimethyl benzyl ammonium chloride (CAS Reg. No. 139–08–2); *n*-tetradecyl dimethyl ethylbenzyl ammonium chloride (CAS Reg. No. 27479–29–4).

(b) The additive meets the following specifications: pH (5 percent active solution) 7.0–8.0; total amines, maximum 1 percent as combined free amines and amine hydrochlorides.

(c) The additive is used as an antimicrobial agent, as defined in § 170.3(o)(2) of this chapter, in raw sugar cane juice. It is added prior to clarification when further processing of the sugar cane juice must be delayed.

(d) The additive is applied to the sugar juice in the following quantities, based on the weight of the raw cane:

Component	Parts per million
n-Dodecyl dimethyl benzyl ammonium chloride	0.25–1.0
n-Dodecyl dimethyl ethylbenzyl ammonium chloride	3.4–13.5
n-Hexadecyl dimethyl benzyl ammonium chloride	1.5–6.0
n-Octadecyl dimethyl benzyl ammonium chloride	0.25–1.0

FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

8-04
20 October 2004

INITIAL ASSESSMENT REPORT

APPLICATION A542

NATAMYCIN - EXTENSION OF USE AS A FOOD ADDITIVE

DEADLINE FOR PUBLIC SUBMISSIONS to FSANZ in relation to this matter:
1 December 2004
(See 'Invitation for Public Submissions' for details)

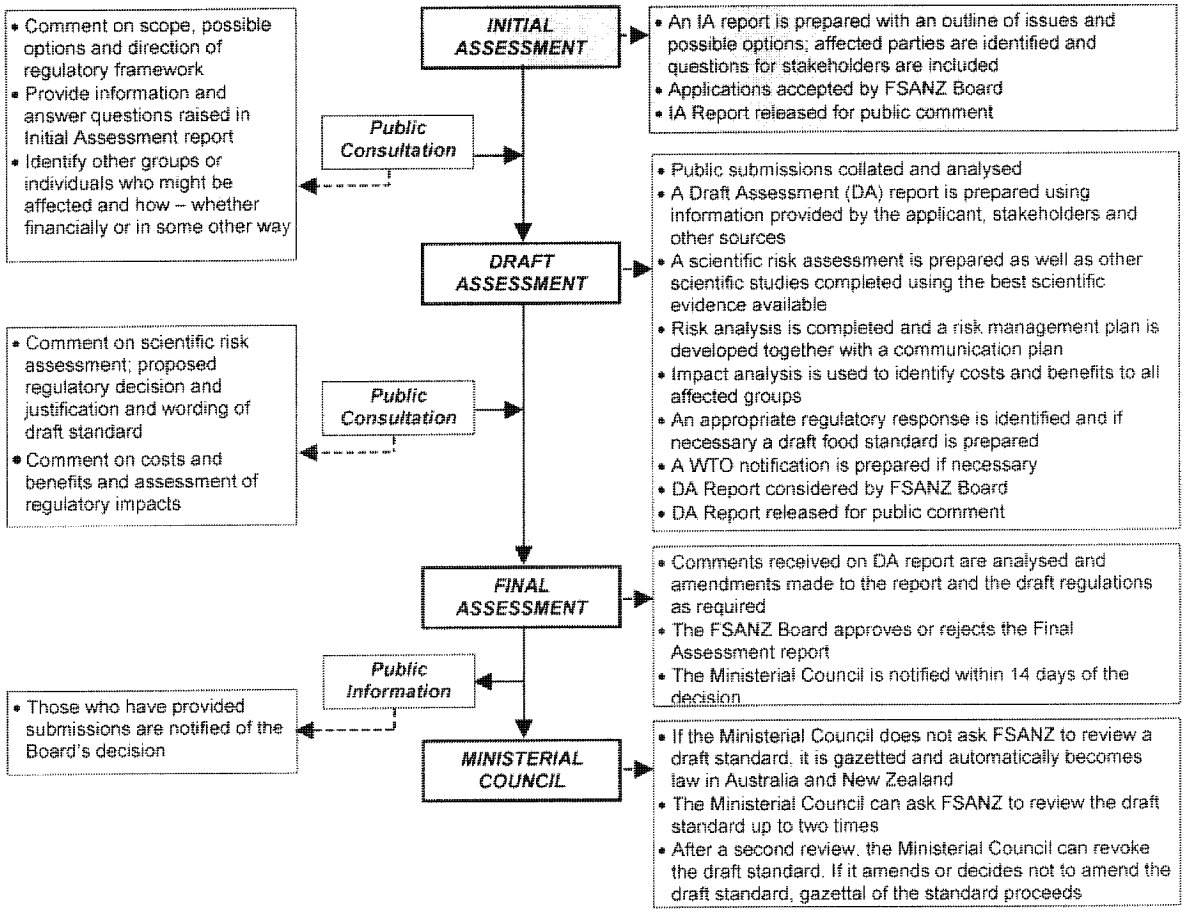
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

FSANZ's role is to protect the health and safety of people in Australia and New Zealand through the maintenance of a safe food supply. FSANZ is a partnership between ten Governments: the Commonwealth; Australian States and Territories; and New Zealand. It is a statutory authority under Commonwealth law and is an independent, expert body.

FSANZ is responsible for developing, varying and reviewing standards and for developing codes of conduct with industry for food available in Australia and New Zealand covering labelling, composition and contaminants. In Australia, FSANZ also develops food standards for food safety, maximum residue limits, primary production and processing and a range of other functions including the coordination of national food surveillance and recall systems, conducting research and assessing policies about imported food.

The FSANZ Board approves new standards or variations to food standards in accordance with policy guidelines set by the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council) made up of Commonwealth, State and Territory and New Zealand Health Ministers as lead Ministers, with representation from other portfolios. Approved standards are then notified to the Ministerial Council. The Ministerial Council may then request that FSANZ review a proposed or existing standard. If the Ministerial Council does not request that FSANZ review the draft standard, or amends a draft standard, the standard is adopted by reference under the food laws of the Commonwealth, States, Territories and New Zealand. The Ministerial Council can, independently of a notification from FSANZ, request that FSANZ review a standard.

The process for amending the *Australia New Zealand Food Standards Code* is prescribed in the *Food Standards Australia New Zealand Act 1991* (FSANZ Act). The diagram below represents the different stages in the process including when periods of public consultation occur. This process varies for matters that are urgent or minor in significance or complexity.



INVITATION FOR PUBLIC SUBMISSIONS

FSANZ has prepared an Initial Assessment Report of Application A542, which includes the identification and discussion of the key issues.

FSANZ invites public comment on this Initial Assessment Report for the purpose of preparing an amendment to the Code for approval by the FSANZ Board.

Written submissions are invited from interested individuals and organisations to assist FSANZ in preparing the Draft Assessment for this Application. Submissions should, where possible, address the objectives of FSANZ as set out in section 10 of the FSANZ Act. Information providing details of potential costs and benefits of the proposed change to the Code from stakeholders is highly desirable. Claims made in submissions should be supported wherever possible by referencing or including relevant studies, research findings, trials, surveys etc. Technical information should be in sufficient detail to allow independent scientific assessment.

The processes of FSANZ are open to public scrutiny, and any submissions received will ordinarily be placed on the public register of FSANZ and made available for inspection. If you wish any information contained in a submission to remain confidential to FSANZ, you should clearly identify the sensitive information and provide justification for treating it as commercial-in-confidence. Section 39 of the FSANZ Act requires FSANZ to treat in-confidence, trade secrets relating to food and any other information relating to food, the commercial value of which would be, or could reasonably be expected to be, destroyed or diminished by disclosure.

Submissions must be made in writing and should clearly be marked with the word 'Submission' and quote the correct project number and name. Submissions may be sent to one of the following addresses:

Food Standards Australia New Zealand
PO Box 7186
Canberra BC ACT 2610
AUSTRALIA
Tel (02) 6271 2222
www.foodstandards.gov.au

Food Standards Australia New Zealand
PO Box 10559
The Terrace WELLINGTON 6036
NEW ZEALAND
Tel (04) 473 9942
www.foodstandards.govt.nz

Submissions should be received by FSANZ **by 1 December 2004**.

Submissions received after this date may not be considered, unless the Project Manager has given prior agreement for an extension.

While FSANZ accepts submissions in hard copy to our offices, it is more convenient and quicker to receive submissions electronically through the FSANZ website using the Standards Development tab and then through Documents for Public Comment. Questions relating to making submissions or the application process can be directed to the Standards Liaison Officer at the above address or by emailing slo@foodstandards.gov.au.

Assessment reports are available for viewing and downloading from the FSANZ website. Alternatively, requests for paper copies of reports or other general inquiries can be directed to FSANZ's Information Officer at either of the above addresses or by emailing info@foodstandards.gov.au.

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Executive Summary

FSANZ received an Application (A542) on 21 June 2004 from Danisco Australia Pty Ltd, submitted by Axiome Pty Ltd, seeking to amend Standard 1.3.1 – Food Additives, of the *Australia New Zealand Food Standards Code* (the Code), to approve the extended use of natamycin (pimaricin) as a food additive to a maximum level of 15 mg/kg in each of the following food categories:

- breads and bakery products;
- fruit and vegetable preparations, including pulp;
- dairy and fat based desserts, dips and snacks; and
- sauces and toppings (including mayonnaises and salad dressings).

This Application is a Group 3 (cost-recovered) Application.

Natamycin (INS 235) is a naturally occurring antimicrobial agent produced by the bacterium *Streptomyces natalensis* and related species. The Applicant wishes to broaden the use of natamycin as a preservative, which is a technological function listed in Schedule 5 to Standard 1.3.1.

This Initial Assessment Report is not a detailed assessment of the merits of the Application but rather an assessment of whether the Application should undergo further consideration according to criteria laid down in the *Food Standards Australia New Zealand Act 1991* (the FSANZ Act).

This Application has been assessed against the requirements of section 13 of the FSANZ Act and accepted for the following reasons:

- The Application seeks approval for extensions of use for natamycin, a permitted food additive.

- Preservative permissions are provided by Schedule 1 of Standard 1.3.1. Therefore, the Application relates to a matter that warrants a variation to Standard 1.3.1, if further assessment supports such a variation.

- This Application is not so similar to any previous application that it ought not be accepted.

- There are no other measures than a variation to the Code available to permit the extensions of use of this preservative.

This Initial Assessment Report includes a summary of information supplied in the Application with relevant issues identified so that interested parties can make submissions to assist in the assessment.

The Code currently permits 15 mg/kg of natamycin on cheese surfaces and 1.2 mg/dm^2 on uncooked fermented manufactured meats in Australia and New Zealand.

Evaluation of a broader use of natamycin will involve consideration of recent toxicological reviews, estimated dietary exposures based on the proposed new uses and the potential for development of antimicrobial resistance. Natamycin is permitted as an antimicrobial preservative in more than 70 countries, mainly for processed meat products, cheese and other dairy products. South Africa permits the widest range of uses, including cheese and cheese products, yoghurts, processed meat products, fish products, wine and fruit wine, fruit juices, fruit pulp and some canned foods. Natamycin is permitted in the USA in cheese, some dairy foods, non-standardised salad dressings and soft tortillas.

The Codex Alimentarius General Standard for Food Additives (Codex Stan 192-1995, Rev. 4 – 2003) includes pimaricin (syn. natamycin) in categories 08.2.1.2 Cured (including salted) and dried non-heat treated processed meat, poultry and game products in whole pieces or cuts to 6 mg/kg, and 08.3.1.2 Cured (including salted) and dried non-heat treated processed meat, poultry and game to 20 mg/kg.

The Application fulfils the requirements for an Initial Assessment and therefore FSANZ has decided to accept the Application. Submissions are now invited to assist in assessing the Application at Draft Assessment.

1. Introduction

FSANZ received an Application (A542) on 21 June 2004 from Danisco Australia Pty Ltd, submitted by Axiome Pty Ltd, seeking amendments to Standard 1.3.1 – Food Additives, of the Code to approve the use of natamycin (pimaricin) as a food additive to a maximum level of 15 mg/kg in each of the food categories:

- breads and bakery products;
- fruit and vegetable preparations, including pulp;
- dairy and fat based desserts, dips and snacks; and
- sauces and toppings (including mayonnaises and salad dressings).

The Applicant wishes to use natamycin (pimaricin) as a preservative, which is a technological function listed in Schedule 5 to Standard 1.3.1. Natamycin (INS 235) is a naturally occurring antimicrobial agent produced by the bacterium *Streptomyces natalensis* and related species.

The primary aim, as stated by the Applicant, is to provide natamycin as an alternative antimicrobial preservative to sorbates, benzoates, propionates and sulphites, and thereby provide many advantages and benefits compared to these compounds.

Work on this Group 3 (cost-recovered) Application commenced on 21 June 2004.

2. Regulatory Problem

Food additives, including preservatives, are required to undergo a pre-market safety assessment before approval for use in Australia and New Zealand.

Food additives are regulated by Standard 1.3.1. Natamycin is currently permitted in Schedule 1 of Standard 1.3.1 as a preservative for use on cheese surfaces to a maximum level of 15 mg/kg and fermented, uncooked processed comminuted meat products to a maximum level of 1.2 mg/dm^2 in a surface sample. This Application is to broaden the use of natamycin and therefore a safety assessment considering these proposed new uses will be required.

Natamycin is an effective antimicrobial preservative against yeasts and moulds, exhibiting a wide spectrum of activity and effectiveness at very low concentrations. Natamycin has strong cidal activity towards susceptible microorganisms and is particularly effective against fungi, which may produce mycotoxins.

In the food categories where the extension of use is requested, other preservatives are currently permitted, but all are claimed by the Applicant to have limitations. The other preservatives are generally only inhibitory in their action, limited in the range of microorganisms affected and often used at their maximum permitted levels. Even at maximum permitted levels, spoilage problems commonly occur and adverse flavours can result due to high usage levels. The Applicant claims that natamycin offers many advantages over these preservatives, including a much wider spectrum of activity, very low use levels, no adverse flavour effects, and generally, vastly superior effectiveness and improved product shelf-life.

3. Objective

The objective of this assessment is to determine whether it is appropriate to amend the Code to permit the extension of use of natamycin as a preservative in a wider variety of foods in Australia and New Zealand.

In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives, which are set out in section 10 of the FSANZ Act. These are:

- the protection of public health and safety;
- the provision of adequate information relating to food to enable consumers to make informed choices; and
- the prevention of misleading or deceptive conduct.

In developing and varying standards, FSANZ must also have regard to:

- the need for standards to be based on risk analysis using the best available scientific evidence;
- the promotion of consistency between domestic and international food standards;
- the desirability of an efficient and internationally competitive food industry;
- the promotion of fair trading in food; and
- any written policy guidelines formulated by the Ministerial Council.

4. Background

4.1 Overview

The contamination of foods by spoilage microorganisms and opportunistic pathogens have always created significant problems for the food industry. The growth in popularity of processed, convenience foods, urbanisation and globalisation have also contributed to increased awareness of potentially serious consequences resulting from microbial contamination. Food spoilage and food poisoning outbreaks cause economic losses and raise public health and safety concerns.

Various processing, formulation and packaging techniques, in combination with Good Manufacturing Practices and food safety programs are employed to control microbial contamination of food. Some foods are more susceptible to microbial contamination due to their inherent nature or composition, processing requirements or usage. For these foods antimicrobial preservatives are widely permitted and used.

Natamycin is an effective antimicrobial preservative against yeasts and moulds, exhibiting a wide spectrum of activity and effectiveness at low concentrations. Natamycin has been used for over 30 years as an antimicrobial food preservative and is currently approved in more than 70 countries.

Natamycin was re-evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2001 and confirmed as safe for its intended use and use levels with respect to cheese and processed meats proposed in the draft Codex Alimentarius General Standard for Food Additives (GSFA) in 2001. JECFA reconfirmed the existing Acceptable Daily Intake (ADI) of 0-0.3 mg/kg/bw.

The Applicant contends that the use of natamycin in the food categories requested in this Application would not lead to intakes in excess of the JECFA ADI. Furthermore the use of natamycin in these foods would provide improved protection against microbial spoilage, benefiting both consumers and manufacturers by reducing product losses, extending shelf-life and protecting public health and safety. An indirect benefit could be reduced use of other antimicrobial preservatives.

4.2 Work Plan Classification

This Application had been provisionally rated as Category of Assessment 3 (level of complexity) and placed in Group 3 (cost-recovered) on the FSANZ standards development Work Plan. This Initial Assessment confirms these ratings. Further details about the Work Plan and its classification system are given in *Information for Applicants* at www.foodstandards.gov.au.

5. Relevant Issues

5.1 Identity and Purity of Natamycin

Natamycin, also known as pimaricin, has;

INS Number: 235

Chemical Abstract (CAS) Number; 7681-93-8; and

JECFA specification in Food and Nutrition Paper series 52 Addendum 9 (2001).

Natamycin is a naturally occurring antimicrobial agent produced by the bacterium *Streptomyces natalensis* and related species. Natamycin acts by disrupting cell membranes of yeasts and moulds, causing leakage and eventual lysis. Natamycin therefore tends to be cidal rather than inhibitory in action to yeasts and moulds. It is active at low concentrations against a wide spectrum of yeasts and moulds, including mycotoxin-producing fungi. Natamycin is not active against bacteria.

5.2 Proposed New Food Uses

The Application states that they wish to use natamycin as a food additive to a maximum level of 15 mg/kg in each of the food categories:

- breads and bakery products;
- fruit and vegetable preparations, including pulp;
- dairy and fat based desserts, dips and snacks; and
- sauces and toppings (including mayonnaises and salad dressings).

The Applicant contends there will be no adverse dietary implications for the use of natamycin in the food categories requested. As part of the re-evaluation by JECFA in 2001, dietary intake of natamycin was reviewed from data supplied by a number of countries, including Australia. The intakes estimated were significantly lower than the JECFA ADI.
The dietary intake in Australia and New Zealand for the requested extensions of use for natamycin will be investigated at Draft Assessment.

5.3 Safety Assessment

5.3.1 Toxicological Assessment

The recent JECFA evaluation in WHO Food Additive Series: 48: "Safety Evaluation of Certain Food Additives and Contaminants – Natamycin (Pimaricin)" is available.

JECFA confirmed the previously established ADI of 0-0.3 mg/kg bw for natamycin, which was based on observations of gastrointestinal effects in humans.

The safety of the extended use of natamycin will be assessed in the context of the proposed new food uses at Draft Assessment.

5.3.2 Dietary Exposure Assessment

JECFA noted that the estimated intake of natamycin based on maximum levels of use in cheese and processed meats proposed in the draft Codex GSFA does not exceed the ADI.

Further dietary modelling will be required in the Draft Assessment Report for the product categories in which natamycin is requested for use in this Application.

5.3.3 Antimicrobial Properties

Natamycin is an antimicrobial agent produced by the bacterium *Streptomyces natalensis*. It belongs to the group of polyene macrolide antimycotics. Natamycin acts by complexing with sterols present in the cell membranes of yeasts and moulds and disrupting the membrane. Permeability of the membrane is increased, causing leakage from the cell and eventual lysis. Natamycin tends to be cidal in action rather than inhibitory to yeast and mould growth. Natamycin is active against a wide range of yeasts and moulds, including mycotoxin-producing fungi and strains pathogenic to humans, however it is reportedly not active against bacteria.

In addition to food applications, it has also been used therapeutically (via topical application) to treat several types of fungal infections of the eye. The emergence of antimicrobial resistance, particularly through misuse of antibiotics, has been a major human health issue in recent years. The World Health Organization recently developed a 'WHO Global Strategy for Containment of Antimicrobial Resistance' to address this issue. The use of antimicrobial agents in foods, such as natamycin, therefore, will need to be examined in light of any potential for antimicrobial resistance.

The Applicant claims that there is no development of antimicrobial resistance through the use of natamycin and that this is supported by the JECFA evaluation report. This issue will be further evaluated for Draft Assessment, including consultations with the National Health and Medical Research Council (NHMRC) External Advisory Group on Antibiotic Resistance.

5.4 Other International Regulatory Approvals

The Applicant supplied a detailed list of natamycin regulatory approvals and states that natamycin is permitted as an antimicrobial preservative in more than 70 countries, mainly for processed meat products, cheese and other dairy products. South Africa permits the widest range of uses, including cheese and cheese products, yoghurts, processed meat products, fish products, wine and fruit wine, fruit juices, fruit pulp and some canned foods. Natamycin is permitted in the USA in cheese, some other dairy foods, non-standardised salad dressings and soft tortillas.

FSANZ has also undertaken a preliminary search of various international regulations and standards and will review the permissions around the world at Draft Assessment.

The Codex GSFA (revision 4 -2004) includes pimaricin (syn. natamycin) in categories; 08.2.1.2 Cured (including salted) and dried non-heat treated processed meat, poultry and game products in whole pieces or cuts to 6 mg/kg; and 08.3.1.2 Cured (including salted) and dried non-heat treated processed meat, poultry and game to 20mg/kg. The Notes (3 and 81) associated with the Codex GSFA permissions are for surface treatment and equivalent to 1 mg/dm2 surface application to a maximum depth of 5 mm respectively. A previous draft of the GSFA (2001) provided a listing for natamycin to 40 mg/kg in category 01.6 for cheese.

6. Regulatory Options

FSANZ is required to consider the impact of various regulatory (and non-regulatory) options on all sectors of the community, which includes consumers, food industries and governments in Australia and New Zealand. The benefits and costs associated with the proposed amendment to the Code will be analysed using regulatory impact principles at Draft Assessment.

There are no options other than a variation to the Code for this Application. Therefore the two regulatory options available for this Application are:

Option 1. Not approve the extended use of natamycin in a wider range of foods.

Option 2. Approve the extended use of natamycin in a wider range of foods.

7. Impact Analysis

7.1 Affected Parties

The affected parties to this Application include the following:

1. those sectors of the food industry wishing to produce and market food;
2. consumers; and
3. Australian, State, Territory and New Zealand Government agencies that enforce food regulations.

7.2 Impact Analysis

In the course of developing food regulatory measures suitable for adoption in Australia and New Zealand, FSANZ is required to consider the impact of all options on all sectors of the community, including consumers, the food industry and governments. The regulatory impact assessment identifies and evaluates, though is not limited to, the costs and benefits of the regulation, and its health, economic and social impacts.

The regulatory impact of the proposed change will be assessed at Draft Assessment.

8. Consultation

8.1 Public consultation

The Initial Assessment Report is not a detailed assessment of this Application but rather an assessment of whether the Application should undergo further consideration. FSANZ is seeking public comment in order to assist in assessing this Application at Draft Assessment. A further round of public comment will occur after the Draft Assessment Report is completed to assist in the Final Assessment.

FSANZ is seeking public comment to assist in assessing the Application. Comments on, but not limited to, the following would be useful:

- technological justification for extending the use of natamycin;
- if there are any safety considerations with its proposed use;
- likely costs and benefits of extending the use of natamycin; and
- affected parties to this Application.

8.2 World Trade Organization (WTO)

As members of the World Trade Organization (WTO), Australia and New Zealand are obligated to notify WTO member nations where proposed mandatory regulatory measures are inconsistent with any existing or imminent international standards and the proposed measure may have a significant effect on trade.

This issue will be fully considered at Draft Assessment and, if necessary, notification will be recommended to the agencies responsible in accordance with Australia's and New Zealand's obligations under the WTO Technical Barrier to Trade (TBT) or Sanitary and Phytosanitary Measure (SPS) Agreements. This will enable other WTO member countries to comment on proposed changes to standards where they may have a significant impact on them.

9. Conclusion and Recommendation

This Application has been assessed against the requirements of section 13 of the FSANZ Act and accepted for the following reasons:

- The Application seeks approval for extensions of use for natamycin, a permitted food additive.

- Preservative permissions are provided by Schedule 1 of Standard 1.3.1 – Food additives. Therefore, the Application relates to a matter that warrants a variation to Standard 1.3.1, if further assessment supports such a variation.

- This Application is not so similar to any previous application that it ought not be accepted.

- There are no other measures than a variation to the Code available to permit the extensions of use of this preservative.

- No other relevant matters are apparent at this stage.

It is recommended that this Application now be progressed to Draft Assessment.

WHO FOOD ADDITIVES SERIES: 48

SAFETY EVALUATION OF CERTAIN FOOD ADDITIVES AND CONTAMINANTS

NATAMYCIN (PIMARICIN)

First draft prepared by Dr Antonia Mattia[1], Dr Carl Cerniglia[2] and Janis Baines[3]

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1. EXPLANATION

Natamycin (pimaricin) is a polyene macrolide antibiotic produced by submerged aerobic fermentation of *Streptomyces natalensis* and related species. Fermentation is conducted for several days, and the antibiotic is isolated either by broth extraction or by extraction of the mycelium. It is used as a food additive to control the growth of yeasts and moulds on the surface of cheese and other non-sterile products, such as meat and sausages.

Natamycin was evaluated by the Committee at its twelfth and twentieth meetings (Annex 1, references *17* and *41*). At its twentieth meeting, the Committee established an ADI of 0–0.3 mg/kg bw. Natamycin was evaluated at the present meeting at the request of the Codex Committee on Food Additives and Contaminants.

The Committee considered information on the current uses of natamycin, biological data not previously evaluated, and data on its intake.

The activity of natamycin against yeasts and moulds, but not bacteria, makes it convenient for use in foods that undergo a ripening period after processing. Its low solubility in water and most organic solvents makes it suitable for the surface treatment of foods. Natamycin is used topically in veterinary medicine to treat mycotic infections, such as ringworm in cattle and horses. Previously, it was used topically against fungal infections of the skin and mucous membranes in humans. Its medical use is now confined to topical treatment of corneal fungal infections and the prevention of such infections in users of contact lens.

2. BIOLOGICAL DATA

2.1 Biochemical aspects: Absorption, distribution, and excretion

Rats

The distribution of natamycin was studied by autoradiographic and bioautographic techniques. In the autoradiographic study, five female Wistar rats (TNO, specific pathogen-free) were each given a single dose of 50 mg/kg bw of [^{14}C]natamycin (50 mg in 5 ml of 1% amylum) orally. In the bioautographic study, four female rats were each given a single dose of 50 mg/kg bw (70 mg in 7 ml of 1% amylum) orally. No information on the purity of the compound was provided. Before treatment, the animals were fasted for 24 h but were given a 5% glucose drinking-water solution. One animal in each group was killed by immersion in a solid CO_2 and acetone mixture under mild ether anaesthesia 1, 2 (autoradiographic study only), 4, 8, and 24 h after treatment. Whole-body sections of the animals were cut in a cryostat at –20 °C. In the autoradiographic study, sections were freeze-dried (48 h) and exposed on photographic plates at –20 °C for 93 days (a few for 150 days). In the bioautographic study, the antibiotic activity of the sections was evaluated by exposure on Whiffen agar plates inoculated with *Saccharomyces cerevisiae* strain ATCC 9763 for 5, 10, 15, or 20 min (20, 40, 60, and 120 min for sections from the animals killed 24 h after treatment). After exposure, the agar plates were incubated at 30 °C for 20 h and photographed.

In the autoradiographic study, radiolabel was confined to the gastrointestinal tract after 93 days' exposure (1 h, oesophagus, stomach, small intestine; 2 h, oesophagus, stomach, small intestine, caecum; 4 h, stomach, small intestine, caecum, colon; 8 h, stomach, intestine; 24 h, caecum, colon). After 150 days' exposure, radiolabel was visible only faintly after magnification of the pictures, in the liver, kidneys, and fatty tissue, in addition to the gastrointestinal tract. In the bioautographic study, the antibiotic activity of natamycin was restricted to the gastrointestinal tract (1 h, stomach, small intestine; 4 h, stomach, small intestine, caecum; 8 h, stomach, small intestine, caecum) and lasted less than 24 h. No antibiotic activity was observed in the colon. The results of the autoradiographic study indicate that natamycin is minimally absorbed into the bloodstream and excreted almost entirely in the faeces. The lack of antibiotic activity and the presence of radiolabel in the caecum and colon 24 h after dosing are consistent with the breakdown of natamycin into microbiologically inactive compounds by bacterial flora in the caecum and colon (Blankwater & Hespe, 1979).

A series of experiments was conducted to study the excretion and resorption of [^{14}C]natamycin and its degradation products in normal and cholestatic Wistar rats (induced by tying the bile ducts with a ligature). In the first series, the excretion pattern of radiolabelled compound was investigated in groups of three young male Wistar rats given [^{14}C]natamycin at a dose of 0.1, 1, or 10 mg/kg by quantifying the amount of radiolabel in the urine and faeces at 24-h intervals for 72 h and in expired breath hourly for up to 7 h. Another group received a single dose of 10 mg/kg bw intraperitoneally. A similar experiment was performed in which 10 mg/kg bw of the degradation products of [^{14}C]natamycin, obtained by acid hydrolysis to simulate that in the stomach, were administered orally or intraperitoneally to three Wistar rats, and their urine, faeces, and expired breath were analysed as described above.

Separate experiments were conducted to determine the elimination of [^{14}C]-natamycin in the bile by giving 10 mg/kg bw to two rats orally and to four rats by intraperitoneal injection. Bile was obtained via a cannula in the bile duct at 1-h intervals for 7 h and analysed for radiolabel. In a similar experiment, the elimination of [^{14}C]natamycin via the bile was determined after oral administration of 10 mg/kg bw, in which 0.1 ml of bile obtained from rats not treated with natamycin was placed in the duodenum of treated animals. Bile was collected hourly for 7 h and analysed for radiolabel.

A series of analyses was also carried out to quantify the radiolabel in the stomach, small intestine, caecum, and large intestine of groups of two animals 1, 2, 4, 8, and 24 h after administration of 10 mg/kg bw [^{14}C]natamycin. Sections of the stomach, small intestine, caecum, and large intestine were extracted in methanol, and the extracts were analysed for radiolabel by thin-layer chromatography. One rat was given the non-radioactive form of the test material and killed after 4 h. The concentration of natamycin was analysed in each section of the gastrointestinal tract by high-performance liquid chromatography. The results were compared with those obtained with the radioactive form. In each experiment, all animals were fasted for 20 h before treatment. Water was available during fasting.

When 10 mg/kg bw [^{14}C]natamycin were administered orally to normal or cholestatic rats, most of the radiolabel (93–103%) was found in the faeces. Cholestatic rats had about 5% more radiolabel in their urine than normal rats at this dose. The results were similar in rats treated with 0.1 or 1 mg/kg bw. When natamaycin was delivered by intraperitoneal injection at a dose of 10 mg/kg bw, about 16% of the radiolabel was found in urine and about 76% in faeces by 72 h, indicating significant elimination in the bile. Most of the elimination (63%) occurred within 24 h after administration of natamycin. Intraperitoneal administration of acid-hydrolysed [^{14}C]natamycin resulted in approximately twice as much radiolabel in the urine (61%) as in faeces (30%), showing that hydrolysis transforms natamycin into breakdown products which are more hydrophilic than intact natamycin and thus have less affinity for bile. In contrast, after oral administration of acid-hydrolysed [^{14}C]natamycin, most of the radiolabel was recovered in faeces (94% as compared with 6.7% in urine); thus, hydrolysis did not appear to result in significant systemic absorption. Little radiolabel associated with either intact or acid-hydrolysed natamycin was eliminated as $^{14}CO_2$ in expired breath (< 1%) after either oral or intraperitoneal administration.

In experiments to determine the amount of radiolabel in bile after an oral or intraperitoneal dose of 10 mg/kg bw [^{14}C]natamycin, 40% of the total radiolabel was recovered over 7 h from the bile of rats treated intraperitoneally and only 1% from the bile in rats treated orally. When 'blank' bile was administered duodenally each hour for 7 h to animals treated orally, the amount of radiolabel recovered in the bile was similar to that recovered in animals not given bile.

In the stomach and small intestine, natamycin was mostly untransformed, as indicated by thin-layer chromatography. Most degradation took place in the large intestine. The degradation products were more hydrophobic than natamycin and were found from about 4 h after treatment. Most of the dose of 10 mg/kg was degraded about 8 h after treatment, suggesting that elimination is relatively rapid. Biotrans-formation was attributed to the bacterial flora in the caecum and small intestine.

Overall, no more than 5–7% of the total radioactive dose was absorbed after oral administration of [^{14}C] natamycin, and approximately 90% of the administered compound passed through the gastrointestinal tract without resorption and was eliminated in the faeces (Meier & Hespe, 1979).

Dogs

The resorption and excretion of natamycin were studied by autoradiography in dogs given the compound in plastic coating on cheese at 0.75–0.88 mg/kg, in gelatin capsules at 1.00–1.03 mg/kg, or in a 1% starch suspension at 0.95–1.0 mg/kg. In another experiment, [^{14}C]natamycin was administered intravenously in 5 ml propylene glycol at a concentration of 1 mg/ml. In the experiments with cheese, a radioactive plastic coating with a natamycin content of approximately 2% was applied to one side of 20-g blocks of cheese. A single batch of natamycin was labelled with ^{14}C by incorporating labelled sodium

acetate as the substrate in the usual fermentation process. The quantity of applied radioactive natamycin was determined by weighing the blocks of cheese before and after application of the radioactive plastic coating. Tests were carried out with blocks that had been stored at 4 °C for various periods. Four female beagle dogs, weighing 10–12.5 kg, were used in these experiments. Three of the four dogs were used in multiple tests, but at least 2 weeks were allowed to elapse between experiments to ensure complete elimination of radiolabelled material from the previous experiment. Before dosing, the animals were fasted for about 16 h but were given drinking-water. Th animals were housed individually in metabolism cages after dosing, and faeces and urine were collected daily for 2–5 days. The samples were processed appropriately, and radiolabel was measured with a liquid scintillation counter. The plastic coating was analysed by thin-layer chromatography to quantify natamycin and reaction products formed during storage.

After oral administration, most of the radiolabel was eliminated in the faeces within 24 h, with less than 4% of the total dose in urine. This pattern of excretion was consistent with all three forms of orally administered natamycin. Storage of the cheese at 4 °C for various lengths of time (1–57 days) had no effect on the pattern of radiolabel observed. Thin-layer chromatographic analysis confirmed the presence of [^{14}C]natamycin in the cheese coating after up to 56 days at 4 °C. Approximately equal amounts of radiolabel were measured in faeces and urine after intravenous administration of natamycin in propylene glycol, suggesting that resorption occurred via biliary elimination. The amount of radiolabel recovered was < 100% after adminis-tration in an oral capsule or a suspension and was > 100% after administration as a cheese coating. This difference was probably due to uncertainties in the experimental procedure. It is not clear from this study if excretion of natamycin in the faeces after oral administration was due to the lack of absorption from the gastrointestinal tract or to resorption of systemic natamycin via bile (Hespe & Meier, 1980).

Humans

Little information was available on the absorption, distribution, excretion, or metabolism of natamycin in humans. No natamycin (< 1 µg/ml) could be detected in the blood after ingestion of 500 mg by human subjects (Anonymous, 1968). This finding corroborates the statement that natamycin is not absorbed from the gut in animals or humans (Raab, 1972).

2.2 Toxicological studies

2.2.1 Acute toxicity

The available data on the acute toxicity of natamycin are summarized in Table 1. They suggest that male rats are more sensitive to the acute toxicity of orally administered natamycin than females (Levinskas et al., 1966). However, in a study by van Eken and Wubs (1976) to determine the LD_{50} values for natamycin and three of its potential metabolites after intraperitoneal administration to mice, females were more susceptible to the lethal effects (Table 1). The LD_{50} values of the metabolites of natamycin in this study were higher than that for natamycin, indicating that they are less acutely toxic than the parent compound. LD_{50} values of 3200, 3700, and > 4000 mg/kg were reported for aponatamycin (n = 2), mycosamine hydrochloride (n = 2), and dinatamycinolidediole (range-finding study), respectively, which are potential metabolites of natamycin.

Table 1. Acute toxicity of natamycin

Species	Sex	Route	LD50 (g/kg bw)	Reference
Mouse	NR	Oral	1500 2500	Anonymous (1965)
Rat	Male Female	Oral	2700 4700	Levinskas et al. (1966)
Guinea-pig	Female	Oral	450	Struyk et al. (1958)
Rabbit	Male	Oral	1400	Levinskas et al. (1966)
Dog	NR	Oral	1000	Anonymous (1965)
Mouse	Male Female	Intraperitoneal	1600 420	van Eeken & Wubs (1976)

NR, not reported

In rabbits, doses of natamycin ž 500 mg/kg bw caused diarrhoea, and the animals that died had haemorrhagic gastric mucosa. Complexing of natamycin with one-third its weight of a modified polysaccharide increased its toxicity sixfold, and when it was fed to rats natamycin was detected in their blood (Raab, 1972).

2.2.2 Short-term studies of toxicity

Rats

Oral administration of natamycin at doses of 50–70 mg/kg bw per day for 5–10 weeks had no effect on the growth, blood, or tissues of rats. A daily oral dose of 150 mg/kg bw for 9 weeks caused some growth inhibition, and a daily dose of 500 mg/kg bw caused 30% of the rats to die within 2 weeks (Struyk, 1958).

Groups of 20 male and 20 female rats were fed diets containing natamycin at a concentration of 0, 125, 500, 2000, or 8000 mg/kg for 94–96 days. None of the five deaths observed could be attributed to treatment. Growth was retarded and food intake was diminished at the two highest concentrations. The results of haemato-logical examinations and organ weights were within normal limits, and no gross or microscopic lesions were found that could be attributed to natamycin (Levinskas et al., 1966).

Dogs

Groups of three male and three female beagle dogs received diets containing natamycin at a concentration of 0, 125, 250, or 500 mg/kg for 2 years. All but one dog that receiving 250 mg/kg survived for 2 years; the death was unrelated to exposure to natamycin. No effect was seen on food intake, but males receiving the highest concentration did not grow as rapidly as controls initially, and after 15 months, when the dietary intake was reduced, some animals were unable to maintain a satisfactory body weight. The results of haematological and clinical chemical studies revealed no abnormalities. No effects of significance were found on organ weights, and gross and microscopic examination showed no pathological changes (Levinskas et al., 1966).

Groups of two male and two female beagle dogs were given diets containing natamycin at a target concentration of 0, 375, or 750 mg/kg (equivalent to 0, 12, and 25 mg/kg bw per day) for 3 months. The natamycin was obtained in micronized form and was 90.5% pure. The animals were monitored for clinical changes, body weight, food consumption, haematological, clinical chemical, and urinary alterations, electrocardiography (wave intervals and heart rate at weeks 0, 4, 8, and 12), ophthalmology, and pupillary reactions. After being killed by an intravenous overdose of pentobarbital, all animals were necropsied, and the weights of the thymus, heart, liver, kidneys, adrenals, spleen, and testes were measured and gross lesions noted. The tissues preserved in buffered formaldehyde saline and examined microscopically were brain, thyroid, thymus, lung, heart, liver, kidneys, adrenals, spleen, pancreas, lymph nodes, urinary bladder, ovaries, testes, stomach, ileum, colon, jejunum, caecum, and oesophagus. The statistical evaluations included analysis of variance and the Student *t* test. A signed statement indicated that the study had been conducted in compliance with regulations for Good Laboratory Practice as specified in the Code of Federal Regulations (Title 21, part 58) of the USA and the OECD. A signed and dated quality assurance statement indicated that the findings had been audited throughout the study.

No dose- or treatment-related effects were seen in males or females with respect to mortality rate, food consumption, body weight, haematological, clinical chemical, or urinary end-points, electrocardiography, ophthalmology, absolute and relative organ weights, gross pathology, and histopathology. The only effect attributed to treatment was diarrhoea, which occurred most frequently in animals at the high dose but was also observed in controls and animals at the low dose. The diarrhoea was attributed to local irritation of the gastrointestinal tract. Because of the frequent occurrence of diarrhoea at 750 mg/kg, the authors noted that it would be difficult to expose animals to higher doses. No NOEL could be identified (van Eeken et al., 1984).

2.2.3 Long-term study of toxicity and carcinogenicity

Rats

Groups of 35–40 male and female rats received diets containing natamycin at a concentration of 0, 125, 250, 500, or 1000 mg/kg for 2 years. The animals remained in good health, and their survival was unaffected by treatment. Inhibition of growth rate and diminished food intake were seen only for animals of each sex receiving the highest concentration. The results of haematological investigations and determination of organ weights and gross and microscopic lesions showed no differences between treated and control groups. The numbers and types of tumours found in natamycin-treated rats were not significantly different from those in untreated animals (Levinskas et al., 1966).

2.2.4 Genotoxicity

In vitro

Studies were conducted to evaluate the mutagenic potential of natamycin, its products of degradation (i.e. aponatamycin, natamycinolidediol, and mycosamine hydrochloride), and Delvocid (a 50% suspension of natamycin in water) in *Bacillus subtilis*, *Salmonella typhimurium*, and *Escherichia coli*. *B. subtilis* was exposed in a standard *rec* assay (spot diffusion method) according to Kada (citation not provided). *E. coli* strains WP2*uvr*A$^-$ and WP2 and *S. typhimurium* strains TA1535, TA1538, TA98, and TA100 were evaluated in the spot test for reverse mutation. All the tests were carried out by plating a 50-µl spot containing the appropriate dilution of natamycin on a petri dish with the appropriate microbial strain. The spot tests were carried out within 3 h of exposure and after storage for 1, 3, 7, or 14 days and 1, 2, or 4 months (It was not clear whether all tests were conducted at all intervals). The plate

incorporation assay was used to evaluate the mutagenicity of Delvocid at concentrations up to 1% alone (without addition of an exogenous metabolic activation system from rodent liver) and in combination with up to 0.2 mol/L nitrite in *E. coli* WP2*uvrA*$^-$ and WP2 *trp*$^-$ and *S. typhimurium* TA98 and TA100, with or without addition of exogenous metabolic activation. Nitrite was tested with Delvocid, as other studies have shown that nitrite in combination with some food preservatives forms reaction products that interact with DNA. The design of these tests is shown in Table 2. In each spot test, negative controls were included with solvent or buffer alone and positive controls with a known mutagen (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) or mixtures of sorbic acid and nitrite at pH 3.5 or 4.5. For the plate incorporation assays, benzidine was used as the positive control. No statistical analyses were reported. The author reported that no positive responses were observed in the spot tests in any of the three test systems. Results for individual plates and summary data were not reported for the spot tests. The authors concluded from the plate incorporation assays that Delvocid did not induce reverse mutation when tested alone or with nitrite in any of the strains of *S. typhimurium* or *E. coli* tested. The author commented on the slight positive response with nitrite at about 0.2 mol/L and concluded that Delvocid did not enhance the mutagenic effect (Khoudokormoff, 1977; Khoudokormoff & Gist-Brocades, 1978).

Table 2. Experimental design of study reported by Khoudokormoff (1977)

Material	Concentrations tested (%)	Bacterial system	Metabolic activation	Nitrite concentration
Spot test[a]				
Natamycin[b]	0.1–1[c]	*S. typhimurium*, *E. coli*, *B. subtilis*[d]	No[e]	≤ 400 mg/kg
Aponatamycin	0.5	*S. typhimurium*, *E. coli*, *B. subtilis*[d]	No[e]	≤ 400 mg/kg
Pimaricinolidediol	0.5	*S. typhimurium*, *E. coli*, *B. subtilis*[d]	No[e]	≤ 400 mg/kg
Mycosamine hydrochloride	0.5	*S. typhimurium*, *E. coli*, *B. subtilis*[d]	No[e]	≤ 400 mg/kg
Delvocid[b,f] hydrochloride	2	*S. typhimurium*, *E. coli*, *B. subtilis*[d]	No[e]	≤ 400 mg/kg
Plate incorporation assay on top agar				
Delvocid	0.04–1	*E. coli*, *S. typhimurium*[g]	No	None
	0.04–1	*E. coli*, *S. typhimurium*[g]	No	0.01–0.2 mol/L
	0.4	*E. coli*, *S. typhimurium*[g]	Yes	0.01–0.5 mol/L
	0.04–1	*E. coli*, *S. typhimurium*[g]	Yes	0.5 mol/L

a Carried out within 3 h of exposure and after storage for 1, 3, 7, or 14 days and 1, 2, or 4 months

b Tested at pH 2.5–6.5

c Only range provided

d *E. coli* strains WP2 *trp*$^-$ and WP2*uvr*A$^-$ and *S. typhimurium* strains TA1535, TA1538, TA98, and TA100

e Reported that a metabolic activation system was added 'if desired'; no further details were provided

f Also tested in the presence of a cheese coating (WL30) at pH 4.3

g *E. coli* strains WP2 and WP2*uvr*A$^-$ and *S. typhimurium* strains TA98 and TA100

The Committee noted that the reporting of the results of these studies, described as preliminary, had limitations which prevented verification of the author's conclusions. For example, the bacterial strains used were not assayed for the appropriate phenotypic markers or plasmids, the criteria for a positive response were not reported, summary and individual data were not reported for the spot test, and no statistical analyses were performed. Sufficient information was not provided to indicate that the studies were adequately sensitive to detect positive responses in all strains tested. Furthermore, the assays were conducted in a single trial with one plate per dose. The usefulness of these studies is therefore limited.

Natamycin at a concentration of 1% and its known degradation products (aponatamycin, dinatamycinolidediol, and mycosamine) at 0.5% and at pH and nitrite conditions similar to those in preserved food products such as cheese and sausages, were reported to have no mutagenic activity in *B. subtilis* under the conditions tested. No actual data were presented to verify this statement (Khoudokormoff, 1978).

In vivo

Groups of 10 male rats taken from the second litters of the F_1 generation in a three-generation study of reproductive toxicity (see below) were fed on control diet until sexually mature, when they received natamycin at 0, 5, 15, 50, or 100 mg/kg bw daily for 7 days by gastric intubation. Each rat was mated each week for 8 consecutive weeks with two virgin untreated females. Each female was killed and examined 13 days after mating. No differences were found between control and test animals in respect of the numbers of implantation sites or live or dead fetuses or the mutagenic index (Cox et al., 1973).

Five males and five females were selected at random from the five litters produced in the same three-generation study. The animals were given colchicine 3–4 h before being killed, and a bone-marrow preparation was made for examination for aberrant chromatin material. The number of abnormalities in the metaphase chromosomal preparations of test groups did not differ significantly from that in sham-treated controls (Cox et al., 1973).

2.2.5 Reproductive toxicity

(a) Multigeneration studies

Rats

Groups of 10 female and five male rats receiving diets containing natamycin at a concentration of 0 or 1000 mg/kg were mated after 181 and 223 days. Other groups were mated after 48, 174, and 250 days on the diets; four control and four test female young from the second mating were fed on the same diet as their parents and mated when 107 days of age. The pups of natamycin-treated animals had lower mean body weights at weaning than control pups, but examination of the results of the 54 matings showed that their fertility, gestation, lactation, and viability indices were similar to or better than those of the controls. There was a low incidence of abnormalities among pups in this study, but none could be attributed to treatment (Levinskas et al., 1963; Levinskas, 1966).

Groups of 10 male and 20 female rats were given a diet containing natamycin providing a dose of 0 (two groups), 5, 15, 50, or 100 mg/kg bw per day for 11 weeks. These formed the F_0 generation of a three-generation study of reproductive toxicity, two litters being produced in each generation. Animals at 100 mg/kg had an increased number of fetuses born dead, a decrease in the number born alive, and a decrease in the number surviving at 21 days. The weight of pups was depressed in the second litters of the F_0 and F_1 generations and both litters of the F_2 generation. However, the fertility, gestation, viability, and lactation indices were within normal limits for both litters of all three generations. The doses of 5, 15, and 50 mg/kg had no detectable effect on growth or reproduction (Cox et al., 1973).

(b) Developmental toxicity

Rats

Groups of 20 female rats from the second litters of the F_1 generation of the three-generation study of reproductive toxicity were reared to maturity on control diet and mated with untreated males. The females were given the same dose of natamycin as their parents (0, 5, 15, 50, or 100 mg/kg bw per day) by gastric intubation during the 6–15 days of gestation and were killed and examined on day 20. No differences were found between control and test animals in respect of the numbers of pregnancies, live litters, implantation sites, resorption sites, live and dead fetuses, or skeletal and soft tissue abnormalities (Cox et al., 1973).

Rabbits

Groups of 10–12 female rabbits were given natamycin at a dose of 0, 5, 15, or 50 mg/kg bw per day by gavage on days 6–18 of gestation. They were examined on day 29, and the numbers of corpora lutea, implantation sites, resorption sites, and live and dead fetuses were recorded. No adverse effects on nidation or maternal or fetal survival were found. The number of abnormalities seen in the soft or skeletal tissues did not differ from that occurring spontaneously in controls (Bailey & Morgareidge, 1974).

An aqueous suspension of Delvocid (50% natamycin) was administered to groups of 20–26 mated female Dutch belted rabbits by gavage at a dose of 5, 15, or 50 mg/kg bw per day on days 6–18 of gestation. Two control groups were used: a vehicle control that received an equal volume of sterile saline daily by gavage on days 6–18 of gestation and a positive control group given 2.5 mg/kg bw of 6-aminonicotinamide by gavage on day 9 of gestation. The does were observed daily for signs of toxicity, and body weights were recorded on days 0, 6, 9, 12, 15, 18, and 29 of gestation. On day 29, all surviving does were killed, and the numbers of corpora lutea, implantation sites, resorption sites, and live and dead fetuses, sex of fetuses, and fetal body weights were evaluated at autopsy. The survival rate of the fetuses was determined, and they were examined for external, soft-tissue, and skeletal anomalies. According to the protocol, the study was conducted in compliance with proposed Good Laboratory Practice regulations (21 CFR 3), but there is no indication as to whether quality assurance or quality control

procedures were in place.

Treated does showed no clinical signs of toxicity. One at the low dose, two at the intermediate dose, and five at the high dose died or were killed when moribund. Accordingly, the maternal mortality rates were 0% (0/20), 5% (1/20), 9% (2/22), and 19% (5/26) in the four groups, respectively. The cause of these deaths was not indicated in the report. One doe at the intermediate dose delivered young prematurely (the day before scheduled removal). There were no clear treatment-related signs of toxicity. The following parameters were comparable in treated groups and the vehicle control group: mean maternal body weight, pregnancy rate, number of implantation sites, number of resorption sites, numbers of live and dead fetuses, male to female ratio of fetuses, per cent viability, and incidence of soft-tissue anomalies. Maternal body-weight gain was not calculated. In addition, although the number of corpora lutea in each doe and the occurrence of external anomalies were determined, these data were not summarized or analysed statistically. The average body weight of live fetuses in the group at the intermediate dose was significantly lower than that of the vehicle control group. The groups at the two higher doses showed a significant increase relative to the vehicle control group in the incidence of extra sternebrae. The authors noted that the effect on fetal body weight was not dose-related, and they considered the extra sternebrae to be a developmental variation and not an indication of frank teratogenicity (Knickerbocker & Re, 1978, 1979).

The results of this study were difficult to interpret owing to maternal mortality, problems associated with gavage of rabbits, and because the digestive system of rabbits is sensitive to antibiotics. However, there was evidence that the extra sternebrae observed in fetuses of does at the intermediate (15 mg/kg bw per day) and high (50 mg/kg bw per day) doses of natamycin were variations rather than malformations (Manson & Kang, 1994). Consequently, this study was not considered suitable for deriving an ADI.

2.2.6 Special studies

(a) Allergic effects

No allergic sensitization occurred among 111 patients being treated with natamycin for a variety of conditions (Gruyper, 1961, 1964). No history of allergic reactions was found in 73 workers engaged for an average of 5 years in the manufacture of natamycin, and no allergic reactions were found in the 71 who were tested with cutaneous or intradermal challenge doses (Malten, 1967). Repeated patch tests on 102 patients with various forms of eczema failed to demonstrate any sensitizing potential of natamycin (Malten, 1968).

(b) Degradation products

(i) Acute toxicity

Similar breakdown products of natamycin occur in simulated gastric juice, 0.5% citric acid, and urine, and it appears likely that breakdown products in stored apples resemble those produced in gastric juice. The breakdown products are tetraenes related to natamycin, principally aglycone dimerized and/or decarboxylated; whether these are absorbed remains to be tested (Brik, 1975). Approximately 50% natamycin is broken down within 1 h in simulated gastric juice, and the losses from the stomachs of fasted and non-fasted rats were 33–43% and 0–31% respectively (Morgenstern & Muskens, 1975).

The results of studies of the acute toxicity of the decomposition products of natamycin kept under various conditions after intraperitoneal administration to mice are presented in Table 3.

Table 3. Acute toxicity of natamycin decomposition products

Treatment of suspension	Decomposition (%)	LD_{50} in mice (mg/kg bw)
pH 2.2 with citric acid	74	200
pH 6.3 in the dark	13	200–400
pH 6.3 in the light	80	400–600
pH 8.5 (NaOH)	0	150–250
pH 8.5 (NaOH)	5	450
pH 10.4 with 'soda'	100	> 800
pH 6.3 with 0.1% H_2O_2	9	200–400
pH 5.0 in ultra-violet light	0	170

From Ottens (1965)

(ii) Short-term studies of toxicity

Rats

Groups of 15 male and 15 female rats were given diets containing 5% water, 5% of 0.5% citric acid, 500 mg/kg natamycin, or 5% of a solution of acid-degraded natamycin (suspended in 0.5% citric acid until only 14% of the activity remained) for 98 days. No animals died, and their weight gain was unaffected by treatment; no adverse effects were seen in haematological tests or on the absolute weights of the liver and kidneys. Minor differences in relative organ weights were considered to be coincidental and not due to treatment. Microscopic examination of a wide range of organs showed no lesions due to treatment (Hutchison et al., 1966).

Slices of cheese were treated with 0.05% and 5% suspensions of natamycin and left to dry at room temperature. The antimicrobial activity of the two cheeses declined to less than 20% and 60–80% during the 3-week storage period before they were incorporated into rat diet, and the final dietary concentrations of natamycin plus degraded natamycin were 3.6 and 360 mg/kg. Groups of 15 and 30 male and female rats received diets containing fresh cheese dressed with 0, 0.05, or 5.0% natamycin or cheese dressed with 0, 0.05, or 5% suspensions and stored for 3 weeks. The test lasted 7 weeks. No effects that could be attributed to natamycin degradation products were found on behaviour, appearance, morbidity, mortality, food consumption, body-weight gain, haematological indices, liver function, organ weights, or macro- or microscopic appearance of the animals (Wieriks, 1966).

Groups of 10 male and 10 female rats were fed for 3 months on diets containing the peel of apples which had been untreated, freshly treated with natamycin, or treated with natamycin and stored for 2–8 weeks to allow degradation to take place. In a similar experiment, sausage skins untreated, freshly treated, or stored with natamycin were fed to rats. The doses of natamycin and its degradation products cannot be calculated, but the apple-skin diet provided rats with approximately 0, 50, and 1250 times the probable

human intake, and the sausage-skin diet provided approximately 0, 1000, and 25 000 times the human intake. Some minor abnormalities were found, but none related to growth rate, mortality rate, haematological indices, serum enzymes, liver function, organ weights, or gross or microscopic appearance could be attributed to the intake of natamycin breakdown products (Wieriks, 1971).

2.2.7 Microbiological effects

Limited information on the microbiological effects of natamycin, including fungal resistance, was included in the previous monograph (Annex 1, reference 42). In that monograph, it was stated that natamycin is active against a wide range of mycotic organisms such as dermatophytes and other fungi, yeasts, and yeast-like organisms (including strains pathogenic to humans, animals, and plants and saprophytic varieties). Standard tests have shown that it has no activity on bacteria or on actinomycetes. There is no evidence that mycotoxin-forming species are unusually resistant to natamycin (Raab, 1972). No yeast or yeast-like organisms have been reported to have primary resistance to natamycin, although some dermatophytes are resistant. It is more difficult to induce resistance to natamycin in yeasts than in bacteria (Khoudokmoff & Petru, 1974), and the resistance that could be obtained appeared to be due to selection of naturally more resistant strains and not to adaptation. The resistant cultures had reduced pathogenicity (Athar & Winner, 1971). No evidence of resistance has been recorded in clinical use of natamycin. In studies of its cross-resistance with other antimicrobials, amphotericin B but not natamycin showed cross-resistance with nystatin, filipin, endomycin, and candidin (Stout & Pagano, 1956; Littman et al., 1958; Bodenhoff, 1968; Walter & Heilmeyer, 1969). Nystatin- and amphotericin-B resistant organisms were susceptible to natamycin (Sörensen et. al., 1959), and a wide selection of nystatin-resistant yeasts were normally susceptible to natamycin (Hejzlar & Vymola, 1970). Cross-resistance between natamycin and nystatin and amphotericin appeared to occur *in vitro* (Athar & Winner, 1971).

More information on fungal resistance has become available since the previous review, and that pertinent for assessing potential resistance, including a discussion of the mechanism of action of polyene antibiotics and fungi in the human gastrointestinal tract, is summarized below.

(a) Mechanism of action

The polyenes constitute a large group of antibiotics with various molecular structures, which interact with fungal membranes in an especially interesting way (Franklin & Snow, 1998). The approximately 200 polyenes are all produced by *Streptomyces* spp. The antifungal activities of natamycin and other polyenes are dependent on their binding to cell membrane sterols, primarily ergosterol, the principal sterol in fungal membranes, thereby making them leaky (Hamilton-Miller, 1974; Norman et al., 1976; McGinnis & Rinaldi, 1985; Carlile & Watkinson, 1994). As polyene macrolide antibiotics like amphotericin B, nystatin, and natamycin have a much greater affinity for ergosterol than for cholesterol, the mammalian membrane sterol, they are selectively antifungal. The polyenes form complexes with sterols and apparently disrupt membrane function by this mechanism. The oomycete fungi and bacteria are insensitive to these antibiotics because their membranes lack sterols. At low concentrations, selective changes in membrane permeability may occur. Leakage of potassium ions is the first detectable event, and, at high concentrations, leakage of amino acids and other metabolites occurs.

The polyenes have a large lactone ring with a rigid lipophilic chain containing three to seven conjugated double bonds and a flexible hydrophilic portion bearing several hydroxyl groups. The length of the chromophore gives the characteristic ultra-violet spectrum for each compound and contributes to the instability of some polyenes to heat, light, and pH. Most polyenes have a sugar unit, typically the amino sugar mycosamine, which is linked by the glycosidic bond to the □ carbon atom of the chromophore. Amphotericin B contains seven conjugated double bonds, and natamycin contains four, so these

antimicrobial agents are known as heptaenes and tetraenes, respectively. Nystatin is classified as either a pseudoheptaene or a tetraene (McGinnis & Rinaldi, 1985).

The typical polyene structure has both a hydrophobic and hydrophilic face. The polyenes insert themselves into the cell membrane by associating with sterols (the hydrophobic face) and are thought to cause rearrangement of the sterols, so that a group of four to eight polyene molecules forms a ring with the hydrophilic faces in the centre. Thus, they form a polar pore through which small ions like K^+ and H^+ can pass freely, disrupting the cell's ionic control (Griffin 1994; Deacon 1997). Polyenes can also directly affect enzymatic sequences involved in the synthesis of membrane constituents at the level of the early cyclic precursors in the ergosterol biosynthetic pathway (Mukhtar, et al., 1994). The accumulation of these precursors results from a decrease in the *trans*-methylation reaction that requires *S*-adenosylmethione as the donor of the methyl group and zymosterol as the substrate for methylation. Bacteria are not susceptible to natamycin as their membranes are devoid of sterols. Accordingly, the reported minimum inhibitory concentrations (MICs) of natamycin against bacteria are high, those for *Staphylococcus aureus*, *Streptococcus faecalis*, *Streptococcus haemolyticus*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* all being > 250 mg/kg.

(b) Fungi in the human gastrointestinal tract

The microflora in the human gastrointestinal tract form an extremely complex, yet relatively stable ecological community, populated with over 10^{11} bacterial cells per gram of content and containing more than 400 bacterial species (Cerniglia & Kotarski, 1999). There are fewer bacteria than fungi. Up to 10^5 colony forming units of yeasts have been reported in stool samples from healthy subjects (Bernhardt, 1998).

The yeasts found are *Candida albicans* (the commonest), *C. glabrata* (*Torulopsis glabrata*), *C. tropicalis*, *C. guillermondii*, *C. krusei*, *C. inconspicua*, *C. parapsilosis*, *C. lusitaniae*, and *C. kefyr* (*C. pseudotropicalis*). *Rhodotorula* spp., *Trichosporon*, *Saccharomyces cerevisiae*, *Geotrichum candidum*, *Aspergillus* spp., *Cryptococcus* spp., and *Mucor* spp. are rarely found in the intestine (Bernhardt, 1998). The metabolic activity of *Candida* spp. in the gastrointestinal tract is very low owing to the anaerobic conditions and limited nutrients. Yeasts of the normal flora can invade the tissues of patients whose immune defences have been suppressed by disease or in persons with an altered intestinal microflora. The therapeutic use of antimicrobials can suppress the normal bacterial flora, and this is responsible in part for the increase in the number of yeast infections, particularly gastrointestinal candidosis (Blaschke-Hellmessen et al., 1996; Kreisel, 1999). The polyenes are not absorbed from the gastrointestinal tract, but are sometimes given by mouth to combat fungal growth in the gut, which most commonly results from the use of broad-spectrum antibacterials that deplete the normal bacterial flora of the gut and allow yeasts and fungi to multiply and cause opportunistic infection (Scheurlen, 1996).

The use of natamycin as an antifungal agent in food may result in trace quantities of antimicrobial residues that interact with endogenous microflora. No data were available on the effect of natamycin on the human intestinal microflora. As bacteria are not affected by polyenes, it can be concluded that natamycin residues would have no potentially harmful effects. Furthermore, as yeasts are found in small quantities in the human gastrointestinal tract, the risk of trace exposure of fungi to natamycin would be minimal.

(c) Fungal resistance to natamycin

Natural resistance against polyenes such as natamycin does not occur among fungi, because of the mode

of action of these chemical agents (Khoudokormoff, 1984). Moreover, in contrast with the main polyenes used clinically, such as amphotericin B and nystatin, the fungistatic and fungicidal minimal concentrations of natamycin differ only negligibly (Table 4), further reducing the opportunity for establishment of resistance (Sorensen et al., 1959). Induction of polyene- and especially natamycin-resistant mutants is difficult (Athar & Winner, 1971). Such mutants invariably show reduced metabolic and growth rates *in vitro*, and in the absence of polyenes readily revert to normal metabolism, growth, and sensitivity to natamycin. One way of obtaining such resistant isolates is by successive sub-culturing *in vitro* in the presence of gradually increasing concentrations of a polyene. Typically, such isolates are resistant up to the highest concentration to which they are exposed, and the conditions are not likely to result from technical application of natamycin as a food preservative.

Table 4. Minimal inhibitory concentrations (MICs) of amphotericin B and natamycin when tested in vitro against 28 fungal isolates

Organism	Amphotericin B		Natamycin	
	Mean (µg/ml)	Range (µg/ml)	Mean (µg/ml)	Range (µg/ml)
Group 1 (10 isolates)				
Candida albicans	0.36	0.2–0.6	3.3	1.6–4.7
Candida parapsilosis	2.4	0.8–3.1	5.2	4.7–6.2
Candida krusei	1.6	1.6	1.6	1.6
Rhodotorula spp.	6.2	6.2	2.3	2.3
Group 2 (10 isolates)				
Fusarium solani	20	3.1–50	4.2	3.1–6.2
Group 3 (8 isolates)				
Aspergillus fumigatus	3.1	3.1	3.1	3.1
Aspergillus niger	4.3	2.3–6.2	2.3	1.6–3.1
Penicillium spp.	4.7	4.7	2.3	2.3
Penicillium lilacinus	4.7	4.7	9.4	9.4
Rhizopus spp.	ND	2.3–> 50	9.4	6.2–12
Scopulariopsis brevicaulis	> 50	> 50	3.1	3.1

From Stern (1978)

The antifungal action of polyene antibiotics is based on their linkage with sterols in the cytoplasmic

membrane of the fungal cell wall, which distends the wall. The sensitivity of fungal cells to the drug depends on the characteristics of the sterol (Littman et al., 1958; Molzahn & Woods, 1972; Subden et al., 1977). *Candida* strains resistant to nystatin contain more ergosterol than sensitive ones (Athar & Winner, 1971; Safe et al., 1977). Sensitivity to polyene antibiotics is a consistent feature of wild-type fungal strains. Prolonged therapy with an antibiotic results in increased resistance to it. Induced resistance to polyene antibiotics has been observed in *Candida, Torulopsis*, and *Cryptococcus* strains (Macura, 1991).

Although there is a potential risk of development of resistance among microbial flora as a consequence of prolonged, repeated application of natamycin, the studies reported indicate that the level of resistance would be low.

Attempts to induce resistance to natamycin in *C. albicans* by serial passage on Sabouraud maltose agar showed that resistance developed gradually. After 25 passages, the MIC was increased from 2.5–12 to 12–50 µg/ml. Comparison of the polyene antibiotics natamycin and fungicidin indicated that strains that are resistant to fungicidin are sensitive to natamycin (Hejzlar & Vymola, 1970; Table 5).

Table 5. Correlation between minimal inhibitory concentrations (MICs) of natamycin and fungicidin in some strains of Candida spp.

Strain	MIC (µg/ml)	
	Natamycin	Fungicidin
Candida albicans 1	10	5.0
albicans 2	6.2	> 100
albicans 135	5.0	12
albicans 32/V	3.1	3.1
Candida crusei 182	10	6.2
crusei 196	10	3.1
crusei 87/V	10	12
Candida tropicalis 11	6.2	10
tropicalis 94	5.0	12
tropicalis	3.1	> 100
Candida albicans 36/V	2.5	> 100
albicans 69/V	5.0	> 100
albicans 101/V	6.2	1.6

albicans 137/V	> 100	6.2
albicans 138/V	12	12
albicans 141/V	6.2	> 100
albicans	12	3.1
albicans 165/V	12	2.1

From Hejzlar & Vymola (1970)

Natamycin has been given orally for the treatment of intestinal candidosis at a daily dose of up to 400 mg. It was highly active against yeast-like fungi (MIC, 1.5 µg/ml) but less effective against dermatophytes (MIC, 3.0–100 µg/ml). Strains resistant to natamycin are rare, but the effectiveness of this drug in the treatment of vaginal candidosis has decreased (Lovgren & Salmela, 1978). The MIC values were between 2.9 and 31 µg/ml for strains isolated from untreated women but 9.8–64 µg/ml for strains from women who had been treated previously.

Delvocid, a 50% natamycin preparation, has been used for more than 20 years for preserving cheese and sausages (Jay, 1996). Surveys in cheese warehouses and in dry sausage factories where Delvocid had been used for up to 9 years showed no change in the composition or the sensitivity of the contaminating fungal flora (de Boer & Stolk-Horsthuis, 1977; de Boer, 1979; Hoekstra & Van der Horst, 1998).

de Boer and Stolk-Horsthuis (1974) isolated yeasts and moulds from various cheese warehouses in which natamycin was used. All of the isolated fungi but one were inhibited at low concentrations of natamycin (0.5–8 µg/ml). In a similar study in 1976, in which eight warehouses where natamycin had been used and two in which it had never been used were surveyed, 26 strains were isolated and tested for sensitivity to natamycin; no insensitive yeasts or moulds were found.

Laboratory experiments intended to induce tolerance to natamycin in strains isolated from cheese warehouses indicated that after 25–30 transfers to media containing increasing concentrations of natamycin none of the strains had become less sensitive to natamycin (Table 6).

Table 6. Induction of tolerance to natamycin of moulds isolated from cheese warehouses

Strain	No. of transfers	MIC of natamycin (µg/ml)	
		Initial	After a given no. of transfers
Penicillium viridicatum Westling	30	8	10
Penicillium viridicatum Westling	30	8	10
Penicillium chrysogenum Westling	31	2	2
Aspergillus versicolor (Vuill.)	25	4	1

Tiraboschi			
Penicillium viridicatum Westling	30	8	12
Cladosporium cladosporioides (Fres.) de Vr.	30	2	2
Aspergillus versicolor (Vuill.) Tiraboschi	25	4	2
Penicillium verrucosum Dierekx var. *cyclopium* (Westling)	31	2	2
Aspergillus versicolor (Vuill.) Tiraboschi	25	2	1
Penicillium viridicatum Westling	29	6	10
Penicillium verrucosum Dierckx var. *cyclopium* (Westling)	31	2	2
Penicillium verrucosum Dierckx var. *cyclopium* (Westling)	31	2	2
Cladosporium cladosporioides (Fres.) de Vr.	27	2	6
Penicillium citreo-viride Biourge	28	4	4
Penicillium verrucosum Dierckx var. *cyclopium* (Westling)	25	4	2
Penicillium brevi-compactum Dierckx	30	4	2
Beauveria alba (Limber) Saccas	23	8	4
Penicillium roseo-purpureum Dierckx	29	8	10
Scopulariopsis asperula	25	8	4
Penicillium cf. lividum Westling	25	4	2
Aspergillus versicolor (Vuill.) Tiraboschi	25	4	2
Acremomium sclerotigenum	23	8	8
Penicillium viridicatum Westling	30	8	10
Penicillium viridicatum Westling	29	8	12

Penicillium nigricans (Bain.) Thom	30	4	2
Aspergillus versicolor (Vuill.) Tiraboschi	25	2	2

From De Boer & Stolk-Horsthuis (1977)

The sensitivity to natamycin of yeasts and moulds isolated in dry sausage factories where natamycin had been used for several years was compared with that of isolates from factories where natamycin had never been applied. No significant differences were found (de Boer et al., 1979).

In experiments with the plant pathogens *Cladosporium cucumerinum* and *Fusarium oxysporum*, the frequency of emergence of resistance was 1 in 10^7. Eighteen resistant strains were selected for further study, and the natamycin-resistant strains were divided into those with lower and higher levels of resistance. Greater resistance appeared to be associated with decreased fitness *in vitro* (radial growth and sporulation on agar media) and *in vivo* (pathogenicity). The authors suggested a link between increased resistance and decreased fitness (Dekker & Gielink, 1979).

Reduced sensitivity to polyenes can be induced *in vitro*, but this may be of no practical significance. The resistance of several subcultures in the presence of increasing concentrations of a polyene antimycotic was associated with slower growth and diminished virulence, so that any resistant cells that appear during polyene antimycotic treatment may succumb to the body defence mechanisms.

2.3 Observations in humans

Nausea, vomiting, and diarrhoea have been observed occasionally after an oral dose of 300–400 mg of natamycin daily; no changes in peripheral blood cells were observed (Anonymous, 1966). A group of 10 patients with systemic mycoses received oral doses of 50–1000 mg/day for 13–180 days. Nausea, vomiting, and diarrhoea occurred in those receiving 600–1000 mg/day (Newcomer et al., 1960).

3. INTAKE

Natamycin is proposed in the Codex draft General Standard for Food Additives (GSFA) for use in food groups 1.6 'Cheese' at 40 mg/kg, in 8.2.1.2 'Cured and dried non-heat treated processed meats, poultry and game products' at 6 mg/kg, and in 8.3.1.2 'Cured and dried non-heat comminuted meat, poultry and game products' at 20 mg/kg.

Data on intake of natamycin were submitted by Australia, Germany, New Zealand, and by the manufacturer, whose submission included limited data on intake in the United Kingdom and the USA.

3.1 Screening of additives by the budget method

The budget method can be used to assess whether the use of natamycin should be restricted to specific food groups. The calculations indicated that the theoretical maximum level of use of natamycin is 24 mg/kg, assuming it is used in only half the solid food supply and that the ADI is 0–0.3 mg/kg bw. This theoretical level is lower than the proposed level of use in cheese in the draft GSFA but higher than that proposed for meats, indicating that use of natamycin should be restricted.

As natamycin is proposed for use in two single food groups, the reverse budget method can indicate the maximum amount of each food group that can be consumed before the ADI is exceeded, assuming use in only one food group. If use is assumed to be only in cheese, up to 450 g could be consumed per day at a concentration of 40 mg/kg, assuming an ADI of 0–0.3 mg/kg bw and an average body weight of 60 kg. For cured meats, up to 900 g could be consumed per day.

Consumption of these amounts of either food group on a daily basis is unlikely. The maximum amounts reported for cheese consumers were 99 g/day for the Australian population and 108 g/day for the New Zealand population at the 95th percentile of consumption, 62 g/day for the adult population of the United Kingdom at the 97.5th percentile of consumption, and 45 g/day for the population of the USA at the 90th percentile of consumption. Dietary records do not distinguish whether the cheese consumed had been cut, shredded, or grated but report all cheese consumed, either directly or indirectly in mixed foods.

The maximum amounts reported for consumption of comminuted meat were 170 g/day for the Australian population and 210 g/day for the New Zealand population, both at the 95th percentile of consumption.

3.2 Individual dietary records

The intake of natamycin estimated from individual dietary records was available for five countries (Table 7). The estimates were all well below the ADI, when either draft GSFA or national use levels were assumed.

Table 7. Intake of natamycin estimated from individual dietary records

Country and reference	Population group	Natamycin intake (mg/kg bw per day)	% ADI[a]	Assumptions	Survey	Date o survey
Australia (Australia–New Zealand Food Authority, 2001a)	All respondents Consumers only Consumers only Consumers only All respondents Consumers only Consumers only Consumers only	Mean, 0.017 Mean, 0.026 Median, 0.016 95th percentile, 0.081 Mean, 0.005 Mean, 0.009 Median, 0.006 95th percentile, 0.028	5.6 8.5 5.5 27 1.5 3.0 2.0 9.2	GSFA permissions Australia New Zealand Food Standards Code permissions: cheese, 15 mg/kg; salami, 1.2 mg/kg	National survey, single 24-h recall; sample,13 858; age, ≥ 2 years; mean weight, 67 kg; calculations based on individual body weights	1995
New Zealand (Australia–New Zealand Food Authority, 2001b)	All respondents Consumers only Consumers only Consumers only All respondents Consumers only Consumers only Consumers only	Mean, 0.013 Mean, 0.022 Median, 0.015 95th percentile, 0.068 Mean, 0.003 Mean, 0.008 Median, 0.005 95th percentile, 0.021	4.4 7.3 5.1 22 1.1 2.5 1.7 7.1	GSFA permissions Australia New Zealand Food Standards Code permissions for cheese and salami	National survey; sample, 4636; single 24-h recall; age, ≥ 15 years; mean weight, 71 kg; calculations based on individual body weights	1997

United Kingdom (DSM Food Specialities, 2001)	Adult consumers	Mean, 0.014 90th percentile, 0.027 97.5th percentile, 0.041	4.6 9.0 14	Consumers represent 75% of the population; cheese only; GSFA level, 40 mg/kg[a]	National survey; 7-day record; adults 16–64 years; sample, 2197; assumed weight, 60 kg	1986–87
USA (DSM Food Specialities, 2001)	Consumers only Consumers only Consumers only Consumers only	Mean, 0.015 90th percentile, 0.032 Mean, 0.007 90th percentile, 0.016	5.0 11 2.3 5.3	Consumers represent 46% of the population; cheese only. GSFA level, 40 mg/kg[a] Use level, 20 mg/kg	Department of Agriculture Continuing Survey of Food Intakes; 2-day intake (one 24-h record plus one self-reported 1-day intake; weighted data; assumed weight, 60 kg	1994
Germany (DSM Food Specialities, 2001)	Consumers only, 4–10 years Consumers > 10 years	Mean, 0.015 90th percentile, 0.032 97.5th percentile, 0.051 Mean, 0.01 90th percentile, 0.021 97.5th percentile, 0.031	5.0 11 17 3.3 7.0 10	Use level: cheese, 20 mg/kg; meats, 6 mg/kg (8.2.1.2) to 20 mg/kg (8.3.1.2)	National Food Intake Survey; sample, 15 838 (1359 4–10 years; 14 479 > 10 years)	1985–89 except April 1986–April 1987[b]

a Intakes estimated from data given in submission from DSM Food Specialities (2001), assuming draft GSFA permission

b After Chernobyl accident

The estimates based on proposed GSFA levels of use from Australia and New Zealand were for cheese and meat sources. The mean intake for Australian consumers was estimated to be 0.026 mg/kg bw per day, or 9% of the ADI; that for New Zealand consumers was 0.022 mg/kg bw per day, or 7% of the ADI. Cheese contributed 72% of the total natamycin intake in Australia and 67% of that in New Zealand.

Estimates of the intake of natamycin in the United Kingdom and the USA were based on the proposed GSFA use in cheese. The mean estimated intakes were slightly lower than those reported for Australia and New Zealand: 0.014 mg/kg bw per day, or 5% of the ADI, for consumers in the United Kingdom and 0.015 mg/kg bw per day, or 5% of the ADI, for consumers in the USA. The intakes of consumers at high percentiles based on draft GSFA levels of use of natamycin ranged from 0.03 to 0.08 mg/kg bw per day (11–27% of the ADI).

National estimates of natamycin intake, submitted by Australia, Germany, New Zealand, and the USA, were all well below the ADI (Table 7), the mean intakes of consumers being 0.008–0.015 mg/kg bw per day (2.5–5% of the ADI), as the national permitted levels of use were much lower than those proposed in the draft GSFA, and use of natamycin was further restricted: 15 mg/kg in cheese and 1.2 mg/kg in salami in Australia and New Zealand; 20 mg/kg in cheese, 6 mg/kg in meats (8.2.1.2), and 20 mg/kg in meats (8.3.1.2) in Germany; and 20 mg/kg in cheese in the USA.

3.3 Evaluation of intake estimates

The submissions indicated that the intake of natamycin was well below the ADI and that the ADI was not likely to be exceeded even by consumers at high percentiles. The higher estimates for consumers at high percentiles in Australia and New Zealand (27% and 23% of the ADI, respectively) were due to use of single 24-h recall data, which tend to result in overestimates of the habitual intake of consumers at high percentiles. In the surveys of food consumption in the United Kingdom and the USA, the amounts were averaged over a number of days (3 and 7, respectively), which would tend to decrease the reported daily consumption of all foods and of occasionally consumed foods, such as salami type meats, in particular (Gibney, 1999; Lambe et al., 2000).

4. COMMENTS

Toxicological data

The Committee considered eight studies that had not been evaluated previously; these studies had been conducted more than 20 years earlier. A study of single intraperitoneal administration was considered to be irrelevant to the safety assessment of an ingested substance. The results of two studies of genotoxicity in three bacterial systems (*Bacillus subtilis, Salmonella typhimurium*, and *Escherichia coli*) were negative.

Two studies in rats and one in dogs given radiolabelled material for investigation of the distribution and elimination of the compound supported the previous conclusion that natamycin is excreted primarily in the faeces, with minimal absorption. The only adverse effect reported in a short-term study of toxicity in dogs was diarrhoea, which occurred most frequently in animals at the high dose (equivalent to 25 mg/kg bw per day); however, the usefulness of this study was limited as only two dogs were tested.

In a study of developmental toxicity, an aqueous suspension of 50% natamycin was given to groups of 20–26 mated rabbits at a dose of 0, 5, 15, or 50 mg/kg bw per day by gavage on days 6–18 of gestation. The maternal mortality rate was 0%, 5%, 9%, and 19% at the four doses, respectively. No clinical signs of toxicity were observed in the does, and the cause of death was unknown. Mean maternal body weight, pregnancy rate, number of implantation sites, number of resorption sites, numbers of live and dead fetuses, per cent viability, and incidence of soft-tissue anomalies were comparable in treated groups and a control group given the vehicle only. The fetal body weight at the intermediate dose was lower than that of the vehicle control group. The incidence of extra sternebrae was increased at the two higher doses in comparison with the vehicle control group, but not in a dose-related manner. However, in view of the known and unusual sensitivity of the gastrointestinal tract of rabbits to poorly absorbed substances and to compounds with antimicrobial activity, this study was not considered suitable for deriving the ADI.

Microbiological data

The antifungal activities of natamycin and other polyenes depend on their binding to cell membrane sterols, primarily ergosterol, the principal sterol in fungal membranes. Oomycete fungi and bacteria are insensitive to these antibiotics because their membranes lack ergosterol.

Use of natamycin as an antifungal agent in food may result in exposure of the endogenous microflora to trace quantities of antimicrobial residues. The human intestinal microflora are a complex mixture of more than 400 bacterial species, composed primarily of bacterial cells at a concentration of 10^{11}–10^{12} colony forming units per gram. Fungi are much less abundant in the human gastrointestinal tract than bacteria, up to 10^5 colony forming units per gram of yeast being reported in stool samples from healthy subjects. As bacteria are not affected by polyenes, natamycin residues should not harm them, and as

yeasts are found in low quantities the consequences of exposure to traces of natamycin would be minimal.

Several studies in experimental animals indicated a lack of antibiotic activity in the colon, suggesting that natamycin was degraded into microbiologically inactive compounds by bacterial flora. However, no data are available on the degradation of natamycin by human intestinal microflora. In one study, natamycin was present in faecal specimens of volunteers who ingested 500 mg of the compound, indicating that the compound is incompletely absorbed or degraded.

As emergence of antibiotic resistance is a concern, the Committee evaluated the possible development of resistance among microflora as a consequence of ingestion of natamycin. A 50% natamycin preparation has been used for over 20 years to preserve cheese and sausages. Surveys in cheese warehouses and in dry-sausage factories where the preparation has been used showed no change in the composition or the sensitivity of the contaminating fungal flora. All but one of the species of yeasts and moulds isolated in cheese warehouses where natamycin was used were inhibited at the same low concentrations (0.5–8 µg/ml). In another study, 26 fungi were isolated in eight warehouses where natamycin was used and two warehouses where it never had been used and tested for sensitivity to the compound; no insensitive yeasts or moulds were found. The results of laboratory experiments intended to induce resistance to natamycin in strains isolated from cheese warehouses indicated that, after 25–30 transfers to media with increasing concentrations of natamycin, none of the strains had become less sensitive. When the sensitivity of yeasts and moulds isolated from dry-sausage factories where natamycin had been used for several years was compared with that of isolates from factories where natamycin had never been applied, no significant differences were demonstrated.

Induction of polyene-resistant and especially natamycin-resistant mutants *in vitro* is difficult. Such mutants invariably show reduced metabolic and growth rates and, in the absence of polyenes, readily revert to normal metabolism, growth, and sensitivity to natamycin. One means of obtaining resistant isolates is successive sub-culturing *in vitro* in the presence of gradually increasing concentrations of the polyene. Typically, such isolates are resistant only up to the highest concentration to which they have been exposed. After 25 passages, the microbiological inhibitory concentration of *Candida albicans* was minimally increased from 2.5–12 to 12–50 µg/ml.

Assessment of intake

Application of the budget method indicated that further assessment of the intake of natamycin was required. The draft GSFA proposes restricted use of natamycin in cheese (category 1.6) and dried, non-heat-treated meat groups (categories 8.2.1.2 and 8.3.1.2) only, so that the intake would not be expected to exceed the ADI.

Submissions from Australia, Germany, New Zealand, the United Kingdom, and the USA indicated that the intakes at the mean and high levels of consumption were well below the ADI, although the estimates for the United Kingdom and the USA covered cheese consumption only. The estimated mean intakes for consumers ranged from 0.01 to 0.03 mg/kg bw per day (representing 3 and 9% of the ADI in Germany and the United Kingdom, respectively), and those for high consumers were 0.03–0.08 mg/kg bw per day (representing 9 and 27% of the ADI in Australia and the United Kingdom, respectively), if it is assumed that natamycin was used at 40 mg/kg in all cheese products and 20 mg/kg in all cured meat products, as proposed in the draft GSFA. The estimated intakes of natamycin were lower when national use levels were assumed.

5. EVALUATION

Although use of natamycin as an antifungal agent in food may result in exposure of the endogenous flora to trace quantities of antimicrobial residues, bacteria in the human gastrointestinal tract are not affected by polyenes, and the Committee concluded that disruption of the colonization barrier is not a concern. Fungi are found in much smaller amounts than bacteria in the human gastrointestinal tract, and the negative results in studies of acquired resistance indicate that selection of natamycin-resistant fungi is not an issue.

The Committee noted the finding of extra sternebrae in the study of developmental toxicity in rabbits, in which a dose-related increase in the mortality rate was reported. It considered that administration of an antimicrobial agent to rabbits by gavage was inappropriate. In addition, extra sternebrae have been described as a skeletal variation rather than a frank indication of teratogenicity. Thus, the Committee did not consider this result to be evidence that natamycin is teratogenic.

The Committee confirmed the previously established ADI of 0–0.3 mg/kg bw for natamycin, which was based on observations of gastrointestinal effects in humans. The Committee noted that the estimated intake of natamycin based on maximum levels of use in cheese and processed meats proposed in the draft GSFA does not exceed this ADI.\

6. REFERENCES

Anonymous (1965) Data on the safety of the use of pimaricin as preservative against mold growth on cheese. Summary of the results of acute and chronic toxicity tests. Unpublished report submitted to WHO by the Royal Netherlands Fermentation Industries Ltd, Delft.

Anonymous (1968) Absorption of pimaricin following oral administration. Unpublished report submitted to WHO by the Royal Netherlands Fermentation Industries Ltd, Delft.

Aparicio, J.F., Colina, A.J., Ceballos, E. & Martin, J.F. (1999) The biosynthetic gene cluster for the 26-membered ring polyene macrolide pimaricin. *J. Biochem. Mol. Biol.*, **274**, 10133–10139.

Athar, M.A. & Winner, H. I. (1971) The development of resistance by Candida species to polyene antibiotics in vitro. *J. Med. Microbiol.*, **4**, 505–517.

Australia New Zealand Food Authority (2001a) Submission on estimated pimaricin intakes to WHO on behalf of the Australian Government.

Australia New Zealand Food Authority (2001b) Submission on estimated pimaricin intakes to WHO on behalf of the New Zealand Government.

Bailey, D.E. & Morgareidge, K. (1974) Teratogenicity test with pimaricin. Unpublished report No. 1-1052 submitted to WHO by Food and Drug Research Laboratories Inc.

Bernhardt, H. (1998) Fungi in the intestine—Normal flora or pathogens? *Z. Arztliche Fortbild. Qual.*, **92**, 154–156.

Blankwater, Y.J. & Hespe, W. (1979) Autoradiographic and bioautographic study of the distribution of oral natamycin in the rat. Unpublished report No. 20.502, dated 8 May 1979 from Gist-Brocades NV, Delft.

Blaschke-Hellmessen, R., Buchmann, H. & Schwarze, R. (1996) Effect of orally administrated polyene

antimycotics on the intestinal colonization with yeasts: Possibilities and limitations. *Mycoses*, **39**, 33–39.

de Boer, E. & Stolk-Horsthuis, M. (1977) Sensitivity to natamycin (pimaricin) of fungi isolated in cheese warehouses. *J. Food Prot.*, **40**, 533–536.

de Boer, E., Labots H., Stolk-Horsthuis, M. & Visser, J.N. (1979) Sensitivity to natamycin of fungi in factories producing dry sausage, *Fleischwirtschaft*, **59**, 1868–1869.

Bodenhoff, J. (1968) [Resistance of Candida albicans, with besonderer Berücksichtigung von zwei während einer längeren Zeit mittels antibiotica behandelten Patienten.] *Scand. Dent. J.*, **76**, 279 (in German).

Brik, H. (1975) Natamycin (pimaricin). New high-molecular decomposition products with intact lactone-ring. Unpublished report submitted to WHO by Gist-Brocades NV, Delft.

Brik, H. (1981) Natamycin. *Anal. Profiles Drug Substances*, **10**, 513–561.

Carlile, M.J. & Watkinson, S.C., eds (1994) Fungal cells and vegetative growth. In: *The Fungi*, San Diego, CA: Academic Press, p. 148.

Cerniglia, C.E. & Kotarski, S. 1999. Evaluation of veterinary drug residues in food for their potential to affect human intestinal microflora. *J. Regul. Toxicol. Pharmacol.*, **29**, 238–261.

Cox, G.E., Bailey, D.E. & Morgareidge, K. (1973) Unpublished report No. 1-1052 submitted to WHO by Food and Drug Research Laboratories Inc.

Deacon, J.W., ed. (1997) Prevention and control of fungal growth. In: *Modern Mycology*, 3rd Ed., Oxford: Blackwell Science, pp. 289–290.

Dekker, J. & Gielink, A.J. (1979) Acquired resistance to pimaricin in *Cladosporium cucumerinum* and *Fusarium oxysporum* f.sp. *narcissi* associated with decreased virulence. *Neth. J. Plant Pathol.*, **85**, 67–73.

DSM Food Specialities (2001) Submission on pimaricin to WHO from DSM Food Specialities USA, Inc., King of Prussia, Pennsylvania, USA.

van Eeken, C.J. & Wubs, W. (1976) Acute intraperitoneal toxicity of natamycin and three potential metabolites. Unpublished report No. 15.465, 11 January 1976, submitted to WHO by Gist-Brocades.

van Eeken, C.J., Birtwhistle R.D.R. & Aboulwafa-wan Velthoven, M.J.E. (1984) Three months study in dogs of the toxicity of natamycin by addition to the food. Unpublished report No. 12.401, 24 October 1984, submitted to WHO by Gist-Brocades Research and Development.

Euchi, H., Kamada, Y., Kanagawa, T., Naito, T. & Shiota, H. (1999) Cases of keratomycosis due to *Fusarium* species. *Rinsho Ganka* (*Jpn. J. Clin. Ophthamol.*), **53**, 609–611.

Farkas, J. & Kiss, I. (1976) The effect of pimaricin on the microbial flora of cottage cheese in plastic packaging. *Acta Aliment.*, **5**, 107–118.

Food and Drug Administration (2001) Food additives permitted for direct addition to food for human

consumption: Natamycin (pimaricin). Final rule. *Fed. Reg.*, **66**, 21 CFR Part 172.

Franklin, T.J. & Snow, G.A., eds (1998) Antiseptics, antibiotics and the cell membrane. In: *Biochemistry and Molecular Biology of Antimicrobial Drug Action*, 5th Ed. Dordrecht: Kluwer Academic Publishers, pp. 55–56.

Gibney, M.J. (1999) Dietary intake methods for estimating food additive intake. *Regul. Toxicol. Pharmacol.*, **30**, S31–S33.

Griffin, D.A., ed. (1994) Fungicides. In: *Fungal Physiology*, 2nd Ed., New York: Wiley-Liss, Inc., pp. 416–417.

Grupper, C. (1961) Personal communication from the Hôpital Saint-Louis, Paris.

Grupper, C. (1964) Pimaricin in the treatment of superficial mucocutaneous monoliasis. In: Proceedings of the International Congress on Tropical Dermatoses, Naples, June 1964.

Hamilton-Miller, J.M.T. (1974) Fungal sterols and the mode of action of the polyene antibiotics. In: Perlman, D., ed., *Advances in Applied Microbiology*, New York: Academic Press, pp. 109–134.

Hejzlar, M. & Vymola, F. (1970) Comparative study of pimaricin and fungicidin activity *in vitro*. *J. Hyg. Epidemiol. (Praha)*, **14**, 211–213.

Hespe, W. & Meier, A.M. (1980) Studies involving dogs in regard to the resorption of radioactivity following the oral administration of ^{14}C-pimaricin, applied on cheese, in comparison to other oral forms of administration. Unpublished report No. 20.531, dated 4 February 1980, submitted to WHO by Gist-Brocades NV, Haarlem.

Hoekstra, E.S. & Van der Horst, M.I. (1998) Survey of the fungal flora in Dutch cheese factories and warehouses. *J. Food Mycol.*, **1**, 13–22.

Holly, R.A. (1981) Prevention of surface mold growth on Italian dry sausage by natamycin and potassium sorbate. *Appl. Microbiol.*, **41**, 422–429.

Holly, R.A. (1986) Effect of sorbate and pimaricin on surface mold and ripening of Italian dry salami. *Lebensm-Wiss. Technol.*, **19**, 59–65.

Hutchison, E.B., Ribelin, W.E. & Levinskas, G.J. (1966) 98-day study in the rat. Unpublished report submitted to WHO by American Cyanamid Co.

Jay, J.M. ed. (1996) *Modern Food Microbiology*, 5th Ed., New York: Chapman & Hall, pp. 293–294.

Khoudokormoff, B. (1977) Short term microbial tests on mutagenicity of pimaricin (natamycin) and its products of degradation. Unpublished preliminary results, archive No. 10.545, 29 June 1977, submitted to WHO by Gist-Brocades NV.

Khoudokormoff, B. (1978) Potential carcinogenicity of some food preservatives in the presence of traces of nitrite. *Mutat. Res.*, **53**, 208–209.

Khoudokormoff, B. (1984) Are resistance development and morphological changes possible after use of

natamycin? *Wein-Wiss.*, **39**, 45–50.

Khoudokormoff, B. & Petru, M. (1974) On the possible development of antibiotic resistance amongst fungi with special reference to the use of pimaricin as a preservative in the food industry. Unpublished report submitted to WHO by Gist-Brocades NV, Delft.

Knickerbocker, M. & Re, T.A. (1978) Teratologic evaluation of pimaricin in Dutch belted rabbits. Unpublished report from Food and Research Laboratories, Inc., Waverly Research Center, NY). FDRL Report No. 5906, 22 November 1978. Submitted to WHO by Gist-Brocades, Delft.

Knickerbocker, M. & Re, T.A. (1979) Teratologic evaluation of pimaricin in Dutch belted rabbits (amendment). Unpublished report from Food and Research Laboratories, Inc., Waverly Research Center, NY). FDRL Report No. 5906, 26 April 1979. Submitted to WHO by Gist-Brocades, Delft.

Kreisel, W. (1999) Fungi in the intestine. Clinical significance. *Schweiz. Rundsch. Med. Prax.*, **88**, 5–10.

Lambe, J., Kearney, J., Leclercq, C., Berardi, D., Zunft, H.F.J., De Henauw, S., De Volder, M., Lamberg-Allardt, C.J.E., Karkkainen, M.U.M., Dunne, A. & Gibney, N.J. (2000) Enhancing the capacity of food consumption surveys of short duration to estimate long term consumer-only intakes by combination with a qualitative food frequency questionnaire. *Food Addit. Contamin.*, **17**, 177–187.

Lavingia, B. & Dave, D. (1986) Comparative study of amphotericin-B, pimaricin and gentian violet on ocular fungi. *Indian J. Ophthalmol.*, **34**, 73–77.

Levinskas, G.J., Shaffer, C.B., Bushey, C., Kinde, M.L., Stackhouse, D.W. & Vidone, L.B. (1963) Two-year feeding to rats. Unpublished report from the Central Medical Department, American Cyanamid Co. Submitted to WHO by

Levinskas, G.J., Ribelin, W.E. & Shaffer, C.B. (1966) Acute and chronic toxicity of pimaricin. *Toxicol. Appl. Pharmacol.*, **8**, 97–109.

Littman, M.L., Pisano, M.A. & Lancaster, R.M. (1958) Induced resistance of Candida species to nystatin and amphoteracin B. In: *Antibiotics Ann.* 981. New York: Medical Encyclopedia.

Lovgren, T. & Salmela, I. (1978) *In vitro* sensitivity of *Trichomonas vaginalis* and *Candida albicans* to chemotherapeutic agents. *Acta Pathol. Microbiol. Scand. Sect. B*, **80**, 155–158.

Lüock, H. & Cheesman, C.E. (1978) Mould growth on cheese as influenced by pimaricin or sorbate treatments. *S. Afr. J. Dairy Technol.*, **10**, 143–146.

Macura, A.B. (1991) Fungal resistance to antimycotic drugs. A growing problem. *Int. J Dermatol.*, **30**, 131–183.

Majewski, S. & Macua, A.B. (1978) Efficacy of topical antifungal treatment in denture stomatitis. *Mykosen*, **21**, 403–406.

Malten, K.E. (1967) Report of an investigation concerning possible allergic side effects of pimaricin in humans. Unpublished report from the Instituut voor Genceskunde en Maatschappij, Nijmegen. Submitted to WHO by Gist-Brocades NV, Delft.

Malten, K.E. (1968) Report on investigation into possible sensitising side effects of pimaricin in human beings. Unpublished report from the Instituut voor Geneeskunde en Maatschappij, Nijmegen. Submitted to WHO by Gist-Brocades NV, Delft.

Manson, J.M. & Kang, Y.J. (1994) In: Hayes, J.W., ed., *Principles and Methods of Toxicology*, 3rd Ed., New York: Raven Press, p. 1003.

Masterton, G., Sengupta, S.M. & Schofield, C.B.S. (1975) Natamycin in genital candidosis in men. *Br. J. Vener. Dis.*, **51**, 210–212.

McGinnis, M.R. & Rinaldi, M.G. (1985) Antifungal drugs: Mechanisms of action, drug resistance, susceptibility testing, and assays of activity in biological fluids. In: Lorian, V., ed., *Antibiotics in Laboratory Medicine*, 2nd Ed., Baltimore: Williams & Wilkins, pp. 223–281.

Meier A.M. & Hespe, W. (1979). The metabolism of pimaricin in rats. II. Investigation, with the help of ^{14}C-pimaricin, of its resorption, its decomposition in the gastrointestinal tract, and its elimination. Unpublished report 20.504, 28 May 1979.

Mendes, M.V., Aparicio, J.F. & Martin, J.F. (1999) Complete nucleotide sequence and characterization of pSNA1 from pimaricin-producing *Streptomyces natalensis* that replicated by a rolling circle mechanism. *Plasmid*, **43**, 159–165.

Molzahn, S.W. & Woods, R.A. (1972) Polyene resistance and the isolation of sterol mutants in *Saccharomyces cerevisiae*. *J. Gen. Microbiol.*, **72**, 339–348.

Morgenstern, A.P. & Muskens, G.J.A.M. (1975) Further data on the toxicity of the decomposition products of pimaricin. Unpublished report submitted to WHO by Gist-Brocades NV, Delft

Morris, H.A. & Castberg, H.B. (1980) Control of surface growth on blue cheese using pimaricin. *Cult. Dairy Prod. J.*, **15**, 21–23.

Mukhtar, H., Hakkou, A. & Bonaly, R. (1994) Studies on the activity of *Kluyveromyces lactis* S-adenosylmethionine: □ 24-sterol methyltransferase in presence of polyenic antifungal agents. *Mycopathologia*, **126**, 75–83.

Newcomer, V.D., Sternberg, T.H., Wright, E.T., Reisner, R.M., McNall, E.G. & Sorensin, L.J. (1960) The treatment of systemic diseases with orally administered pimaricin: Preliminary report. *Ann. N.Y. Acad Sci.*, **89**, 240–246.

Nilson, K.M., Shahani, K.M., Vakil, J.R. & Kilara, A. (1974) Pimaricin and mycostatin for retarding cottage cheese spoilage. *J. Dairy Sci.*, **58**, 668–671.

Norman, A.W., Spielvogel, A.M. & Wong, R.G. (1976) Polyene antibiotic-sterol interaction. *Adv. Lipid Res.*, **14**, 127–170.

Novak, E.K., Barbarics, E., Vincze, I. & Zala, J. (1984) *In vitro* studies on the food preservative antifungal polyene antibiotic pimaricin (natamycin). In: Kiss, I., Deck, T. & Incaze, K., eds, *Microbial Association and Interactions in Food*, Dordrecht: Kluwer..

O'Day, D.M., Ray, W.A., Robinson, R.D., Head, W.S. & Savage, A.M. (1987) *In vitro* and *in vivo*

susceptibility of *Candida* keratitis to topical polyenes. *Invest. Ophthalmol. Visual Sci.*, **28**, 874–880.

Oldenkamp, E.P. (1979) Natamycin treatment of ringworm in cattle in the United Kingdom. *Vet. Rec.*, **105**, 554–556.

Oldenkamp, E.P. & Spanoghe, L. (1977) Natamycin-S treatment of ringworm in cattle. *Tijdschr. Diergeneeskd.*, **102**, 124–125.

Ottens, H. (1965) Unpublished report submitted to WHO by Royal Netherlands Fermentation Industries, Delft.

Pedersen, J.C. (1992) Natamycin as a fungicide in agar media. *Appl. Environ. Microbiol.*, **58**, 1064–1066.

Pugazhenthi, T.R, Dhanalakshmi, B., Narasimhan, R., Shibu, A.V. & Madhan, S. (1999) Effect of anti-mycotic agents on *Penicillium citrinum* in cheese. *Indian Vet. J.*, **76**, 537–539.

Raab, W. P. (1972) *Natamycin (Pimaricin). Its Properties and Possibilities in Medicine*, Stuttgart: Georg Thieme Publishers.

Reynolds, J.E.F., ed. (1996) *Martindale. The Extra Pharmacopoeia*, 31st Ed., London: Pharmaceutical Press.

Rusul, G. & Marth, E.H. (1988) Growth and aflatoxin production by *Aspergillus parasiticus* in a medium at different pH values and with or without pimaricin. *Z. Lebensm. Unters. Forsch.*, **187**, 436–439.

Sacjdeva, S. & Singh, S. (1985) Pimaricin—A potential preservative in the cheese industry. *Indian Dairyman*, **37**, 587–596.

Safe, L.M., Safe, S.H., Subden, R.E. & Morris, D.C. (1977) Sterol content and polyene antibiotic resistance in isolates of *Candida krusei*, *Candida parakrusei*, and *Candida tropicalis*. *Can. J. Microbiol.*, **23**, 398–401.

Scheurlen, M. (1996) Pathogenicity of fungi in the intestines—Current status of the discussion. *Fortschr. Med.*, **114**, 319–321.

Shahani, K.M., Bullerman, L.B., Evans, T.A. & Arnold, R.G. (1977) Prevention of toxin mold growth in cheese by pimaricin. *Arch. Inst. Pasteur Tunis*, **43**, 511–520.

Sörensen, L.J., McNall, E.G. & Sternberg, T.H. (1959) The development of strains of *Candida albicans* and *Coccidioides immitis* which are resistant to amphotericin B. In: *Antibiotics Annual 1958–1959*, New York: Medical Encyclopedia, pp. 920–923.

Stern, G.A. (1978) *In vitro* antibiotic synergism against ocular fungal isolates. *Am. J. Ophthalmol.*, **86**, 359–367.

Stout, H.A. & Pagano, J.F. (1956) Resistance studies with nystatin. In: Antibiotics Ann.: 704. New York

Struyk, A.P., Hoette, I., Drost, G., Waisvisz, J.M., van Eek, T. & Hoogerheide, J.C. (1958) Pimaricin, a

new antifungal antibiotic. In: Welch, H. & Marti-Ibanez, F., eds, *Antibiotics Annual 1957–1958*, New York: Medical Encylopedia, Inc., pp. 878–885.

Subden, R.E., Safe, L., Morris, D.C., Brown, R.G., and Safe S. (1977) Eburicol, lichesterol, ergosterol, and obtusifoliol from polyene antibiotic-resistant mutants of *Candida albicans*. *Can. J. Microbiol.*, **23**, 751–754.

Walter, A.M. & Heilmeyer, L. (1969) Antibiotika Fibel. Stuttgart: Thieme Verlag.

Wieriks, J. (1966) Pimaricin in cheese: A toxicity test of seven weeks in rats. Unpublished report from the Royal Netherlands Fermentation Industries Ltd, submitted to WHO.

Wieriks, J. (1971) Pimaricin in apples: A toxicity test of three months in rats. Unpublished report from the Royal Netherlands Fermentation Industries Ltd, submitted to WHO.

See Also:
Toxicological Abbreviations

NATAMYCIN

Prepared at the 61st JECFA (2003) and published in FNP 52 Add 11 (2003) superseding specifications prepared at the 57th JECFA (2001) and published in FNP 52 Add 9 (2001) superseding specifications for pimaricin prepared at the 20th JECFA (1976), published in FNP 52 (1992). An ADI 0-0.3mg/kg bw was established at the 20th JECFA (1976).

SYNONYMS Pimaricin; INS No. 235

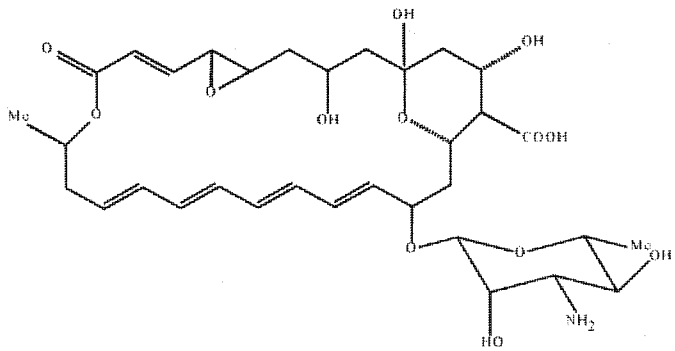
DEFINITION A fungicidal antimycotic of the polyene macrolide group. It is produced by several species of *Streptomyces*. The commercial product may contain up to three moles of water.

Chemical names A stereoisomer of 22-(3-Amino-3,6-dideoxy-β-D-mannopyranosyloxy)-1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28-trioxatricyclo[22.3.1.0^{5,7}]octacosa-8,14,16,18,20-pentaene-25-carboxylic acid

C.A.S. number 7681-93-8

Chemical formula $C_{33}H_{47}NO_{13}$

Structural formula



Formula weight 665.74

Assay Not less than 95.0% calculated on the dried basis

DESCRIPTION White to creamy-white, almost odourless, crystalline powder

FUNCTIONAL USES Fungicidal preservative

CHARACTERISTICS

IDENTIFICATION

Solubility (FNP 5) Practically insoluble in water, in lipid and in mineral oils; slightly soluble in methanol; soluble in glacial acetic acid and dimethylformamide.

Colour reaction On adding a few crystals of the sample, on a spot plate, to a drop of
- concentrated hydrochloric acid, a blue colour develops;
- concentrated phosphoric acid, a green colour develops, which changes into pale-red after a few minutes

Infrared absorption The infrared spectrum of a potassium bromide dispersion of the sample corresponds with the reference infrared spectrum in Appendix A.

Ultraviolet absorption	A solution of 5mg/l of the sample in 0.1% glacial acetic acid in methanol has absorption maxima at about 290, 303 and 318 nm, a shoulder at about 280 nm and exhibits minima at about 250, 295.5 and 311 nm. See Appendix B.
PURITY	
Loss on drying (FNP 5)	Not more than 8.0% (60°, over P_2O_5, pressure less than 5 mm Hg)
Specific rotation (FNP 5)	$[\alpha]_D^{20}$: + 250° to + 295° (1% w/v solution in glacial acetic acid)
pH (FNP 5)	5.0 - 7.5 (1.0% w/v suspension in demineralised water)
Sulfated ash (FNP 5)	Not more than 0.5% Test 2 g of the sample (Method I)
Lead (FNP 5)	Not more than 2 mg/kg Determine using an atomic absorption technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the method described in FNP 5,"Instrumental Methods"

METHOD OF ASSAY <u>High Performance Liquid Chromatography</u>

(Note: Throughout this *Assay*, protect from direct light all solutions containing natamycin)

Mobile phase: Dissolve 3.0 g of ammonium acetate and 1.0 g of ammonium chloride in 760 ml of water, and mix. Add 5.0 ml of tetrahydrofuran and 240 ml of acetonitrile, mix, and filter through a 0.5-µm or finer porosity filter. Make adjustments if necessary to meet the system suitability requirements.

Standard preparation: Transfer about 20 mg of natamycin Reference Standard, accurately weighed, to a 100-ml volumetric flask. Add 5.0 ml of tetrahydrofuran, and sonicate for 10 min. Add 60 ml of methanol, and swirl to dissolve. Add 25 ml of water, and mix. Allow to cool to room temperature. Dilute with water to volume, mix, and filter through a membrane filter of 5-µm or finer porosity.

Resolution solution: To prepare a mixture of natamycin and natamycin methyl ester, dissolve 20 mg of natamycin in a mixture of 99 ml of methanol and 1 ml of 0.1 N hydrochloric acid, and allow to stand for 2 h.

Note: use this solution within 1 h.

Assay preparation: Transfer about 20 mg of natamycin, accurately weighed, to a 100-ml volumetric flask. Proceed as directed under "Standard preparation", beginning with "add 5.0 ml of tetrahydrofuran...."

Chromatographic system (see High-Performance Liquid Chromatography, FNP 5):

Use a high performance liquid chromatograph equipped with an ultraviolet detector measuring at 303 nm and a 4.6-mm x 25-cm column packed with octadecylsilanized silica (Supelcosil LC 18 or equivalent). The flow rate is about 3 ml/min. Chromatograph the "standard preparation", and record the peak responses. The column efficiency should not be less than 3000 theoretical plates and the tailing factor should be between 0.8 and 1.3. The relative standard deviation for three replicate injections of the standard preparation is not more than 1.0 %.

Chromatograph the "resolution solution". The relative retention times are about 0.7 for Natamycin and 1.0 for its methyl ester. The resolution (R) between Natamycin and its methyl ester is not less than 2.5:

$$R = 2(t_2-t_1)/(W_2+W_1)$$

where:

t_2 and t_1 are the retention times of natamycin methyl ester and natamycin, respectively

W_2 and W_1 are the width of the corresponding peaks at their bases extrapolated to the baseline.

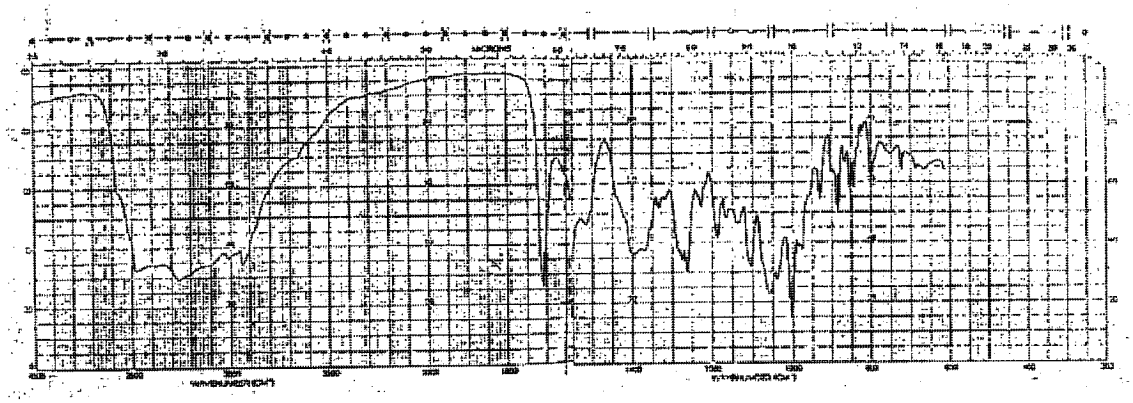
Procedure: Separately inject about 20 µl for each of the "standard preparation" and the "assay preparation" into the chromatograph, and record the peak areas of the major peaks. Calculate the percentage of Natamycin in the portion taken by the formula:

$$0.1(W_S P_S / W_U)(r_U / r_S)$$

in which W_S is the weight, in mg, of Natamycin Reference Standard taken to prepare the "Standard preparation"; P_S is the stated content, in µg/ml, of Natamycin Reference Standard; W_U is the weight, in mg, of Natamycin taken to prepare the "Assay preparation"; and r_U and r_S are the peak area responses obtained with the "Assay preparation" and the "Standard preparation", respectively.

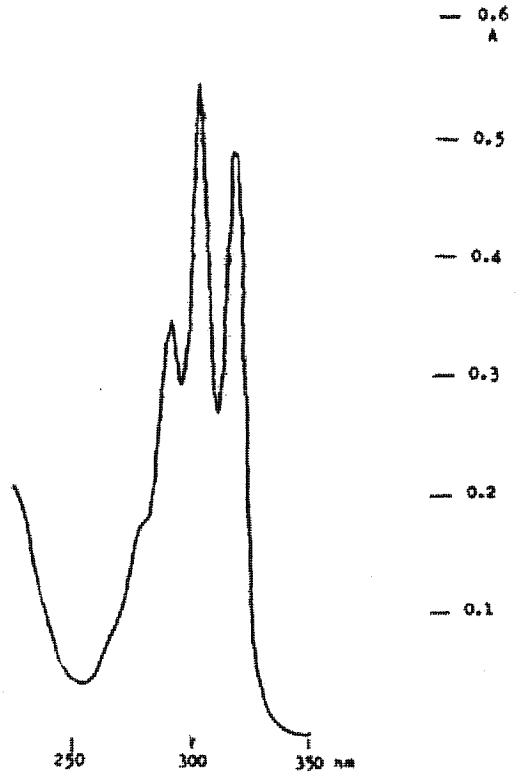
Appendix A

Reference Infrared Spectrum (1.3 mg solid in 300 mg potassium bromide) for natamycin



Appendix B

Ultraviolet absorption spectrum of natamycin
Concentration: 5 µg/ml in methanol/glacial acetic acid mixture



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Support for this project was provided by U.S. Food and Drug Administration Contract No. 223-99-2321. The views presented in this report are those of the Institute of Medicine Committee on Food Chemicals Codex and are not necessarily those of the funding agency.

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Library of Congress Cataloging-in-Publication Data

Food chemicals codex / Committee on Food Chemicals Codex, Food and Nutrition Board, Institute of Medicine.-- 5th ed.
p. cm.
"Effective January 1, 2004."
Includes index.
ISBN 0-309-08866-6 (hardback)

1. Food additives--Standards--United States. 2. Food additives--Analysis. I. Institute of Medicine (U.S.). Committee on Food Chemicals Codex.
TP455.F66 2003
664'.06'021873--dc21 2003010423

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The serpent has been a symbol of long life, healing, and knowledge among almost all cultures and religions since the beginning of recorded history. The serpent adopted as a logotype by the Institute of Medicine is a relief carving from ancient Greece, now held by the Staatliche Museen in Berlin.

of a typical spectrum as shown in the section on *Infrared Spectra* using the same test conditions as specified therein.
Acid Value Between 2 and 13.
Angular Rotation Between −60° and −98°.
Refractive Index Between 1.519 and 1.528 at 20°.
Saponification Value Between 9 and 35.
Solubility in Alcohol Passes test.
Specific Gravity Between 0.985 and 1.014.

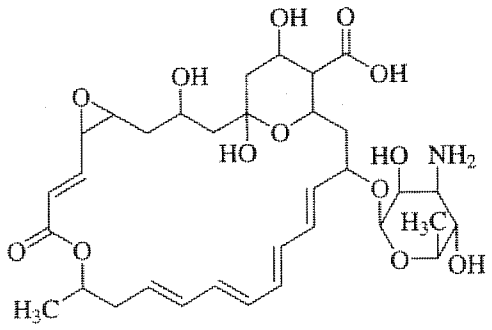
TESTS

Acid Value Determine as directed under *Acid Value*, Appendix VI.
Angular Rotation Determine as directed under *Optical (Specific) Rotation*, Appendix IIB, using a 100-mm tube.
Refractive Index Determine as directed under *Refractive Index*, Appendix IIB, using an Abbé or other refractometer of equal or greater accuracy.
Saponification Value Determine as directed under *Saponification Value*, Appendix VII, using about 5 g of sample, accurately weighed.
Solubility in Alcohol Determine as directed under *Solubility in Alcohol*, Appendix VI. One milliliter dissolves in 10 mL of 90% alcohol, occasionally with opalescence or turbidity.
Specific Gravity Determine by any reliable method (see *General Provisions*).

Packaging and Storage Store in a cool place protected from light in full, tight containers that are made from steel or aluminum and that are suitably lined.

Natamycin

Pimaricin



$C_{33}H_{47}NO_{13}$ Formula wt 665.73

INS: 235 CAS: [7681-93-8]

DESCRIPTION

Natamycin occurs as an off white to cream colored powder that may contain up to 3 moles of water. It melts with decomposition at about 280°. It is practically insoluble in water, slightly soluble in methanol, and soluble in glacial acetic acid and in dimethylformamide.

Function Antimycotic.

REQUIREMENTS

Identification Transfer 50 mg of sample, accurately weighed, into a 200-mL volumetric flask, add 5.0 mL of water, and moisten the sample. Add 100 mL of a 1:1000 solution of glacial acetic acid in methanol, and shake by mechanical means in the dark until dissolved. Dilute to volume with the acetic acid–methanol solution, and mix. Transfer 2.0 mL of this solution into a 100-mL volumetric flask, dilute to volume with the acetic acid–methanol solution, and mix. The ultraviolet absorption spectrum of the solution so obtained exhibits maxima and minima at the same wavelengths as those of a similar solution of USP Natamycin Reference Standard, concomitantly measured.
Assay Not less than 97.0% and not more than 102.0% of $C_{33}H_{47}NO_{13}$, calculated on the anhydrous basis.
Lead Not more than 2 mg/kg.
Optical (Specific) Rotation $[\alpha]_D^{20°}$: Between +276° and +280°.
pH Between 5.0 and 7.5.
Water Between 6.0% and 9.0%.

TESTS

Assay (**Note**: Throughout this *Assay*, protect all solutions containing Natamycin from direct light.)

Mobile Phase Dissolve 3.0 g of ammonium acetate and 1.0 g of ammonium chloride in 760 mL of water, and mix. Add 5.0 mL of tetrahydrofuran and 240 mL of acetonitrile, mix, and filter through a 0.5-µm or finer porosity filter. If necessary, make adjustments to meet the system suitability requirements.

Standard Preparation Transfer about 20 mg of USP Natamycin Reference Standard, accurately weighed, into a 100-mL volumetric flask. Add 5.0 mL of tetrahydrofuran, and sonicate for 10 min. Add 60 mL of methanol, and swirl to dissolve. Add 25 mL of water, and mix. Allow to cool to room temperature. Dilute to volume with water, mix, and filter through a membrane filter of 5-µm or finer porosity.

Resolution Solution Dissolve 20 mg of sample in 99:1 (v/v) methanol:0.1 *N* hydrochloric acid mixture, and allow to stand for 2 h.

Note: Use this solution within 1 h.

Assay Preparation Transfer about 20 mg of sample, accurately weighed, into a 100-mL volumetric flask. Proceed as directed under *Standard Preparation*, beginning with "add 5.0 mL of tetrahydrofuran. . . ."

Chromatographic System (See *Chromatography*, Appendix IIA.) Use a high-performance liquid chromatograph equipped with an ultraviolet detector measuring at 303 nm and a 25-cm × 4.6-mm (id) column, or equivalent, packed with octadecylsilanized silica (Supelcosil LC 18, or equiva-

lent). The flow rate is about 3 mL/min. Chromatograph the *Standard Preparation*, and record the peak responses. The column efficiency is not less than 3000 theoretical plates, the tailing factor is between 0.8 and 1.3, and the relative standard deviation for three replicate injections is not more than 1.0%. Chromatograph the *Resolution Solution*. The resolution between the sample and its methyl ester is not less than 2.5. The relative retention times are about 0.7 for Natamycin and 1.0 for its methyl ester.

Procedure Separately inject about 20 µL each of the *Standard Preparation* and the *Assay Preparation* into the chromatograph, and record the peak areas of the major peaks. Calculate the percentage of Natamycin in the portion of sample taken by the formula

$$0.1(W_S P_S/W_U)(r_U/r_S),$$

in which W_S is the weight, in milligrams, of USP Natamycin Reference Standard taken to prepare the *Standard Preparation*; P_S is the stated content, in micrograms per milligram, of USP Natamycin Reference Standard; W_U is the weight, in milligrams, of sample taken to prepare the *Assay Preparation*; and r_U and r_S are the peak area responses obtained with the *Assay Preparation* and the *Standard Preparation*, respectively.

Lead Determine as directed in the *Flame Atomic Absorption Spectrophotometric Method* under *Lead Limit Test*, Appendix IIIB, using a 10-g sample.

Optical (Specific) Rotation Determine as directed under *Optical (Specific) Rotation*, Appendix IIB, using a solution containing 100 mg of sample in each 10 mL of glacial acetic acid.

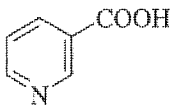
pH Determine as directed under *pH Determination*, Appendix IIB, using an aqueous suspension of sample containing 10 mg/mL.

Water Determine as directed under *Water Determination*, Appendix IIB.

Packaging and Storage Store in tight, light-resistant containers in a cool place.

Niacin

Nicotinic Acid; 3-Pyridinecarboxylic Acid



$C_6H_5NO_2$ Formula wt 123.11

CAS: [59-67-6]

DESCRIPTION

Niacin occurs as white or light yellow crystals or as a crystalline powder. One gram of sample dissolves in 60 mL of water. It is freely soluble in boiling water and in boiling alcohol and also in solutions of alkali hydroxides and carbonates. It is almost insoluble in ether.

Function Nutrient.

REQUIREMENTS

Identification

A. The infrared absorption spectrum of a mineral oil dispersion of the sample, previously dried at 105° for 1 h, exhibits maxima only at the same wavelengths as those of a similar preparation of USP Niacin Reference Standard.

B. Dissolve about 50 mg of sample in 20 mL of water, neutralize to litmus paper with 0.1 *N* sodium hydroxide, and add 3 mL of cupric sulfate TS. A blue precipitate gradually forms.

C. Determine the absorbance of a solution containing 20 µg of sample in each milliliter of water, in a 1-cm cell, at 237 nm and 262 nm, using water as the blank. The ratio A_{237}/A_{262} is between 0.35 and 0.39.

Assay Not less than 99.5% and not more than 101.0% of $C_6H_5NO_2$, calculated on the dried basis.

Loss on Drying Not more than 1.0%.

Melting Range Between 234° and 238°.

Residue on Ignition Not more than 0.1%.

TESTS

Assay Dissolve about 300 mg of sample, accurately weighed, in about 50 mL of water, add phenolphthalein TS, and titrate with 0.1 *N* sodium hydroxide. Perform a blank determination (see *General Provisions*). Each milliliter of 0.1 *N* sodium hydroxide is equivalent to 12.31 mg of $C_6H_5NO_2$.

Loss on Drying Determine as directed under *Loss on Drying*, Appendix IIC, drying the sample at 105° for 1 h.

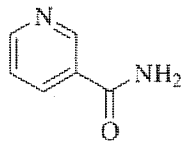
Melting Range Determine as directed under *Melting Range or Temperature*, Appendix IIB.

Residue on Ignition Determine as directed under *Residue on Ignition*, igniting a 1-g sample.

Packaging and Storage Store in well-closed containers.

Niacinamide

Nicotinamide



$C_6H_6N_2O$ Formula wt 122.13

CAS: [98-92-0]

DESCRIPTION

Niacinamide occurs as a white, crystalline powder. One gram dissolves in about 1 mL of water, in about 1.5 mL of alcohol,

Fungal Sterols and the Mode of Action of the Polyene Antibiotics

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I. Introduction

When I started work on mycological topics in 1970, I was given the brief to investigate the mode of action of polyene antibiotics and resistance to them among pathogenic yeasts. After a few weeks in the library, I came to two conclusions: first, that the mode of action was already known; and second, that resistance did not occur (at least in nature). These same conclusions could very well be reached by anyone else who studied the question for a short time only, and they could be excused for turning to some apparently more fruitful field. However, on more mature reflection, it became apparent that my first conclusion was untrue, and my second (apparently true) of potentially staggering import. Further reading and subsequent and concurrent experimental work showed me that a full study of the mode of action of the polyene antibiotics leads to a biophysical world entirely removed from conventional medical mycology. Experiments designed to probe the mode of action

of polyenes have revealed, often for the first time, vital information on basic membrane function and structure, and have thus paved the way for fundamental discoveries in this most crucial field. In much the same way, studies on antibiotics that inhibit protein synthesis have vastly increased knowledge on protein synthesis in general. It is gratifying for workers in mycological disciplines, who are often regarded as the Cinderellas of Microbiology, that such experiments are the result of their early and continuing interest in these antibiotics.

Problems of drug resistance have been a continual worry to the medical bacteriologist since antimicrobial therapy started in earnest in the 1930's with the introduction of the sulfonamides. Mycologists have been spared this problem, and an explanation of why this might be so is of the greatest interest and importance. A short analysis of this problem is presented toward the end of the present review.

Various aspects of sterols in relation to polyene activity are considered here; first, the distribution, nature, and function of sterols is discussed; then their role in the mode of action of the polyenes; and finally the part they have to play in resistance mechanisms in yeasts.

II. Sterols in the Microbial Kingdom

A. Bacteria

Sterols are notably absent from bacteria (Asselineau and Lederer, 1960) with the exception of the nitrogen-fixing *Azotobacter chroococcum* which has a sterol content of 10 mg/100 gm. Schubert *et al.* (1964, 1968) were able to isolate minute amounts of cholesterol, campesterol (a homolog of cholesterol), β-sitosterol, and stigmasterol from *Escherichia coli*, but the total sterol yield was only 400 μg/100 gm. Sterols are important in the classification of Mycoplasmataceae, the two genera *Mycoplasma* and *Acholeplasma* being distinguished by the ability of the latter to grow in the absence of added cholesterol. However, *A. laidawii* is capable of incorporating exogenous sterol, a phenomenon which has helped to adduce important evidence as to the mode of action of the polyene antibiotics (see Section V) (Feingold, 1965; Weber and Kinsky, 1965).

B. Yeasts and Fungi

The existence of sterols in the fungal kingdom has been known for many years. The most widely distributed sterol, ergosterol, was so named after the mold ergot, from which it was first isolated more than 90 years ago (Tanret, 1879); before the end of the 19th century, ergosterol had also been found in *Penicillium* and *Mucor*. As ergosterol is a commercially important natural product, being a precursor of vitamin D, much attention has been paid to finding the best source of this compound. Dulaney *et al.* (1954) investigated 558 yeast cultures, and found that *Saccharomyces carlsbergensis*, *S. cerevisiae*, and *S. oviformis* contained at least 2% by weight of sterols; brewer's yeast and ale yeasts were the most consistent in this respect. Appleton *et al.* (1955) screened more than 50 strains from a total of more than 30 genera of molds and yeasts and found a low sterol content (<1%) in all except *Neurospora*, *Penicillium*, *Zygosaccharomyces*, and *Saccharomyces*. Species of the latter genus then gave the most consistently high values.

A feature which occurs throughout the work on the sterol content of yeasts is the wide quantitative variations obtained, not merely, as might be expected, from species to species and from strain to strain reported from different laboratories, but also on occasions in the same strain grown at different times under apparently identical conditions (see El-Refai and El-Kady, 1968a,b). Strains of *Saccharomyces* containing up to 5% of their dry weight of sterol, much of it ergosterol, are not uncommon.

Because of its extremely wide use commercially in the baking and brewing trades, *Saccharomyces* has been the most widely studied yeast in respect to its production of sterols. A detailed chemical study of the sterols of *S. cerevisiae* has been recently published by Barton *et al.* (1972); in addition to the major components ergosterol (the largest single component) and ergo-5,7,22,24(28)-tetraen-3-β-ol (24,28 dehydroergosterol), they found at least 12 minor components, the chief of which was fecosterol. Hunter and Rose (1972) similarly found ergosterol and 24(28)-dehydroergosterol in approximately equal amounts as major components, with zymosterol (<5% of the total sterol), fecosterol, and an unidentified C_{30} diunsaturated sterol as minor components. The latter authors also found that most of sterol fraction (80–85%) was composed of esterified material. Adams and Parks (1967) have found in *S. cerevisiae* an ergosterol fraction that resists extraction by conventional means (alkaline hydrolysis followed by extraction into an organic solvent); this fraction requires acid treatment before being extractable.

Under the actual conditions used for commercial production of yeast (e.g., in the manufacture of baker's yeast) ergosterol accounts for as much as 80% of the total sterol. Under laboratory conditions, however, the content of 24(28)-dehydroergosterol may come to as much as 74% (Breivik and Owades, 1957).

III. Factors Affecting the Sterol Content of Fungal Cells

A. Aerobiosis

It is now clearly established that sterol metabolism is dependent upon oxygen availability. The reason for this seems to be that the conversion of squalene to sterols requires oxygen; both the oxidative cyclization of squalene to lanosterol and the conversion of the latter to cholesterol require molecular oxygen. Thus, squalene can be shown to accumulate in yeast cells grown anaerobically, and is converted to sterol when aerobiosis is resumed. *Saccharomyces cerevisiae* requires a suitable sterol in order to grow anaerobically; a very wide range of sterols can substitute for the naturally occurring ergosterol. Requirements for such sterols are that the molecule must be planar, have a long C-17 alkyl side chain and a hydroxyl group (Proudlock *et al.*, 1968). By no means all yeasts, however, are incapable of growing anaerobically; *Schizosaccharomyces japonicus* grows very well anaerobically unsupplemented, but anaerobic cells are virtually devoid of ergosterol (Bulder, 1971). A similar situation obtains for *Mucor genevensis*, in which squalene accumulates anaerobically (Gordon *et al.*, 1971).

B. Growth and Morphological Phase

There are several instances of reports of alterations in sterol content, both qualitative and quantitative, occurring during the growth cycle of various yeasts and fungi. In *M. genevensis*, Gordon *et al.* (1971) found that ergosterol comprised about 80% of the total sterol content during the exponential phase of growth, and that the stigmasterol content rose to 40% in the stationary phase. Capek and Simek (1972) found that the sterol content (mainly ergosterol) of the dermatophytes *Microsporum gypseum* and *Trichophyton mentagrophytes* increased as the cultures aged. Bianchi (1967) reported a higher proportion of the lipid fraction from juvenile (logarithmic phase) than from mature yeast cells of *Candida albicans* was composed of sterol and sterol esters; four sterols were separated by thin-layer chromatography, but only cholesterol was tentatively identified. Between them, these two fractions always outweighed the other lipid components of the cell wall (triglycerides, free fatty acids, and phospholipids). The same author has also compared the sterol contents of *C. albicans* cells in the yeast form with those of the filamentous phase; although the total sterol content was greater when the organism was in the mycelial form, the proportions of the various lipid fractions were markedly different. Anaerobiosis, which markedly decreases the ergosterol content of *M. genevensis* cells (see

previous section), is one of the factors which predisposes toward the yeastlike development of this species (Gordon *et al.*, 1971). However, it is the opinion of these workers that this decrease in sterol, and the concomitant alteration in fatty acid content, is an effect rather than the cause of the morphological change.

C. Medium Composition

Inasmuch as a certain amount of dimorphism may be induced by alteration of medium [e.g., use of a liquid medium to obtain the filamentous form of *C. albicans*: (Bianchi, 1967; Hamilton-Miller, 1972a)], the physical composition of medium may be said to affect the sterol content of yeast cells, but more attention in this respect has been paid to the affects of different sources of carbon and nitrogen. Thus, various authors have claimed the following carbon sources to be the most favorable for the production of sterols by yeast: maltose, glucose, sucrose, and pyruvate; similarly, many nitrogen sources have also been investigated, NH_4^+ being favored by some, peptone by others (see El-Refai and El-Kady, 1968a,b). Concentrations of nutriments may also be crucial. The situation is complicated not only by the multiplicity of sources of nitrogen and carbon available—giving the possibility of virtually limitless permutations and combinations to be tested—and the differing strains and cultural conditions, but also by the fact that ergosterol is a valuable commercial product and hence what has often been sought, understandably, is maximum yields of sterol per volume of culture rather than maximum yield per gram dry weight of cells. It is thus clear that the true importance of the nature and concentration of nutriments and the production of sterols by yeast and fungi is very difficult to assess.

Dulaney (1957) has shown how the ergosterol content of *S. cerevisiae* may be raised to as much as 10%, w/w; this was done by growing the organisms to maximum cell yield, harvesting and resuspending in fresh medium for a "refermentation" phase during which ergosterol enrichment occurs.

D. Temperature

The true effect of alterations in temperature on the cellular processes in microorganisms is very difficult to assess except by the use of continuous culture. In batch culture, changing the incubation temperature is accompanied by an alteration in growth rate, so that any overall effects may be the result of either the altered growth rate, the altered temperature, or a combination of the two. It should be noted that the above

argument also applies, although perhaps not to such an extent, to alterations in nutriments. Hence, from the strictly scientific point of view, one should reject virtually all the studies made on the effects of altering the incubation temperature on sterol production, while bearing in mind that, from an applied point of view (obtaining the maximum overall yields of sterol), such studies are of obvious importance.

The problem of dissociating changes in growth rate from alterations in temperature is solved by the use of continuous culture, using a fermenter—a piece of apparatus regrettably much more familiar to scientists in industry than to those in universities. By this technique, the growth rate (dilution rate) of the culture can be adjusted independently of the temperature, so that a constant growth rate can be achieved over a range of temperatures and, conversely, growth rates can be varied at any one desired temperature. An excellent illustration of the application of this technique to the current problem is the work of Hunter and Rose (1972). They found that, although in batch culture lowering the growth temperature had little or no effect on the content of sterols and sterol esters, lowering the growth rate at fixed temperature caused an increase in the amount of sterol esters, whereas decreasing the incubation temperature while maintaining the same growth rate brought about a decrease in total and esterified sterols. Parks and Starr (1963) found that growing *S. cerevisiae* at 42°C caused a sharp reduction in sterol content.

IV. Physiological Role of Sterols

Sterols seem to have only one major function in the majority of fungi, a structural role in maintaining membrane integrity and permeability. Studies at the molecular level have shown that sterols have a marked condensing effect upon experimental membranes composed of phospholipid, reducing their permeability to polyol compounds, such as glucose, glycerol, and erythritol, and to water (see Demel *et al.*, 1972b). Incorporation of cholesterol into the membrane of cells which usually lack sterols was found to decrease the leakiness of the cells (Child *et al.*, 1969).

In one group of fungi, however, the oomycetes, sterols do have an additional role. Members of this group, typified by the genera *Pythium* and *Phytophthora*, do not contain sterols under normal cultural conditions (Schlösser *et al.*, 1969); vegetative growth can occur in the absence of sterols, but they are required for sexual reproduction and the formation of normal sporangia and zoospores. Hendrix (1964) found that ergosterol, stigmasterol, phytosterol, and cholesterol allowed the formation of oospores, and that the latter two compounds also increased growth rates. Divalent cations (Lenney and Klemmer, 1966) are additionally

required for sexual maturation. As they contain no sterols, oomycetes are resistant to the antifungal action of polyenes, but Defago *et al.* (1969) reported that polyenes in high concentrations (0.5–1 mg/ml) induced oogenesis in *Pythium*, and that this effect was reversed by cholesterol. Sterols can be incorporated into the cell membranes of *Pythium*, and the resulting cells are sensitive to polyenes (Schlösser and Gottlieb, 1966).

The general necessity for sterol biosynthesis to fungal growth is shown by the fact that, when *S. cerevisiae* is grown anaerobically to a state of total ergosterol depletion, no intracellular membranous organelles can be seen (Morpurgo *et al.*, 1964). It is of interest, too, that certain drugs known to inhibit sterol biosynthesis in animals also possess some antifungal activity (Elliott, 1969; Hamilton-Miller, 1973b).

It is interesting to speculate whether the fact that oomycetes lack sterol confers any ecological advantage on them over other fungal types. It could be argued that the oomycetes will be at a disadvantage due to a slower growth rate and their inability to reproduce sexually in the absence of sterols, but that they will enjoy a telling advantage in the presence of *Streptomyces* spp., which produce polyene antibiotics. While there is no doubt that organisms capable of producing polyenes are widespread in the soil (see Hamilton-Miller, 1973a), the question of whether antibiotics are actually produced under natural conditions is still far from resolved (see Hill, 1972).

V. Sterols and the Mode of Action of Polyenes

It is now firmly established that the presence of sterols is a prerequisite for sensitivity to polyene antibiotics. The evidence for this has been reviewed frequently, most recently by the present author (Hamilton-Miller, 1973a), and it would not serve any useful purpose in the present context to repeat it. At the multicellular level, the action of polyenes on a wide variety of organisms has been studied: mycoplasmas, yeasts and filamentous fungi, chloroplasts, protozoa, flatworms, flies and moths, amphibian tissues (muscle fibers, skin, and bladder), chick intestine, and various mammalian tissues. It is at the fractionated-cell and molecular levels, however, that the greatest strides have been made recently toward an understanding not only of the intimate details of the nature of the interaction of polyene and sterol, but also of membrane function as a whole. It is the results of this type of experiment that will be reviewed here.

These studies, most of which emanate from the Utrecht Laboratory of L. L. M. van Deenen, are chiefly biophysical in nature, and interpretation of the findings made may offer some difficulty to the reader who

is more usually occupied with medical or microbiological matters. It will therefore be appreciated that the following summary is much condensed and simplified.

Various systems, at different levels of organization, have been used in the studies, and the order of review (in ascending order of complexity), although not chronologically correct, appears apt.

It is noticeable that the majority of these studies involve filipin, which has been conclusively shown to be much the most active of the common polyene antibiotics as far as membrane activity is concerned in *in vitro* experiments. Much of the work was done before it was reported (Bergy and Eble, 1968) that filipin was a highly heterogeneous mixture composed of several compounds with similar molecular structures but distinct properties. The main component of the complex, filipin III, comprises about 53% of the total. Some of the latter work was done using filipin III, but some workers have continued to use the complex. Thus, all results with "filipin" may not be strictly comparable. Some pertinent differences between the biological properties of the various filipin fractions are discussed by Sessa and Weissmann (1968b).

A. Studies in Solution

The earliest work carried out on the problem of sterol–polyene interaction was based on the observation of changes in absorption spectra that occurred when sterols were added to aqueous preparations of polyenes. Thus, Lampen *et al.* (1960) and Gottlieb *et al.* (1961) deduced that cholesterol and ergosterol combined with nystatin, antimycoin, and filipin, greatest spectral changes occurring with filipin. Norman *et al.* (1972a,b) have recently repeated these experiments, and found that filipin, lucensomycin (Etruscomycin), amphotericin B, nystatin, and pimaricin interact with cholesterol, the magnitude of the interaction being in the order given. The precise chemical nature of the sterol greatly affected the degree of action, an optimum effect being obtained with compounds containing a cholestane ring and a Δ-22 double bond. Thus, it appears that the interaction is hydrophobic. Sterol esters were found to be of low activity in this respect.

Polyenes are known to form suspensions of micelles rather than true solutions in aqueous solvents; e.g., Lampen *et al.* (1959) and Norman *et al.* (1972b) found that E_m for filipin was consequently lower in water than in methanol, and the Beer-Lambert law is not obeyed. Thus, experimental results based on spectrophotometric measurements are open to the criticism that changes in spectra may be due merely to the fact that polyenes are less soluble in dilute aqueous solutions of sterols than in pure water. However, change in micellar size may be argued to be a direct effect, and, if specific for a certain sterol, is clearly of potential physiological significance; similarly, where qualitative changes occur the only explanation is interaction.

These hypothetical difficulties may be overcome, however, by measuring a parameter which is (unlike extinction coefficient) not concentration dependent. Such a parameter is partial quantum efficiency, measured spectrofluorimetrically. As a criterion for fluorescence appears to be an uncharged molecule, original experiments were carried out using the nonionized pentaenes filipin and lagosin (Norman *et al.*, 1972a; Schroeder *et al.*, 1971; Bittman and Fischkoff, 1972). The combination of sterols with these compounds was found in general to decrease fluorescence and partial quantum efficiency, thereby offering direct evidence for the interaction of such compounds as cholesterol, β-stigmasterol, ergosterol, and β-cholestenol with the antibiotics. The intrinsic fluorescence of amphoteric polyenes, such as nystatin, amphotericin B, pimaricin and its homolog lucensomycin, is extremely low. However, it was found that fluorescence increased (in some cases substantially) when the antibiotics combine with cholesterol and other β-OH sterols (Crifo *et al.*, 1971; Strom *et al.*, 1973a,b; Schroeder *et al.*, 1972; Norman *et al.*, 1972a), thus again providing evidence in favor of the sterol-polyene interaction hypothesis. By means of another fluorimetric parameter, fluorescence polarization, Bittman and Fischkoff (1972) and Strom *et al.* (1973a), were able to show that when polyene antibiotics combine with the sterols the rigidity of the polyene molecule increases. Schroeder *et al.* (1972) reported that the stoichiometry of the filipin/cholesterol interaction was 1:1; they also found that filipin may, after recrystallization from organic solvents, exist in forms that react with sterols only after some considerable period of time in aqueous solution.

B. Differential Calorimetry

Norman *et al.* (1973a,b) have investigated the effects of the incorporation of cholesterol into, and the subsequent addition of polyene antibiotics to, dispersions of lecithin. The lecithins used in these cases, 1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine and 1,2-dielaidoyl-*sn*-glycero-3-phosphorylcholine, each display a phase change at about 13°C which is characterized by a sharp absorption of heat as the lecithin changes from a crystalline to a liquid crystalline structure. The heat is required to melt the hydrocarbon chains. When sterols are incorporated into the lecithin dispersion, the phase change becomes less endothermic (de Kruyff *et al.*, 1972); it is interesting that cholesterol had a much greater effect on the energy content of this phase transition than did epicholesterol or sterols lacking a 3-OH group or a 17 side chain.

Demel *et al.* (1972b) further showed that such sterols—which have also been shown to be those that interact the best with the polyenes—also have the greatest effect on the permeability of liposomes (see next paragraph).

This system clearly offers scope for the investigation of the interaction of polyenes with sterols, as the combination of the two will result in a greater or lesser negation of the effect of the sterol on the heat content of the phase transition of lecithin. By differential scanning calorimetry, Norman *et al.* (1972a,b) have shown that filipin, lucensomycin, pimaricin, nystatin, and amphotericin B all interact with cholesterol, and that the antibiotics by themselves had no effect on the phase transition of lecithin. Under the conditions used (viz. highly concentrated solutions), the stoichiometry of the interaction varied from 1.2 to 7.9 molecules of cholesterol complexed per polyene molecule (Norman *et al.*, 1972a).

C. Experiments with Monolayers

The interaction of polyenes and sterols can also be quantitated by measuring changes in surface pressure occurring when polyenes (which behave as soluble compounds under the experimental conditions obtaining) are injected beneath monolayers of lipids or lipid mixtures at varying initial surface pressures. These changes are brought about because interactions alter the spatial orientation of the lipid molecules with respect to the air–water interface; Demel *et al.* (1967) discovered that cholesterol had a condensing effect on monolayers of certain lecithins.

Demel *et al.* (1965), using this technique, found that nystatin was less active than filipin, which reacted more strongly with cholesterol than with ergosterol, and virtually not at all with phospholipids. Monolayers prepared from material extracted from bacterial protoplast membranes were not affected by filipin, in contrast to similar monolayers made from erythrocyte membranes. In an extension of this study, Demel *et al.* (1968a) found that lucensomycin, amphotericin B, and pimaricin behaved like filipin and nystatin; they also showed that the activity of filipin was decreased if the phospholipid:cholesterol ratio of the monolayer was increased. Filipin at high concentrations interfered with monolayers of lecithin alone, but this interaction, also reported by Sessa and Weissman (1967) using a different system (see below), was not considered to be of physiological significance.

The facts that (i) monolayers of cholesteryl acetate were virtually unaffected by filipin, (ii) filipin at low concentrations reacted with monolayers of cetyl alcohol ($CH_3 \cdot (CH_2)_{14} \cdot CH_2OH$), and (iii) 5 *M* urea, which is known to disrupt hydrogen bonds, diminishes the effect of filipin on cholesterol monolayers, suggested that, in this heterogeneous system as opposed to in solution (see above), hydrophilic bonding may be important in the interaction of sterols with polyenes (see Demel *et al.*, 1972a).

D. Experiments with Bilayers

Van Zutphen *et al.* (1966) were the first to exploit the fact that stable bilayer lipid films possess certain dimensional, mechanical, electrical, and permeability properties, which are characteristic of the membranes of living cells, in order to investigate the sterol–polyene interaction. Such bilayer films had been developed a few years previously; they are formed from solutions of lipids across a small hole in a membrane, contained in a small cell. The cell is then filled with an appropriate solution, such as 0.1 *M* NaCl, and the passage of ions across the film can be studied by introducing the marker on one side of the membrane, and measuring its concentration subsequently on the other side. In their early work, van Zutphen *et al.* (1966) in fact merely studied the macroscopic stability of bilayer films in the presence of filipin; they found that lecithin films were stable for more than 1 hour in the presence of c. 4×10^{-5} *M* filipin and nystatin, while bilayers comprised of equal parts of cholesterol and lecithin disrupted after 5 and 25 minutes, respectively, when these polyenes were added. Films made of 10 parts of lecithin to 1 of cholesterol were more stable. The concentration of polyene used in these experiments was about 10 times that required to prevent the growth of *Candida albicans* (see Hamilton-Miller, 1973a), and some 5000 times greater than that which had an effect on lipid monolayers (see preceding section); this discrepancy is doubtless due to the rather crude criterion of activity. In a later, more extended study, van Zutphen *et al.* (1971) investigated the effects of filipin, nystatin, lucensomycin, pimaricin, and amphotericin B on bilayers of various compositions. In addition to showing the disrupting effects of the antibiotics, which roughly paralleled their physiological activities in terms of hemolytic abilities (Kinsky, 1963), it was found that the electrical dc resistance of lecithin/cholesterol black films could also be markedly decreased (up to 1000-fold) by polyene treatment. Bilayers exposed to filipin (components II, III, or the complex) became selectively permeable to Ca^{2+} ions, while those treated with nystatin or amphotericin B became, under appropriate conditions, anion selective, although being of increased permeability to cations as well. High concentrations of filipin (0.1 m*M*) or nystatin (10 m*M*) also slightly lowered the resistance of, and disrupted, bilayers composed of lecithin alone. Lippe (1968) found that the permeability of mixed bilayers to an uncharged molecule, thiourea, was increased, while the film remained completely stable, 10-fold and 4-fold by 1 μM and 0.2 μM amphotericin B, respectively [cf.

antiyeast activity of amphotericin B c. 0.5 μM (Hamilton-Miller, 1973a)]. Andreoli and Monahan (1968) and Andreoli *et al.* (1969) used bilayers made from sheep erythrocyte lipids, and their observations on the action of nystatin and amphotericin B on such films are in general agreement with those made by van Zutphen *et al.* (1966, 1971)—thus, electrical resistance was decreased one millionfold, permeability to Cl^- and $CH_3 \cdot COO^-$ was increased greatly and to Na^+, K^+, and Li^+ to a lesser extent. On the basis of their findings, they calculated that amphotericin B causes the formation in this type of membrane of pores of equivalent radii 0.7 to 1 nm. The results of Finkelstein and Cass (1968) are also generally in line with those listed above; they made the further observations that, in nystatin-treated bilayers, membrane conductance decreased extraordinarily with increasing temperature, the process having a Q_{10} of about 1000, and also that the conductance was proportional to the tenth power of the nystatin concentration. Both the latter authors and Andreoli *et al.* (1968, 1969), however, were unable to detect changes in ion permeability or electrical properties after filipin treatment, although bilayers were readily lysed by this compound.

Cass *et al.* (1970) and Holz and Finkelstein (1970) investigated the changes in permeability to ions, water, and nonelectrolytes in lipid membranes induced by amphotericin B and nystatin, and concluded that the behavior of such systems was consistent with the formation of nonstatic pores, of diameter about 0.8 nm, which coalesce and reform continuously. The permeability to water and small hydrophilic solutes of polyene-treated bilayers approximated closely to that of normal erythrocytes.

E. Liposomes

If lecithin is allowed to swell in water, or if solutions (in solvents such as decane or chloroform) of lecithin, dicetylphosphate, and, if desired, sterols are mixed, allowed to evaporate and then suspended in an aqueous menstruum, the result is the formation of spherical structures consisting of several bimolecular layers of mixed lipids separated by aqueous compartments, varying in diameter (according to the method of homogenization) from 0.5 to 50 μm (Sessa and Weissmann, 1968a). Such bodies, now generally called liposomes, are very useful as model membranes, and have been widely utilized for the study of the interaction of polyenes and sterols. Liposomes are, by nature, much less permeable to cations than to anions, and are liable to osmotic swelling, as water is freely diffusible through the membranes. The permeability of lecithin liposomes is reduced by the incorporation of cholesterol, but this sterol effect is markedly dependent upon the chemical nature of

the lecithin (Demel *et al.*, 1968b; de Gier *et al.*, 1968). Cholesterol seems to make the phospholipid molecules pack more tightly, thereby increasing the stability of the membrane. It is, presumably, this packing effect with which polyenes interfere in order to have their effect on permeability. One of the most convenient methods of applying liposomes involves the trapping of a marker such as glucose, CrO_4^{2-}, or $H_2PO_4^-$, removal of external marker by dialysis or gel filtration, and measurement of its transfer from the disperse to the continuous phase after treatment of the suspension with a polyene. A disadvantage inherent in the use of liposomes is that high concentrations of antibiotics (> 10 μM) are necessary, a fact which gave rise to some early confusion when Weissmann and Sessa (1967) reported that filipin was active against liposomes composed of lecithin only—a cognate phenomenon to that found with bilayers. Reaction also occurred with liposomes made from sphingomyelin, cardiolipin, and phosphatidylcholine, and was not due to the nature of the charge on the liposome (Sessa and Weissmann, 1967). This paradox was resolved when it was found that filipin II, which comprises 25% of the filipin complex, was responsible for this phenomenon (Sessa and Weissman, 1968b). The results obtained with this techinque using liposomes of lecithin and lecithin/cholesterol in general provide evidence which supports the sterol–polyene interaction theory; thus, using a sensitive assay for glucose, Kinsky *et al.* (1968) showed that the filipin-mediated release of this substance was much more rapid from cholesterol/lecithin liposomes than from lecithin liposomes. Neither they nor Weissmann and Sessa (1967), however, were able to show interaction between either pimaricin or lucensomycin and cholesterol-containing liposomes. Recent experiments by HsuChen and Feingold (1973) indicate the importance of the nature of phospholipid; they found that cholesterol incorporation into egg lecithin liposomes enhanced the sensitivity of the liposome to amphotericin B (as found above), but that the sensitivity of dipalmitoyl lecithin liposomes was in fact decreased by the incorporation of cholesterol. This phenomenon is explained by postulating that the membrane must be in an ordered state for a polyene to cause permeability changes therein, and that cholesterol, while making liposomes of egg lecithin more ordered, decreases the order of dipalmitoyl lecithin liposomes.

Liposomes containing sterols have been shown both to bind and to alter the ultraviolet absorption spectra of polyene antibiotics. Norman *et al.* (1972a) demonstrated this for filipin, lucensomycin, and amphotericin B, but the phenomenon was not apparent using nystatin or pimaricin. The same workers showed that the presence in the sterol of a 3β-OH group is an absolute requirement for interaction with polyenes, in contrast to the situation in solution (see above), where 3α-OH and 3 keto

sterols, as well as sterol esters, do react, although less well than 3β-OH compounds. Similarly, liposomes of appropriate composition alter the fluorescence properties of polyenes: Drabikowski *et al.* (1973) and Strom *et al.* (1973a) reported that the fluorescence intensities of solutions of filipin and lucensomycin, respectively, were enhanced in the presence of liposomes made from cholesterol and lecithin. The finding in respect to filipin is of interest in view of the quenching of fluorescence by "soluble" cholesterol (see above).

Liposomes can also be made from material extracted from naturally occurring membranes. De Kruyff *et al.* (1972) and McElhaney *et al.* (1973) have indicated that such liposomes have permeability properties very similar to those of the cells from which they were derived—in these cases, *A. laidlawii*. This model, in the hands of Demel *et al.* (1972b) and de Kruyff *et al.* (1972, 1973), shows quite clearly that the structural requirements for sterols to give optimal reductions in permeability to nonelectrolytes (e.g., erythritol and glycerol) as well as electrolytes (e.g., Rb^+), in both whole cells of *A. laidlawii* and derived liposomes, are identical with those for maximum interaction with the polyenes. This is indeed an intriguing finding, which indicates why polyenes can have such a devastating effect on cellular permeability, combining most avidly with the very sterols whose presence most affect permeability.

F. Studies with Disrupted Natural Membranes

Most of these studies have been carried out using erythrocyte ghosts, which are very easy to prepare in bulk, and are known to contain about 50 mole % of their total lipid as cholesterol and are thus equivalent to liposomes composed of equal parts of cholesterol and lecithin, or membrane preparations from *A. laidlawii*. The convenience of the latter is that it may be preconditioned to contain a particular sterol, or none. Crifo *et al.* (1971) and Strom *et al.* (1973b) found that the fluorescence of lucensomycin was enhanced by erythrocyte ghosts; treatment of the ghosts with formaldehyde or extraction with methanol/chloroform did not remove this effect. The enhancement occurred much more rapidly than when free cholesterol was used. Norman *et al.* (1972a,b) reported that erythrocyte ghosts alter the absorption spectra of filipin, lucensomycin, and amphotericin B, as did membranes from *A. laidlawii* enriched with cholesterol; membranes lacking cholesterol, or enriched with the 3α isomer epicholesterol, did not bring about spectral changes.

Electron microscope examination of red cells lysed with filipin and negatively stained revealed the presence of pits about 12 nm in diameter (Kinsky *et al.*, 1966). This sort of evidence is fully consistent with the

theory (Kinsky *et al.*, 1967b) that filipin acts by inducing a lamellar to micellar phase change in membranes. These electron micrographic studies have also been able to show conclusively that the action of saponins, which are hemolytic agents whose activity is blocked by sterols, is entirely different to that of polyenes. A later study (Verkleij *et al.*, 1973), using a freeze-etch technique as opposed to negative staining, showed that the pits were not in fact pores which penetrated the membrane, but were rather areas of aggregated material, presumably composed of filipin-cholesterol complexes. Lesions of an identical type were seen in *A. laidlawii* membranes and liposomes damaged by filipin. No parallel phenomenon was observed in membranes which had been in contact with amphotericin B, but the postulated size of "pores" produced under these conditions—0.5–1 nm in diameter (Andreoli *et al.*, 1969)—would not be visible after freeze-etching.

Membranes from *A. laidlawii*, if they contained cholesterol, caused changes in the absorption spectrum of filipin, lucensomycin, and amphotericin B and also bound the antibiotics (Norman *et al.*, 1972a,b). Similar findings were made for filipin interacting with membranes obtained from disrupted rabbit sarcoplasmic reticulum (Drabikowski *et al.*, 1973); such binding was associated with an increase in the fluorescence of filipin.

G. Experiments with Whole Cells and Functional Subcellular Fractions

1. Erythrocytes

Kinsky *et al.* (1962) were the first to show that polyenes caused hemolysis; filipin was the most active compound, followed by amphotericin B and nystatin. In further experiments, Kinsky (1962, 1963) showed that several polyenes lysed erythrocytes and caused permeability changes to *N. crassa* protoplasts, but did not affect bacterial protoplasts; the polyenic compound vitamin A (retinol)—which, it should be noted, is not a macrolide—lysed red cells and bacterial protoplasts, but not fungal protoplasts. Kinsky *et al.* (1967a) correlated the hemolytic and chemical properties of various filipin derivatives, the perhydro compound (obtained by catalytic hydrogenation), the irradiated derivative and the saponified adduct (the lactone ring having been broken by aqueous NaOH). All these derivatives had drastically decreased hemolytic and antifungal activities, and were later shown (e.g., Demel *et al.*, 1968a) also to have diminished power to combine with sterols. A further degradation product of filipin (structurally much less altered than the compounds mentioned above), the tetraenic epoxide (Rickards *et al.*, 1970), is also devoid of sterol-combining power (Schroeder *et al.*, 1972). Sessa

and Weissman (1968b) found that the hemolytic and sterol-combining activities of the four components of filipin ran in parallel. An important point, stressed by Kinsky *et al.* (1967a), is that it is the ratio of antibiotic to sterol, rather than the absolute concentration of antibiotic, which determines the rate and extent of hemolysis.

2. Mitochondria

The function of mitochondria, isolated from a sensitive organism, *N. crassa*, was not affected by polyenes, although the antibiotics were bound (Kinsky *et al.*, 1965). This lack of sensitivity was attributed to the high phospholipid to sterol ratio in the mitochondrial membrane (c. 40:1), compared to microsomes (7:1), the latter fraction accounting for the bulk of antibiotics binding in this species. These phospholipid:sterol ratios are virtually identical to those reported in mammalian tissues (Parsons and Yano, 1967), and should be compared with the figures for parenchymatous cell membranes (c. 1.3:1 Dod and Gray, 1968), and erythrocyte membranes (1:1).

Weissman *et al.* (1966) reported that polyenes disrupted lysosomes, but did not affect mitochondria, from rabbit cells.

3. Tissues from Cold-Blooded Animals

Lippe and Giordana (1967) reported a differential effect on amphotericin B on the large and small intestine of *Testudo*; the mucosal but not the serosal side of the large intestine became more permeable to thiourea after exposure to the antibiotic. A similar type of effect was noted using toad bladder; Lichtenstein and Leaf (1965) observed that amphotericin B acted on the mucosal side of the bladder only, mimicking to some extent the action of antidiuretic hormone. Leung and Eisenberg (1973) have reported an effect of nystatin on frog skeletal muscle, the result of which is an increase in membrane conductance.

4. Insect Larvae

The experiments of Sweeley *et al.* (1970) and Schroeder and Bieber (1971/1972) have shown that filipin kills the larvae of the house fly and interferes with cholesterol metabolism in the larvae of the wax moth. The administration of cholesterol can reverse some of these deleterious effects. The disturbance of phospholipid metabolism reported may not be a direct effect of polyene, but a consequence of the altered cholesterol metabolism.

5. Microorganisms

Experiments elucidating the role of sterols in the mode of action of the polyenes will be described very briefly, as they have been covered

in detail elsewhere. That exogenously added sterols can protect sensitive organisms from the action of polyenes has been demonstrated by Lampen *et al.* (1960) and Gottlieb *et al.* (1960) for *S. cerevisiae*, and by Zygmunt and Tavormina (1966) for *C. albicans*. Resistant organisms that do not contain sterols become sensitive when they have incorporated sterols into their membranes: this was shown for *A. laidlawii* by Feingold (1965) and Weber and Kinsky (1965), for *Schizosaccharomyces japonicus* by Bulder (1971), and for *Pythium* spp. by Schlösser and Gottlieb (1966) and by Child *et al.* (1969).

H. Discussion

All the diverse and ingenious systems which have been used to probe the mode of action of the polyene antibiotics have given consistent results and have satisfied their users that their behavior resembles that of living cells. Filipin, the antibiotic which shows the greatest membrane-disrupting activity, in terms of lysis of erythrocytes, protoplasts, and black films, and alteration in pressure relationships in monolayers and permeability changes, is also the compound which reacts most strongly with sterols, and whose antiyeast activity is reversed most easily by sterols (Zygmunt and Tavormina, 1966). The general order agreed upon by the various groups using the different techniques is roughly filipin $>>$ amphotericin B ($>$lucensomycin, pimaricin) $>$nystatin; lucensomycin and pimaricin are in parentheses because by no means all the studies included them. While this order correlates to some extent with the relative toxicities of the antibiotics (thus, filipin is too toxic for human use; nystatin is used topically whereas amphotericin B can be used parenterally), it does not tally at all with the order of antiyeast activity, which is amphotericin B $\gg$ nystatin $>$ filipin = pimaricin (Hamilton-Miller, 1973a). One is inevitably led to the conclusion that the results of the experiments described above throw more light quantitatively upon the toxicity of the polyenes than upon their precise mode of antimicrobial activity. It is still not clear which of the many sequelae of membrane damage is ultimately responsible for yeast cell death (Lampen, 1969).

The atomic architecture of the sterols with which polyenes preferentially complex has been worked out very clearly. The differences between requirements in solution (where the interaction was primarily by hydrophobic forces) and on the surface of membranous structures (where it is hydrophilic forces that seem to predominate) are of particular interest. While something is known of the stoichiometry of the interaction, there is little information about the precise chemical and physical nature of the sterol–polyene complex. Gale (1973) has recently put forward

a tentative scheme for the reaction of polyenes and membranes *in vivo*. From a physiological point of view, and with the medical microbiologist in mind, perhaps the most significant findings in this respect are that cholesterol and ergosterol are good "receptors" for polyenes, and that cholesteryl esters are much less effective. Again, there is not total agreement when the "polyene avidity" league table for sterols is compared with the ability of sterols to inhibit the antiyeast activity of polyenes. Thus, Gottlieb *et al.* (1960) and Zygmunt and Tavormina (1966) found that cholesterol acetate effectively reversed the action of filipin on *C. albicans*, and that stigmasterol, the most active compound in *in vitro* tests in models, was not as good an antipolyene agent as was ergosterol. It is of passing interest to note that the actions of two other antibiotics, mycobacillin (a polypeptide) and pyrrolnitrin (a halogenated pyrrole), are to some extent nullified by the presence of certain lipids, including phospholipids and sterols (Haldar and Bose, 1973).

Another point which bears strongly upon the physiological activity of the polyenes and which is brought out by the experiments described above is that it is not only the qualitative and quantitative nature of the sterol in a membrane that is important, but also the sterol:phospholipid ratio, and the chemical nature of that phospholipid. Further, the multiplicity of polyene to sterol molecules also affects the final issue. With all these variables in mind, it can be seen that mechanisms of selective toxicity will be at best difficult and at worst impossible to work out precisely.

Overall, however, the quality and the quantity of the research done on the mode of the action of the polyenes is most impressive, if only as an exercise in applied biophysics, and has given rise to two, at least, potentially valuable spin-offs, namely, the nystatin-modified erythrocyte (Cass and Dalmark, 1973) and the muscle fiber with high conductance (Leung and Eisenberg, 1973), both of which may prove to be of extreme value to the physiologists.

VI. Resistance to the Polyene Antibiotics

A search of the literature has revealed only two examples of the occurrence of apparent polyene resistance: Bodenhoff (1968) reported on two cases in which drug-resistant *C. albicans* appeared *in vivo* after the prolonged use of polyenes, and Hejzlar and Vymola (1970) describe an apparent increase in the incidence of low-level nystatin resistance in clinically isolated yeasts. Thus, from a clinical point of view, the problem of polyene resistance is nonexistent. There are no other antibiotics in common therapeutic use about which a similar statement could be made. Before experimental work is reviewed in this section, it seems worth while to speculate briefly on this remarkable fact.

First, the extent of its veracity must be probed. It must be accepted that, at least in the United Kingdom, most medical mycology is carried out in laboratories geared to microbiological investigations in general, the bulk of which involves bacteria and viruses. The presence in such routine laboratories of a trained mycologist is not regarded as necessary. Bearing in mind that the speciation of yeasts is certainly not as easily carried out as is bacterial identification, there is a distinct chance that strains of potentially pathogenic yeasts may be misidentified (or, most commonly, not identified at all), and therefore not reported upon. Further, sensitivity testing to the polyenes is not always routinely carried out, as it is generally assumed that sensitivity is universal. Perhaps the advent of new antimycotics, such as clotrimazole and 5-fluorocytosine, to which there is a range of sensitivities (Hamilton-Miller, 1972c), may lead to a reappraisal of the whole question of the sensitivity testing of clinically isolated yeasts. The problem of misidentification, however, is by no means so easy to rectify. As pointed out previously by the reviewer (Hamilton-Miller, 1972a), if resistant variants have even slightly unusual properties (and the limited evidence available suggests that this might indeed be so), they may very well be misidentified even by experienced mycologists. However, whatever doubts one may cast on the lack of resistance to polyenes, for practical purposes it must be assumed that the happy situation exists whereby the vast majority, at least, of pathogenic yeasts and fungi have retained their sensitivity throughout 15 years or more of polyene usage. Why, then, has resistance not arisen so far?

It is known that many (but probably not all) *Candida* strains are capable, genetically, of giving rise to mutants with markedly lower sensitivity to the polyenes, especially to amphotericin B. Multistep resistant mutants, made in the usual laboratory fashion of serial subculture in the presence of increasing concentrations of antibiotics, have been characterized from several *Candida* species. Also, single-step mutants of *C. albicans* have been isolated either with or without the necessity of mutagenesis (see Hamilton-Miller 1973a for reference to the above), but the multistep pathway seems much more common. The only reports of polyene-resistant dermatophytes have so far come from one group (Capek and Simek, 1972), and under *in vitro* conditions only. Perhaps dermatophyte infections are more often treated with Whitfield's ointment or with griseofulvin than with nystatin, but nevertheless there must have been considerable opportunities for the emergence of resistant mutants, which the pathogens do not seem to have grasped. The lack of resistance is even more surprising when one considers the enormous topical use of antifungal antibiotics, topical use of an antibacterial compound often sounding its death knell.

Official figures (Annual Report for 1970, 1971) show that 2.9

million prescriptions, worth £900,000, were written in 1970 in the United Kingdom for "antibacterial and fungicidal agents" prepared for use on the skin and mucocutaneous junctions: a substantial proportion of those prescriptions must have been for preparations containing nystatin. Any attempted explanation for the lack of acquisition of resistance to the polyenes must take into account two predominant factors—first, the opportunistic nature of the pathogen, and second, the physical properties of the antibiotics. While a true study of the nature of polyene resistance is, by its very nature, impossible, such observations as have been made on strains made resistant *in vitro* tend to support the view that resistance (at least the multistep variety) is associated with diminished virulence (see Athar and Winner, 1971, and references therein). A further lowering of an already low virulence may not allow the organism to continue its parasitic existence, even in the compromised host, so that any resistant cells that appear under selection during antibiotic treatment may succumb to lost bodily defense mechanisms. As to the second point: the only clear qualitative difference between the polyenes, to which resistance does not occur, and all other antibiotics, to which resistance does occur, is that the polyenes are virtually insoluble in water. This fact may have very important repercussions; as is well known, multistep resistance is encouraged by the use of too small doses of antibiotics, and prevented by the use of massive dosages. Even in very dilute aqueous solution, it seems probable that polyene antibiotics exist in micelles, i.e., packets or quanta of molecules. Thus, a subinhibitory concentration of a polyene antibiotic may well be one in which there are fewer quanta than there are organisms, and one can visualize the situation that any cell that comes in contact with a quantum will perish. In this way, an all-or-none phenomenon will exist, despite superficial appearances to the contrary, as cells will either die or not be exposed at all to the antibiotic, thus effectively preventing the selection of mutants. It is interesting in this respect that Lampen *et al.* (1959) found that yeast cells absorb far more nystatin than is necessary to kill them. If this hypothesis is correct, namely, that the lack of resistance to polyenes is a consequence of their lack of solubility in water, then the introduction of soluble derivatives of amphotericin B (see Keim *et al.*, 1973) is to be deplored. Time alone will tell.

VII. The Role of Sterols in Resistance to the Polyenes

Polyene resistance has been demonstrated not only in *Candida* spp. and dermatophyte strains, as discussed in the preceding section, but has also been found in other organisms of mycological interest, namely *Saccharomyces cerevisiae* and *Neurospora crassa*. In the former species,

nystatin-resistant mutants were selected, without prior mutagenesis, by Ahmed and Woods (1967) and Patel and Johnston (1968), while Molzahn and Woods (1973) used ethyl methanesulfonate to obtain their mutants. Grindle (1973) isolated polyene-resistant *N. crassa* variants after treatment with nitrous acid or ultraviolet light.

Patel and Johnston (1971) suggested that the low-level (3.5-fold) resistance occurring in tetraploid *S. cerevisiae* was due to the difference in surface area to volume (A/V) ratio in these cells as compared to haploids, rather than to a biochemical mechanism. Unfortunately, this interesting suggestion was not supported by any hard evidence. In an attempt to resolve this question, the present reviewer (Hamilton-Miller, 1973c) calculated that A/V ratios for tetraploid yeast cells were 2.7 times larger than for haploid cells, so this "physiological" theory of nystatin-resistance is indeed a possibility for polyploid strains of *S. cerevisiae*, but cannot be proved in the absence of sterol analyses.

In all other cases of reported polyene resistance, where sterol assays have been carried out, changes, qualitative, quantitative, or both, in sterol patterns have been observed. Thus, Woods (1971) and Molzahn and Woods (1972) found a new sterol in nys-1 and pol-1 mutants, and reduction in the content of ergosterol and its dehydro analog, with replacement by a second new sterol in nys-3 mutants. The pol-2 mutants (Molzahn and Woods, 1972) appeared to lack sterol altogether.

Mutants studied by Bard (1972) lacked ergosterol and his nyr-15 strain resembled the nys-3 mutant mentioned above. Most of the *N. crassa* mutants examined by Grindle (1973) lacked ergosterol: types I and II were devoid of sterols; type III had greatly reduced levels of ergosterol; and types IV, V, and VI lacked ergosterol and contained other sterols instead. In *C. albicans* mutants, Hamilton-Miller (1972b) found that sterol patterns in strains YL and YS resembled those in the nys-1 mutants of Woods (1971), but that all 4 mutant strains studied contained more ergosterol than the wild-type. The multistep mutants of various *Candida* species studied by Athar and Winner (1971) seemed to be ergosterol-deficient, but lack new sterols (Athar, 1969).

It is apparent from the above that alterations in sterol patterns play a crucial role in resistance to the polyenes. Lampen *et al.* (1959) showed that the binding of polyene was a prerequisite for the killing of cells, and that a low-level resistant strain of *C. albicans* absorbed less antibiotic than did a sensitive strain. It is logical, therefore, to consider the ways in which alterations in sterol content, both qualitative and quantitative, could cause changes in the amount of polyene absorbed by yeast cells.

The simplest example is given by the nyr-1 and -3, pol-2, -4, and -5 mutants of *S. cerevisiae*, as well as petites (Woods, 1971), the types I, II, and III *N. crassa* strains, and the "trained" variants of *Candida*

studied by Athar and Winner (1971). All these strains either contain decreased amounts of ergosterol compared with the wild type, or none at all, and there are no other changes in sterol composition. Clearly, if it is to ergosterol that polyenes absorb, such organisms will have increased resistance by virtue of having fewer binding sites.

Second, there are the strains in which some or all of the ergosterol has been replaced by another sterol, one which binds polyenes less well. Examples in this category are the nys-1 and -3, pol-1 and -3, nyr-15, ole 2-1, ole 2-2 and ole3ole4 *S. cerevisiae* mutants, and the *N. crassa* IV, V, and VI mutants. It has been suggested (Thompson *et al.*, 1971; Bard, 1972) that the nys-3 and the ole series contain zymosterol and lanosterol, respectively. Both these sterols differ sufficiently from ergosterol to mean that the binding of polyenes will be substantially decreased.

The remaining strains—the mutagen-induced *C. albicans* variants—were found to contain more ergosterol than the wild type, whether the sterol content was expressed as gravimetrically, as molecules per cell, or as molecules/μm^2 of cell surface (Hamilton-Miller, 1973c), and strains YL and YS contained a new sterol as well (Hamilton-Miller, 1972b). In order to explain resistance in these cases on a biochemical basis—the physiological hypothesis of Patel and Johnston (1971) being unlikely to apply in this instance (Hamilton-Miller, 1973c)—it must be postulated that the ergosterol is reoriented (or, in the case of strains YS and YL, masked by the new sterol) so that polyene binding is made more difficult on steric and/or thermodynamic grounds. Alternatively, one can postulate a change in the ratio of phospholipid to sterol in the membrane, as such changes have been seen to alter the lability of membranes to polyenes. Again, the ergosterol may be esterified in such strains.

It must be stressed that the above is only a tentative effort to delineate possible ways in which changes in sterol patterns may affect resistance; it is unlikely that any single explanation applies to any one particular case. Further work needs to be done, especially on uptake of antibiotic by resistant organisms, before any hard and fast statements can be made. In this respect, much more information can be obtained by working with laboratory-induced resistant strains rather than naturally occurring variants, as one has the wild type as a control throughout.

VIII. Conclusions

Sterols in yeast have taken on a new importance in the past few years. Previously, as indicated in Section III, interest in this topic was almost completely a commercial one, as *S. cerevisiae* was a valuable and easily accessible source of ergosterol, a precursor of many important products in the pharmaceutical industry. Now that it has been established

that polyene-resistant mutants of yeasts may have altered sterol patterns, the pharmaceutical industry has new access to a potentially rich source of rare chemicals and important precursors. Furthermore, the presence of sterol-deficient mutants is inevitably a bonus to workers in the field of sterol and steroid biosynthesis, whose understanding of the relevant metabolic pathways will be furthered by a study of various polyene-resistant and sterol-dependent (Karst and Lacroute, 1973) mutants.

In the long run, however, it may transpire that the greatest contribution made to the fund of human knowledge from a study of the mode of action of the polyene antibiotics and the involvement therein of sterols will lie in the field of membrane structure and function, which is perhaps the most vital and challenging problem in biology today.

References

Adams, B. G., and Parks, L. W. (1967). *J. Cell. Physiol.* **70**, 161–168.

Ahmed, K. A., and Woods, R. A. (1967). *Genet. Res.* **9**, 179–193.

Andreoli, T. E., and Monahan, M. (1968). *J. Gen. Physiol.* **52**, 300–325.

Andreoli, T. E., Dennis, V. W., and Weigh, A. M. (1969). *J. Gen. Physiol.* **53**, 133–156.

Annual Report for 1970. (1971). Department of Health and Social Security. HM Stationery Office, London.

Appleton, G. S., Kieber, R. J., and Payne, W. J. (1955). *Appl. Microbiol.* **3**, 249–251.

Asselineau, J., and Lederer, E. (1960). *In* "Lipide Metabolism" (K. Bloch, ed.), pp. 337–406. Wiley, New York.

Athar, M. A. (1969). Ph.D. Thesis, University of London.

Athar, M. A., and Winner, H. I. (1971). *J. Med. Microbiol.* **4**, 505–517.

Bard, M. (1972). *J. Bacteriol.* **111**, 649–657.

Barton, D. H. R., Kempe, U. M., and Widdowson, D. A. (1972). *J. Chem. Soc., Perkin Trans.* **I**, 513–522.

Bergy, M. E., and Eble, T. E. (1968). *Biochemistry* **7**, 653–658.

Bianchi, D. T. (1967). *Antonie van Leeuwenhoek; J. Microbiol. Serol.* **33**, 324–332.

Bittman, R., and Fischkoff, S. A. (1972). *Proc. Nat. Acad. Sci. U.S.* **69**, 3795–3799.

Bodenhoff, J. (1968). *Odontol. Tidskr.* **76**, 279–294.

Breivik, O. N., and Owades, J. L. (1957). *Agr. Food Chem.* **5**, 360–363.

Bulder, C. J. E. A. (1971). *Antonie van Leeuwenhoek; J. Microbiol. Serol.* **37**, 353–358.

Capek, A., and Simek, A. (1972). *Folia Microbiol. (Prague)* **17**, 239–240.

Cass, A., and Dalmark, M. (1973). *Nature (London), New Biol.* **244**, 47–49.

Cass, A., Finkelstein, A., and Krespi, V. (1970). *J. Gen. Physiol.* **56**, 100–124.

Child, J. J., Defago, G., and Haskins, R. H. (1969). *Can. J. Microbiol.* **15**, 599–603.

Crifo, C., Strom, R., Santoro, A., and Mondovi, B. (1971). *FEBS Lett.* **17**, 121–126.

Defago, G., Child, J. J., and Haskins, R. H. (1969). *Can. J. Microbiol.* **15**, 509–514.

de Gier, J., Mandersloot, J. G., and van Deenen, L. L. M. (1968). *Biochim. Biophys. Acta* **150**, 666–675.

de Kruyff, B., Demel, R. A., and van Deenen, L. L. M. (1972). *Biochim. Biophys. Acta* **255**, 331–347.

de Kruyff, B., De Greef, W. J., van Eyk, R. V. W., Demel, R. A., and van Deenen, L. L. M. (1973). *Biochim Biophys. Acta* **298**, 479–499.

Demel, R. A., van Deenen, L. L. M., and Kinsky, S. C. (1965). *J. Biol. Chem.* **240**, 2749–2753.
Demel, R. A., van Deenen, L. L. M., and Pethica, B. A. (1967). *Biochim. Biophys. Acta* **135**, 11–19.
Demel, R. A., Crombag, F. J. L., van Deenen, L. L. M., and Kinsky, S. C. (1968a). *Biochim. Biophys. Acta* **150**, 1–14.
Demel, R. A., Kinsky, S. C., Kinsky, C. B., and van Deenen, L. L. M. (1968b). *Biochim. Biophys. Acta* **150**, 655–665.
Demel, R. A., Bruckdorfer, K. R., and van Deenen, L. L. M. (1972a). *Biochim. Biophys. Acta* **255**, 311–320.
Demel, R. A., Bruckdorfer, K. R., and van Deenen, L. L. M. (1972b). *Biochim. Biophys. Acta* **255**, 321–330.
Dod, B. J., and Gray, G. M. (1968). *Biochim. Biophys. Acta* **150**, 397–404.
Drabikowski, W., Lagwinska, E., and Sarzala, M. C. (1973). *Biochim. Biophys. Acta* **291**, 61–70.
Dulaney, E. L. (1957). U.S. Patent 2,817,624.
Dulaney, E. L., Stapley, E. O., and Simpf, K. (1954). *Appl. Microbiol.* **2**, 371–379.
Elliott, C. G. (1969). *J. Gen. Microbiol.* **56**, 331–343.
El-Refai, A. H., and El-Kady, I. A. (1968a). *Z. Allg. Mikrobiol.* **8**, 355–360.
El-Refai, A. H., and El-Kady, I. A. (1968b). *Z. Allg. Mikrobiol.* **8**, 361–366.
Feingold, D. S. (1965). *Biochem. Biophys. Res. Commun.* **19**, 261–267.
Finkelstein, A., and Cass, A. (1968). *J. Gen. Physiol.* **52**, 145S–172S.
Gale, E. F. (1973). *Brit. Med. J.* **3**, 33–38.
Gordon, P. A., Stewart, P. R., and Clarke-Walker, G. D. (1971). *J. Bacteriol.* **107**, 114–120.
Gottlieb, D., Carter, H. E., Wu, L. C., and Sloneker, J. H. (1960). *Phytopathology* **50**, 594–603.
Gottlieb, D., Carter, H. E., Sloneker, J. H., Wu, L. C., and Gaudy, E. (1961). *Phytopathology* **51**, 321–330.
Grindle, M. (1973). *Mol. Gen. Genet.* **120**, 283–290.
Haldar, A., and Bose, S. K. (1973). *J. Antibiot.* **26**, 358–361.
Hamilton-Miller, J. M. T. (1972a). *J. Med. Microbiol.* **5**, 425–440.
Hamilton-Miller, J. M. T. (1972b). *J. Gen. Microbiol.* **73**, 201–203.
Hamilton-Miller, J. M. T. (1972c). *Sabouraudia* **10**, 276–283.
Hamilton-Miller, J. M. T. (1973a). *Bacteriol. Rev.* **37**, 166–196.
Hamilton-Miller, J. M. T. (1973b). *Chemotherapy (Basel)* **18**, 154–161.
Hamilton-Miller, J. M. T. (1973c). *Microbios* **8**, 209–213.
Hejzlar, M., and Vymola, F. (1970). *J. Hyg., Epidemiol., Microbiol., Immunol.* **14**, 211–213.
Hendrix, J. W. (1964). *Science* **144**, 1028–1029.
Hill, P. (1972). *J. Gen. Microbiol.* **70**, 243–252.
Holz, R., and Finkelstein, A. (1970). *J. Gen. Physiol.* **56**, 125–145.
HsuChen, C-C., and Feingold, D. S. (1973). *Biochem. Biophys. Res. Commun.* **51**, 972–978.
Hunter, K., and Rose, A. H. (1972). *Biochim. Biophys. Acta* **260**, 639–653.
Karst, F., and Lacroute, F. (1973). *Biochem. Biophys. Res. Commun.* **52**, 741–747.
Keim, G. R., Poutsiaka, J. W., Kirpan, J., and Keysser, C. H. (1973). *Science* **179**, 584–585.
Kinsky, S. C. (1962). *J. Bacteriol.* **83**, 351–358.
Kinsky, S. C. (1963). *Arch. Biochem. Biophys.* **102**, 180–188.
Kinsky, S. C., Avruch, J., Permutt, M., Rogers, H. B., and Schonder, A. A. (1962). *Biochem. Biophys. Res. Commun.* **9**, 503–507.

Kinsky, S. C., Gronau, G. R., and Weber, M. M. (1965). *Mol. Pharmacol.* **1**, 190–201.
Kinsky, S. C., Luse, S. A., and van Deenen, L. L. M. (1966). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **25**, 1503–1510.
Kinsky, S. C., Demel, R. A., and van Deenen, L. L. M. (1967a). *Biochim. Biophys. Acta* **135**, 835–843.
Kinsky, S. C., Luse, S. A., Zopf, D., van Deenen, L. L. M., and Haxby, J. (1967b). *Biochim. Biophys. Acta* **135**, 844–861.
Kinsky, S. C., Haxby, J., Kinsky, C. B., Demel, R. A., and van Deenen, L. L. M. (1968). *Biochim. Biophys. Acta* **152**, 174–185.
Lampen, J. O. (1969). *Amer. J. Clin. Pathol.* **52**, 138–145.
Lampen, J. O., Morgan, E. R., Slocum, A., and Arnow, P. (1959). *J. Bacteriol.* **78**, 282–289.
Lampen, J. O., Arnow, P. M., and Safferman, R. S. (1960). *J. Bacteriol.* **80**, 200–206.
Lenney, J. F., and Klemmer, H. W. (1966). *Nature (London)* **209**, 1265–1366.
Leung, J., and Eisenberg, R. S. (1973). *Biochim. Biophys. Acta* **298**, 718–723.
Lichtenstein, N. S., and Leaf, A. (1965). *J. Clin. Invest.* **44**, 1328–1342.
Lippe, C. (1968). *J. Mol. Biol.* **35**, 635–637.
Lippe, C., and Giordana, B. (1967). *Biochim. Biophys. Acta* **135**, 966–972.
McElhaney, R. N., de Gier, J., and Ven der Neut-Kok, E. C. M. (1973). *Biochim. Biophys. Acta* **298**, 500–512.
Molzahn, S., and Woods, R. A. (1972). *J. Gen. Microbiol.* **72**, 339–348.
Morpurgo, G., Serlupi-Crescenzi, G., Tecce, G., Valente, F., and Venettacci, D. (1964). *Nature (London)* **201**, 897–899.
Norman, A. W., Demel, R. A., de Kruyff, B., Geurts van Kessel, W. S. M., and van Deenen, L. L. M. (1972a). *Biochim. Biophys. Acta* **290**, 1–14.
Norman, A. W., Demel, R. A., de Kruyff, B., and van Deenen, L. L. M. (1972b). *J. Biol. Chem.* **217**, 1918–1929.
Parks, L. W., and Starr, P. R. (1963). *J. Cell. Comp. Physiol.* **61**, 61–65.
Parsons, D. F., and Yano, Y. (1967). *Biochim. Biophys. Acta* **135**, 362–364.
Patel, P. V., and Johnston, J. R. (1968). *Appl. Microbiol.* **16**, 164–165.
Patel, P. V., and Johnston, J. R. (1971). *J. Appl. Bacteriol.* **34**, 449–458.
Proudlock, J. W., Wheeldon, L. W., Jollow, D. J., and Linnane, A. W. (1968). *Biochim. Biophys. Acta* **152**, 434–437.
Rickards, R. W., Smith, R. M., and Golding, B. T. (1970). *J. Antibiot.* **23**, 603–612.
Schlösser, E., and Gottlieb, D. (1966). *J. Bacteriol.* **91**, 1080–1084.
Schlösser, E., Shaw, P. D., and Gottlieb, D. (1969). *Arch. Microbiol.* **66**, 147–153.
Schroeder, F., and Bieber, L. L. (1971/1972). *Chem. Biol. Interact.* **4**, 239–249.
Schroeder, F., Holland, J. F., and Bieber, L. L. (1971). *J. Antibiot.* **24**, 846–849.
Schroeder, F., Holland, J. F., and Bieber, L. L. (1972). *Biochemistry*, **11**, 3105–3111.
Schubert, K., Rose, G., Tümmler, R., and Ikekawa, N. (1964). *Hoppe-Seyler's Z. Physiol. Chem.* **339**, 293–296.
Schubert, K., Rose, G., Wachtel, H., Hörhold, C., and Ikekawa, N. (1968). *Eur. J. Biochem.* **5**, 246–251.
Sessa, G., and Weissmann, G. (1967). *Biochim. Biophys. Acta* **135**, 416–426.
Sessa, G., and Weissmann, G. (1968a). *J. Lipid Res.* **9**, 310–318.
Sessa, G., and Weissmann, G. (1968b). *J. Biol. Chem.* **243**, 4364–4371.
Strom, R., Crifo, C., and Bozzi, A. (1973a). *Biophys. J.* **13**, 568–580.
Strom, R., Crifo, C., and Santoro, A. (1973b). *Biophys. J.* **13**, 581–593.
Sweeley, C. C., O'Connor, J. D., and Bieber, L. L. (1970). *Chem. Biol. Interact.* **2**, 247–253.
Tanret, C. (1879). *Ann. Chim. Phys.* [5] **17**, 493–512.

Thompson, E. D., Starr, P. R., and Parks, L. W. (1971). *Biochem. Biophys. Res. Commun.* **43**, 1304–1309.
van Zutphen, H., van Deenen, L. L. M., and Kinsky, S. C. (1966). *Biochem. Biophys. Res. Commun.* **22**, 393–398.
van Zutphen, H., Demel, R. A., Norman, A. W., and van Deenen, L. L. M. (1971). *Biochim. Biophys. Acta* **241**, 310–330.
Verkleij, A. J., de Kruyff, B., Gerritsen, W. F., Demel, R. A., van Deenen, L. L. M., and Ververgaert, P. H. J. (1973). *Biochim. Biophys. Acta* **291**, 577–581.
Weber, M. M., and Kinsky, S. C. (1965). *J. Bacteriol.* **89**, 306–312.
Weissmann, G., and Sessa, G. (1967). *J. Biol. Chem.* **242**, 616–625.
Weissmann, G., Hirschhorn, R., Pras, M., and Bevans, V. (1966). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **25**, 358.
Woods, R. A. (1971). *J. Bacteriol.* **108**, 69–73.
Zygmunt, W. A., and Tavormina, P. A. (1966). *Appl. Microbiol.* **14**, 865–869.

Methods of Numerical Taxonomy for Various Genera of Yeasts

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I. Theory of Classification of Yeasts

The methods of classification of yeasts are fully described elsewhere (Kreger-van Rij, 1969; van der Walt, 1970a), but some introductory comment is required. The current system of classification depends on the following characters (see also Table I).

1. Form of asexual reproduction: by multilateral and narrow-based, or bipolar and broad-based budding, or binary fission. Usually no difficulties arise in this test, but Barnett (1960) has noted the occasional difficulties in determining the difference between multilateral and bipolar budding.

2. Formation of spores. Classification of yeasts has evolved from the methods of mycology rather than of bacteriology. Therefore the mycologists' obsession with the type of sexual spores, if formed, is of fundamental importance in yeast taxonomy. This is unfortunate for several

TABLE I
Tests for Identification of Yeasts[a]

Morphology	Characteristics of vegetative reproduction
	Size and shape of cells
	Size, shape, and number of spores
	Formation of pseudomycelium
	Appearance of colonies
Physiology	Pellicle formation on liquid media
	Fermentation of sugars
	Assimilation of carbon compounds
	Assimilation of nitrate (and possibly nitrite, ethylamine, or lysine)
	Tests of minor importance
	Growth in absence of vitamins
	Maximum temperature of growth
	Growth in high osmotic pressure
	Resistance to cycloheximide (Actidione)

[a] After van der Walt (1970a).

Polyene Antibiotic–Sterol Interaction[1]

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I. Introduction

The discovery in 1950 by Hazen and Brown of the compound nystatin led in a short time to the development of a new area of research in the field of antibiotics (Hazen and Brown, 1951). It shortly became appreciated that nystatin belonged to a new class of antibiotics. The chief distinguishing feature of this class of antibiotics was the observation that they had no effect on bacteria but were toxic to fungi.

[1] Supported in part by United States Public Health Service Grants AM-09012 and AM-14,750.

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Other representative compounds, such as filipin, amphotericin B, and pimaricin, were subsequently discovered. It soon became apparent that, besides a similarity of biological action, all these compounds also had certain common chemical structural features. Accordingly, the class of substances of polyene antibiotics was born.

There is now a multifaceted interest in the use of polyene antibiotics—ranging from their application for certain clinical purposes, to their employment as larvicidal or chemosterilant substances (Sweeley *et al.*, 1970), to their use as biochemical probes for membrane structure–function relationships. The polyene compounds amphotericin B and nystatin are the chief substances employed clinically. They have been employed in relieving dermatophytic infections and treating monilial infections, particularly in obstetrical and gynecological cases. Additionally the hypocholesterolemic activity of orally administered polyene antibiotics (Schaffner and Gordon, 1968) has been reported, as has their possible use in treating prostatic hypertrophy (Gordon and Schaffner, 1968).

Tsao has reviewed the use of polyene antibiotics as an aid in the isolation of certain pathogenic fungi (Tsao, 1970). By the incorporation of polyene antibiotics in growth media, studies on the isolation of economically important pathogenic fungi, especially soil-borne members, have been greatly facilitated.

Finally there has been in recent years a burgeoning interest concerning the biochemical mode of action of the polyene antibiotics. As will become apparent later in this article, there is ample evidence to support the concept that the polyenes have biological activity mainly by virtue of their interaction with the sterol component of membranes. As a consequence of the interaction of the polyene compound with the membrane sterol, a series of biochemical and physiological changes are initiated in the membrane, which may under extreme conditions ultimately lead to the death of the organism in question. It has now become possible to utilize polyene compounds, particularly filipin, to study certain aspects of membrane structure and properties, e.g., permeability to ions, effects in membrane resistance and capacitance, or mobility of membrane components.

It is the purpose of this article to (a) review the chemistry and structure of the class of polyene antibiotics, (b) discuss the biology of the action of the polyenes in the affected organism and in both natural and artificial membrane systems, and finally (c) discuss in some detail the proposed biochemical mechanism of their action. In recent years the following excellent review articles pertaining to the gen-

eral field of polyene antibiotics have appeared: Kinsky (1967, 1970), Gottlieb (1971), Gottlieb and Shaw (1967, 1970), Lampen (1966), Oroshnik and Mebane (1963), Vining (1960), and Hamilton-Miller (1973).

II. Structures and Chemical Properties of Polyene Antibiotics

A. Background

Hazen and Brown (1951) made the first discovery of a polyene antibiotic when they isolated the compound nystatin. Since that time more than 70 other compounds have been isolated and identified as belonging to the similar structural class of polyenes. All these substances are characterized by having intense ultraviolet (UV) absorption spectra. The absorption spectra of most of these compounds are quite similar (for example see Fig. 1). The chromophores responsible for this UV absorption have been unequivocally identified as being

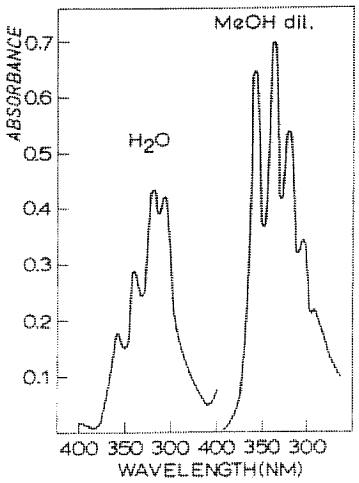


FIG. 1. Ultraviolet absorption spectra of filipin in methanol and water. From a stock solution of filipin in dimethylformamide, 50 μl were added separately to 5.0 ml of methanol and H_2O. After thorough mixing, the ultraviolet absorbance spectrum was measured. The concentration of filipin in each sample was 1.23×10^{-5} *M*. The absorption maxima in methanol (MeOH) for peaks 1 to 4 were 356, 337, 321, and 311 nm, respectively. In water the absorption maxima for peaks 1 to 4 were 356, 338, 320, and 311 nm.

Table I

PROPERTIES OF IMPORTANT POLYENE ANTIBIOTICS PRODUCED BY *Streptomyces* SPECIES

Antibiotic	Class	Elemental analysis	Structure known	Neutral, basic zwitterionic	Mycosamine present	Produced by species	References
Ascosin	Heptaene	—	—	Z	—	*S. canescus*	Hickey *et al.* (1952)
Amphotericin A	Tetraene	$C_{44}H_{67}O_{14}N$	—	Z	—	*S. nodosus*	Vandeputte *et al.* (1955–1956)
Amphotericin B	Heptaene	$C_{46}H_{73}O_{18}N$	Yes	Z	+	*S. nodosus*	Mechlinski *et al.* (1970), Ganis *et al.* (1971)
Aurenin	Pentaene	$C_{33}H_{54}O_{11}$	Yes	N	—	*S. aureorectus*	Ushakova *et al.* (1970)
Candidin	Heptaene	$C_{47}H_{71}O_{17}N$	Yes	Z	+	*S. viridoflavus*	Borowski *et al.* (1971), Taber *et al.* (1954), Vining and Taber (1956)
Capacidin	Pentaene	$C_{54}H_{85}O_{18}N_2$	—	—	—	*S. gourgeroti*	Brown and Hazen (1960)
Chainin	Pentaene	$C_{33}H_{54}O_{10}$	Yes	—	—	*S. chainia*	Pandey *et al.* (1972)
Endomycin	Tetraene	—	—	—	—	*S. endus*	Vining and Taber (1957)
Etruscomycin	Tetraene	$C_{36}H_{57}O_{14}N$	Yes	Z	+	*S. lucensis*	Arcamone *et al.* (1957), Arcamone and Perego (1959)
Eurocidin A	Pentaene	$C_{40}H_{65}O_{15}N$	Yes	Z	+	*S. albireticuli*	Horii *et al.* (1970)
Eurocidin B	Pentaene	$C_{39}H_{63}O_{15}N$	Yes	Z	+	*S. erocidicus*	Horii *et al.* (1970)
Filipin	Pentaene	$C_{35}H_{58}O_{11}$	Yes	N		*S. filipinensis*	Whitfield *et al.* (1955), Ceder and Ryhage (1961), Pandey and

Endomycin	Tetraene	—	—	—	—	*S. endus*	Vining and Taber (1957)
Etruscomycin	Tetraene	$C_{50}H_{77}O_{14}N$	Yes	Z	+	*S. lucensis*	Arcamone *et al.* (1957), Arcamone and Perego (1959)
Eurocidin A	Pentaene	$C_{40}H_{65}O_{15}N$	Yes	Z	+	*S. albireticuli*	Horii *et al.* (1970)
Eurocidin B	Pentaene	$C_{39}H_{63}O_{15}N$	Yes	Z	+	*S. erocidicus*	Horii *et al.* (1970)
Filipin	Pentaene	$C_{35}H_{58}O_{11}$	Yes	N		*S. filipinensis*	Whitfield *et al.* (1955), Ceder and Ryhage (1964), Pandey and Rinehart (1970)
Fungichromin	Pentaene	$C_{35}H_{58}O_{12}$	Yes	N	—	*S. cellulosae* *S. roscoluteus*	Dhar *et al.* (1960)
Nystatin	Tetraene	$C_{47}H_{75}O_{18}N$	Yes	Z	+	*S. noursei*	Ikeda *et al.* (1967)
Mycoticins A and B	Pentaene	$C_{67}H_{60}O_{10}$	Yes	N	—	*S. ruber*	Wasserman *et al.* (1967)
Pimaricin	Tetraene	$C_{33}H_{47}O_{13}N$	Yes	B	+	*S. natalensis*	Ceder and Ryhage (1964), Golding *et al.* (1966)
Perimycin	Heptaene	$C_{47}H_{75}O_{14}N$	—	B	Perosamine	*S. coelicolor*	Borowski *et al.* (1960)
Rimocidin	Tetraene	$C_{37}H_{59}O_{13}N$	Yes	N	—	*S. rimosus*	Davisson *et al.* (1951), Cope *et al.* (1966)
Tetrin	Tetraene	$C_{31}H_{59}O_{12}N$	—	—	—	—	Gottlieb and Pote (1960)
Tetrin A	Tetraene	$C_{34}H_{51}O_{13}N$	Yes	Z	+	S. (?)	Pandey *et al.* (1971)
Tetrin B	Tetraene	$C_{34}H_{51}O_{14}N$	Yes	Z	+	S. (?)	Rinehart *et al.* (1971)
Trichomycin	Heptaene	$C_{61}H_{86}O_{21}N_2$	Yes (?)	Z	+	*S. hachijoensis*	Nakano (1961), Hattori (1962)

conjugated double bonds. In fact, the number of conjugated double bonds present is the basis for the chemical classification of these substances. Table I lists the properties of many of the more thoroughly studied polyene antibiotics.

All these polyene antibiotics have in common a significant toxic effect against yeast and filamentous fungi with essentially no deleterious effect on bacteria. The basis for this biological distinction will be discussed in the following section. All these compounds are produced by the actinomycetes, in particular the genus *Streptomyces*. An elaborate screening program has been developed to test the production of new compounds by various species of *Streptomyces*. It has been claimed that, if a streptomycete produces an antifungal compound, there is a 95% probability that the compound will prove on examination to be a polyene (Ball *et al.*, 1957). These substances are normally extracted from the mycelia of the organism or in some instances from the whole culture broth by aqueous solutions of organic solvents. The isolation of pure polyene compounds has usually proved to be extremely difficult because they are present initially at very low concentrations. Also their solubility properties are such that they are neither very water soluble nor organic solvent soluble. Common techniques employed for their purification have included differential crystallization, countercurrent distribution, and in some instances partition column chromotography. To date, thirteen polyene antibiotics have been isolated and purified to an extent where subsequent prolonged chemical analyses have resulted in structure assignment. Structures of the polyenes most frequently encountered in the biological literature are shown in Fig. 2. In addition the chemical structures of the tetraenes aurenin (Ushakova *et al.*, 1970) and rimocidin (Cope *et al.*, 1966) and the pentaenes chainin (Pandey *et al.*, 1972), mycoticins A and B (Wasserman *et al.*, 1967), and eurocidin A and B (Horii *et al.*, 1970) have been reported.

B. Chemical Properties

It has been found that all the polyene antibiotics analyzed to date have certain common structural features in addition to their conjugated double-bond system. They are all characterized by a macrolide ring, which is a large ring of carbon atoms whose closure is effected by the formation of an internal ester or lactone. The presence of the lactone confers a highly characteristic peak on the infrared spectra of these compounds. Ring sizes have been found to vary from 12–14 up to 35–37 carbon atoms. The conjugated double-bond sys-

tem is contained exclusively within the limits of the cyclic lactone. The analysis of the characteristic ultraviolet spectra of the polyene antibiotics and also the X-ray crystallographic analysis of an *N*-iodoacetyl derivative of amphotericin B (Ganis *et al.*, 1971) indicates that all the double bonds are in the trans conformation. Certain theoretical discussions have been advanced suggesting that there should be $(4n + 4)$ carbons in the macrolide ring, where n is the number of double bonds in the conjugated system (Oroshnik and Mebane, 1963). The possibility also exists that the polyene antibiotics may, under certain circumstances, undergo conformational changes where some or all of the trans double bonds are converted to cis isomers.

The second highly characteristic feature of the polyene antibiotics concerns the plethora of hydroxyls present on the molecule. They are usually distributed along the macrolide ring on alternate carbon atoms. The number of hydroxyls has been found to vary from 6 to 14. The presence of such a large number of hydroxyl groups has posed challenging problems to the chemist who is concerned with their precise location. Also the presence on the one hand of the polar hydroxyl groups and on the other hand of multiple hydrophobic double bonds confer upon the polyene antibiotics an additional characteristic chemical property, that of being amphipathic. It is likely that this amphipathic feature plays an important role in the mode of action of these substances as they interact in various biological systems. Inspection of the structures given in Fig. 2 reveals that these hydrophilic and hydrophobic components reside on opposite sides of the macrolide ring.

The amphipathic character does confer interesting solubility properties on the polyene molecules. Purified polyene antibiotics show, as a rule, limited solubility in both water or such nonpolar organic solvents as alcohols, esters, or ethers. In contrast they may be dissolved by very polar organic solvents, such as dimethylsulfoxide or dimethylformamide. These solutions may then be diluted with water to produce concentrations of antibiotics of up to 50–100 μg/ml. It is likely that the polyene compounds are not in "true solution," but are present only as micelles. Stock solutions of the antibiotics in dimethlyformamide and dimethylsulfoxide are stable if extreme care is taken to exclude light and oxygen (Whitfield *et al.*, 1955).

Elemental analyses of the polyene antibiotics indicate that they contain only carbon, hydrogen, oxygen, and, in some instances, nitrogen. Many, but not all, of the polyene antibiotics have a net charge conferred upon them by virtue of the presence of either a single amino group, single carboxyl group, or both. In the latter instance they have a zwitterionic character.

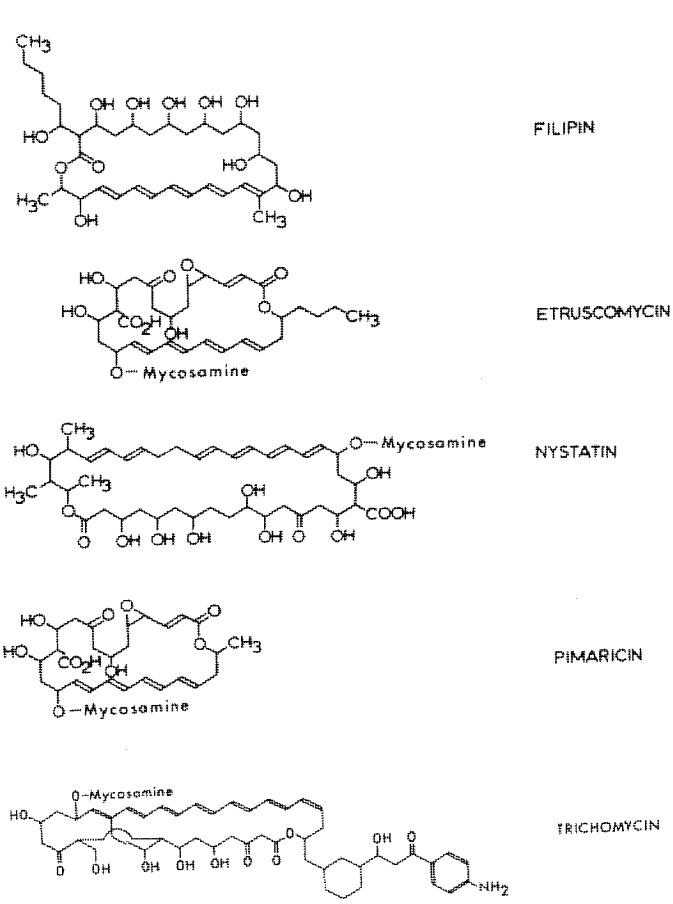
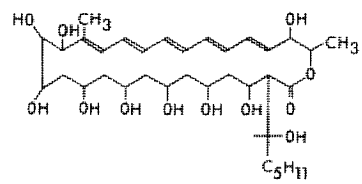
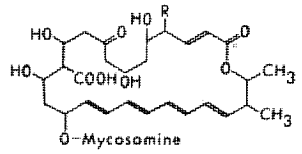


FIG. 2. Structures of polyene antibiotics for which a complete chemical structure has been proposed. The literature references from which the structures are taken are as follows: filipin (Ceder and Ryhage, 1964), etruscomycin or lucensomycin (Arcamone and Perego, 1959), nystatin (Ikeda *et al.*, 1967), pimaricin (Golding *et al.*, 1966), trichomycin (Hattori, 1962), fungichromin or lagosen (Dhar *et al.*, 1960), tetrins A and B (Pandey *et al.*, 1971; Rinehart *et al.*, 1971), amphotericin B (Mechlinski *et al.*, 1970; Ganis *et al.*, 1971), and candidin (Borowski *et al.*, 1971).

The amino group present in some of the polyene antibiotics is associated with an amino sugar that is connected to the macrolide ring through a glycosidic bond. In all instances the carbohydrate moiety has been found to be mycosamine (see Fig. 3). Its elemental analysis indicated it to be $C_8H_{15}O_4N$; its structure was ultimately deduced to be 3-amino-3, 6-dideoxymannose (Walters *et al.*, 1957). This is a 3-amino analog of D-rhammose or 6-deoxy-D-mannose. The structure was unequivocally established in 1961 by its chemical synthesis

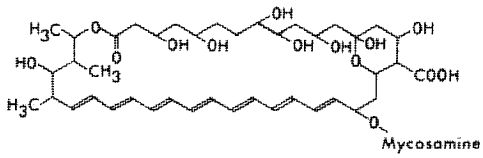


FUNGICHROMIN

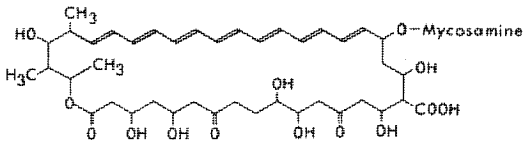


TETRIN A R=H

TETRIN B R=OH



AMPHOTERICIN B



CANDIDIN

FIG. 2. *(continued).*

(Bond Saltza *et al.*, 1961). Mycosamine has been found in the polyene antibiotics pimaricin, etruscomycin, trichomycin, candicidin, candidin, and tetrins A and B. The α-pyranose ring form of mycosamine is preferentially more stable than the α-furanose ring structure.

The most characteristic, and at the same time useful, physical property of the polyene antibiotics is their ultraviolet absorption spectra. The UV spectra of all the polyene have a regular series of sharp peaks of absorption, which are separated by sharp troughs, all in the range of 360–280 nm. Oroshnik and Mebane (1963) have given an extensive tabulation of the exact absorption maxima for many of the polyene antibiotics. Oroshnik *et al.* (1955) identified the ultraviolet absorbing system as being due to conjugated double bonds rather than polyenes. Careful analysis of the UV spectra indicates that there

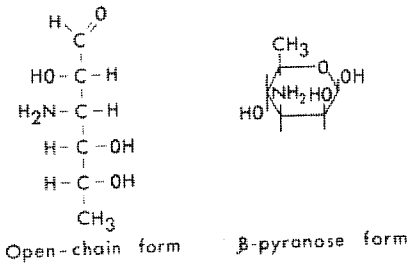


FIG. 3. Structure of mycosamine.

are several distinct classes of chromophores: tetraenes, pentaenes, hexaenes, and heptaenes. The molar extinction coefficients of the various absorption maxima range from 2 to 8 × 10^4. Thus it is a simple matter to measure the absorption of aqueous solutions of polyene antibiotics which are only 0.5 to 1.0 μM.

As shown in Fig. 1, for filipin there is a difference in the molar extinction coefficients of the various UV maxima depending whether the antibiotic is dissolved in an organic solvent or an aqueous solvent. This phenomenon was first reported without comment by Lampen *et al.* (1960). Norman *et al.* (1972a) have found that the exact diminution of the spectra of equivalent concentrations of filipin in water as compared to methanol is determined in a complex manner by a number of variables which include: (a) the concentration of filipin; (b) the manner of introduction of filipin dissolved in dimethylformamide into the water; (c) the length of time of vigorous mixing of the aqueous solution; (d) the temperature; and (e) the age of the stock solution of filipin dissolved in dimethylformamide. It is suspected that a portion of this reduction of ultraviolet absorbance of filipin in water is due to either micelle or aggregate formation. It was noted by these workers that filipin in organic solvents followed the Beer–Lambert relationship over a range of 1 to 400 × 10^{-6} M, but that in aqueous solutions, filipin did not follow the Beer–Lambert law over the same range of concentration. In spite of these technical difficulties, Norman *et al.* (1972b) have been able to utilize the ultraviolet-absorbing properties of the polyene antibiotics to carry out studies on their biological and biochemical mode of action.

C. Specific Polyene Antibiotics

In the following sections there is a brief description of the prominent structural features of the polyene antibiotics of biological inter-

est which have been chemically characterized. The reader is referred to Fig. 2, where their structures are presented.

1. *Amphotericin B*

Amphotericin B is the only polyene antibiotic for which both chemical structure and absolute configuration are known. Mechlinski *et al.* (1970) and Ganis *et al.* (1971) prepared an *N*-iodoacetyl derivative of amphotericin B which was used as the heavy atom derivative necessary for X-ray single-crystal analysis. Also the *N*-iodoacetyl derivative was found to be biologically active so that it may be permissible to equate the structure and conformation of this crystalline derivative with that of the solvent dissolved, biologically active amphotericin B. Previous work had indicated that 1 mole of amphotericin B contained 1 mole of mycosamine and had additionally a free carboxyl group. The macrolide ring structure contains 37 carbon atoms. There are seven double bonds and seven free hydroxyls on the ring structure. One very interesting feature, appreciated for the first time as a consequence of the X-ray analysis, was the presence of a six-membered ketal ring which is included as an appendage to the macrolactone ring. Amphotericin B also was found to contain 14 asymmetric centers, all of which are present in the macrolide lactone ring. One additional unique feature that was determined from the X-ray analysis was the demonstration that the mycosamine was bonded via a α-glycosidic rather than an α-glycosidic linkage to the hydroxyl group at carbon 19. The six-membered ketalic ring as well as the mycosamine pryanose ring are both in the "chair" conformation. The hydroxyls at carbons 8, 15, and 35 were found to be equatorial, while those at carbons 3, 5, 9, and 11 were in the axial configuration.

There are several other interesting features of the crystal structure that became evident from the X-ray analysis. The molecule was found to be definitely amphipatic, with all the hydroxyl groups and the carbohydrate moiety on one side of the lactone ring opposite the chromophoric double-bond groups, located on the opposite side of the ring. In the crystalline form, where multimolecules of amphotericin are present, the X-ray data indicated that the long sequences of conjugated double bonds in various molecules were packed closely together, in essence forming narrow hydrophobic channels (ca. 1.6 × 16 Å). The hydrophobic channel in turn was found to be surrounded by the hydrophilic groups of the lactone rings, which also were organized to create channels. It will be interesting to compare

the configuration of amphotericin B with that of the other polyene antibiotics as they become elucidated.

2. *Filipin*

Filipin belongs to the class of pentaene antibiotics. It has nine hydroxyl groups and no ionizable functional groups. Thus it is one of the few polyene antibiotics that are noncharged or neutral in character. The biological activities of filipin have been rather more thoroughly studied than those of any of the other polyene antibiotics.

The pentaene, nonahydroxy lactone structure originally proposed by Ceder and Ryhage (1964) for the filipin complex may possibly have to undergo some revision. Bergy and Eble (1968) showed that crystalline filipin can be resolved into 4 components, designated filipins I, II, III, and IV, which constitute 4, 25, 53, and 18%, respectively, of the original material. All four components had the characteristic pentaene chromophore. Filipin I was reported to be a heptahydroxy, II an octahydroxy, and III and IV nonahydroxy compounds (Pandey and Rinehart, 1970). The molecular formulas and number of hydroxyl groups assigned to filipins III and IV agree with the structure originally proposed for the filipin complex (Ceder and Ryhage, 1964) (see Fig. 2). Both the conjugated double-bond system and the intact macrolide ring structure have been found to be essential for biological activity, since either alkaline hydrolysis of the lactone or hydrogenation of the double bonds results in inactivation (Kinsky *et al.*, 1967a).

3. *Nystatin*

The complete chemical structure of nystatin became available in 1967 (Ikeda *et al.*, 1967). Nystatin is a tetraene macrolide that contains the carbohydrate moiety mycosamine. In addition it has two other double bonds not conjugated with the four that qualify it as a tetraene; it has ten hydroxyl groups and one free carboxyl. The exact site of attachment of the mycosamine moiety to the ring is not yet known with certainty. It is possible that nystatin may have been derived originally from a heptaene polyene and that, by saturation of one of the double bonds in the series of seven, the isolated diene and tetraene series were produced. The ring structure for nystatin (36 carbons) is certainly comparable in size to many other heptaene antibiotics. The biosynthesis of nystatin has been studied via the administration of various ^{14}C-labeled precursors to *Streptomyces noursei*. Such studies indicated that nystatin was likely derived from 16 acetate and 3 propionate units.

4. *Pimaricin*

Some controversy has existed concerning the precise chemical structure of pimaricin. Patrick *et al.* (1958) made the first suggestion with regard to its structure. This was subsequently modified by Ceder (1964) and more recently by Golding *et al.* (1966). The presently accepted structure of pimaricin is given in Fig. 2. Characteristic features include 4 conjugated double bonds, which qualify it as a tetraene, a mycosamine moiety, a free carboxyl, two ketone functional groups, an epoxide, and the presence of only three free hydroxyls (not counting those on the mycosamine).

5. *Trichomycin*

Prior to the very recent work on the X-ray crystallographic analysis of the conformation of amphotericin B, trichomycin was the only heptaene for which there was any definitive structural information. Hattori (1962) proposed the structure of trichomycin given in Fig. 2. Characteristic features include the seven conjugated double bonds, the carbohydrate mycosamine, two ring structures, one with 27 carbons and one with 12 carbons, approximately five free hydroxyl groups, and a side chain consisting of *p*-aminoacetophenone. It is to be anticipated that further studies will be reported in the near future which will clarify certain aspects of the trichomycin structure.

6. *Candidin*

The chemical structure of the antibiotic candidin has recently been reported by Borowski *et al.* (1971) and is given in Fig. 2. The characteristic features are seven conjugated double bonds, the carbohydrate mycosamine, five free hydroxyls (not counting those on the sugar), three ketone functional groups, and a free carboxyl. The possibility exists that candidin may be capable of forming an internal cyclic hemiketalic structure analogous to that reported for amphotericin B.

7. *Fungichromin*

The structure of fungichromin (lagosin) has been independently determined by two separate groups, the Oxford workers (Dhar *et al.*, 1960) and the M.I.T. group (Cope *et al.*, 1962). The characteristic features are a 28-membered macrolide ring containing five conjugated double bonds and nine free hydroxyls. There are no amino or carboxyl functional groups or sugar moiety.

8. *Tetrin A and Tetrin B*

The structures of tetrins A and B have recently been reported by Pandey *et al.* (1971) and Rinehart *et al.* (1971). Their structures are closely related; they differ only by an interchange of a hydrogen and hydroxyl group. The characteristic features of both the antibiotics are the presence of four conjugated double bonds, a free carboxyl group, three free hydroxyls, not including those on the mycosamine carbohydrate moiety, and one ketone functional group. Noteworthy is the absence of an epoxide functional group, since it is present in the closely structurally related compounds pimaricin and etruscomycin.

9. *Etruscomycin*

The structure of etruscomycin was reported by Arcamone *et al.* (1957; Arcamone and Perego, 1959). The characteristic features are four double bonds, four hydroxyls, the sugar mycosamine, a ketone group, and a free carboxyl group.

III. Biological Properties of Polyene Antibiotics

A. Effects on Membrane Permeability

1. *Background*

In the early 1960s several laboratories independently presented evidence that polyene antibiotics could mediate a change in the cellular permeability of a number of organisms, thus promoting a leakage of important cellular constituents and ultimately lysis and death of the cell. Kinsky (1961a,b) found that at low concentrations (ca. 1×10^{-6} *M*), the polyenes caused a rapid decrease in the dry weight of mycelial mats. This atrophy of mycelial material was accompanied by the leakage of amino acids, sugars, and other metabolites from the cytoplasm into the culture media. It was found that only the polyene antibiotics nystatin, amphotericin B, and filipin could produce this specific effect, and that other metabolic inhibitors (azides, iodoacetamide) or nonpolyene antibiotics (cycloheximide, viridin) were without effect.

Similarly, Caltrider and Gottlieb (1961) found in studies where filipin was placed in the growth media of *Saccharomyces cerevisiae* or *Neurospora crassa* that there was a leakage of vital cytoplasmic constituents from the cells. Other parallel studies on "leakage" effects

indicated that nystatin treatment of yeast protoplasts resulted in the loss of K^+ and NH_4^+ ions (Sutton *et al.*, 1961; Marini *et al.*, 1961).

These studies demonstrated that polyene antibiotic treatment caused an increase rate of leakage of cellular constituents from certain organisms, suggesting that there were changes in the permeability of the boundary membranes of these organisms. However, it was not possible to state unequivocally that the changes in membrane permeability resulted as a consequence of the action of the polyene at this specific locus. It was conceivable that the polyene antibiotic was entering the cell and by virtue of its interaction there, was causing secondary permeability changes or inhibiting endogenous aerobic and anaerobic metabolism. Gottlieb *et al.* (1961) reported that filipin inhibited both the aerobic and anaerobic metabolism of glucose, acetate, and endogenous substrate in intact yeast cells (*Saccharomyces cerevisiae*). However, no effect was observed on the rate of respiration when filipin or other polyene antibiotics were added to cell free preparations of yeast or other organisms (Gottlieb *et al.*, 1961; Sutton *et al.*, 1961). The report of Sutton *et al.* (1961) and Marini *et al.* (1961) concerning the leakage of K^+ and NH_4^+ ions from yeast cells and concomitant inhibition of glycolysis prompted further work. They found that at neutral pH, addition of K^+ and NH_4^+ to the media could reverse this inhibition. This suggested to these workers that the metabolic effects of the polyenes were a result of the leakage of some vital cytoplasmic constituents due to an alteration of cellular permeability and not ot come direct inhibition of glycolysis or some other critical metabolic events.

2. *Site of Action—the Plasma Membrane*

Since the plasma membrane is the integral structural unit of the cell responsible for the maintenance of selective permeability, it seems likely that this would be the principal site of action for the polyenes. Lampen and co-workers (Lampen and Arnow, 1959; Lampen *et al.*, 1959) found that the assimilation of the polyene nystain by fungal cells was an essential factor in the subsequent inhibition of growth of these cells. However, it was found that, in bacteria, nystatin at concentrations as high as 100 μg/ml had little or no effect on the cell growth and that, in fact, they accumulated only minimal amounts of the antibiotic. It appeared that only cells that accumulated appreciable amounts of the polyenes were sensitive to these antibiotics. These results led to the hypothesis that the polyenes interact with membrane sterols. This would explain why the fungi

were selectively sensitive and the bacteria were not, since bacteria contain no sterols in their membranes.

3. *Sterol-Binding Hypothesis*

Insight into the possible sterol polyene antibiotic relationship was first provided by Gottlieb and co-workers in a classic series of studies (Gottlieb *et al.*, 1958, 1961; Caltrider and Gottlieb, 1961). They found that addition of sterols to the growth media of *Saccharomyces cerevisiae* prevented the characteristic filipin-induced growth inhibition and permeability changes. Thus, there appeared to be a competition between filipin and sterol. It was not clear initially whether the reversal of the polyene inhibition mediated by exogenous sterol in the growth media was due to: (a) the possibility that filipin inhibited the biosynthesis of sterol necessary for membrane competence; (b) the ability of filipin to compete internally with sterol for an important cofactor necessary for membrane competence; or (c) it being simply a physicochemical interaction between the polyene antibiotic and the added sterol which prevented the antibiotic from interacting with the sterol of the organism under study. Subsequent studies have shown that the results obtained by Gottlieb and co-workers could most easily be explained by the following explanation: the addition of exogenous sterol to the growth media which contained the polyene antibiotic might, by simple competition, prevent the reaction or interaction of the antibiotic with the membrane-bound sterol. Lampen *et al.* (1962) were able to demonstrate a correlation between the binding of nystatin to subcellular fractions of *Saccharomyces cerevisiae* and the sterol content of the particular fraction. These results would be most consistent with the hypothesis that there was a physical–chemical interaction between the sterol and the polyene.

Precedents for such physical interactions between sterols and other molecules already existed. It is well known that digitonin can react with cholesterol to form a noncovalently bonded tight complex of digitonin and cholesterol (Haslam and Klyne, 1953). In this respect, it is interesting to note that the studies of Lampen *et al.* (1962) demonstrated that binding sites for polyene antibiotics existed on the membrane of organisms which contained sterol and that these sites could be removed by treatment with digitonin. Digitonin has also been shown to inhibit polyene binding to the fungal membrane by Ghosh and Ghosh (1963a,b) and by Kinsky (1964). Ghosh and Ghosh found that absorption of nystatin and amphotericin B by *Can-*

dida albicans was inhibited by digitonin. Studies with *Mycoplasma laidlawii*, now known as *Acholeplasma laidlawii*, provided interesting evidence for the sterol hypothesis. The simultaneous studies of Feingold (1965) and Weber and Kinsky (1965) utilized an interesting property of this microorganism. The organism is unable to synthesize sterols de novo, but when it is grown on a medium containing sterols it will incorporate the sterol into the cell membrane. It was found that filipin had no effect on the growth of the cells raised in the sterol-deficient medium; however, when the organism was grown in a sterol-enriched medium, filipin and amphotericin B caused inhibition of growth. Also work relating to *Pythium* sp. indicated that only when the sterol is present in the organism is this organism sensitive to filipin (Schlosser and Gottlieb, 1966, 1968; Schlosser *et al.*, 1969). Thus, there is a wide range of studies which all suggest or point to the interaction of polyene antibiotic with membrane-bound sterols as the basis for conferring sensitivity on an organism to this class of compounds.

B. Selective Toxicity to Various Organisms

The polyene antibiotics have been shown to be toxic to a number of organisms. In support of the sterol-binding hypothesis of the polyene antibiotics and their selective toxicity, it was found that only those organisms containing sterols in their plasma membranes were susceptible to polyene treatment. The polyenes have been shown to inhibit various biological activities of algae, protozoa, planaria, insects and mammals, as well as most mycelial fungi and yeasts. However, bacteria, blue-green algae, and certain fungi are found to be resistant. It is of interest that these prokaryotic organisms, the bacteria, and blue-green algae which are resistant to the polyene antibiotics are also generally found to be devoid of membrane sterols. Likewise a number of fungi that do not contain membrane sterols are also resistant to these antibiotics. Of similar interest is the finding that isolated mitochondria are also resistant to polyene interaction. Although it is known that mitochondrial membranes contain sterols, the reason for the lack of inhibition may be that sterols constitute only a small fraction of total mitochondrial lipids.

However, as a general rule, the selective toxicity of the polyenes can be predicted by the sterol hypothesis—i.e., only those organisms known to contain sterols should be sensitive to the antibiotics. A number of eukaryotic organisms known to contain membrane sterols have been found to be sensitive. Hunter and McVeigh (1961) and

Lampen and Arnow (1961) have shown that the higher algae are sensitive to the polyenes amphotericin A and nystatin.

Ghosh *et al.* (1960) have found that nystatin produced an inhibition of both endogenous and exogenous respiration in the protozoan *Leishmania donovani*. This effect could not be attributed to inhibition of the enzymes of the glycolytic or respiratory pathways directly, but was most likely due to the loss of vital intracellular constituents as a result of cell lysis (Ghosh and Chatterjee, 1961, 1963a). Subsequently, it was shown by these investigators (Ghosh and Chatterjee, 1962, 1963b) that nystatin is rapidly absorbed by the cell and preferentially bound to those fractions containing the highest sterol content. This binding effect could be inhibited by the presence of digitonin or cholesterol in the incubation media.

Similarly Seneca and Bergendahl (1955) and Johnson *et al.* (1962) have found the polyenes toxic to snails and planaria, respectively. Extensive work has been carried out on the mammalian erythrocyte and the fungi *Mycoplasma laidlawii* or *Acholeplasma laidlawii*, and these studies are further discussed in Section V, A.

Most of the above-mentioned work has dealt with simple organisms or subcellular components that possessed only one outer limiting membrane. Therefore the conclusions reached are greatly simplified and generally support the hypothesis that the presence of sterols in the membrane is a necessary condition for polyene sensitivity. However, when more complex tissues were selected for study, consisting of a variety of cell types and membrane barriers, this simplistic all-or-none effect does not hold in all cases. Sweeley *et al.* (1970) in studying the effects of polyene toxicity in insect larvae, fed the polyene filipin in the diet and found that death was due, not to any alteration of the alimentary canal membrane, but to the complexing of dietary cholesterol. Thus the larvae for whom cholesterol is a vitamin died of cholesterol starvation.

In a number of studies on the intestinal transport of various solutes in a number of vertebrate species, it was found that the polyenes (a) reacted only with the mucosal membrane surface, not the serosal surface; (b) were sometimes more reactive with tissue obtained from the large intestine than from the small; (c) did not appear to cause a general alteration of the membrane, resulting in a general permeability change. It was also found that (d) pretreatment of the serosal membrane surface with a general proteolytic enzyme made the serosal surface more susceptible to polyene treatment. All these results suggest that although the presence of sterols in a membrane is a necessary condition for polyene sensitivity, it is not the only requirement.

IV. Molecular Basis of Polyene Antibiotics—Interaction with Membrane Sterols

A. Spectrophotometric Studies

As emphasized previously, polyene antibiotics have a highly characteristic UV absorption spectrum, the absorption maxima having large molar extinction coefficients. Lampen *et al.* (1960) reported that when sterols were added to aqueous solutions of the polyene antibiotics, filipin or nystatin, the UV absorbance values decreased significantly. They suggested that this might be indicative of a lower solubility of the polyene antibiotic, and they noted that addition of organic solvents reversed the changes seen in the aqueous media. There was some suggestion with filipin, which had the largest spectral changes, that a direct interaction had occurred between the added sterol and the polyene. Also Gottlieb *et al.* (1961) reported similar results. However, the results obtained by both groups were not as striking as that reported below; accordingly, it was more difficult to make definitive conclusions from the earlier studies about the possibility of a direct interaction between the sterol and the antibiotic.

No further advances were made for the next 10 years until simultaneously Kleinschmidt *et al.* (1972), Schroeder *et al.* (1971, 1972, 1973), and Norman *et al.* (1972a,b) all reported striking effects on the polyene UV absorption spectrum after addition of sterol to aqueous solutions of the antibiotic. A typical example of the spectral changes seen when cholesterol is added to an aqueous solution of filipin is given in Fig. 4.

Schroeder *et al.* (1972, 1973) studied the fluorescence spectrum of filipin rather than its UV absorption spectrum; the results obtained are essentially in agreement with those of the other two groups, who studied only the UV spectral changes. They reported that filipin in water had a characteristic fluorescence spectrum, which was markedly decreased by the addition of cholesterol. They also measured changes in the partial quantum efficiency and calculated "corrected" fluorescence values. They found that addition of cholesterol decreased the partial quantum efficiency and corrected fluorescence values by 36% and 62%, respectively. Such changes were interpreted as being indicative of a direct molecular interaction between the sterol and filipin. They also found that addition of cholesterol increased the partial quantum efficiency and corrected fluorescence values of pimaricin more than 80-fold. In contrast, little or no change

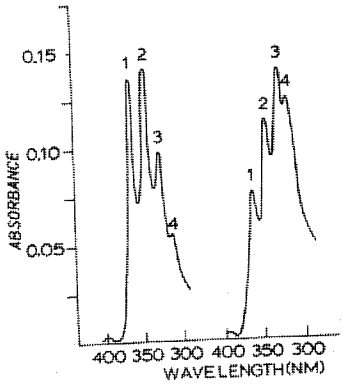


FIG. 4. Spectrum of filipin–cholesterol complex. To 10 ml of a 1.2×10^{-5} *M* aqueous solution of filipin was added 25 μl of cholesterol in ethanol so that the final concentration of sterol was 10 μg/ml or 2.8×10^{-5} *M*. The ultraviolet absorption spectrum was measured as shown on the right-hand side; the adsorption maxima of peaks 1, 2, 3, and 4 are 356, 338, 320, and 310 nm, respectively. This solution was then diluted by half with methanol, and the spectrum was determined again as shown on the left-hand side. Before methanol dilution the ultraviolet absorbance ratio of peak 3 to peak 1 was 2.47; after dilution, it was 0.83. The spectrum obtained from the filipin in methanol is corrected for dilution 1:1.

was noted on the fluorescence emission spectra when cholesterol was added to aqueous solutions of nystatin or amphotericin B.

Norman *et al.* (1972a) carried out a detailed study of the spectral changes that occur when a variety of sterols were added to filipin. The conclusion drawn was that filipin can interact with sterol in a stereochemically and stoichiometrically defined manner to produce a filipin–sterol complex or adduct. As shown in Fig. 4, filpin has a characteristic UV absorption spectrum with four maxima. The ratio of absorbance of peak 3 (λ = 320 nm) to peak 1 (λ = 356 nm is 0.7 in organic solvents and 0.8 in aqueous solution. It was found that addition of cholesterol caused a striking spectral change (see Fig. 4) in aqueous solutions of filipin. The consequences of this change were such that the absorption maxima did not shift, but the ratio of absorbance of peak 3 to peak 1 changed from a value of 0.8 to a value of approximately 2.5. Clearly the cholesterol had to come into close association with the conjugated double-bond system of the filipin molecule to cause or induce such a dramatic spectral change. The formation of the complex, as defined by the spectral change, was specific only for sterols; no spectral changes were noted upon addition of bovine serum albumin, lecithin, galactose, 4 *M* NaCl, 4 *M* urea, or variation of pH from 2 to 10. In contrast, the addition of a nonionic

detergent Triton X-100, or organic solvents which were miscible with water, such as dioxane or methanol, abolished the spectral changes and by inference meant that they broke or dissociated the filipin–cholesterol complex.

Similar spectral changes were noted when filipin was added to aqueous preparations of liposomes which contained cholesterol (Norman *et al.*, 1972a). In the absence of liposomally bound sterol, there was no spectral change. The spectral change was also evident in red cell ghost membranes and in cells and membrane fractions obtained from *A. laidlawii* grown in the presence of cholesterol (Fig. 5). Most significantly, no spectral change was evident in cells or membranes obtained from *A. laidlawii* grown in the absence of cholesterol or in the presence of epi(3α-hydroxyl)cholesterol (Norman *et al.*, 1972b; DeKruijff *et al.*, 1974a,b). Thus, in summary, the filipin spectral change has been shown to be totally dependent upon the presence of sterol irrespective of whether it was free in solution, bound in a liposomal bilayer, or present in a naturally occurring membrane.

A study of the sterol structural requirements necessary for this optimal interaction indicated that, with the free sterol in water, the interaction is primarily hydrophobic in nature, the presence of a cholestane ring structure and a Δ-22 double bond producing the most favorable interaction (Norman *et al.*, 1972a). Most interestingly, an additional structural requirement is evident in the liposomal system and membrane system in that it was shown unequivocally that a 3β-hydroxyl on the steroid nucleus was critically essential. That is to say, cholesterol with a 3β-OH on the steroid nucleus was effective whereas epicholesterol with a 3α-OH was totally unable to produce

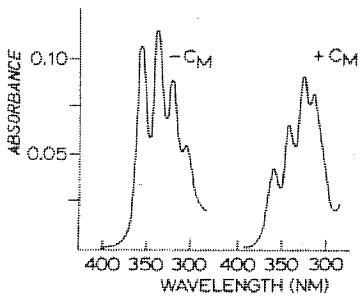


FIG. 5. Interaction of filipin with cells of *Acholeplasma laidlawii*. Cell suspensions of *A. laidlawii* grown in the absence of sterol or in the presence of cholesterol were obtained. Identical suspensions of the cells in 0.20 *M* sucrose were placed in the reference and sample cuvettes of a split-beam spectrophotometer. After a stable base line was obtained, filipin was added in 10 μl of Tris-acetate, pH 7.0, to the sample cuvette, giving a final concentration of 9.4×10^{-6} *M* of filipin.

the characteristic spectral change in filipin. Essentially similar observations were noted by Kleinschmidt *et al.* (1972), who studied spectral changes that occurred upon filipin addition to aqueous preparations of liposomes that contained a variety of sterols. They correlated spectral changes with changes in permeability of the liposomes, which contained either bound glucose or bound 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB).

Also the laboratory of Bittman (Bittman and Blau, 1972; Bittman and Fischkoff, 1972; Bittman *et al.*, 1974a,b) has employed ultraviolet, fluorescence, and circular dichroism spectral studies and stopped-flow kinetic and equilibrium studies in combination with electron microscope methods to investigate the nature of the interaction between polyene antibiotics and steroids. They reported (Bittman *et al.*, 1974b) that the rate and degree of binding of filipin III to cholesterol-containing liposomes depended upon both the absolute concentration of steroid and its mole percent in the bilayer. Changes in the UV and fluorescence polarization of filipin III in vesicles with differing lipid compositions were used to determine apparent binding constants. At 25°C the K_D values for the binding of filipin to sterol-lecithin lysomes were cholesterol, 0.80 μM, epicholesterol, 13 μM, and ergosterol 2.5 μM whereas to vesicles composed of only lecithin the K_D was 144 μM.

From the foregoing results, it is apparent that filipin is capable of interacting in a stereospecific manner with sterols to form some kind of loose adduct or associated complex which results in different UV-absorbing properties for the filipin moiety. Norman *et al.* (1972b) and DeKruijff *et al.* (1974a,b) and DeKruijff and Demel (1974) have also carried out similar studies with four additional polyene antibiotics—etruscomycin, pimaricin, nystatin, and amphotericin B—while Bittman *et al.* (1974a) have extended their studies to amphotericin B. Each of these antibiotics has its own characteristic UV absorption spectra in aqueous and organic solvents. The addition of free cholesterol to aqueous solutions of these antibiotics resulted in a general lowering in the extinction coefficient of the 3–4 absorption maxima. The order of effectiveness of interaction with cholesterol as judged by this criterion was filipin > amphotericin B > etruscomycin > nystatin = pimaricin. Additionally, it was observed that etruscomycin and amphotericin B, like filipin, were also capable of interacting with sterol bound to liposomes or to membrane-bound sterol of erythrocyte ghosts or *Acholeplasma* membrane-bound cholesterol as denoted by UV spectral changes. No evidence of interaction was observed in the absence of cholesterol. Additionally,

neither nystatin nor pimaricin showed little evidence of interaction, as judged by an absence of UV spectral change.

B. Differential Scanning Calorimetric Studies

As a consequence of the foregoing studies, it was felt that several polyene antibiotics, particularly filipin, were capable of interacting directly with sterol whether it be free in solution or membrane found. Norman *et al.* (1972b) additionally observed in studies employing *Acholeplasma* or erythrocyte ghost membranes that there was a good correlation between spectral change and binding of the antibiotic in question to the membrane fraction. When suspensions of membrane fractions in aqueous solutions of polyene antibiotics were sedimented, it was observed that the polyene antibiotics were bound tightly to the membranes and sedimented with them in only those instances where there was evidence of a spectral change. This observation plus other studies on effects of polyene antibiotics on membrane permeability led to the suggestion that the interaction of the polyene antibiotic with the membrane might lead to measurable changes in the physical properties of the membrane preparation. This possibility was tested directly by carrying out differential scanning calorimetric studies using preparations of chemically synthesized lecithin with a defined fatty acid composition (Norman *et al.*, 1972b). Such lecithins exhibit sharp phase transitions over a narrow temperature range, characteristic of the conversion from the crystalline L (α) to liquid–crystalline L (β) phase (Ladbrooke and Chapman, 1969). The exact temperature of the phase transition is determined by both the fatty acid composition and polar group. This transition is believed to reflect melting of the fatty acid hydrocarbon chains from an ordered to a more random structure. It was also known that the addition of cholesterol, up to 50 mole percent, to the synthetic lecithin abolishes the phase transitions (Ladbrooke *et al.*, 1968). Apparently, incorporation of cholesterol leads to fluidization of the hydrocarbon chains.

It was found (Norman *et al.*, 1972a) that addition of filipin to lecithin–cholesterol dispersions caused a complete reappearance of the phase transition characteristic of pure lecithin. In a control experiment, filipin addition to a pure lecithin dispersion caused no alteration in the amount of energy required for a phase transition. Filipin addition to a lecithin–cholesterol dispersion leads to formation within the dispersion of the putative filipin–cholesterol complex. Formation of this complex abolishes the fluidizing effect that the

sterol has on the hydrocarbon chains, and as a consequence they are capable of undergoing the phase transition from an ordered status to the melted or random status. Thus, the filipin addition to a lecithin–cholesterol dispersion is capable of mediating a striking change in one of the physical properties of the sample. Parallel differential scanning calorimetric studies carried out by Norman *et al.* (1972b) indicated that etruscomycin, pimaricin, nystatin, and amphotericin B, like filipin, were also capable of causing the appearance of the phase transitions of lecithin–cholesterol dispersions.

More recently, DeKruijff *et al.* (1974b) extended these differential scanning calorimetric studies to an examination of the effects of polyenes on the energy content of the gel liquid–crystalline phase transition in cholesterol-containing membranes obtained from *A. laidlawii* grown on elaidic acid. These workers obtained conclusive proof that polyene antibiotics form complexes with cholesterol in the *A. laidlawii* cell membrane.

Although it is not known definitely whether such a change in phase transition could specifically lead to a more permeable liposome bilayer, these results are highly suggestive of the concept that filipin addition to sterol-containing liposomes or membranes can lead to a change in molecular architecture of the systems. Such structural changes might provide the basis for a physical description of the filipin-mediated increases in permeability of (a) liposomes to glucose and chromate (Kinsky *et al.*, 1968; Sessa and Weissman, 1968), (b) intestinal mucosal tissue in vitamin D-deficient chicks to only calcium (Adams *et al.*, 1970), and (c) a black lipid film to primarily calcium (Van Zutphen *et al.*, 1966, 1971).

C. Stoichiometric Studies of the Filipin–Sterol Complex

The first effort to define the stoichiometry of the interaction of filipin with cholesterol was carried out in monolayer studies by Demel *et al.* (1965). They found that one filipin molecule per one hundred molecules of a monolayer of sterol could cause a significant pressure increase. It is likely, however, that their results were "system dependent," since the collapse pressure of cholesterol in the monolayer was already reached at filipin concentrations as low as 6×10^{-9} *M*.

Additional studies on the stoichiometry of filipin–cholesterol interaction have been obtained in several laboratories by taking advantage of the aforementioned UV and fluorescence spectral changes.

Katzenstein (1973) used aqueous solutions of filipin–cholesterol, and Spielvogel (1973) utilized lipsomal solutions in the presence of filipin; both observed that there appeared to be a 1:1 cholesterol:filipin relationship to produce a maximum spectral change. Their results agree with the earlier more preliminary studies of Norman *et al.* (1972a,b). The results of Schroeder *et al.* (1972, 1973) are in excellent agreement with these results. Utilizing their fluorescence measurement, quantum efficiency, and "corrected" fluorescence measurements, they ascertained that the stoichiometry of the filipin:cholesterol interactions was 1:1 within their experimental error. They also obtained evidence in their fluorescence studies that the filipin molecule was capable of existing in two conformations. Only one of these conformations was capable of interacting with sterol under their experimentally defined conditions to produce a fluorescence change. However, Bittman *et al.* (1974b), who also employed fluorescence measurements, while agreeing with the stoichiometry of the filipin–sterol complex, did not find evidence supporting the existence of two conformational species of free filipin.

Norman *et al.* (1972b) were also able to make some estimates of stoichiometry from their differential scanning calorimetric measurements. They calculated that the number of cholesterol molecules that could be complexed or associated with filipin, etruscomycin, pimaricin, nystatin, and amphotericin B was, respectively, 1.2, 0.6, 1.7, 1.2, and 3.9 after correction for the amount of cholesterol in the liposome not available for the polyene antibiotic (see DeKruijff *et al.*, 1974a). Thus, these results are in reasonable agreement with those obtained from the direct spectral measurements. The calorimetric measurements suffer from the fact that the experiments have to be carried out in very highly concentrated solutions, in which the polyenes can aggregate to varying degrees. It is very likely that this extent of aggregation is different from that which exists in dilute concentrations of filipin in aqueous solution.

Most recently Demel and co-workers (DeKruijff *et al.*, 1974a,b; Dekruijff and Demel, 1974) further examined, in an unusually thorough study, the specificity of membrane permeability and the temperature dependence of the polyene–sterol complex formation in membranes of *A. laidlawii* and lecithin liposomes for the series filipin, amphotericin B, etruscomycin, nystatin, and pimaricin. They observed that when cholesterol was present in the membrane, the different polyene antibiotics produced both permeability changes and gross morphological changes—as monitored by freeze-etch electron microscopy—which were different for the various antibiotics.

They obtained quantitative evidence that (a) filipin disrupted the membrane structure so that both small (K^+) and large (glucose-6-phosphate dehydrogenase) molecules leaked out; (b) amphotericin B, nystatin, and etruscomycin produced specific permeability changes that were consistent with the formation of aqueous pores 8 Å in diameter, after complexation with the sterol; and (c) pimaricin was unable to produce permeability changes.

These experimental data became the factual basis on which DeKruijff and Demel (1974) proposed specific and detailed molecular models of the individual polyene–sterol complexes which accommodated essentially all the known data. In essence these postulates define the necessary degree of precision required for the stoichiometry measurement described above. The amphotericin–B-cholesterol complex was envisioned as a circular arrangement of 8-amphotericin B molecules interdigitated by 8 cholesterol molecules. The outside of the complex is hydrophobic whereas the inside is exclusively hydrophilic due to the multiple hydroxyl groups asymmetrically located on amphotericin B. Two such complexes (half pores) will generate a pore which is capable of traversing a membrane with an aqueous channel 8 Å in diameter.

The etruscomycin–cholesterol and nystatin–cholesterol complexes were also postulated to exist as pores of essentially the same diameter. However the pimaricin–cholesterol complex would not be able to form a conducting pore since twice the length of the "half pores" is considerably less than the cross-sectional width of a lipid bilayer.

Finally the filipin–cholesterol complex is believed not to be able to form "half pores." Instead, the orientation of the two components is such that an aggregate of filipin-cholesterol some 150–250 Å in diameter occurs in the hydrophobic core of the bilayer. The presence of this aggregate then leads to gross membrane fragmentation. The reader is referred to DeKruijff and Demel (1974) for an excellent representation of the various complexes that have been constructed from space-filling molecular models.

It may be possible in the future to further confirm the stoichiometry of these polyene antibiotics with a sterol. If it should prove possible to crystallize a definite compound or complex, then it might be anticipated that X-ray crystallographic studies could be carried out. Only then would it be known conclusively whether filipin or other polyene antibiotics are capable of interacting directly with the sterol. Moreover, it would be possible to ascertain whether the polyene (P) sterol (S) was of the type of (PS) as compared with $(PS)_n$.

V. Polyene Antibiotics in the Study of Membrane Structure–Function Relationships

A. Natural Membranes

The availability of agents that interfere specifically with the biosynthesis and/or structure of complex macromolecules has played an important role in the elucidation of the structure of nucleic acids, proteins, and bacterial cell wall components. Early studies on the mode of action of polyene antibiotics suggested that a primary consequence of their action in biological systems was to cause permeability changes. It has now been shown that only organisms that contain sterols are affected by the polyenes and exhibit the characteristic sensitivity to these compounds. Recently it has been suggested and demonstrated that these compounds can be utilized as useful tools for investigating membrane architecture and functional properties.

1. *Fungal Plasma Membrane*

A number of studies with the pleuropneumonialike organism *Mycoplasmia laidlawii*, now referred to as *A. laidlawii*, have provided direct evidence that the presence of sterols is a prime prerequisite for polyene sensitivity. This unique organism has the ability to grow and synthesize membranes with or without a sterol component, since it cannot biosynthesize its own sterols. However, when the growth medium of the *Acholeplasma* contains sterol, it grows normally but also incorporates the exogenous sterol in an integral fashion into its membranes. Thus it is possible to control the sterol content of the membrane and to test the biological interaction of the polyenes with these natural membranes. The early experiments of Weber and Kinsky (1965) and Feingold (1965) utilizing filipin and amphotericin B, respectively, readily verified the need for sterol for polyene activity. It was found that filipin and amphotericin B had no effect on *A. laidlawii* growth when the cells were cultured on a medium devoid of cholesterol. However, when this same organism was grown on a cholesterol-enriched medium, filipin exhibited its typical lytic action and prohibited cell growth. Both groups observed that cells grown on a cholesterol-rich medium gradually became resistant to the polyenes when switched to a cholesterol-free medium and grown at 37°C; however, polyene sensitivity was retained when the incubation was carried out at 4°C. The change in polyene sensitivity appeared to parallel the dynamic reassembly or turnover of membrane

components as evident by changes in the cholesterol content of the cell (Feingold, 1965) and change in the sensitivity to other lytic agents, such as digitonin, which is known to complex with cholesterol (Weber and Kinsky, 1965).

Precedents for such physical interaction between sterols and other molecules already existed. It is well known that digitonin can react with cholesterol to form a noncovalently bonded tight complex of digitonin and cholesterol (Haslam and Klyne, 1953). In this respect, it is interesting to note that the studies of Kinsky *et al.* (1966) demonstrated that binding sites for polyene antibiotics existed on the membrane of organisms that contained sterol and that those sites could be removed by treatment with digitonin (Lampen *et al.*, 1962).

Kinsky (1963) reported that there was no demonstrable decrease in the total ergosterol content, or a change in the ratio of unesterified to esterified sterol, in species of *Neurospora* which had been exposed to damaging concentrations (30% decline in dry weight) of filipin, amphotericin B, or nystatin. It was concluded from these studies that the polyenes do not act like detergents resulting in the selective extraction of the sterol from the plasma membrane. Freeze-etch electron microscopy demonstrated that the polyene antibiotic filipin, but not amphotericin B, induces the formation of aggregates 150–250 Å in diameter (or "pits") in cholesterol-containing membranes from *A. laidlawii*. However, it is suggested that it is unlikely that enhanced permeability of the membrane is through these aggregates. These aggregates cannot be considered as pores since no "through and through" holes were visible on the etched faces (Verkleij *et al.*, 1973; DeKruijff and Demel, 1974).

In a most thorough study, Bittman *et al.* (1974a) correlated filipin III interactions with the membrane-bound sterol of *Tetrahymena pyriformis* W as monitored by UV, fluorescent, and circular dichroism (CD) spectral studies with negative-staining electron micrographic-monitored changes in morphology of the organisms membranes. They found it possible to relate polyene-mediated pit formation with changes in the spectral properties of the antibiotic. They proposed that pit formation occurs by a process of micelle-mediated fusion of lipid bilayers.

2. *Erythrocytes*

Application of the polyenes to humans in the treatment of mytotic infections may also lead to hemolytic anemia and renal damage. Kinsky *et al.* (1962) and Kinsky (1963) have demonstrated that amphotericin B and filipin induce a rapid hemolysis of rat and human

erythrocytes. These results are consistent with the sterol hypothesis since erythrocytes membranes are known to contain cholesterol. The extent of hemolysis was found to be dependent on the concentration ratio of antibiotic to cell; the effect of different osmotic stabilizers to polyene lysis indicates that mammalian erythrocytes and fungal protoplasts are analogous in their response to polyene antibiotic treatment (Kinsky, 1963). Additionally, Kinsky *et al.* (1967b) found that perhydrofilipin, UV-irradiated filipin, or filipin which had its lactone ring opened, were ineffective in terms of causing permeability changes. Thus the permeability changes are dependent upon the presence of an intact polyene molecule.

It was found that the antibiotics caused increasing damage to the membrane in the following order: nystatin, pimaricin, candidin, amphotericin B, etruscomycin, and filipin (Kinsky, 1963). Filipin appears to cause a structural change in sterol-containing membranes when examined microscopically. Electron microscopic examination of natural and artificial membranes, negatively stained with phosphotungstate showed the appearance of pits following filipin treatment (Kinsky *et al.*, 1966, 1967b). Rat erythrocyte ghosts obtained by lysis of cells with filipin showed pits approximately 100–200 Å in diameter (Kinsky *et al.*, 1966). These pits were apparent in erythrocyte membranes prepared by lysis with not only filipin, but also amphotericin B or nystatin. They were not found in erythrocyte ghosts obtained by lysis with water. Treatment with filipin of artificial lipid dispersions or monolayers containing lecithin and sterol resulted in the formation of pits in the film. No pits were observed in monolayers containing only lecithin (Kinsky *et al.*, 1966, 1967b). Freeze-etch studies carried out on rat erythrocytes by Verkleij *et al.* (1973) and Tillack and Kinsky (1973) revealed the same results as those reported earlier for the work on *A. laidlawii*. Although structural alterations (pits, doughnut-shaped craters, and protusions) in the erythrocyte membrane were apparent, examination of the inner and outer fracture faces did not indicate that these structural changes had resulted in holes or pores through the membrane.

3. *Subcellular Organelles*

It has been suggested by Kinsky *et al.* (1966) that, although the presence of sterol is a necessary criterion for polyene sensitivity, it is not the only factor involved. A number of subcellular membrane systems appear to be unaffected by polyene treatment. Micrographic examination of protozoa and fungi (Ghosh and Chatterjee, 1963a; Gale, 1963) show that nuclei and mitochondria remain intact, even at

high concentrations of polyenes which are sufficient for lysis and leakage of the cell. Studies with isolated organelles, such as mitochondria from rat liver of *Neurospora*, have shown that none of the functions of this organelle (e.g., oxidative phosphorylation, electron transport, and ion transport) are affected by incubation with high concentrations of polyene (Kinsky *et al.*, 1965). *Neurospora* mitochondria shown to contain ergosterol were still viable after binding filipin. However, the total sterol content is low in the mitochondria and constitutes only a small portion of the total lipids present (Kinsky *et al.*, 1965). It may be possible that other lipids play a critical role in determining whether or not membrane is sensitive to the polyene, even when cholesterol is present in the membrane. It has been suggested by Kinsky *et al.* (1966) and Van Zutphen *et al.* (1966) that the relative proportion of cholesterol to phospholipid may be the key relationship in this selection factor. Recently Hsu Chen and Feingold (1973) reported that the presence of cholesterol in liposomes derived from egg lecithin were more sensitive than those derived from dipalmitoyl or distearoyl lecithins to nystatin or amphotericin B. In fact, the latter two suppressed the activity of the antibiotics. Thus it appears that polyene antibiotics toxicity may also be dependent on the fatty acid composition of the phospholipid.

Similar results have been reported when whole organ tissues were tested in various vertebrates. Lippe and Giordana (1967) and Adams *et al.* (1970) have found that the polyenes were selective for only various segments of the alimentary canal tissue (e.g., small intestine versus colon), the membrane surface treated (mucosal versus serosal), and differences between species reactivity when the same segment of tissue was tested. It has also been suggested by Mondavi *et al.* (1971) that certain polyenes, such as filipin and lucensomycin, have a greater affinity for malignant cell membranes than those of normal cells.

4. *Complex Transport Systems*

A number of complex transport systems have been investigated utilizing polyene antibiotics as a specific membrane tool. Sharp *et al.* (1966) were able to effectively use a polyene antibiotic, amphotericin B, in evaluating the role of aldosterone in sodium transport in the toad bladder. Sharp and Leaf (1966) found that the aldosterone response could be mimicked by the polyene antibiotic amphotericin B when the latter was placed only in the mucosal solution. They suggested that this effect was caused by an altered permeability of the mucosal plasma membrane and inferred that aldosterone was

causing a similar effect via an induction mechanism. Lichtenstein and Leaf (1966) found that other small molecules (potassium chloride and thiourea) were transported more rapidly after amphotericin B or nystatin treatment, but that net water flux was unaffected. Mendoza, Handler, and Orloff (1967) have also found that amphotericin B increases the osmotic permeability of the toad bladder.

A further confirmation of the permeability effect of amphotericin B on sodium transport was reported by Sharp *et al.* (1966). They found that amphotericin B increases the short-circuit current (i.e., Na^+ flux) and potential difference across the membrane which results in a secondary stimulation of sodium transport due to the oxidation of pyruvate, acetoacetate, glucose, oxoaloacetate, or lactate. This is similar to the effect following aldosterone treatment, in which there is an increase in aerobic metabolism associated with the stimulation of Na^+ transport by this hormone. It is known that this effect is dependent upon sodium being present in the mucosal bathing medium. Thus amphotericin B elicits its effect by reducing the permeability barrier to sodium at the mucosal surface and then secondarily increases aerobic metabolism in a manner mimicking the corticoid steroid aldosterone.

Bentley (1968) found that amphotericin B increased Na^+ flux only at high sodium concentration and that this stimulation of transport was insensitive to cyanide or iodoacetate poisoning. In contrast, the hormones vasopressin and aldosterone elicited their response at much lower Na^+ concentrations. Therefore Bentley proposed two pathways for sodium transport across the toad bladder: (a) one pathway is mediated by aldosterone, which operates at low substrate concentrations, and is a saturable process that may be considered to be the inducible sodium transport system (Fanestil *et al.*, 1967); (b) the other pathway involves a nonphysiological response mediated by amphotericin B, which is probably indicative of passive diffusional effects.

Lippe and Giordana (1967) studying thiourea transport in the large intestine of the turtle found that amphotericin B preferentially stimulated the thiourea flux but had little or no effect on the flux in the small intestine. Likewise, amphotericin B also only preferentially increased permeability of the membrane when placed on the mucosal surface, but not on the serosal surface, of the tissue. They concluded that this was linked to some unknown difference in the cell membrane composition, possibly a protein. Preincubation with proteolytic enzyme(s) rendered this previously insensitive serosal surface sensitive to amphotericin B treatment (Lippe *et al.*, 1968).

In an interesting series of experiments, Norman and co-workers (Adam *et al.*, 1970; Wong *et al.*, 1970) employed filipin as a membrane selective tool to study some aspects of vitamine D-mediated intestinal calcium transport. Dietary vitamin D treatment causes a 2- to 3-fold increase in the rate of translocation of calcium across the small intestine. These investigatore further characterized this system as being an active, cation-oriented process which is (a) sensitive to cold, (b) inhibited by *N*-ethylmaleimide, and (c) has an effective calcium carrier at the microvillar side of the cell. The vitamin D-deficient (rachitic) chick exhibited none of these characteristics. It was found that addition of filipin ($\sim 10^{-5}$ *M*) *in vitro* to the mucosal surface of intestinal cells from vitamin D-deficient chicks stimulates the transport of calcium 2- to 3-fold, but has little or no stimulatory effect on intestinal cells from vitamin D-repleted animals. The effect of filipin was found to be specific for calcium with little or no effect on Rb^+, SO_4^{2-}, P_i, serine, thiourea, or water transport. This specific translocation of calcium was shown not to be due to some ionophoretic property of the polyene or general permeability change of the membrane.

The incubation of filipin *in vitro* with intestines from vitamin D-deficient animals mimicked many aspects of dietary vitamin D treatment. The conclusion of these studies was that filipin is apparently able to effect a structural reorganization of vitamin D-deficient mucosal membrane so that a previously inactive calcium transport system becomes active.

Thus there is a wide range of studies which all suggest or point to the interaction of polyene antibiotics with membrane-bound sterol as the basis for conferring sensitivity on an organism to this class of compounds. However, from such biological studies, the molecular basis of action of the polyene antibiotic is not directly ascertainable. Also the consequences of the action of the polyene upon the membrane are not readily discernible. Why does the membrane become more permeable? At the present time there is no generally accepted model of membrane structure, nor is there a detailed understanding of the precise arrangement of proteins, lipids, and particularly sterol in the membranes. It is quite likely that different membranes are highly specialized and differentiated for different biological functions. An additional challenging question concerns the function of sterols in membranes. The view is emerging that sterols play an essential role in stabilizing the structure of cell membranes and in that process make the membranes less permeable. Any of the following review articles provide a discussion of current concepts relating to

membrane structure–function relationships: Danielli and Davison (1952), Robertson (1959), Kavanaugh (1966), Korn (1966), and Bangham (1972).

B. Effects on Artificial Membranes

In an effort to obtain more detailed information as to the possible mode of action of the polyene antibiotics with membrane sterol, studies have been carried out with a variety of model membrane systems. These include liposomes, "black film" bilayers, and monolayer systems. The results from each system will be discussed separately.

1. *Studies with Liposomes*

Liposomes (liquid spherules, liquid crystals, smectic mesophases, Bangosomes) are dispersions of lipids in aqueous solutions. Depending on the procedure for formation, multilayered onionlike spherules are obtained or unilamellar vesicles are formed. The permeability properties of liposomes have been studied either by trapping radioactive markers and following their release or by using the observation that liposomes behave as ideal osmometers (Bangham *et al.*, 1965) and as such exhibit linear swelling rates when placed in a hypertonic solution. Liposomes consisting mainly of uninterrupted bilayers exhibit a low permeability for ions but high permeabilities for small nonelectrolytes, which is in agreement with the passive diffusion pattern of biological membranes and consistent with our current concept of membrane permeation (Bangham *et al.*, 1967). In contrast to biological membranes, liposomes offer the advantage that the lipid composition can be varied under controlled conditions and the influence of these variations on membrane permeability can be investigated. Some of the most extensively studied parameters are the variation of the fatty acid composition of the phospholipids (Papahadjopoulos and Watkins, 1967), the use of charged phospholipids (Haest *et al.*, 1972), and the absence and presence of sterols (DeKruijff *et al.*, 1972). The latter point is of course of particular interest in view of the proposed mechanism of action for polyene antibiotics. DeKruijff *et al.* (1972) studied the glycerol and erythrytol permeability of liposomes prepared from lipids isolated from sterol-free or cholesterol-containing *A. laidlawii* lipids. They observed that incorporation of cholesterol into the bilayer strongly reduced the permeability of these liposomes for these two compounds. Additionally they observed a striking structural requirement for the sterol

moiety. Epicholesterol (3α-hydroxycholesterol) was not capable of reducing the membrane permeability.

Studies by Papahadjopoulos *et al.* (1972) demonstrated that cholesterol also reduces the permeability of ions through liposomes. The differential effect of cholesterol on the activation energy of diffusion on cations on the one hand and anions and glucose on the other hand has been used as a basis to propose a difference in their permeation mechanism.

Liposomal systems have been used by a variety of workers to study the effects of polyene antibiotics on their permeability. Weissman and Sessa (1967) and Sessa and Weissman (1968) have studied the rate of release of glucose and the ions phosphate and chromate, which were trapped inside liposomes that either did or did not have cholesterol included in the membrane structure. They reported that nystatin, amphotericin B, etruscomycin, and filipin could enhance the rate of release of these various compounds. They placed the antibiotics in the aqueous solution of the liposomes at the high concentration of 10^{-4} to 10^{-3} *M*. In contrast to the results of other workers, they noted that only amphotericin B and nystatin, but not filipin or etruscomycin, were twice as effective in cholesterol-containing liposomes. Kinsky *et al.* (1968) carried out similar studies and were able to demonstrate very nicely that the presence of cholesterol in a liposomal membrane markedly enhanced the ability of the polyene antibiotics, in particular filipin, to increase the rate of leakage of entrapped glucose. In these studies the antibiotic was present at a concentration of 10^{-6} to 10^{-5} *M*. It is quite likely that at the higher levels of antibiotic certain nonspecific effects may arise that do not require specifically the presence of sterol.

Sessa and Weissman (1968) in a later study, carried out a comparison of the effects of filipins II and III on liposomal permeability. They observed that the individual filipin compounds had varying activities with regard to stimulating the release of chromate from liposomes. Filipin III, which is believed to be the major component of the crude filipin "complex," showed the most specificity with regard to a cholesterol requirement, and filipin II was capable of eliciting permeability increases in the absence of sterol.

Kinsky *et al.* (1966, 1967b) have carried out an electron micrographic study of the effects of filipin on lecithin or lecithin–cholesterol liposomes. They made the interesting observation, using the technique of negative staining, that filipin was capable of creating "pits" in the lecithin–cholesterol dispersons. Similar pitting was also noted on filipin-treated rat erythrocyte membranes. It was apparent from

these studies that the polyene antibiotics have the capability of bringing about a structural rearrangement of the lipids present in both the natural and artificial membranes. The change was so great that it could be observed via electron micrographic analysis. The effects were found to be totally dependent upon the presence of cholesterol, and these workers suggested that the lipid rearrangement which occurred was the basis for the molecular action of polyene antibiotics. It is now known, though, from electron micrograph freeze-etch studies that these pits are not pores (Tillack and Kinsky, 1973; Verkleij *et al.*, 1973).

More recently, both DeKruijff *et al.* (1974a) and Bittman *et al.* (1974a) have been able to correlate the rate of interaction of filipin and other polyenes with liposomal sterol with the rate of antibiotic-induced permeability changes of the liposomes. DeKruijff *et al.* (1974a) proposed that the permeability changes with filipin must be the result of a fragmentation of the membrane caused by the presence or formation of the 150–250 Å diameter filipin–cholesterol complexes which can be visualized with electron microscopy. DeKruijff and Demel (1974) envision the filipin–cholesterol complex as an aggregate of 150–250 Å in diameter oriented in the hydrophobic core of the liposomal membrane, perpendicular to the array of the membrane. This aggregate they suggest is composed of two regular arrays of stacked filipin molecules such that the exterior of this aggregate is hydrophobic owing to the presence of the double bonds of filipin to which stoichiometric amounts of cholesterol are bound. As discussed earlier, the presence of this aggregate leads not to pore formation, but to membrane fragmentation and concomitant increase in membrane permeability. This paper by DeKruijff and Demel (1974) is clearly a landmark in the polyene antibiotic literature.

2. *Studies with Black Film Bilayers or "Thin" Lipid Membranes*

"Thin" lipid membranes are very thin films which are formed across an opening in a septum that separates two aqueous phases. They are believed to consist of essentially a unit membrane. The films are so thin that under normal conditions they reflect light and visually appear black when viewed against a dark background. Accordingly, they are also known as "black films." Henn and Thompson (1969) have reviewed their properties in some detail.

Since the black film acts as a partition between two compartments, this system has the advantage of permitting electrical measurements to be made of potential difference or capacitance changes that may exist across the bilayer. Owing to the fact that the thin lipid mem-

branes are very fragile and small (their cross sectional area is approximately 0.5–1.0 nm), it has not been readily possible to use them to study the actual transfer of solute across the membrane. Van Zutphen *et al.* (1966) first utilized black film preparations for studying the effects of polyene antibiotics. They found that black films consisting of lecithin–cholesterol, but not those consisting only of cholesterol, were sensitive in terms of survival to the presence of very low concentrations (10^{-7} *M*) of filipin. Maximum instability to the presence of the polyene antibiotic occurred at a sterol, phospholipid ratio of 1:1. Nystatin was also found to decrease the stability of lecithin–cholesterol black films.

This work was followed shortly by the work of Finkelstein and Cass (1968), Holz and Finkelstein (1970), Cass *et al.* (1970), Andreoli and Monahan (1968), and Andreoli *et al.* (1969). These workers carried out extensive electrical measurements on their black film preparations and studied the consequences of adding various polyene antibiotics. They observed that nystatin and amphotericin B increased the permeability of these black films to various ions, water, and other nonelectrolytes. They suggested that these antibiotics might create aqueous pores in the membranes through which the materials could migrate. Thus, the addition of polyene antibiotics could mediate changes in the electrical properties of the membrane by causing an asymmetric distribution of a charged ion. The antibiotic concentrations of 10^{-7} to 10^{-5} *M* radically lowered the dc resistance, by some 10^{+5}- to 10^{+6}-fold. Andreoli *et al.* (1969) and Andreoli and Monahan (1968) found that several other factors were observed to influence the extent of the antibiotics' effects on the electrical properties of the black film. These were (a) the temperature of the system, (b) the concentration of the antibiotic, and (c) most important, whether or not there was sterol present. Antibiotics had no effect on the dc resistance across black films that did not contain cholesterol above a critical minimal concentration in the black film.

Also of great interest was the observation by these same workers that nystatin and amphotericin B had a selective effect in that the permeability of the anions chloride and acetate increased much more than that of the cations sodium or potassium. These particular antibiotics made the black film systems apparently selectively permeable to anions. This is an important observation and suggests that polyene antibiotics could conceivably have selective effects on particular membranes with regard to mediating permeability increases. It should be emphasized, though, that Andreoli and Monahan (1968) and Andreoli *et al.* (1969) and Finkelstein and Cass (1968) demon-

strated additional effects of the polyene antibiotics in facilitating the passage of certain nonelectrolytes, such as water, thiourea, glycerol, erythritol, but not glucose or sucrose.

Dennis *et al.* (1970) carried out studies to determine structural requirements for their so-called pore formation and attempted to identify some factors that might contribute to its apparent anion selectivity. Modification of the carboxyl or amino groups of amphotericin B (*N*-acetyl amphotericin B or a methyl ester of amphotericin B) did not affect the anion selectivity. Thus it seemed that the hydroxyl groups or carboxyl groups are not involved in the anion selectivity exhibited by amphotericin B and its derivatives. This is difficult to understand since according to the fixed charge theory, a positive charge distribution in the molecule would be expected to exhibit anion selectivity (Teorell, 1953). Since a recent report (Papahadjopoulos *et al.*, 1972) demonstrated that the chloride movement through thin lipid layers is electrically silent, the anion selectivity reported for amphotericin B and nystatin needs reevaluation, possibly by comparison with tracer studies.

Van Zutphen *et al.* (1971) carried out somewhat similar studies concerning the stability and electrical resistance of lipid bilayers to ascertain whether the effects measured would depend on (a) the concentration of cholesterol in the film-forming solution, (b) the concentration of polyene antibiotics added, or (c) the method of addition of the polyene. In addition they studied whether filipin caused any preferential permeability changes in the black film system. They observed that the filipin complex, filipin II, filipin III, nystatin, etruscomycin, and pimaricin (all at the concentration of 10^{-5} *M*) were able to disrupt bimolecular lipid films which had lecithin and cholesterol present in the 1:1 molar ratio. No effects were observed with these antibiotics on cholesterol-free black films (at a concentration of antibiotic of 4 to 6 $\times$ 10^{-5} *M*). When UV-irradiated filipin or perhydrofilipin were added to the black film system at a concentration of 4 $\times$ 10^{-5} *M* no disruptive effects were noted either in the presence or in the absence of cholesterol. The dc resistance of lecithin–cholesterol bilayers formed in the presence of 0.1 *M* NaCl, which were then treated with either filipin complex, filipin II, filipin III, nystatin, or pimaricin, was 10,000–100,000 times lower in the presence of the cation calcium, but was not affected by the presence of a variety of other cations and anions. The results of transference number measurements were interpreted to indicate that lecithin–cholesterol bilayers, when treated with these polyenes, became cation selective. This was a most interesting observation, both in light of the preced-

ing observation concerning the anion selectivity induced by amphotericin B and in light of the observation of Adams *et al.* (1970) and Wong *et al.* (1970) concerning the ability of filipin, *in vitro*, to increase the permeability to calcium of intestinal mucosal cells obtained from rachitic chicks, but not of mucosal cells obtained from normal chicks. Van Zutphen *et al.* (1971) also noted that amphotericin B was capable of making black films selectively permeable to nitrate, sulfate, or phosphate; i.e., they became anion selective. Again it should be pointed out that these selectivities should be verified by tracer techniques. In the case of filipin it was found that filipin would increase the leakage of divalent cations and anions to the same extent in the liposomal system (Spielvogel, 1973).

The transference number calculations are based on the assumption that the electric charge is transferred by ions and that the driving force for this transport is a difference in salt concentration or electrical potential in the two aqueous phases bathing the surfaces of the membrane. The decrease in the dc resistance of lecithin–cholesterol bilayers, in the presence of polyene antibiotics and calcium, once again could possibly be explained by the formation of channels or pores within the membrane phase or alternatively by the functioning of the polyene as carriers. However, in view of the electron microscopic freeze-etch studies, discussed earlier, the formation of pores does not apparently occur. One attractive mechanism to explain the transfer of ions through black film has been proposed by Hladky and Haydon (1970). Hladky and Haydon suggested that aqueous pores could be present in black film, but that they remain open for less than 100 msec. Thus they envision a dynamic opening and closing of the putative pores. Pores of this nature might not be detectable by static electron micrographic techniques.

In examining the question of polyenes functioning as ionophores, it is useful to compare the effect of polyene antibiotics with those of the ionophore valinomycin. (a) Valinomycin is soluble in hydrocarbons and can by virtue of its binding capacity actually increase the ion concentration in this phase. Polyenes on the other hand are ampholites that would orient themselves in the solvent interphase and could not be shown to exhibit a binding capacity for ions (Spielvogel, 1973). (b) The polyene antibiotics require the presence of sterols for their effect on permeability, whereas the presence of sterols in membranes decreases the permeability changes mediated by valinomycin. (c) The membrane conductance in thin layers rises linearly with valinomycin concentrations, while the conductance rises exponentially for the polyene antibiotics. (d) The temperature

dependence of the conductance are different for polyene antibiotics and valinomycin. (e) Valinomycin exhibits an exclusive cation selectivity $Rb^+ > K^+ > Cs > Na^+ > Li$ (Diamond and Wright, 1969) which is consistent with a selectivity sequence predicted by the alkali selectivity theory by Eisenman (1961). The polyene antibiotics show some discrimination between anions and cations but are also effective in increasing the permeability of small electrolytes.

It is clear that these data suggest that polyene antibiotics must probably act by a different mechanism than the carrier ionophoretic mechanism proposed for valinomycin.

3. *Studies with Monolayer System*

Monolayers or lipid monomolecular layers have also been a model membrane system extensively used to explore structure–function relationships of the action of polyene antibiotics. It should be appreciated that a monolayer is in essence only one half of a membrane. Demel *et al.* (1965) carried out the first studies investigating the interaction of various polyene antibiotics with monolayers of varying composition. They found that filipin and nystatin both increased the surface pressure of monolayers which were composed entirely of either ergosterol or cholesterol but had no effect on monolayers that contained only phospholipids. In these studies, the antibiotics were added at a concentration of approximately 10^{-8} *M*. Further studies by these workers (Demel *et al.*, 1968) indicated that filipin produced the largest increase in surface pressure while etruscomycin, amphotericin B, pimaricin, and nystatin produced lesser effects, in the order given. As will be recalled, this was the same order of effectiveness that was noted for the action of these polyene antibiotics in causing membrane lysis. Once again it was noted that perhydrofilipin, or UV- irradiated filipin, was not capable of causing pressure increases in the cholesterol-containing monolayer. Additional studies on the structural requirements for the greatest interaction indicated that if the sterol was esterfied or that the presence of 5 *M* urea in the aqueous substrate greatly lowered the pressure increases produced when filipin was added. Demel *et al.* (1968) interpreted these results as indicating the possible role of hydrogen bonds in the stabilization of the putative polyene–sterol complex. In mixed monolayers of lecithin and cholesterol, the surface pressure increase obtained with filipin was diminished proportionately as the phospholipid content was raised. This observation was claimed to support the concept that the polyene sensitivity of the membrane may be dependent on the ratio of phospholipid to sterol present.

VI. Concluding Remarks

It is apparent from the foregoing discussion that our present understanding of the mode of action of polyene antibiotics in many ways parallels both the development of molecular biology and the related increasingly sophisticated techniques available to the modern chemist for physical and chemical characterization. Considering that the general class of polyene antibiotics was discovered only in 1951 (Hazen and Brown, 1951), tremendous strides have been made in the intervening 25 years. There are now well over 70 members of the class of polyenes, all with highly complicated chemical structures; of these fourteen have had their complete structure elucidated, and for one (amphotericin B) the complete three-dimensional organization of its 138 atoms is known. Similarly, the biology of polyenes has advanced from the first gross observations on their toxicity to certain organisms, to demonstration that the "toxicity" was mediated through changes in membrane permeability and that in particular there was an absolute requirement for a large proportion of sterol in the membrane. These concepts were further refined by investigation at several levels of membrane organization—natural membranes (with and without sterol), artificial membranes (liposomes and black films), and simple membrane models (monolayers)—using a vast array of molecular biological techniques including UV circular dichroic, and fluorescence spectroscopy, stopped-flow kinetics, positive, negative, and freeze-etch electron microscopy, and differential scanning calorimetry, to name a few. From all this has been synthesized a highly detailed, stereospecific molecular model for polyene–antibiotic interaction. One must await future studies and developments to learn whether this model can be further refined and observe whether it adequately explains the many facets of polyene action.

ACKNOWLEDGMENT

The authors are very grateful to Professor Peter H. Tsao of the Department of Plant Pathology, University of California, Riverside, for many fruitful discussions concerning the planning and preparation of this manuscript.

References

Adams, T. H., Wong, R. G., and Norman, A. W. (1970). *J. Biol. Chem.* **245**, 4432.
Andreoli, T. E., and Monahan, M. (1968). *J. Gen. Physiol.* **52**, 300.
Andreoli, T. E., Dennis, V. W., and Weigl, A. M. (1969). *J. Gen. Physiol.* **53**, 133.

Arcamone, F., and Perego, M. (1959). *Ann. Chim. (Rome)* **49**, 345.
Arcamone, F., Bertazzoli, D. D., Canevazzi, G., Di Marco, A., Ghione, M., and Grein, A. (1957). *Microbiol.* **4**, 119.
Ball, S., Bessell, C. J., and Mortimer, A. (1957). *J. Gen. Microbiol.* **17**, 96.
Bangham, A. D. (1972). *Annu. Rev. Biochem.* **41**, 753.
Bangham, A. D., Standish, M. M., and Watkins, J. C. (1965). *J. Mol. Biol.* **13**, 238.
Bangham, A. D., De Gier, J., and Greville, D. G. (1967). *Chem. Phys. Lipids* **1**, 225.
Bentley, P. J. (1968). *J. Physiol. (London)* **196**, 703.
Bergy, M. E., and Eble, T. E. (1968). *Biochemistry* **7**, 653.
Bittman, R., and Blau, L. (1972). *Biochemistry* **11**, 4831.
Bittman, R., and Fischkoff, S. (1972). *Proc. Nat. Acad. Sci. U. S.* **69**, 3795.
Bittman, R., Chen, W. C., and Anderson, O. R. (1974a). *Biochemistry* **13**, 1364.
Bittman, R., Chen, W. C., and Blau, L. (1974b). *Biochemistry* **13**, 1374.
Bond Saltza, M. H., Reid, J., Dutcher, J. D., and Wintersteiner, O. (1961). *J. Amer. Chem. Soc.* **83**, 2785.
Borowski, E., Schaffner, C. P., and Lechevalier, H. (1960). *Antimicrob. Ag. Annu.* p. 532.
Borowski, E., Falkowski, L., Golik, J., Zielinski, J., Ziminski, T., Mechlinski, W., Jereczek, E., Kolodziejczyk, P., Adlercreutz, H., Schaffner, C. P., and Neelakatan, S. (1971). *Tetrahedron Lett.* **22**, 1987.
Brown, R., and Hazen, E. L. (1960). *Antibiot. Chemother. (Washington, D. C.)* **10**, 702.
Caltrider, P. G., and Gottlieb, D. (1961). *Trans. Ill. State Acad. Sci.* **54**, 189.
Cass, A., Finkelstein, A., and Krespi, V. (1970). *J. Gen. Physiol.* **56**, 100.
Ceder, O. (1964). *Acta Chem. Scand.* **18**, 126.
Ceder, O., and Ryhage, R. (1964). *Acta Chem. Scand.* **18**, 558.
Cope, A. C., Bly, R. K., Burrows, E. P., Ceder, O. J., Ciganek, E., Gilles, B. T., Porter, R. F., and Johnson, H. (1962). *J. Amer. Chem. Soc.* **84**, 2170.
Cope, A. C., Axen, U., and Burrows, E. P. (1966). *J. Amer. Chem. Soc.* **88**, 4221.
Danielli, J. F., and Davison, H. (1952). *In* "The Permeability of the Plasma Membrane," Chapter 2, pp. 57-71. Cambridge Univ. Press, London and New York.
Davisson, J. W., Tanner, F. W., Jr., Finlay, A. C., and Solomons, I. A. (1951). *Antibiot. Chemother. (Washington, D. C.)* **1**, 289.
DeKruijff, B., and Demel, R. A. (1974). *Biochim. Biophys. Acta* **339**, 57.
DeKruijff, B., Demel, R. A., and Van Deenen, L. L. M. (1972). *Biochim. Biophys. Acta* **255**, 331.
Dekruijff, B., Gerritsen, W. J., Oerlemans, A., Demel, R. A., and Van Deenen, L. L. M. (1974a). *Biochim. Biophys. Acta* **339**, 30.
Dekruijff, B., Gerritsen, W. J., Oerlemans, A., Van Dijck, P. W. M., Demel, R. A., and Van Deenen, L. L. M. (1974b). *Biochim. Biophys. Acta* **339**, 44.
Demel, R. A., Van Deenen, L. L. M., and Kinsky, S. C. (1965). *J. Biol. Chem.* **240**, 2749.
Demel, R. A., Crombag, F. J. L., Van Deenen, L. L. M., and Kinsky, S. C. (1968). *Biochim. Biophys. Acta* **150**, 1.
Dennis, V. W., Stead N. W., and Andreoli, T. E. (1970). *J. Gen. Physiol.* **55**, 375.
Dhar, M. L., Thaller, V., and Whiting, M. C. (1960). *Proc. Chem. Soc., London* p. 310.
Diamond, J. M., and Wright, E. M. (1969). *Annu. Rev. Physiol.* **31**, 581.
Drabikowski, W., Lagwinska, E., and Surzala, M. G. (1973). *Biochim. Biophys. Acta* **291**, 61.
Eisenman, G. (1961). *In* "Membrane Transport and Metabolism" (A. Kleinzeller and A. Kotyk, eds.), pp. 163-179. Academic Press, New York.

Fanestil, D. D., Porter, G. A., and Edelman, I. S. (1967). *Biochim. Biophys. Acta* **135**, 74.
Feingold, D. S. (1965). *Biochem. Biophys. Res. Commun.* **19**, 261.
Finkelstein, A., and Cass, A. (1968). *J. Gen. Physiol.* **52**, 145.
Frazier, H. S. (1962). *J. Gen. Physiol.* **45**, 515.
Gale, G. R. (1963). *J. Bacteriol.* **80**, 151.
Canis, P., Avitabile, G., Mechlinski, W., and Schaffner, C. P. (1971). *J. Amer. Chem. Soc.* **93**, 4560.
Ghosh, A., and Ghosh, J. J. (1963a). *Ann. Biochem. Exp. Med.* **23**, 101.
Ghosh, A., and Ghosh, J. J. (1963b). *Ann. Biochem. Exp. Med.* **23**, 603.
Ghosh, B. K., and Chatterjee, A. N. (1961). *Ann. Biochem. Exp. Med.* **21**, 343.
Ghosh, B. K., and Chatterjee, A. N. (1962). *Antibiot. Chemother.* **12**, 221.
Ghosh, B. K., and Chatterjee, A. N. (1963a). *Ann. Biochem. Exp. Med.* **23**, 173.
Ghosh, B. K., and Chatterjee, A. N. (1963b). *Ann. Biochem. Exp. Med.* **23**, 309.
Ghosh, B. K., Haldar, D., and Chatterjee, A. N. (1960). *Ann Biochem. Exp. Med.* **20**, 55.
Golding, B. T., Rickards, R. W., Meyer, W. E., Patrick, J. B., and Barber, M. (1966). *Tetrahedron Lett.* **30**, 3551.
Gordon, H. W., and Schaffner, C. P. (1968). *Proc. Nat. Acad. Sci. U. S.* **60**, 1201.
Gottlieb, C. (1971). *In* "Morphological and Biochemical Events in Plant-parasite Interaction" (S. Akai and S. Ouchi, eds.), pp. 153-179. Phytopathol. Soc. Japan, Tokyo.
Gottlieb, D., and Pote, H. L. (1960). *Phytopathology* **50**, 817.
Gottlieb, D., and Shaw, P. D., eds. (1967). "Mechanism of Action," Vol. I, p.785. Springer-Verlag, Berlin and New York.
Gottlieb, D., and Shaw, P. D. (1970). *Annu. Rev. Phytopathol.* **8**, 371.
Gottlieb, D., Carter, H. E., Sloneker, J. H., and Amman, A. (1958). *Science* **128**, 361.
Gottlieb, D., Carter, H. E., Sloneker, J. H., Wu, L. C., and Gaudy, E. (1961). *Phytopathology* **51**, 321.
Haest, C. W. M., De Gier, J., Op Den Kamp, J. A. F., Bartels, P., and Van Deenen, L. L. M. (1972). *Biochim. Biophys. Acta* **255**, 720.
Hamilton-Miller, J. T. M. (1973). *Bacteriol. Rev.* **37**, 166.
Haslam, R. M., and Klyne, W. (1953). *Biochem. J.* **55**, 340.
Hattori, K. (1962). *J. Antibiot.* **15B**, 39.
Hazen, E. L., and Brown, R. (1951). *Proc. Soc. Exp. Biol. Med.* **76**, 93.
Henn, F. A., and Thompson, T. E. (1969). *Annu. Rev. Biochem.* **38**, 241.
Hickey, R. J., Corum, C. J., Hidy, P. H., Cohen, I. R., Nager, U. F. B., and Kropp, E. (1952). *Antibiot. Chemother. (Washington, D. C.)* **2**, 471.
Hladky, S. B., and Haydon, D. A. (1970). *Nature (London)* **225**, 451.
Holz, R., and Finkelstein, A. (1970). *J. Gen. Physiol.* **56**, 125.
Horii, S., Shima, T., and Ouchida, A. (1970). *J. Antibiot.* **22**, 102.
Hsu Chen, C. C., and Feingold, D. S. (1973). *Biochem. Biophys. Res. Commun.* **51**, 972.
Hunter, E. O., Jr., and McVeigh, J. (1961). *Amer. J. Bot.* **48**, 179.
Ikeda, M., Suzuki, M., and Djerassi, C. (1967). *Tetrahedron Lett.* 38, 3745.
Johnson, W., Miller, C. A., and Brumbaugh, J. H. (1962). *Physiol. Zool.* **35**, 18.
Katzenstein, I. (1973). M. S. Thesis, University of California, Riverside.
Kavanaugh, J. L. (1966). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **25**, 1096.
Kinsky, S. C. (1956). *Proc. Nat. Acad. Sci. U. S.* **48**, 1049.
Kinsky, S. C. (1961a). *Biochem. Biophys. Res. Commun.* **4**, 353.
Kinsky, S. C. (1961b). *J. Bacteriol.* **82**, 889.

Kinsky, S. C. (1963). *Arch. Biochem. Biophys.* **102,** 180.
Kinsky, S. C. (1964). *Antimicrob. Ag. Chemother.* pp. 384–394.
Kinsky, S. C. (1967). *In* "Antibiotics" (D. Gottlieb and P. D. Shaw, eds.), Vol. I, pp. 122–141 and 749–750 (addenda). Springer-Verlag, Berlin and New York.
Kinsky, S. C. (1970). *Annu. Rev. Pharmacol.* **10,** 119.
Kinsky, S. C., Avruch, J., Permutt, M., Rodgers, H. B., and Schonder, A. A. (1962). *Biochem. Biophys. Res. Commun.* **9,** 503.
Kinsky, S. C., Gronau, G. R., and Weber, M. M. (1965). *Mol. Pharmacol.* **1,** 190.
Kinsky, S. C., Luse, S. A., and Van Deenen, L. L. M. (1966). *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **25,** 1503.
Kinsky, S. C., Demel, R. A., and Van Deenen, L. L. M. (1967a). *Biochim. Biophys. Acta* **135,** 835.
Kinsky, S. C., Luse, S. A., Zopf, D., Van Deenen, L. L. M., and Haxby, J. (1967b). *Biochim. Biophys. Acta* **135,** 844.
Kinsky, S. C., Haxby, J., Kinsky, C. B., Demel, R. A., and Van Deenen, L. L. M. (1968). *Biochim. Biophys. Acta* **152,** 174.
Kleinschmidt, M. G., Chough, K. S., and Mudd, J. B. (1972). *Plant Physiol.* **49,** 852.
Korn, E. (1966). *Science* **153,** 1491.
Ladbrooke, B. D., and Chapman, D. (1969). *Chem. Phys. Lipids* **3,** 304.
Ladbrooke, B. D., Williams, R. M., and Chapman, D. (1968). *Biochim. Biophys. Acta* **150,** 333.
Lampen, J. O. (1966). *In* "Biochemical Studies of Antimicrobial Drugs" (B. A. Newton and P. E. Reynolds, eds.), pp. 111-130. Cambridge Univ. Press, London and New York.
Lampen, J. O., and Arnow, P. (1959). *Proc. Soc. Exp. Biol. Med.* **101,** 792.
Lampen, J. O., and Arnow, P. (1961). *J. Bacteriol.* **82,** 247.
Lampen, J. O., Morgan, E. R., Slocum, A., and Arnow, P. (1959). *J. Bacteriol.* **78,** 282.
Lampen, J. O., Arnow, P. M., and Safferman, R. S. (1960). *J. Bacteriol.* **80,** 200.
Lampen, J. O., Arnow, P. M., Borowska, Z., and Laskin, A. I. (1962). *J. Bacteriol.* **184,** 1152.
Lichtenstein, N. S., and Leaf, A. (1966). *J. Clin. Invest.* **8,** 1328.
Lippe, C., and Giordana, B. (1967). *Biochim. Biophys. Acta* **135,** 966.
Lippe, C., Giordana, B., and Caprarao, V. (1968). *In* "Membrane Models and the Formation of Biological Membranes" (L. Bous and B. A. Pethica, eds.), pp. 160-165. North-Holland Publ., Amsterdam.
Marini, F. P., Arnow, P., and Lampen, J. O. (1961). *J. Gen. Microbiol.* **24,** 51.
Mechlinski, W., Schaffner, C. P., Ganis, P., and Avitabile, G. (1970). *Tetrahedron Lett.* **44,** 3873.
Mendoza, S. A., Handler, J. S., and Orloff, J. (1967). *Amer. J. Physiol.* **213,** 1263.
Mondavi, B., Strom, R., Agro, A. F., Caiafa, P., Sole, P. D., Bozzi, A., Rotilio, G., and Fanelli, A. R. (1971). *Cancer Res.* **31,** 505.
Nakano, H. (1961). *J. Antibiot., Ser. A* **14,** 68.
Norman, A. W., Demel, R. A., DeKruijff, B., and Van Deenen, L. L. M. (1972a). *J. Biol. Chem.* **247,** 1918.
Norman, A. W., Demel, R. A., DeKruijff, B., Guerts-van-Kessel, W. S. M., and Van Deenen, L. L. M. (1972b). *Biochim. Biophys. Acta* **290,** 1.
Oroshnik, W., and Mebane, A. D. (1963). *Fortschr. Chem. Org. Naturst.* **21,** 17.
Oroshnik, W., Vining, L. C., Mebane, A. D., and Taber, W. A. (1955). *Science* **121,** 147.
Pandey, R. C., and Rinehart, K. L. (1970). *J. Antibiot.* **23,** 414.
Pandey, R. C., German, V. F., Nishikawa, Y., and Rinehart, K. L. (1971). *J. Amer. Chem. Soc.* **93,** 5738.

Pandey, R. C., Narasimhachari, N., Rinehart, K. L., and Millington, D. S. (1972). *J. Amer. Chem. Soc.* **94,** 4306.
Papahadjopoulos, D., and Watkins, J. C. (1967). *Biochim. Biophys. Acta* **135,** 639.
Papahadjopoulos, D., Nir, S., and Ohki, S. (1972). *Biochim. Biophys. Acta* **266,** 561.
Patrick, J. B., Williams, R. P., Wolf, C. F., and Webb, J. S. (1958). *J. Amer. Chem. Soc,* **80,** 6688.
Rinehart, K. L., Tucker, W. P., and Pandey, R. C. (1971). *J. Amer. Chem. Soc.* **93,** 3747.
Robertson, J. D. (1959). *Biochem. Soc. Symp.* **16,** 3.
Schaffner, C. P., and Gordon, H. W. (1968). *Proc. Nat. Acad. Sci. U. S.* **61,** 36.
Schlosser, E., and Gottlieb, D. (1966). *J. Bacteriol.* **91,** 1080.
Schlosser, E., and Gottlieb, D. (1968). *Arch. Mikrobiol.* **61,** 246.
Schlosser, E., Shaw, P. D., and Gottlieb, D. (1969). *Arch. Mikrobiol.* **66,** 147.
Schroeder, F., Holland, J. F., and Bieber, L. L. (1971). *J. Antibiot.* **24,** 846.
Schroeder, F., Holland, J. F., and Bieber, L. L. (1972). *Biochemistry* **11,** 3105.
Schroeder, F., Holland, J. F., and Bieber, L. L. (1973). *Biochemistry* **12,** 4785.
Seneca, H., and Bergendahl, E. (1955). *Antibiot. Chemother. (Washington, D. C.)* **5,** 737.
Sessa, G., and Weissman, G. (1968). *J. Biol. Chem.* **243,** 4364.
Sharp, G. W. G., and Leaf, A. (1966). *Physiol. Rev.* **46,** 593.
Sharp, G. W. G., Coggins, C. H., Lichtenstein, N. S., and Leaf, A. (1966). *J. Clin. Invest.* **45,** 1640.
Spielvogel, A. M. (1973). Ph.D. Dissertation, University of California, Riverside.
Sutton, D. D., Arnow, P. M., and Lampen, J. O. (1961). *Proc. Soc. Exp. Biol. Med.* **108,** 170.
Sweeley, C. C., O'Connor, J. D., and Bieber, L. L. (1970). *Chem. Biol. Interact.* **2,** 247.
Taber, W. A., Vining, L. C., and Waksman, S. A. (1954). *Antibiot. Chemother. (Washington, D. C.)* **4,** 455.
Teorell, T. (1953). *Prog. Biophys. Mol. Biol.* **3,** 305.
Tillack, T. W., and Kinsky, S. C. (1973). *Biochim. Biophys. Acta* **323,** 43.
Tsao, P. H. (1970). *Annu. Rev. Phytopathol.* **8,** 157.
Ushakova, T. A., Frolova, V. I., and Kuzovkov, A. D. (1970). *Proc. Int. Symp. Chem. Natur. Prod., 7th, 1969,* p. 638.
Vandeputte, J., Wachtel, J. L., and Stiller, E. T. (1956). *Antibiotic Ann.* p. 587.
Van Zutphen, H., Van Deenen, L. L. M., and Kinsky, S. C. (1966). *Biochem. Biophys. Res. Commun.* **22,** 393.
Van Zutphen, H., Demel, R. A., Norman, A. W., and Van Deenen, L. L. M. (1971). *Biochim. Biophys. Acta* **241,** 310.
Verkleij, A. J., DeKruijff, B., Gerritsen, W. F., Demel, R. A., Van Deenen, L. L. M., and Ververgaert, P. H. J. (1973). *Biochim. Biophys. Acta* **291,** 577.
Vining, L. C. (1960). *Hindustan Antibiot. Bull.* **3,** 37.
Vining, L. C., and Taber, W. A. (1956). *Can. J. Chem.* **34,** 1163.
Vining, L. C., and Taber, W. A. (1957). *Can. J. Chem.* **35,** 1461.
Walters, D. R., Dutcher, J. D., and Wintersteiner, O. (1957). *J. Amer. Chem. Soc.* **79,** 5076.
Wasserman, H. H., VanVerth, J. E., McCaustland, D. J., Borowitz, I. J., and Kamber, B. (1967). *J. Amer. Chem. Soc.* **89,** 1535.
Weber, M. M., and Kinsky, S. C. (1965). *J. Bacteriol.* **89,** 306.
Weissman, G., and Sessa, G. (1967). *J. Biol. Chem.* **242,** 616.
Whitfield, G. B., Brock, T. D., Amman, A., Gottlieb, D., and Carter, H. E. (1955). *J. Amer. Chem. Soc.* **77,** 4799.
Wong, R. G., Adams, T. H., Roberts, P. A., and Norman, A. W. (1970). *Biochim. Biophys. Acta* **219,** 61.

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Volume 3

Ammonium Compounds to Antioxidants

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CNS. Fever, vomiting, and chills are observed in about 50 % and seem to be mediated by prostaglandines.

Heart. Rarely, arrythmia is observed under infusion [203].

Circulation. Collapse.

Vessels. Thrombophlebitis at the injection site is requently observed, liposomal formulations show a lower phlebotoxicity [204].

Blood/Spleen. Anemia and thrombocytopenia [204] are rarely observed.

Liver. In some cases, hepatotoxicity was observed.

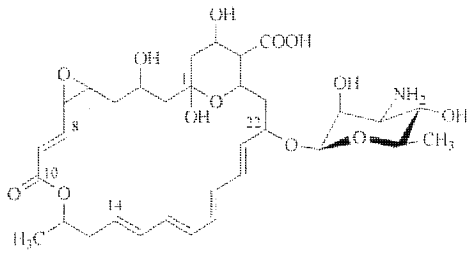
Kidney. Amphotericin B shows a high degree of nephrotoxicity. Cumulative doses of 4 – 5 g generally cause reversible damage to the kidneys [205]. Higher doses mostly cause an irreversible kidney damage with persisting hypopotassemia, hematuria, proteinuria, and azotemia [203–206]. Therefore, serum potassium, magnesium, and creatinine levels have to be frequently monitored.

In the case of intralumbal and/or intrathecal administration, severe side effects of the central nervous system are common. Liposomal formulation of amphotericin B can reduce toxicity problems [207].

Topical application of amphotericin B is highly effective and well tolerated in the treatment of cutaneous and mucocutaneous candidoses. This is also true for inhalation and instillation. Local irritations and allergic reactions may occur in rare cases and are partly due to the galenic formulation.

3.2. Natamycin (Pimaricin)

Natamycin [*7681-93-8*], natural product isolated from culture filtrates of *Streptomyces natalensis* (original isolate from soil of Pietermaritzburg, South Africa) and *Streptomyces chattanoogensis* [208], [209], contains mycosamine as sugar component, like amphotericin B and nystatin. $C_{33}H_{47}NO_{13}$, M_r 665.7. *mp*: does not have an exact melting point and decomposes above 200 °C, colorless substance.



Synonym(s). Pimaricin, antibiotic A 5283, CL 12625, tennecetin.

Solubility. Natamycin is soluble in propylene glycol (20 mg/mL), DMF (50 mg/mL), and *N*-methylpyrrolidone (120 mg/mL); slightly soluble in methanol (2 mg/mL); and practically insoluble in water (0.05 mg/mL). Natamycin is amphoteric, dissolving in dilute acids and bases. However, these solutions are unstable.

Stability. Solutions or suspensions of natamycin are stable for several weeks at pH 5 – 7. The solutions can even be sterilized by heating at 110 °C for 20 min. However, they must be protected from light, which causes rapid decomposition [208], [210].

Synthesis. Fermentative from *Streptomyces* species.

Description. First isolated in 1957 [208], [211], [212].

Formulations. For therapy of human infections: Ointments, powders, creams, lotions, lozenges, dragees, suspensions, and vaginal tablets of different concentration. Also in combination with antibiotics and/or steroids (e.g., neomycine, hydrocortisone).

Trade Names. Deronga (Basotherm), Mycophyt (Gist-Brocades), Myprozine (Lederle), Natacyn (Alcon), Natafucin (Brocades), Pima-Biciron (Basotherm), Pimafucin (Basotherm, Beytout, Byk Procienx, Gist-Brocades), Pimagyn (Doetsch-Grether), Synogil (Basotherm).

Antimycotic Properties. Natamycin is a broad-spectrum polyene antimycotic, MIC values are summarized in Table 7.

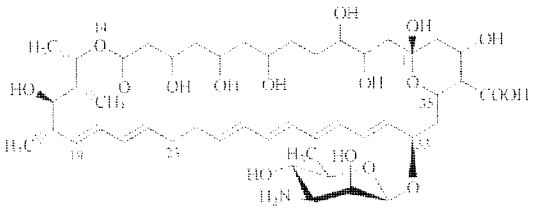
Phamacokinetics. After topical application, no systemic absoption was detectable.

Indications. Cutaneous and mucocutaneous candidosis; in some cases, the substance was also successful for therapy of human dermatophytoses [213–215]. In veterinary medicin, natamycin is used for the therapy of dermatophytoses in cows (generally caused by *Trichophyton verrucosum*) and horses (generally caused by *Trichophyton equinum*) [216], [217]. As natamycin also has antibacterial properties, it is also used as a food preservative.

Side Effects. When applied topically, natamycin is highly effective and well tolerated in the treatment of cutaneous and mucocutaneous candidoses. Local irritations and allergic reactions may occur in rare cases and are mostly due to the galenic formulation. Especially in veterinary medicine, phototoxic effects were observed.

3.3. Nystatin A1

Nystatin (A1) [*1400-61-9*], three biologically active components – Nystatin A1, A2, and A3 – have been described. Nystatin A1 is a natural product isolated from culture filtrates of *Streptomyces noursei* [218], *Streptomyces aureus*, and other *Streptomyces* species. Like amphtericin B it is a 38-membered macrocyclic lactone, from which it differs solely in the position of the hydroxyl groups and in the absence of a double bond at C23 [219]. $C_{47}H_{75}NO_{17}$, M_r 926.1, *mp*: does not have a melting point, begins to decompose above 160 °C and decomposes above 250 °C without melting, yellow powder.



Synonym(s). Polyfungin A1.

Solubility. Nystatin A1 is soluble in pyridine, DMSO, DMF, ethylene glycol (8.7 µg/mL), methanol (11 µg/mL), ethanol (1.2 µg/mL), butanol, dioxane, and water (4.0 µg/mL). The substance is hygroscopic [220].

Stability. The substance is amphoteric, but aqueous and alkaline solutions are unstable. Nystatin shows optimum stability in phosphate – citrate buffers at pH 5 – 7. If kept refrigerated, the pure substance can be stored for several months without loss of activity. For stability, see [221].

Synthesis. Fermentative from *Streptomyces* species.

Description. See [222], [223].

Formulations. Tablets, dragées, lozenges, suspensions, drops, ointments, powders, gels, creams, ovulas, and vaginal tablets of different concentrations. There are also formulations available in combination with antibiotics and/or steroids such as tetracycline, neomycin, gramicidin, and cortisone. Dosages are often expressed in IE (100 000 IE = 22.73 mg nystatin A1).

Trade Names. Adiclair (Ardeypharm), Biofanal (Pfleger), Candex (Dome), Candio-Hermal (Hermal, Merck), Canstat (Lederle), Diastatin (Pfizer), Fungicidin (Spofa), Fungireduct (Azupharma), Herniocid (Mayrhofer), Korostatin (Holland-Rantos), Lederlind (Lederle), Lystin (Mekim), Mikostatin (Squibb), Moronal (Heyden, Squibb), Multilind (Bristol-Myers Squibb, Fair), Mycostatin (Bristol-Myers Squibb, Heyden, Sanofi Winthrop, Westwood-Squibb), Mykinac (NMC), Myco-Posterine N (Kade), Mykundex (Jossa), Nadostine (Pan-Well), Nilstat (Lederle), Nyaderm (Taro), Nysert (Norwich Eaton), Nystacid (Farmos Group), Nystaderm (Dermapharm), Nysta-Dome (Dome), Nystan (Squibb), Nystat-Rx (Pharma-Tek), Nystavescent (Squibb), Nystex (Savage), Oranyst (Taro), O-V Statin (Squibb), Restatin (Remedica), Rivostatin (Rivopharm) Stereomycin (Medica).

Antimycotic Properties. Nystatin A1 is a broad-spectrum polyene antimycotic with in vitro activity against pathogenic yeasts, moulds,

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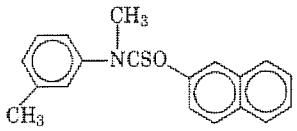
powders are a combination of zinc undecylenate, boric acid, hexachlorophene, and salicylic acid.

Tolnaftate [*2398-96-1*], $C_{19}H_{17}NOS$, which is active against dermatophytes, is the active component in another antifungal powder; it also contains cetylpyridinium chloride and talcum venetum.



tolnaftate

In addition to tolnaftate and cetylpyridinium chloride, the ointment also contains poly(ethylene glycol). This preparation is not active against *C. albicans*.

ANTIMYCOTIC ANTIBIOTICS

Nystatin. This compound (1) belongs to the group of polyene antimycotic antibiotics (Table 1). Since the early 1960s, approximately 200 polyenes have been isolated from different *Streptomyces* strains.

Table 1. Polyene Antimycotic Antibiotics[a]

Structure number	Name	CAS Registry Number	Molecular formula	Trade name
(1)[b]	nystatin	[*1400-61-9*]	[b]	Fungicidin, Biotanal, Diastatin, Candex Mycostatin[c], Mioronal, Multilind, Nystan, Nystarescent
(2)	natamycin	[*7681-93-8*]	$C_{33}H_{47}NO_{13}$	Pimaricin, Mycophyt, Myprozine, Natacyn, Pimatucin, Synogil
(3)	amphotericin B	[*1397-89-3*]	$C_{47}H_{73}NO_{17}$	Amphozone, Fungizone[c], Fungilin, Ampho-Mioronal
(4)[d]	candicidin	[*1403-17-4*]	[d]	Levorin, Candeptin, Candimon, Vanobid
	filipin	[*11078-21-0*]	[e]	Filimarisin
	homycin	[*1403-71-0*]	[e]	Primamycin
(5)	etruscomycin[f]	[*13058-67-8*]	$C_{36}H_{53}NO_{13}$	Etruscomycin
	trichomycin[g]	[*1394-02-1*]	[e]	Cabimicina, Trichonat

[a]See Figure 1.
[b]Nystatin has three biologically active components: A_1, A_2, and A_3. Figure 1 depicts A_1.
[c]Squibb is the U.S. producer.
[d]A 4-component mixture; candicidin D shown in Figure 1 is the primary component.
[e]Multicomponent mixture.
[f]Also known as lucensomycin.
[g]Also known as hachimycin.

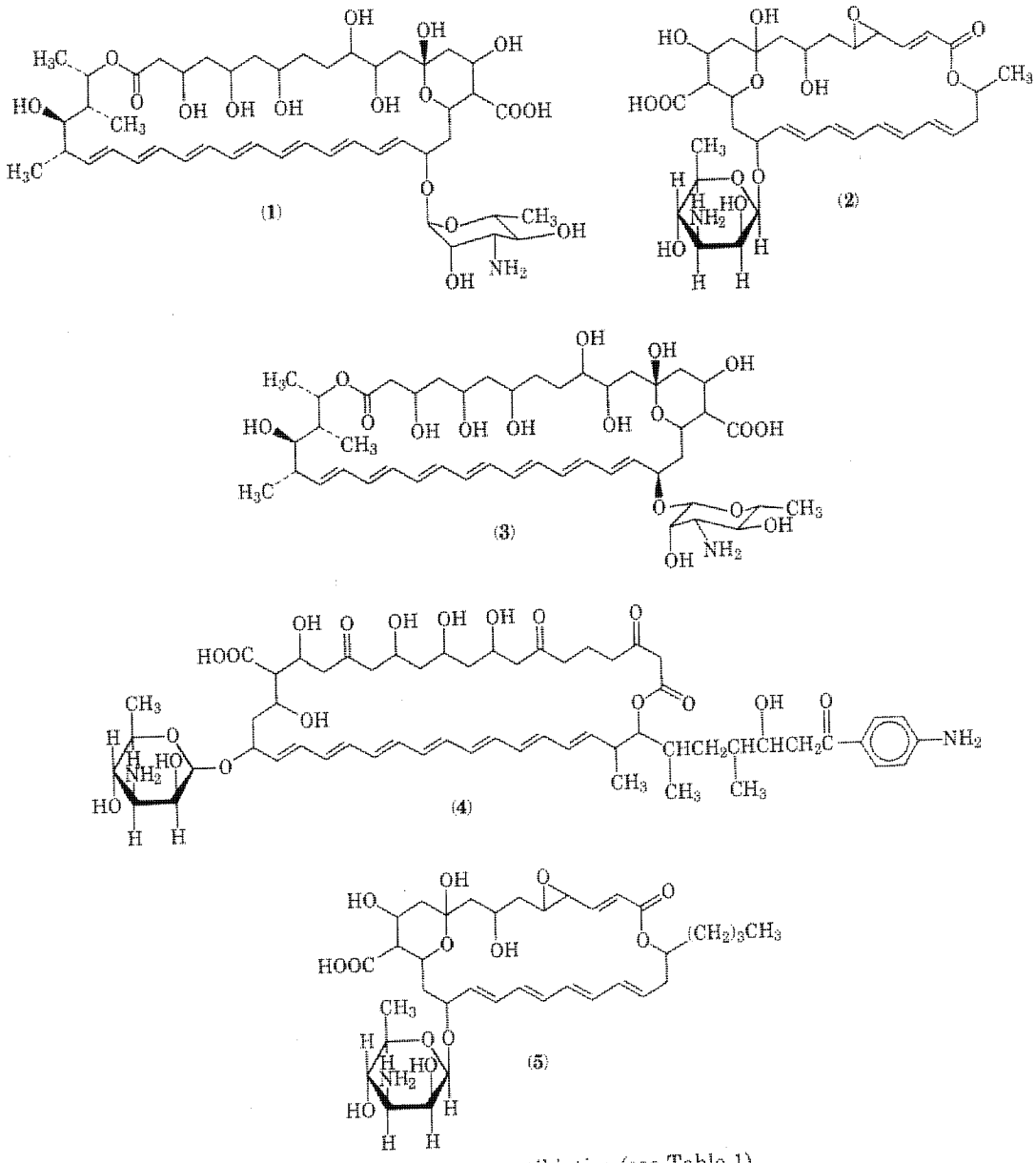


Fig. 1. Polyene antibiotics (see Table 1).

Nystatin is obtained from *Streptomyces noursei*. The polyenes are characterized by a macrolide ring and differ from one another by the number (12–37) of carbon atoms in the ring structure, the number (6–14) of hydroxyl groups, and the presence or absence of a carbohydrate (1). Polyenes alter the membrane permeability of sensitive cells by forming a complex with the sterol present in the membranes. Due to this binding to sterols, potassium ions are lost. Both nystatin and amphotericin B bind more strongly to ergosterol than to cholesterol.

Ergosterol is the principal sterol in the membrane of yeasts and fungi, whereas the cell membrane in mammals contains mainly cholesterol. This difference in binding capacity is probably responsible for the selectivity (1,2). Since bacteria, with the exception of *Mycoplasma* and *Acholeplasma*, contain no sterols; polyenes have no antibacterial activity.

Nystatin has a local fungicidal and fungistatic action against *C. albicans*. This polyene is not water-soluble. It is absorbed to a limited extent from the digestive tract, which limits the field of indications after oral administration to candidoses of the oral cavity, the esophagus, and the intestines. Oral administration of large doses may cause gastrointestinal disorders (nausea, vomiting, diarrhea). Nystatin is too toxic for parenteral administration. More information concerning chemical, mycological, and clinical properties of the polyenes is available (1,3,4).

Nystatin is mainly used to treat vaginal and oral infections and localized skin lesions, including *Candida* intertrigo and *Candida* nappy dermatitis. It may also be used as prophylaxis during treatment with antibiotics.

Nystatin (100,000 IU) is also available in combination with neomycin sulfate [*1405-10-3*] (35,000 IU), polymyxin B sulfate [*1405-20-5*] (35,000 IU), acetarsol [*97-44-9*] (150 mg), and dimethicone [*8050-81-5*] (2,500 mg). One or two ovules per day are inserted vaginally for at least 6–12 days. This combination has an antibacterial, antimycotic, and antitrichomonas action (see also ANTIBIOTICS, OLIGOSACCHARIDES; ANTIPARASITIC AGENTS, ANTIPROTOZOALS).

Natamycin. Natamycin or pimaricin (**2**) is a polyene with only four conjugated double bonds. This tetraene is obtained from *Streptomyces natalensis*. Like the other polyenes, pimaricin induces K^+ release from cells with membranes containing 20–40 mol % ergosterol. This is also the ergosterol concentration in the membrane of *Saccharomyces cerevisiae*.

Natamycin is not water-soluble or soluble in most organic solvents. It is not absorbed in the digestive tract. It is indicated for skin and nail infections with *C. albicans*, intertrigo and fissures at the corners of the mouth (perleche) caused by *C. albicans*, *Candida vulvitis*, and vaginitis. Natamycin plays an important role in the treatment of mycotic keratitis. Natamycin also appears to be active against the protozoan *Trichomonas vaginalis*. Side effects are nausea or diarrhea during oral treatment. Dosage is application of cream several times a day.

Combination creams or ointments contain 3.5 mg neomycin base and 10 mg hydrocortisone [*50-23-7*] per g, in addition to 10 mg natamycin. The combination as a lotion contains 1.75 mg neomycin and 5 mg hydrocortisone/g, in addition to 10 mg natamycin. This combination has an antiinflammatory, antibacterial, and antimycotic action. It is applied 2–4 times per day.

Amphotericin B. Amphotericin B (**3**), an important polyene antibiotic, is administered almost exclusively via the intravenous route and is therefore discussed in more detail under the systemic antimycotics. The vaginal tablets contain 50 mg amphotericin B, and 100 mg tetracycline base per tablet (see also ANTIBIOTICS, TETRACYCLINES). The tablets for oral use contain 50 mg amphotericin B, 250 mg tetracycline base, and 125 mg sodium hexametaphosphate. A combination ointment contains 1 mg fludrocortisone acetate, 2.5 mg neomycin, 0.25 mg gramicidin, and 1 g plastibase in addition to 30 mg amphotericin B (see also ANTIBIOTICS, PEPTIDES).

ANTIMYCOTICS

Since the 1960s, there has been such a rapid evolution in the pharmacotherapy of mycoses that three phases of the development of antimycotic agents can be distinguished: (*1*) compounds that existed before griseofulvin (1939); (*2*) compounds presently available on the market, including itraconazole (1987); (*3*) new antimycotics that are being studied now and will be prescribed tomorrow. Both milestones (griseofulvin and itraconazole) are intended for oral administration. Topical treatment of superficial dermatomycoses (trichophytosis, favus, and microsporosis) has been complemented by systemic oral treatment.

The world market for antimycotics in 1980 was valued at a total of $350 million. About 10% of this came from the sale of systemic (intravenous or oral) antifungals. In 1990, the antifungal world market was estimated at $1560 million of which almost $280 million was accounted for by systemic antifungals. This important increase in market value has induced more interest in research activities in the field of antifungals leading to more and more costly new treatment modalities. The five most prescribed antifungals today are miconazole, clotrimazole, ketoconazole, nystatin, and econazole. As this list indicates, among antifungal drugs the azoles are most significant; roughly 70% of all antifungals used today belong to this chemical category.

Though highly pathogenic, fungi represent only a minority; the importance of opportunistic infections is increasing from year to year. Human resistance against fungal infections is undermined by a number of factors and both exogenous and endogenous factors are capable of increasing the pathogenicity of certain fungi. Fungal growth may be stimulated when the host is treated with antibiotics, oral contraceptives, or cytostatics. Exogenous factors such as protheses, catheters, and valves may give rise to an increased incidence of mycoses. In immunocompromised patients and diabetics, saprophytic fungi may become pathogenic due to disturbances in the internal environment. These patients may present chronic mucocutaneous candidosis (CMC) due to *Candida albicans*. *Aspergillus fumigatus* and *A. niger* may become moderately to highly pathogenic in the presence of previously mentioned predisposing factors.

To mycologists, each fungal infection has something specific, either in its symptomatology or its etiology. However, this is less obvious to practitioners. The incidence and the severity of the pathology are sometimes underestimated. Mycoses may be classified as follows:

Superficial	Deep	
dermatophytosis	aspergillosis	histoplasmosis
trichophytosis	blastomycosis	maduromycosis
microsporosis	candidosis	paracoccidioidomycosis
epidermophytosis	chromomycosis	sporotrichosis
pityriasis	coccidioidomycosis	
candidosis	cryptococcosis	

The physician must decide whether to opt for systemic or topical treatment and which arguments will convince the patient to use one or both forms for the minimum period recommended. It is typical for mycoses that the period of treat-

clopyroxolamine in the Treatment of Mycosis and Its Influence on the Keratinocyte Killing Activity, The Turkish Microbiological Society No. 5, 1986, p. 35.
17. J. D. Stratigos and co-workers, *J. Am. Acad. Dermatol.* **19**(5) (part 1), 850–853 (1987).
18. G. Cauwenbergh, P. De Doncker, P. Schrooten, and H. Degreef, *Int. J. Dermatol.* **25**(8), 541 (1986).
19. A. Polak and D. M. Dixon in R. A. Fromtling, ed., *Recent Trends in the Discovery, Development and Evaluation of Antifungal Agents*, J. R. Prous Science Publishers, SA, 1987, pp. 555–573.
20. A. E. Oxford, H. Raistrick, and P. Simonart, *Biochem. J.* **33**, 240–248 (1939).
21. R. R. Davies in Ref. 3, pp. 49–82.
22. F. Foury and A. Goffeau, *J. Biol. Chem.* **250**, 2354–2362 (1974).
23. H. Vanden Bossche, G. Willemsens, and P. Marichal, *CRC Crit. Rev. Microbiol.* **15**, 57–72 (1987).
24. H. J. Scholer in Ref. 3, pp. 35–106.
25. H. Vanden Bossche and co-workers, *Antimicrob. Agents Chemother.* **17**, 922–928 (1980).
26. J. H. Lewis, H. J. Zimmerman, G. D. Benson, and K. G. Ishak, *Gastroenterology* **86**(3), 503–513 (1986).
27. P. A. J. Janssen and J. E. Symoens, *Am. J. Med.* 80–85 (1983).
28. H. Vanden Bossche, W. Lauwers, G. Willemsens, and W. Cools in F. Schroeder, ed., *European Organisation for Research on Treatment of Cancer*, Monograph 2, Alan E. Liss Inc., New York, 1985, pp. 187–196.
29. J. Van Cutsem, F. Van Gerven, M-A. Van De Ven, M. Borgers, and P. A. J. Janssen, *Antimicrob. Agents Chemother.* **26**, 527–534 (1984).
30. H. Vanden Bossche, D. W. R. Mackenzie, and G. Cauwenbergh, eds., *Aspergillus and Aspergillosis*, Plenum Press, New York, 1988.
31. H. Vanden Bossche in Ref. 19, pp. 207–221.
32. G. Cauwenbergh and P. De Doncker, *Drug Dev. Res.* **8**, 317–323 (1986).
33. P. F. Troke in Ref. 19, pp. 103–112.
34. J. Van Cutsem and P. A. J. Janssen, *28th Interscience Conference on Antimicrobial Agents and Chemotherapy*, Los Angeles, Calif., 1988, p. 141.
35. N. S. Ryder in Ref. 19, pp. 451–459.

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ANTIPROTOZOALS

Diseases caused by protozoa affect more people than those brought on by any other biological cause (1). There are over 60,000 species of protozoa, of which some 10,000 are parasitic. In humans, protozoa chiefly infect the gastrointestinal tract, vagina, urethra, blood, and blood-forming organs. Malaria is the most widespread of the protozoan diseases, and is responsible for the greatest number of deaths due to infection. Although protozoan diseases occur throughout the world, they impact most severely on people of tropical areas where there is widespread malnutrition, minimal health education, and poor sanitation. Fatal protozoal infections are occurring to an increased extent in the more developed countries among immunosuppressed individuals, especially those with AIDS. Domesticated and wild animals are also extensively affected and can harbor and propagate

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Effect of natamycin on the enumeration, genetic structure and composition of bacterial community isolated from soils and soybean rhizosphere

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Received 2 June 2004; received in revised form 27 July 2004; accepted 23 August 2004
Available online 22 September 2004

Abstract

Natamycin is commonly used to control fungal growth on agar media used for bacterial enumeration or strain isolation. However, there is no conclusive report on the possible effect of this antibiotic on bacterial growth or on the diversity of the recovered soil bacteria. Therefore, the possible effects of natamycin on the numbers of bacteria isolated at 12 °C from three different soils and soybean rhizosphere soil were investigated using natamycin concentrations ranging from 0 to 200 mg l^{-1}. Our results demonstrate that natamycin concentrations, which inhibit the growth of fungi on the media, have a small but significant inhibitory effect on the number of bacterial colony forming units. A natamycin concentration of 50–200 mg l^{-1} is required for an efficient control of fungal growth on media in our experimental conditions depending on the soil type. Bacterial community structure was assessed on culturable cells (cells washed from enumeration plates: plate-wash approach) obtained at 12 °C from soybean rhizosphere soil by performing Ribosomal Intergenic Spacer Analysis (RISA) fingerprinting. We demonstrate that all natamycin concentrations used alter the structure of the recovered, culturable bacterial community, compared to control without natamycin. Using ARDRA (amplification of the 16S rDNA gene and restriction analysis) genotyping of individual isolates, some differences were observed between the bacterial isolates obtained in the presence or absence of natamycin. Bacterial isolates recovered in the presence of natamycin are more tolerant (maximal growth rate and lag phase) to this compound than those isolated without natamycin, indicating a possible selection of resistant strains. Therefore, high concentration of natamycin cannot be used for isolation of bacterial strains with the aim of studying biodiversity and could bias a selection of strains for practical applications.

Keywords: Bacterial isolation; Culture medium; Natamycin; Bacterial enumeration; Structure of culturable bacterial community; RISA; ARDRA; Soybean; Rhizosphere

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0167-7012/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2004.08.008

1. Introduction

The isolation of soil bacteria for practical applications like nitrogen fixation, biological control of plant pathogens, or soil bioremediation, is generally performed by culturing on culture media solidified by agar. The composition of the culture medium and the conditions of incubation (temperature) of this first step of isolation can impose bias. The biodiversity of isolates was reported to change with the culture medium used. Phenotypical differences between soil bacterial populations isolated on three different media were observed (Sorheim et al., 1989). Tabacchioni et al. (2000) have reported that 9% of the bacterial strains isolated from corn rhizosphere soil have different genotypes when using two different isolation media. The culture medium could influence the recovery of psychrotolerant bacteria from permafrost (Vishnivetskaya et al., 2000).

In temperate countries, typical winter soil temperatures in the 0–10-cm layer of the surface soil are in the range of 0–15 °C (Cookson et al., 2002) with a mean soil temperature of 14 °C during the period of February to April. However, the selection and screening strategies used to isolate beneficial bacteria for inoculation generally have not taken into account that bacteria will be inoculated in soils at low temperatures (Teperi et al., 1998).

Antifungal agents used in growth media might represent another bias. Due to the relative abundance of fungi and bacteria in soils, it is often necessary to control the growth of some fast-growing fungi on plates. This is particularly critical when trying to isolate bacteria present in low numbers in soil, like for phosphates solubilizing bacteria (Kucey, 1983), or pesticides degraders (Charnay and Fournier, 1994) occurring at 10^1–10^4 g^{-1} soil compared to 10^3–10^4 for fungi. Until recently, the fungicide currently used was cycloheximide, which is considered as very toxic (Martin et al., 2002; Physical & Theoretical Chemistry Laboratory, University of Oxford, UK, 2003). Cycloheximide is an inhibitor of protein synthesis and induces apoptosis in mammalian cells (Busch et al., 2004; Chao et al., 1999).

Natamycin, a product that is safe for humans and utilized in food technology, was proposed by Pedersen (1992) to replace cycloheximide in agar media. The concentrations used vary over an extended range: from 21 to 500 mg l^{-1} (Pedersen, 1992; Engel, 1993; Gomez et al., 1995; Edelstein and Edelstein, 1996; Stack et al., 2002).

To the best of our knowledge, there is no conclusive report on the possible action of natamycin (stimulation or inhibition) on the growth and biodiversity of soil bacteria apart Tsao and Thieleke (1966) who have reported that 100 mg l^{-1} of natamycin could stimulate bacterial growth. This point must be taken into account to develop an efficient strategy of strain selection for application.

In this context, our study aims at evaluating the effect of natamycin on the bacterial growth and diversity of the bacteria recovered on the growth media. The effects of natamycin concentrations ranging from 0 to 200 mg l^{-1} on bacterial growth were tested at 12 °C using three soils with different physicochemical characteristics as well as one soybean rhizosphere soil. In order to characterize the biodiversity observed on culture media supplemented or not with natamycin, two approaches were conducted on bacterial strains isolated from soybean rhizosphere soil: (i) bacterial community structure and composition was assessed by performing RISA (ribosomal intergenic spacer analysis) fingerprinting of whole DNA directly extracted from colonies washed from culture medium plates; (ii) individual colonies grown on the same culture media were characterized by genotyping (ARDRA) and phenotyping (resistance to natamycin).

2. Materials and methods

2.1. Soil and rhizosphere soil

Three soils were studied. The Epoisses soil (Ep) was a clay eutric cambisol (granulometric composition: clay 46.7%, silt 47.4%, and sand 5.9%; 41 g organic matter kg^{-1} soil; pH 7.5) sampled at the INRA Experimental farm situated in Bretennières, 21, France. The Auvillars soil (Au) was a sandy loam luvisoil (granulometric composition: clay 9.7%, silt 29%, sand 61.3%; 18 g organic matter kg^{-1} soil; pH 7.4) sampled at Auvillars sur Saône, 21, France. The Dardilly soil (Da) was a sandy loam cambic arenosoil (granulometric composition: clay 14.9%, silt 19.9%, sand 65.2%; 11 g organic matter kg^{-1} soil; pH 6.15) sampled at

Dardilly, 69, France. All soils were sampled from the 0–10-cm-depth layer, sieved to 4 mm prior to their use. The soils had no known previous history of treatment by natamycin but may have been treated by other fungicides, i.e., $CuSO_4$ (cultivated soils).

Rhizosphere soil was obtained by the following way: soybean seeds (*Glycine max* L. *Merril*) Essor, were surface sterilized in a saturated hypochloride solution (70% active chlorine) for 5 min, rinsed several times with sterile water and sowed in pots containing 2 kg of the Epoisses soil. Plants were grown in a chamber with day/night conditions of 11/13 h at 18/15 °C and lighting at 240 μE s^{-1}. Soil moisture was maintained at 80% of the water holding capacity. After 6 weeks, soil adhering to the roots (rhizosphere soil) of three plants was recovered for subsequent enumeration and biodiversity study of the rhizosphere bacteria.

2.2. Bacterial and fungal enumeration, bacterial strain isolation

Appropriate serial dilutions of the soil suspensions (soil or rhizosphere soil) were plated on 1/10 strength Tryptone Soybean Agar (TSA) medium and containing 0 (N^0), 50 (N^{50}), 100 (N^{100}), or 200 mg l^{-1} natamycin (N^{200}). Media were sterilized at 120 °C for 20 min and γ irradiated natamycin (25 mg l^{-1} solution, Sigma, France) was added after autoclaving. Five plates were inoculated with a $1/10^4$ to $1/10^6$ fold dilution for bacterial enumeration and with a $1/10^2$ to $1/10^4$ fold dilution for fungal enumeration for each media. For fungal enumeration, the medium was supplemented with 500 mg l^{-1} chloramphenicol to prevent bacterial growth. Plates were incubated in the dark at 12 °C (to mimic average soil temperature during the spring under temperate climatic conditions) for 7 days, and numbers of CFU were recorded.

2.3. Resistance of individual isolates to natamycin

Subsequent analysis of bacterial diversity was realized only on the soybean rhizosphere soil obtained from the Epoisses soil.

Forty-three colonies were isolated for N^0 and N^{200} treatments, from three plates of the same dilution, representing approximately 18% of the total CFU. They were pre-cultured in microplates in 1/10 strength Tryptone Soybean Broth (TSB). The growth of bacterial isolates was monitored in 100-well microtiter plates using a Bioscreen® System (Labsystem, Helsinki, Finland). Each well was filled with 300 μl of 1/10 strength TSB medium, containing 0 or 200 mg l^{-1} natamycin and inoculated with 10 μl of the strain pre-cultures. Plates were incubated at 18 °C for 140 h. Optical density ($A_{620\ nm}$) was measured each 2 h. Analyzed data were: (1) maximal growth (growth yield) estimated by the maximal optical density, (2) maximal growth rate estimated by the maximal increase in optical density per unit of time, and (3) apparent lag phase estimated by the time required to observe an increase in optical density. Populations of the two treatments were grouped in five inhibition classes (0–10%, 11–20%, 21–30%, 31–50%, and >50%) corresponding to a decrease of maximal growth or an increase in apparent lag phase between cultures grown on media supplemented or not with natamycinin (expressed as a percentage compared to control culture without natamycin).

2.4. Bacterial community fingerprinting by ribosomal intergenic spacer analysis (RISA)

For each dose of natamycin, bacterial colonies of three plates, each considered as a replicate, were washed and suspended in 4 ml sterile water (plate-wash approach) and then stored at −20 °C for subsequent RISA fingerprinting. DNAs from the washed cells were extracted for all the treatments (N^0, N^{50}, N^{100} and N^{200}) of the Ep rhizosphere soil, by the method of Ranjard et al. (2003), slightly modified. Modifications were the following: cell suspensions (800 μl) were mixed with TES buffer (100 mM Tris–HCl [pH 8.0], 100 mM EDTA [pH 8.0], 100 mM NaCl [pH 8.0]). After three heat chocks (freezing in liquid Nitrogen and 5 min in boiling water), 2% (wt/vol) sodium dodecyl sulfate were added to the suspensions, mixed by hand and incubated at 70 °C for 30 min. Purified DNA samples were quantified according to Ranjard et al. (1998). Image analysis and DNA quantification was performed with ImageQuaNT software (Molecular Dynamics, France).

RISA analysis of bacterial communities was performed on all the treatments using 50 ng of purified template DNA as described by Ranjard et al. (2001). PCR products (25 μl) were then loaded on a 5% nondenaturing acrylamide gel (acrylamide-*N*,*N*-

methylenebisacrylamide, 37.5:1, Bio-Rad, France) in TBE buffer and separated by electrophoresis for 12 h at 60 V and 5 mA (DSG200-02, C.B.S. Scientific, Del Mar, USA). Gels were stained with SYBR green I (FMC Bioproducts, Paris, France) according to the manufacturer's instructions. The banding patterns were photographed using a Storm 860 (Molecular Dynamics). Gels were analyzed using the oneD-Scan software (ScienceTec, Paris, France). The software converted fluorescence data into electrophoregrams where peaks represented PCR fragments. The height of the peaks was calculated in conjunction with the median filter option and the Gaussian integration in oneD-Scan, and represented the relative proportion of the fragments in the total products. Lengths (in base pairs) were calculated by using a 100-bp molecular weight standard (Promega, Paris, France) with bands ranging from 100 to 1500 bp. To convert data obtained from oneD-Scan software rapidly into a table summarizing band presence (i.e., peak) and intensity (i.e., height or area of peak), PrepRISA program (Ranjard et al., 2001) was used. This software allowed us to choose the number of peaks (i.e., all detected populations versus most dominant populations), the profile resolution (between 1 and 10 bp), and the method for evaluating peak intensity (height or area).

2.5. Genotyping of individual isolates by ARDRA (amplified ribosomal DNA restriction analysis)

ARDRA was performed on the forty-three isolates obtained from the Ep rhizosphere soil, already described in Section 2.3. Amplification of the 16S rDNA genes was performed as follows: aliquots of 100 µl of bacterial cultures in 1/10 TSB medium were diluted with Tris–Cl (final concentration of 5 mM and pH 8) and 10 µg of proteinase K were added. Cells suspensions were incubated at 55 °C for 1 h followed by boiling at 95 °C for 10 min. Cell lysates were used as templates to amplify 16S rDNA for each strain using the forward primer 27f (5′ AGA GTT TGA TC(A/C) TGG CTC AG 3′, positions 8–27 of the *Escherichia coli* 16S rDNA and the reverse primer 1492r; 5′ TAC GG(A/T/C) TAC CTT GTT ACG ACT T 3′, positions 1492–1513 of the *E. coli* 16S rDNA sequence). PCR reactions were performed in a total reaction volume of 25 µl, containing: 200 µM of each dNTP, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 0.5µl of the Taq polymerase (Q-Biogene, France), 2.5 µl of template DNA. PCR was performed with a thermocycler (Cycler MJ Research, VWR International, France). PCR cycles were as follows: 1 cycle at 95 °C for 5 min; 35 cycles at 94 °C for 1 min, at 55 °C for 1 min, at 72 °C for 2 min. Final elongation was performed at 72 °C for 15 min. PCR products were analyzed on 1% agarose gels (type II EEO, Sigma Aldrich, France) in TBE buffer. Restriction analysis of the PCR products was performed in a 10 µl reaction containing: 1 µl of 10× restriction enzyme buffer (Incubation Mix II Q-Biogene), 10 U of the restriction endonuclease and 5 µl of PCR product. Reactions were then incubated overnight at 37 °C. Restriction fragments were separated on 3.5% Metaphor agarose gels (FMC, Bioproduct). Gels were photographed after staining with ethidium bromide at 0.5 µg ml^{-1} with the imaging system Bioprint (Vilber Lourmat, France). Sizes of the fragments were estimated using the molecular mass marker VIII (Roche Diagnostic, France) with the Bio 1D++ software (Vilber Lourmat). Final grouping of strains was done also by visual comparison of restriction patterns.

Seventeen strains were analyzed by ARDRA using eight restriction endonuclease enzymes (*Hha*I, *Hae*III, *Hinf*I, *Alu*I, *Dde*I, *Msp*I, *Nde*II, *Rsa*I). Two restriction enzymes (*Hha*I and *Alu*I) were found to be the most discriminative as 16 distinct ARDRA patterns were found using these two enzymes. Therefore, these two enzymes were further used to analyze 43 strains from both treatments by ARDRA.

2.6. Statistical analysis

Variance analyses for colony counts were performed using StatView Version 5 (SAS Institute, USA) with the Fisher's PLSD test at the probability level of 5%.

A principal component analysis (PCA) of the RISA profiles was performed on the data matrix (community submitted to natamycin treatments as rows and bands as columns) generated by PrepRISA software (Ranjard et al., 2001). This method provided an ordination of the bacterial community structure submitted to the different treatments, which were plotted in two dimensions based on the scores in the first two principal components (Ranjard et al., 2003). PCA was performed using the ADE-4 software (Thioulouse et al., 1997).

Table 1
Effect of natamycin on the bacterial counts on 1/10 strength TSA medium (CFU g^{-1} dry soil)

Dose of natamycin (mg l^{-1})	Ep rhizosphere soil	Epoisses (Ep) soil	Dardilly (Da) soil	Auvillars (Au) soil
0	7.4×10^8	1.5×10^7	3.8×10^6	1.1×10^7
	(1.6×10^6)	(3.5×10^5)	(9.3×10^4)	(1.3×10^6)
50	6.5×10^8	1.4×10^7	3.0×10^6	8.1×10^6
	(3.8×10^7)	(5.8×10^5)	(1.2×10^5)	(1.1×10^6)
100	6.0×10^8	1.4×10^7	2.6×10^6	7.9×10^6
	(1.1×10^7)	(3.2×10^5)	(2.5×10^5)	(4.3×10^5)
200	4.6×10^8	1.2×10^7	1.8×10^6	6.4×10^6
	(8.6×10^6)	(4.3×10^5)	(1.8×10^5)	(5.8×10^5)

Data inside parentheses indicate standard error (SE).

Table 3
Effect of natamycin on the fungal counts on 1/10 strength TSA medium (CFU g dry $soil^{-1}$)

Dose of natamycin (mg l^{-1})	Ep rhizosphere soil	Epoisses (Ep) soil	Dardilly (Da) soil	Auvillars (Au) soil
0	9.8×10^5	7.9×10^4	6.2×10^4	7.7×10^4
	(9.7×10^4)	(3.7×10^3)	(4.4×10^3)	(6×10^2)
50	1.8×10^5	$<10^2$	2.3×10^2	$<10^2$
	(3.7×10^4)	(BD)	(1.3×10^2)	(BD)
100	4.1×10^4	$<10^2$	$<10^2$	$<10^2$
	(2.4×10^4)	(BD)	(BD)	(BD)
200	$<10^3$	$<10^2$	$<10^2$	$<10^2$
	(BD)	(BD)	(BD)	(BD)

Data inside parentheses indicate standard error (SE).
BD: below detection limit.

The number of genotypes for each population of N^- and N^1 was estimated by rarefaction analysis using Chao1 as richness estimator plotted against sample size in order to test a posteriori the efficiency of sampling effort to describe the actual diversity. These tests were computed by using the EstimateS program (version 6.0b1 for Windows, 2000). We ran 500 randomizations for all tests, without replacement and with bias correction.

The distribution of isolates from N^0 and N^{200} populations among ARDRA types was compared using the StatXact software (version 3 for Windows, Cytel Software Corporation). The chi-square test for independence was used to compare the structure of the two populations (Laguerre et al., 2003).

3. Results

3.1. Natamycin decreases bacterial counts on TSA medium

Results from bacterial enumeration are given in Tables 1 and 2. ANOVA analysis showed that natamycin has small but significant effects on bacterial counts for all soils studied. Increasing levels of natamycin significantly reduced bacterial numbers on plates for the three soils tested and the rhizosphere soil (Table 2).

The fungal growth was controlled by natamycin in our experimental conditions, but the efficiency of this control depends on soil type (Table 3). Increasing concentrations of natamycin up to 200 mg l^{-1} improved the inhibition of fungal growth (Table 3). Natamycin concentrations of 50 mg l^{-1} of natamycin for Ep and Au soils, and of 100 mg l^{-1} of natamycin for Da soil, are required to control fungi (more than 99.8% inhibition, less than 10^2 CFU g^{-1} soil). However, in Ep rhisospheric soil, natamycin concentrations of 200 mg l^{-1} should be used to control fungi below 10^3 CFU g^{-1} soil. The use of such natamycin concentrations for the different soils significantly decreased bacterial numbers for all soils (Table 2).

In a parallel experiment, we observed that natamycin concentrations up to 200 mg l^{-1} were not sufficient to control fungal growth when plates were

Table 2
Level of significance for bacterial counts between natamycin treatments for the different soil samples [values of the probability (P) from the PLSD Fisher test]

Doses of natamycin (mg l^{-1})	0 and 50	0 and 100	0 and 200	50 and 100	50 and 200	100 and 200
Ep rhizosphere soil	0.0104*	0.0004*	<0.0001*	0.1265	<0.0001*	0.0004*
Epoisses soil	0.308	0.2654	0.0021*	0.916	0.0097*	0.0114*
Dardilly soil	0.0098*	0.0011*	<0.0001*	0.1524	0.0008*	0.0065*
Auvillard soil	0.0828*	0.0622*	0.0109*	0.8588	0.2257	0.2917

* Indicates a significant difference at the 5% level ($P<0.05$).

incubated at 28 °C (data not shown). Moreover, bacterial growth was neither inhibited nor stimulated in the presence of natamycin. This might be due to the poor stability of natamycin during the 7-day incubation period at 28 °C. Thus, higher doses of natamycin should be tested when working at this temperature.

3.2. Resistance of individual isolates to natamycin

As the same effects were observed for the three soils and the rhizosphere soil, further analysis of the effect of natamycin on bacterial diversity was conducted only on bacteria from the Ep rhizosphere soil isolated. Forty-three isolates from each of the N^0 and N^{200} populations were tested for their ability to grow in the presence of natamycin. The results were expressed as a percentage of growth in the presence of natamycin compared to the growth of control culture without natamycin. The isolates were grouped in five classes of growth inhibition by natamycin (Table 4). The distribution of isolates in the five inhibition classes were significantly different between the N^0 and N^{200} populations (P=0.0296 and P=0.0364 when considering maximal growth and apparent lag phase, respectively). The distribution of the isolates were not different between these two populations when considering maximal growth rate. Strains from the N^0 population were more sensitive to natamycin than strains from the N^{200} population since 60.5% and 30.3% of strains exhibited a reduced maximal growth in the presence of natamycin, for N^0 and N^{200} populations, respectively. The same trend was observed for apparent lag phase with 55.9% and 28% of strains with an increased lag phase in the presence of natamycin, for N^0 and N^{200} populations, respectively.

Table 4
Growth inhibition by natamycin of strains from the Ep rhizosphere soil isolated in the presence (N^{200}) or absence (N^0) of this antibiotic

Inhibition classes	Maximal growth*		Apparent lag phase*	
	Strains N^0	Strains N^{200}	Strains N^0	Strains N^{200}
A (0–10%)	17 (39.5%)	30 (69.7%)	19 (44.1%)	31 (72.0%)
B (11–20%)	14 (32.6%)	8 (18.6%)	3 (7%)	0 (0%)
C (21–30%)	4 (9.3%)	1 (2.3%)	2 (4.7%)	3 (7%)
D (31–50%)	7 (16.3%)	2 (4.7%)	6 (14%)	3 (7%)
E (>50%)	1 (2.3%)	2 (4.7%)	13 (30.2%)	6 (14%)

* The distributions of N^0 and N^{200} strains were significantly different at P=0.0296 and 0.0364 for maximal growth and apparent lag phase, respectively.

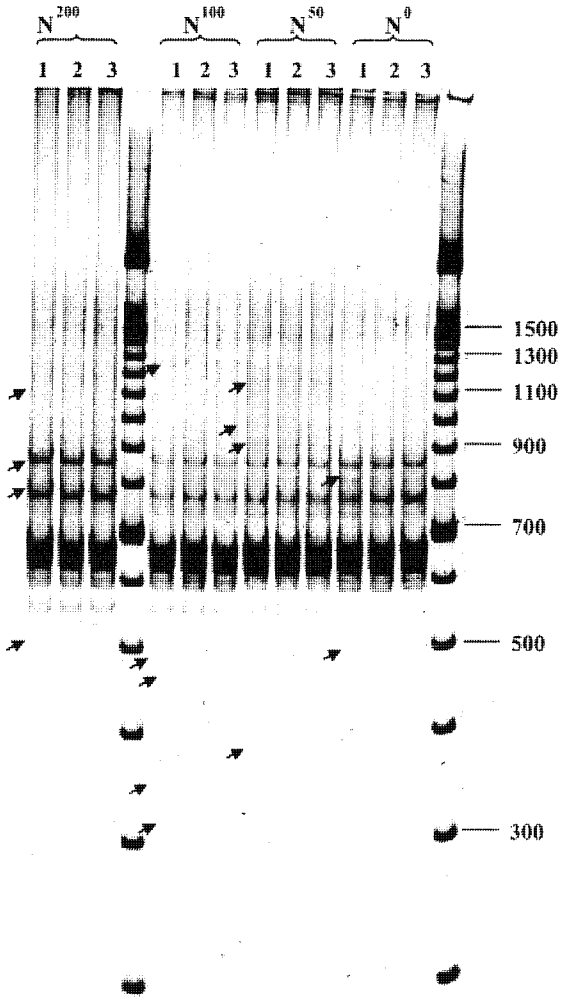

Fig. 1. RISA profiles from the different treatments N^0, N^{50}, N^{100}, and N^{200}, corresponding to bacterial communities isolated on 1/10 strength TSA medium supplemented with 0, 50, 100, and 200 mg l^{-1} of natamycin, respectively. Fragments were resolved on a 5% polyacrylamide gel. 1, 2, and 3 represent the three independent repetitions for each treatment. Arrows indicate discriminating bands between treatments.

3.3. Bacterial community structure, as determined by RISA, is affected by the use of natamycin in culture medium

Fingerprinting of culturable bacterial communities by electrophoretic separation of amplified IGS

sequences between *rrs* and *rrl* genes provided complex profiles with peaks ranging from 300 bp (i.e., 150 bp IGS) to 1400 bp (1250 bp IGS) for the different treatments (Fig. 1). Regardless of the treatments, about 20 bands were detected and integrated for each pattern. The structure of the profiles varied between natamycin treatments in terms of numbers, length polymorphism, and intensity of bands (Fig. 1). Specific or more intense bands appeared in profiles corresponding to the different treatments, as indicated by the arrows in Fig. 1, whereas others bands were less intense or disappeared. The principal component analysis (PCA) allowed us to ordinate bacterial community structure in a factorial map according to natamycin treatment (Fig. 2). Such an analysis confirmed the strong reproducibility of our procedure since it showed more differences between natamycin treatments than between independent repetitions for the same culture conditions. N^0, N^{50}, and N^{200} populations were easily separated on the first axis, which explained 78% of the total variability. Treatment N^{50} was significantly discriminated from the other ones on the second axis, which explained 13% of the variability. Finally, the N^{200} population appears to be the most unique compared to the three other populations (Fig. 2).

3.4. Characterization of individual bacterial isolates by ARDRA reveals that abundance of some phylotypes is affected by natamycin

The continuous analysis of rarefaction curve using the restrictions enzymes *Hha*I and *Alu*I revealed that 43 strains from both N^0 and N^{200} populations were enough to give the maximum of ARDRA types (data not shown). A total of 31 ARDRA types were detected among the N^0 and N^{200} populations, 21 and 23 ARDRA types were found among the N^0 and

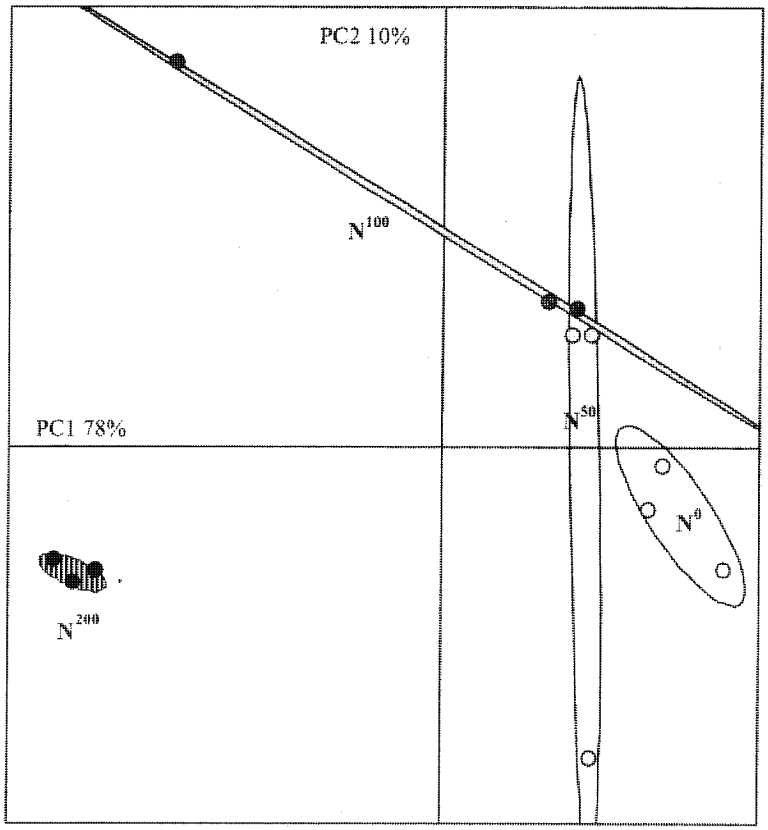

Fig. 2. Principal component (PC1 × PC2) plots generated from RISA profiles of culturable bacterial communities isolated with different natamycin treatments (N^0, N^{50}, N^{100}, and N^{200}). Ellipses represent 90% confidence limits.

Table 5
Distribution of strains from N^0 and N^{200} populations according to their ARDRA types and to their resistance to natamycin

ARDRA type	N^0 population	N^{200} population	ARDRA type	N^0 population	N^{200} population
1	1(E)[a]	0	17	1(B)	1(D)
2	3(A,A,A)	3(A,A,B)	18	1(B)	2(A,A)
3	6(A,A,A,B,B,B)	10(A,A,A,A,B,B,B,B,B,B)	19	2(B,C)	1(A)
4	7(A,B,D,D,D,D,D)	0	20	0	1(A)
5	0	2(A,A)	21	1(C)	1(A)
6	5(A,A,A,B,D)	3(A,A,E)	22	1(B)	0
7	1(C)	3(A,A,A)	23	1(A)	0
8	1(D)	0	24	1(A)	0
9	3(A,B,B)	1(A)	25	1(C)	0
10	1(B)	1(B)	26	2(A,A)	0
11	0	1(C)	27	0	1(D)
12	0	1(A)	28	0	1(A)
13	2(A,B)	2(A,A)	29	0	1(A)
14	1(A)	2(A,A)	30	0	1(A)
15	0	1(A)	31	0	1(E)
16	1(B)	2(A,A)			

[a] Data inside parentheses indicate the number of strains belonging to each inhibition classes by natamycin (see Table 4: inhibition of maximal growth). A, B, and C classes correspond to inhibition by natamycin of less than 30% compared to control culture; D and E classes correspond to inhibition by natamycin of more than 30% compared to control culture.

N^{200} populations, respectively (Table 5). Six (28.6%) and nine (39.1%) ARDRA types, consisting of only one individual, are specific to the N^0 and N^{200} populations, respectively. The ARDRA type 4 is present only in the N^0 population and represents 16.2% of the N^0 isolates. However, the two populations N^0 and N^{200} could not be considered as significantly different (chi-square test for independence P=0.43).

Five strains out of seven from ARDRA type 4, occurring only in the N^0 population, have a maximal growth decreased by more than 30% compared to control, indicating a higher sensitivity to natamycin of strains belonging to this ARDRA type (Table 5). Five out of seven strains from the ARDRA type 4 have lag phase increased by more than 30%, out of which four have lag phase increased by more than 50%, indicating again a higher sensitivity of these strains to natamycin. Our results indicate a possible selection of natamycin-resistant strains, when using this antibiotic in the isolation culture medium.

4. Discussion

The aim of this work was to evaluate the effect of natamycin on the bacterial growth and diversity of the bacteria recovered on the growth media. Natamycin was used as an alternative to cycloheximide for isolating bacteria present in low numbers in the environment (Pedersen, 1992; Engel, 1993; Gomez et al., 1995; Edelstein and Edelstein, 1996; Stack et al., 2002). However, the effect of natamycin on bacterial community structures was never estimated. We choose a 12 °C incubation temperature for isolating bacteria to mimic soil temperature during the spring under temperate climate (conditions for isolating bacteria suitable for practical applications).

Our findings demonstrate that natamycin concentrations varying from 50 to 100 mg l^{-1} are sufficient to control fungal growth in isolation plates using three different soils. In the rhizosphere soil, harboring higher bacterial and fungal populations, natamycin concentrations up to 200 mg l^{-1} are required to control fungi at 12 °C.

Increasing amounts of natamycin reduced significantly bacterial numbers for the three soils and the rhizosphere soil. This means that some bacteria from the soil microflora are not resistant to this antibiotic. Inhibition of bacterial growth has to be considered when using natamycin on isolation plates for bacterial selection studies. Finally, we never observed (at either 12 or 28 °C) bacterial growth stimulation by natamycin, which is contradictory to the findings of Tsao and Thieleke (1966).

In the presence of natamycin in isolation plates, the potential modification of the diversity of the recovered bacteria was monitored. This was done on cells washed from plates and on individual isolates obtained from the rhizosphere soil.

Analysis of the RISA patterns have demonstrated a significant difference between populations with and without natamycin. Up to 100 mg l^{-1} natamycin, the bacterial community structure did not vary significantly. However, the PCA analysis reveals a strong shift in bacterial community structure when the dose of natamycin reaches 200 mg l^{-1}. The differences observed between the RISA profiles were in accordance with the shift observed in the bacterial numbers, indicating that the effect of natamycin at 200 mg l^{-1} is different from those observed at 100 and 50 mg l^{-1} and at 0 mg l^{-1}. All together, our results indicate that high doses of natamycin may induce changes in the diversity of bacteria recovered from plates during the isolation process at 12 °C.

The distribution of isolates among ARDRA types were not significantly different between the N^0 and N^{200} populations, although some changes were observed; that is, the ARDRA type 4 representing 16.2% of the N^0 population did not occur in the N^{200} population. Isolates from ARDRA type 4 are also poorly resistant to natamycin, which could explain their absence in the N^{200} population.

Since ARDRA was performed on individual isolates representing only 18% of the bacterial colonies analyzed by RISA, it is not possible to compare the results obtained by these two methods. We suggest that RISA is probably a more convenient and less time-consuming method than the ARDRA method applied to individual isolates, to monitor small changes of the structure of bacterial populations recovered from plates.

The growth of the strains from the N^0 population is more affected by natamycin than that of strains from the N^{200} population particularly the maximal growth and the lag phase. This result indicates also a possible selection of resistant strains in the presence of natamycin.

The coherence encountered between the effect of natamycin on: (1) plate counts, (2) RISA profiles of culturables bacteria, and (3) growth parameters, allows to conclude that in our experimental conditions, natamycin reduced the number of colonies and induced changes in the genotypic and phenotypic diversity of the culturable bacterial populations. Therefore, the use of natamycin at 12 °C can bias the bacterial isolation procedure and thus should not be used for a diversity study.

Acknowledgements

The authors gratefully acknowledge the technical assistance of Géraldine Depret, Jean-Jacques Giraud, David Lejon, and Virginie Novak. The work was financially supported by the Egypt Embassy, Paris, France.

References

Busch, C.R., Health, D.D., Hubberstey, A., 2004. Sensitive genetic biomarkers for determining apoptosis in the brown bullhead (*Ameiurus nebulosus*). Gene 329, 1–10.

Chao, K.S.C., Hsu, J.S.J., Xu, J., Ezekiel, U.R., Eves, E., Rosner, M., Hsu, C.Y., 1999. Differential effect of cycloheximide on neuronal and glioma cells treated with chemotherapy and radiation. J. Neuro-Oncol. 45, 19–26.

Charnay, M.P., Fournier, J.C., 1994. Study of the relation between carbofuran degradation and microbial or physicochemical characteristics of some French soils. Pestic. Sci. 40, 207–216.

Cookson, W.R., Cornforth, I.S., Rowarth, J.S., 2002. Winter soil temperature (2–15 deg C) effects on nitrogen transformations in clover green manure amended or unamended soils; a laboratory and field study. Soil Biol. Biochem. 34, 1401–1415.

Edelstein, P.H., Edelstein, M.A., 1996. Natamycin as a selective antifungal agent in media for growth of *Legionella* spp. J. Clin. Microbiol. 34, 185–187.

Engel, G., 1993. Hemmung von Hefe- und Schimmelpilzwachstum beim quantitativen Nachweis von Bakterien. Milchwissenschaft 48, 325–327.

Gomez, M., Revellin, C., Hartmann, A., Catroux, G., 1995. Improved enumeration of *Bradyrhizobium japonicum* in commercial soybean inoculants using selective media. Lett. Appl. Microbiol. 21, 142–145.

Kucey, R.M.N., 1983. Phosphate-solubilizing bacteria and fungi in various and virgin Alberta soils. Can. J. Soil Sci. 63, 671–678.

Laguerre, G., Louvrier, P., Allard, M.R., Amarger, N., 2003. Compatibility of rhizobial genotypes within natural populations of *Rhizobium leguminosarum* biovar *viciae* for nodulation of host legumes. Appl. Environ. Microbiol. 69, 2276–2283.

Martin, K.W., Mattick, K.L., Harrison, M., Humphrey, T.J., 2002. Evaluation of selective media for *Campylobacter* isolation when cycloheximide is replaced with amphotericin B. Lett. Appl. Microbiol. 34, 124–129.

Pedersen, J.C., 1992. Natamycin as a fungicide in agar media. Appl. Environ. Microbiol. 58, 1064–1066.

Physical & Theoretical Chemistry Laboratory, University of Oxford, UK. 2003. Safety (MSDS) data for cycloheximide. http://ptcl.chem.ox.ac.uk/MSDS/CY/cycloheximide.html.

Ranjard, L., Poly, F., Combrisson, J., Richaume, A., Nazaret, S., 1998. A single procedure to recover DNA from the surface or inside aggregates and in various size fractions of soil suitable for PCR-based assays of bacterial communities. Eur. J. Soil Biol. 34, 89–97.

Ranjard, L., Poly, F., Lata, J.C., Mougel, C., Thioulouse, J., Nazaret, S., 2001. Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. Appl. Environ. Microbiol. 67, 4479–4487.

Ranjard, L., Lejon, D., Mougel, C., Scherer, L., Merdinoglu, D., Chaussod, R., 2003. Sampling strategy in molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities. Environ. Microbiol. 5, 1111–1120.

Sorheim, R., Torsvik, V.L., Goksoyr, J., 1989. Phenotypical divergences between populations of soil bacteria isolated on different media. Microb. Ecol. 17, 181–192.

Stack, J.A., Harrison, M., Perrett, L.L., 2002. Evaluation of a selective medium for *Brucella* isolation using natamycin. J. Appl. Microbiol. 92, 724–728.

Tabacchioni, S.L., Chiarini, A., Bevivino, C., Cantale, C., Dalmastri, C., 2000. Bias caused by using different isolation media for assessing the genetic diversity of a natural microbial population. Microb. Ecol. 40, 169–176.

Teperi, E., Keskinen, M., Ketoja, E., Tahvonen, R., 1998. Screening for fungal antagonists of seed-borne *Fusarium culmorum* on wheat using in vivo tests. Eur. J. Plant Pathol. 104, 243–251.

Thioulouse, J., Chessel, D., Dolédec, S., Olivier, J.M., 1997. Ade-4: a multivariate analysis and graphical display software. Stat. Comput. 7, 75–83.

Tsao, P.H., Thieleke, D.W., 1966. Stimulation of bacteria and actinomycetes by the polyene antibiotic pimaricin in soil dilution plates. Can. J. Microbiol. 12, 1091–1094.

Vishnivetskaya, T., Kathariou, S., McGrath, J., Gilichinsky, D., Tiedje, J.M., 2000. Low-temperature recovery strategies for the isolation of bacteria from ancient permafrost sediments. Extremophiles 4, 165–173.

ENCYCLOPEDIA OF FOOD TECHNOLOGY AND FOOD SCIENCE SERIES • VOLUME 2

Encyclopedia of Food Technology

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THE AVI PUBLISHING COMPANY, INC.

Westport, Connecticut

1974

anthocyanins. In such cases, the enzyme anthocyanase, which is now available commercially, may be used to lower their concentration.

The determination of the anthocyanin content of a fruit product is simple when only one anthocyanin is present or dominant. The pH of an aqueous extract of the pigment is adjusted to 2 levels, such as pH 1.0 and 5.0, in the case of the anthocyanin pigment of strawberries, and the difference of absorbance at these pH levels is related to the concentration of anthocyanin by means of a reference curve prepared with pure pigment. In the presence of more than one anthocyanin, first separation of the pigments is necessary, which complicates the determination.

PERICLES MARKAKIS

ANTIBIOTICS IN FOODS

The use of antibiotics in foods, and their applications in food technology have been known and studied for many years. Antiobiotics may occur in foods (a) naturally; (b) through direct addition as a food additive to aid in production and keeping quality of the food; or (c) as an unintentional food additive resulting from the carryover of the antibiotic in milk, meat, or eggs as a result of the addition of antibiotics to the feed of animals for growth promotion, or from animal medication for the prevention or treatment of animal diseases.

Naturally-occurring nisin, for example, is an antibiotic which is known to occur naturally, although not frequently, in normal milk. Nisin may also be added directly to the food as an intentional food additive. Gassy fermentations in raw cheese, made with milk containing Clostridia, are controlled, for example, by the use of nisin-producing starter cultures. Such nisin-producing streptococci in milk have been used with success in France for the commercial manufacture of processed cheese. The use of such cultures can result in production of from 50 to 100 units of nisin per gram of cheese.

Nisin

Nisin is one of the antibiotics which is permitted in many countries as a direct food additive. It is a member of a group of closely related polypeptide antibiotics, produced by some strains of *Streptococcus lactis* of the Lancefield group N.

Antibiotic Spectrum.—Some species of Gram-positive bacteria, including bacilli, clostridia lactobacilli, streptococci and staphlococci are sensitive to nisin; however, all enterobacteria and other Gram-negative organisms are not sensitive. Molds and yeasts are insensitive to nisin. The importance of nisin in food preservation is its ability to prevent the outgrowth of bacterial spores in heat-treated foods. The international reference preparation of nisin is defined as 0.001 mg of the pure nisin.

Biological Data.—Nisin was reviewed by the joint FAO/WHO Committee on Food Additives in July 1968, and on the basis of its toxicological evaluation was considered as an acceptable additive, the unconditional acceptable daily intake (ADI) being 0–33,000 units of nisin per kilogram of bodyweight. Nisin is destroyed by digestive enzymes and no nisin was detected in human saliva 10 min after consumption of a chocolate milk containing 200 units of nisin per milliliter. It is readily inactivated by pancreatin at a pH of 8. Nisin has a LD_{50} of over 1,000,000 units per kilogram of body weight of both oral and intraperitoneal (IP) administration in the rat. In the rabbit it has an intramuscular (IM) LD_{50} of 800,000 units, and a subcutaneous LD_{50} of 1,000,000 units.

Two-year chronic toxicity studies are summarized in the FAO/WHO report as well as short-term and other biologic effects including allergy and microbial resistance. Extensive microbiological studies have not shown any cross resistance in organisms which might affect the therapeutic dose of antibiotics. No effect could be demonstrated on the intestinal flora. The antibiotic is destroyed rapidly by proteolytic digestion in the upper part of the GI tract.

Analytical.—The assay of residual nisin in foods can be determined by reverse phase disc assay. The method is sensitive to the presence of 1 unit of nisin per milliliter of supernatant, equivalent to the presence of 5 units per gram of foodstuff in this assay, which employs heat-shocked spores of *Bacillus stearothermophilus*.

Use in Food Technology.—Nisin was first used to control bacterial spoilage of processed cheese spread in the United Kingdom about 1953; since then, its use has been adopted in many countries for use in cheese and certain other foods. The usual recommended level is 20 units per gram. Nisin may occur naturally in milk and milk products.

Its use in cheese and cheese products is to prevent putrefaction caused by relatively heat-stable clostridia. Its use in other foods has been studied, such as in canned vegetables, fruit, fish, meat, milk puddings, etc., as a means of increasing shelf-life, and/or reducing the temperatures or the

time of heat processing required to give the protection obtained in the absence of nisin.

It has been shown that heat-damaged spores have an increased sensitivity to nisin. Information on the mode of action of nisin is presented by several workers and there seems to be general agreement that nisin is sporocidal rather than sporostatic.

The major use of nisin in foods is in cheese and processed cheese products. The principal reason for the use of nisin in these products is to prevent the growth of clostridia and consequent "blowing" caused by clostridia carried through from the original cheese. For this type of use, nisin at levels of 100–400 units per gram of cheese mix has been satisfactory. Nisin can be simply dispersed in water and added to the mix at the same time as the melting salts.

Its use in chocolate milk has been practiced as cocoa powder may be a potential source of spore-forming bacteria. The use of nisin apparently allows for the use of less drastic sterilizing conditions. In this type application, nisin addition can reduce the spoilage at any given processing condition or will allow for some reduction in the severity of processing without increasing the normal spoilage.

In certain countries, nisin is employed as a processing aid in the production of canned milk puddings, such as rice, semolina, and tapioca puddings, to reduce the heat treatment required. In normal production, the heat required for adequate penetration may cause production difficulties and excessive thickening of the pudding. The addition of 50–100 units of nisin per gram has been shown to be an aid in preservation at lower processing temperatures.

Interest is being shown in the possible use of nisin in recombined and reconstituted evaporated milk to control surviving bacterial spores, when less drastic heating is employed in order to produce a more acceptable product.

TABLE A-10

RETENTION OF NISIN IN SKIM MILK HEATED FOR VARIOUS TIMES AND AT VARIOUS TEMPERATURES

	Percentage of Retention		
Temp °C	3 Min (%)	11 Min (%)	40 Min (%)
110.0	84	57	19
115.6	67	38	7
121.1	60	34	4

SOURCE: Fowler and McCann (1971).

TABLE A-11

RETENTION OF NISIN IN FRESHLY PROCESSED FOODS TREATED WITH 100 UNITS OF NISIN PER GRAM

Product	Temp (°C)	Time (Min)	Retention (%)
Canned garden peas	115.6	40	22.0
Canned tomatoes	100	40	72.4
Canned mushrooms	121.1	18	32.7
Processed cheese	93	12	82.0

SOURCE: Fowler and McCann (1971).

Stability.—The stability of nisin in the presence of heat, is largely a function of the pH of the medium. It is quite stable to autoclaving at a pH of 2, but relatively unstable to autoclaving at a pH of 5. The stability is enhanced by the presence of large molecules such as are found in milk solids and broth.

Nisin has a bacteriostatic action, and therefore residual quantities must be present in order to maintain effective preservation. Data on stability during processing is given in Tables A-10 and A-11.

The stability of nisin in a solution during storage at various temperatures, is characterized by the data on stored chocolate milk in Table A-12.

Approved Use.—The FAO/WHO Expert Committee on Food Additives has favorably considered the use of nisin as a direct additive to foods. In the United Kingdom, the use of nisin has been approved for inclusion in cheese, clotted cream, or any canned food. Additionally, any food may contain nisin introduced in the preparation of that food by the use of cheese, clotted cream or canned food containing nisin. In the case of the canned food, the product is defined as contained in a hermetically sealed container which has been sufficiently heat processed to destroy *Clostridium botulinum* in that food or container or which has a pH of less than 4.5. The background for nisin inclusion in cheeses and canned foods is given in the U.K. Food Standard committee 1959 report, paragraph 270 and 278. The use of nisin in canned foods consumed in the United Kingdom is not very extensive, although it is included for technological reasons for some foods exported to tropical countries.

The U.K. Food Additive and Contaminant Committee has just reviewed the use of preservatives in foods and in their report of July 7, 1972 has recommended that these previously approved uses of nisin be allowed to continue. They also reviewed the proposed application of nisin to cream

TABLE A-12

RESIDUAL NISIN ACTIVITY AND ITS STABILITY DURING STORAGE OF CANNED HEAT-PROCESSED CHOCOLATE MILK[1]

Storage Time Months	Residual U/Ml at 4°C	% Storage Loss at 4°C	Residual U/Ml at 22°C	% Storage Loss at at 22°C	Residual U/Ml at 37°C	% Storage Loss at at 37°C
0	17.5	0	17.5	0	17.5	0
3	14.0	20	13.5	23	9.0	49
6	13.5	23	12.5	23	6.0	66
9	13.5	23	11.0	37	3.5	80
12	12.5	29	8.0	54	3.0	83
15	12.0	31	7.5	57	3.0	83
18	12.0	31	6.5	63	2.0	89
24	12.0	31	5.0	71	1.0	94

SOURCE: Fowler and McCann (1971).

[1] Initial content prior to heating: 100 U/ml; potency loss in initial processing: 82.7%.

in flour confectionery, but have recommended against such use. Cream, other than clotted cream, may not contain any preservative, based on the U.K. Cream Regulations of 1970.

In New South Wales, Australia, nisin may be added to cheese, canned tomato pulp, canned tomato juice, canned tomato paste, tomato purée, and canned fruit, provided that in all cases the pH of these items is below 4.5. Addition to canned soups is permitted, provided the product is submitted to heat treatment sufficient to destroy *Clostridium botulinum*.

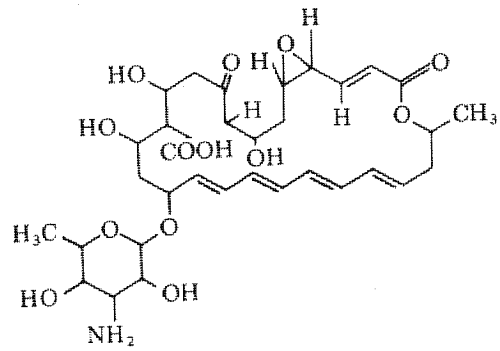
Its use in cheese has been approved in Belgium (2.5 ppm), Czechoslovakia (200 ppm), Finland (100 ppm), Italy (500 ppm), Uruguay (100 ppm), and India at 1000 ppm in cheese and processed cheese. It is also approved in France, Israel (exception of soft white cheese), Jamaica, Mexico, New Zealand (export), Norway (export), South Africa, and Spain. Sweden permits nisin in sterile condensed milk and cream (Group Ie) and cheese (Group If) with no limits.

In vegetables, it is approved in Italy in canned vegetables at 100 ppm, and in Russia in certain canned foods. It is also approved in Russia for certain canned fruits. In Italy it is allowed at 100 ppm in creamed desserts.

In Lebanon, Mozambique, Nigeria, Zambia, and Sudan, nisin is acceptable on the basis of existing legislation elsewhere; there are no specific laws covering food additives in these countries.

Pimaricin

Pimaricin is another antibiotic which is permitted by several countries as a direct food additive for certain foods. It is a fungicidal antibiotic, belonging to the group of polyenic macrolides, more specifically to the tetraenes. It has the following structure:



Pimaricin therefore is closely related to, but not identical with, the other antifungal antibiotics—nystatin, rimocidin, and amphotericin. It is produced by *Streptomyces natalensis Nov. sp.*

Antibiotic Spectrum.—Pimaricin is active against a large number of molds and yeasts, but has no activity against bacteria or viruses. Pimaricin, which is predominantly fungicidal, is effective over a fairly broad pH range. It is also active against fungal spores. Its greatest activity is against actively-multiplying cells, as considerably higher concentrations are required for the destruction of resting cells. For most microorganisms which are sensitive to pimaricin, minimum inhibitory concentration (MIC) ranges from 1 to 15 μg per ml.

The activity of pimaricin against yeasts and molds occurring in foods is considerably greater than that of sorbic acid, pimaricin having MIC of 1–10 μg per ml, while sorbic acid requires approximately 500 μg per ml in most cases.

Biological Data.—Pimaricin is reversibly bound to serum proteins, having a binding strength similar to that of penicillin. There is no evidence of development of resistance to pimaricin, with the exception of the experimental isolation of two strains of *Candida albicans*, which apparently became partially resistant as a result of adaptation. In one of these strains 35 transfers were required to raise the MIC from 1.7 to 15 μg per ml. In this case there was a return from normal sensitivity on withdrawal of pimaricin from the medium. Organisms that become resistant to ampotericin B were also resistant to nystatin, but retained their normal sensitivity to pimaricin. When pimaricin was included in the media it caused no change in sensitivity to erythromycin after 10 transplants different bacterial strains, all sensitive to erythromycin.

Toxicity.—In acute toxicity studies, pimaricin on oral administration showed an LD_{50} of 1500 mg per kilo body weight for rat and mouse, and greater than 1000 for the dog. In additional studies, LD_{50} values of 2730 were found for the female rat and 4670 for the male rat. The LD_{50} for oral dosage yielded in the guinea pig 450 and for the rabbit 1420. Intraperitoneal (IP) dosage yielded an LD_{50} of 250, and intravenous (IV) dosage an LD_{50} of 16.2 for the rat.

In 2-yr chronic toxicity studies, employing dosages of 125, 250, 500, and 1000 ppm of pimaricin in rats, the minimum dosage showing noticeable effect was 1000 ppm which caused a slight inhibition of growth, probably due to diminished appetite.

In 2-year studies with dogs at feeding levels of 125, 250, and 500 ppm, no effects were noticed other than a slight depression in growth in the male dogs at 500 ppm. There were no adverse effects on survival organ weights, or blood, or tissues.

No undesirable allergic effects have been reported, and skin and eye studies reveal no irritant or sensitizing effect of pimaricin. The FAO/WHO Expert Committee on Food additives established a conditional Average Daily Intake (ADI) of 0–0.25 mg of pimaricin per kilo of body weight. On this basis 15 ppm in cheese would be acceptable, assuming a consumption of no more than 1 kilo of cheese a day.

Analytical.—Pimaricin solution in methanol shows ultraviolet absorption spectrogram with minima at 250, 290, and 313 mμ and maxima at 220, 290, 304, and 318 mμ, the latter 3 being used for spectrophotometric assay. The optical density vs. concentration plot for concentrations of 1 to 12 μm per ml yields a straight line; however, with a different slope for each wavelength. The E 1%/1 cm values are 758 at 290 mμ and 1100 at 318 mμ.

Pimaricin has an isoelectric point at a pH of 6.5, and an R_f value of approximately 0.45 when chromatographed on paper (Whatman #1) for 15 hrs at 23° using a 7 to 1 *n*-propanol: water mixture as an eluant.

Assay.—A microbiological assay procedure allowing for the quantitative determination of pimaricin is available employing an agar diffusion test. Small discs of the test material are placed onto the surface of solid Whiffen agar, which has been previously inoculated with *Saccharomyces cerevisiae* ATCC 9763, which is sensitive to approximately 0.5 μg per ml of pimaricin. The clear zones of inhibition found around the discs may represent pimaricin or another fungicide. Other fungicides are differentiated from pimaricin by repeating the test with the inclusion of pimaricinase in the agar.

Pimaricin has been shown to be adsorbed or bound to orange juice pulp; recovery from orange juice depends upon proper sample dilution.

Stability.—In the dry state, pimaricin is an extremely stable compound, provided it is not exposed to sunlight and moisture. In solutions, pimaricin may be relatively unstable particularly when exposed to light. It is more unstable at at high and low pHs, showing greatest stability at a pH of 5 to 7. The unsaturated nature of pimaricin probably accounts for its relative instability in solution and when exposed to light.

When incorporated in food, instability due to light can be effectively reduced through opacity, color, and possibly chemical stabilization. Orange juice, for example, stored for 12 weeks at 2.5°C in the dark showed 77, 74, 70, and 68% retention of pimaricin for pimaricin additions of 20, 10, 5, and 2.5 μg/ml. Chlorophyll addition increases stability twofold. The presence of oxygen increases the decomposition rate, and antioxidants therefore retard the oxidation of pimaricin. It is incompatible with oxidants and rapid destruction results from the presence of peroxide, iodates, bromates, hypochlorates, sulfates, etc.

In stability studies with pimaricin added to cottage cheese through soaking in the wash water, the initial concentrations of pimaricin were from 5 to 10 ppm. At storage temperatures of 40°, 50°, and 60°F, there was a rapid initial loss of residual pimaricin, and no activity could be demonstrated after 3 to 16 days.

In U.K. studies on retention of pimaricin in stored apples dipped with 100 or 200 ppm pimaricin, there were very low residues. In 23 tests of apples stored at 4°C for 51 to 156 days variable results were obtained showing an average

TABLE A-13

EFFECT OF ANTIFUNGAL TREATMENT ON KEEPING QUALITY OF COTTAGE CHEESE

Antibiotic[1] Treatment	Level of Antibiotic in Wash (Ppm)	Average Keeping Time in Days At 40°C No. of Days	At 40°C Improvement (%)	At 50°C No. of Days	At 50°C Improvement (%)	At 60°C No. of Days	At 60°C Improvement (%)
None	0	12.3		6.6		4.3	
Pimaricin	25	16.3	+33	8.3	+26	4.3	0
None	0	16.8		10.0		7.3	
Pimaricin	50	26.8	+60	22.0	+220	15.0	+105
None	0	16.5		6.0			
Mycostatin	10	19.0	+15	10.0	+67		
None	0	12.5				6.0	
Mycostatin	20	21.0	+68			6.0	0

SOURCE: Shahani *et al.* (1959).

[1] Antibiotics added to wash water.

residue of 0.89 ppm of pimaricin. Hand-dipping gave higher residues than machine-dipping.

In pears dipped in 100 ppm of pimaricin, a residue of less than 0.25 ppm was found after 85 days storage at 4°C.

Applications.—*Cheese.*—Pimaricin is employed in some countries in cheese rind to control undesired fungal growth. It does not penetrate into the cheese but is retained on the outer 1 mm of the rind, which is usually not eaten. After a period of 5–10 weeks, the pimaricin essentially disappears; however, during this time the rind hardens and becomes much less susceptible to fungal invasion.

In work conducted at the Netherlands Dairy Research Institute at Ede, four applications methods of pimaricin to hard cheese were studied. These included: (1) pimaricin addition to the brine bath, (2) immersion in an aqueous suspension of pimaricin for 2–4 min after the brine bath, (3) spraying the cheeses with a pimaricin suspension, and (4) covering the cheese with a coating agent containing pimaricin. In all cases a good fungicidal effect was observed.

The recommended application rate for pimaricin in cheese, when applied in cheese coating is 0.005–0.1%, by weight of the coating, 0.005–0.2% of the dipping liquid when applied by dipping, and 0.1–0.2% by weight of the treating liquid for spraying the cheese or for application with a sponge.

Studies have been conducted on the effect of antifungal agents on cottage cheese. By adding antifungal antibiotics (pimaricin and mycostatin) to the second wash water for cheese curd, and holding 5 min before draining, results were obtained as shown in Table A-13. The antibiotics retarded spoilage by yeasts and molds but had little or no effect on microbial spoilage.

Tests were also run (Table A-14) on retardation of cottage cheese spoilage in conjunction with antibiotic retention studies. Cheese washed with antibiotic suspensions of 20 to 100 μg per ml contained 5–10 μg of antibiotic per gram of cheese. In stability testing, the antibiotics lost their activity completely in 3 to 16 days. The rate of loss was greatest in the initial period, and at the higher temperature of 60°F. The improvements due to antifungal agents were greatest at the lowest storage temperature (40°F) and poorest at the highest storage temperature (60°F).

TABLE A-14

EFFECT OF DIFFERENT ANTIFUNGAL TREATMENTS UPON KEEPING QUALITY OF COTTAGE CHEESE

Treatment Method	Average[1] Increase in Keeping Time in Days Pimaricin	Mycostatin
Added through wash water[2]	+5 to 30	+1 to 28
Added through cheese dressing	+up to 39	+up to 20
Mold-infected cheese	+1 to 30	+1 to 17
Yeast-infected cheese	+1 to 16	+1 to 25

SOURCE: Nilson *et al.* (1960).

[1] Average of all treatments and storage temperatures of 40°, 50°, and 60°F.

[2] Antibiotic added to wash water at 20 to 100 μg per ml.

Beverages.—In the case of grape juice, yeast fermentation is prevented by 20 ppm of pimaricin; however, 100 ppm is required to stop active fermentation completely. In wines rich in yeast, 2 ppm kills ½ of the yeast in 1 hr, with practical sterilization being obtained with 10 ppm. In a study of 12 fungicides in the treatment of apple juice, in which the materials were titrated at 10, 20, and 30 ppm, stored at 40°F, and analyzed at periods of 4, 6, 8 and 10 weeks, 30 ppm of pimaricin prevented fermentation for a period of up to 6 weeks. No off-flavor was observed.

In studies with orange juice, it was shown that juice inoculated with natural contaminant spoiled after 1 week of storage, whereas with pimaricin additions as low as 1.25 ppm, it did not spoil in 8 weeks of storage at 2.5–4°C. There were no undesirable effects reported in the pimaricin treatments, even at additions as high as 20 ppm. Pimaricin reduced the yeast population rapidly and the number of viable cells further decreased with storage time. There was a correlation of viable yeast and the pimaricin level used, higher quantities of pimaricin being more fungicidal, particularly with increased storage time. The low levels of 1.25 and 2.5 ppm were judged as having only borderline utility for practical purposes at the temperature employed.

In testing of orange juice concentrates, 10 and 20 ppm of pimaricin held yeast in check at various temperatures for concentrates of 65° and 42° Brix respectively. For periods of testing of 100 days, the inclusion of 10 ppm of pimaricin prevented growth at 10°C, whereas 20 ppm was required to severely retard growth at room temperature.

Fruit.—In the case of whole apples, a 1–2 min immersion in aqueous suspensions of pimaricin containing 500 ppm resulted in a considerable reduction of discards after 8 months' storage. While gloesporium rot in apples is sensitive to pimaricin, if the infection is deep and has penetrated several layers of cells, pimaricin will naturally be unable to control subsequent spoilage.

In evaluation of the effect of dipping apples in a lecithin-pimaricin dipping bath, it was shown that the spots encountered during extended storage, can be materially reduced by such treatment, as illustrated in Table A-15.

Similar results have been reported on dipping of several varieties of apples in studies in the Netherlands. In the United Kingdom, some 56 trials have been conducted in which apples were dipped either by hand or machine in a lecithin-pimaricin mixture for 30–45 sec within 7 days after picking. The test apples were stored under barn storage, cold storage (37–39°F), and storage at 37–39°F with controlled percentage of O_2 and CO_2 in the air.

The recommended amount of pimaricin for the dipping of fruit is 0.02–0.04% by weight of the dipping liquid.

Soft Fruit.—The shelf-life of soft fruits such as currants, plums, strawberries, and raspberries, has been increased by brief immersions of the fruit in aqueous solutions of 50 ppm pimaricin. Pimaricin has been shown to be effective against yeast and mold growth in strawberries, raspberries, cranberries, and cherries.

Other Applications.—Pimaricin has been studied in various baking operations. Rye and white breads were well protected when their surfaces were sprayed with a solution of 100–500 ppm pimaricin. Uncooked doughs were also improved when their surfaces were protected by surface treatment with pimaricin. The keeping time of

TABLE A-15

EFFECT OF DIPPING APPLES IN A LECITHIN-PIMARICIN SUSPENSION ON INCIDENCE AND SEVERITY OF SPOTS IN STORED WHOLE APPLES

Lecithin-Pimaricin Treatment Level		Number of Spots		
Lecithin-Pimaricin Mix[1] (%)	Equiv of Pimaricin (Ppm)	Test No. 1 177 Days @ 4°C 7 Days @ 15°C	Test No. 2 188 Days @ 4°C 13 Days @ 15°C	Test No. 3 179 Days @ 4°C 13 Days @ 15°C
0	0	37	82	55
0.5	20	8	—	17
1.0	40	—	30	13
1.5	60	—	19	14
2.0	80	—	15	9
2.5	100	8	4	9

SOURCE: Stoll (1969).

[1] Soya lecithin, 20% w/v; pimaricin, 0.4% w/v.

fillings for cakes and pies was more than doubled by the addition of 25–50 ppm of pimaricin. Many other foods have also been studied and utility has been demonstrated in the case of margarines, fruit syrups, jams and jellies, pickled products, meat, fish, and poultry.

In the case of sausage, studies have been conducted in the Netherlands which have demonstrated the value of pimaricin. In sausage manufacture, pimaricin has been added as a suspension in one of several steps such as prior to fermentation, by soaking the casing, or dipping the filled sausage, or spraying the filled sausage. Other application methods included pimaricin addition to the brine bath during fermentation, or dipping or spraying the sausages after fermentation, etc. All methods gave improvement in prevention of the mold growth on the surface. No pimaricin could be detected in the sausage meat with the exception of long-term immersion in the brine bath containing pimaricin in which case only traces of pimaricin could be detected in the sausage at a depth of 1–2 mm.

The recommended levels of pimaricin for treatment of hard sausage is 0.05–0.2% by weight of the dipping liquid used for dipping the sausage casing.

Approvals.—Pimaricin has been approved by FAO/WHO for the external application to hard cheese, provided it does not result in a daily intake by humans exceeding 0.25 mg per kilo of body weight, a figure suggested as a conditional ADI. The recommended applications to the rind of cheese are well below such daily intake.

Approval for the use of pimaricin in hard cheese has been given in the Netherlands, Belgium, France, Italy, Sweden, Norway, Spain, and Luxembourg.

The use of pimaricin in hard sausages has been granted in Netherlands Belgium and Spain. Additionally the Netherlands has approved the application of pimaricin to apples and pears.

Nystatin

Nystatin (mycostatin, fungicidin) is another polyene antifungal antibiotic which has some very limited use as a direct food additive. It is used for surface applications on the skin of the banana, and under special circumstances on the surface of meat.

Nystatin is derived from *Streptomyces noursei*, and is closely related to, but not identical with, the other antifungal antibiotics—pimaricin, ramocidin and amphotericin.

Biological Properties.—Nystatin has a fairly broad antifungal spectrum but has no significant activity against bacteria or viruses. Nystatin inhibits endogenous respiration in fungi and their utilization of glucose, whether aerobic or anaerobic. It is used medically for prophylaxis and treatment of monilial infections, both superficial and intestinal. Nystatin is very poorly absorbed from GI tract of man, even when administered in doses as high as 16 gm per day. It has an IP LD_{50} in mice of of about 200 mg per kilo.

Applications.—The use of nystatin as an antifungal for use as a direct food additive has been studied for several applications, such as against fungal spoilage, and in prevention of the decay of peaches thru postharvest treatment.

It has also been studied for possible application in improving the keeping quality of cottage cheese. In these studies direct comparisons were made against pimaricin and details are reported in that section (Tables A-13 and A-14). Nystatin (mycostatin) gave a significant improvement in keeping quality of cottage cheese, but was slightly less effective than pimaricin.

Nystatin finds application in treatment of the skin of bananas. In practice the bananas are dipped in a solution containing up to 400 ppm of nystatin. Residues could be demonstrated on the skin but not in the flesh of the banana. Such use has been approved in Great Britain.

The U.K. Food Additive and Contaminant Committee has just reviewed the use of preservatives in food, and in their report of July 7, 1972 they recommend that this use of nystatin should be abandoned, unless it can be shown to be efficacious and necessary. (No satisfactory alternate method of treatment exists.) Previous antibiotic panels had considered that the use of nystatin on the skin of bananas to control rot was without hazard to the consumer. The present panel concluded that, from a toxicological standpoint, there was no significant danger to health unless the whole banana was used. In the particular circumstances of use, they felt the only persons at risk were those carrying out the dipping or handling of the treated bananas in that they might eventually acquire resistance to nystatin.

The use of nystatin has been reported in the USSR as a surface treatment for certain meats.

The FAO/WHO Expert Committee on Food Additives recommended that the existing restricted external use of nystatin should be permitted but not extended until more adequate studies on its breakdown and toxicology have been reported. No ADI was given.

Tylosin

Tylosin is a macrolide antibiotic which has been proposed and evaluated as a direct food additive. It was proposed as an aid in processing in view of

its activity against clostridia and other spore-forming bacteria. It is produced by *Streptomyces fradiae*, an actinomycete.

Biological Properties.—Its antimicrobial spectrum is essentially Gram positive. It is very active against avian and swine mycoplasma, and finds application in veterinary medicine. It is not used in human medicine.

Tylosin base has an oral LD_{50} of greater than 5000 in the mouse and rat and greater than 800 in the dog.

Application and Approval.—The FAO/WHO Expert Committee on Food Additives in reviewing this antibiotic and its proposed application as a direct food additive, recommended against its use for any purpose which might result in detectable residues in human foods, unless it was necessary to solve an extremely important problem.

In view of the fact that Tylosin shows cross-resistance to some of the therapeutically important macrolide antibiotics used in human medicine, the FAO/WHO Committee felt this would preclude the use of tylosin as a direct food additive, unless required to solve an extremely important problem.

In the United States, several uses of tylosin have been proposed, all relating to destruction of the spores of *Clostridium botulinum*. The use of tylosin lactate as an additive in conjunction with less severe heat processing resulted in a lighter colored and more attractive mushroom product; however, the FDA reported that there may be a delayed spoilage.

In the case of the proposed use in canned dog food of tylosin to permit less vigorous temperature and time processing conditions to avoid caramelization of the buttermilk in a particular type dog food, this use was also found unacceptable by the Committee on Veterinary Medical and Non-Medical Uses of Antibiotics in 1966. The decision was based on the fact that antibiotic use involved a possibility of the appearance in dogs of resistant strains of pathogenic bacteria which could be transmitted to man. In the proposed application in smoked fish, which can be a serious *Cl. botulinum* peril if not properly processed, the Committee suggested that adequate heat processing could suffice together with adequate plant sanitation and management. The Committee's general rejection of the use of tylosin as a feed additive was similar to that of FAO/WHO, and based on data showing such use doses cause the emergence of some resistant organisms and such organisms are frequently cross-resistant to erythromycin and oleandomycin, both therapeutically important antibiotics in human clinical medicine.

Tetracyclines

Tetracyclines have been studied for many years as direct additives to a wide variety of foodstuffs as preservatives in delaying decomposition during transportation, storage, and marketing. This use has been explored for possible application in those countries where adequate refrigeration is not available.

In Milk.—Early studies showed that penicillin, streptomycin, and other narrow spectrums could reduce lactic acid production in stored milk; however, they were of little value against putrefaction. The broad spectrum tetracyclines, when added at 1 ppm, however, could delay the onset of spoilage for 1 day at 98°F. Utility was also demonstrated in pasteurized milk.

In Fresh Fruits and Vegetables.—In fresh fruits and vegetables, bacterial soft-rot has been materially retarded by the use of the tetracyclines or combinations of streptomycin and oxytetracycline.

In Fish and Sea Food.—In view of the rapid decomposition rate of fish, extensive evaluations have been made of the use of the tetracyclines in aiding in the storage and transportation of this perishable foodstuff. Since many of the bacteria involved in fish storage are psychrophilic and can proliferate at refrigerated conditions, the need for preserving aids is great. The tetracycline antibiotics have been shown to be valuable in conjunction with refrigeration as preservatives in fish. The antibiotics are added by addition to the ice used in packing the fish, at levels of 5 ppm, or by dipping or spraying the fish in concentrations of from 10 to 200 ppm of tetracycline. The addition of tetracyclines at a level of 1 ppm to the refrigeration brine is an effective approach. These additions of tetracyclines approximately double the shelf-life of the treated fish.

In dipping fish filets in oxytetracycline solutions for 30 sec, a 10 ppm dip doubled the keeping period, 25 ppm tripled it, and a 50 ppm dip quadrupled the allowable storage period. The tetracyclines have also been employed when fish are caught for the production of fishmeal. Antibiotics have been used in whale fishing. In its early use, the antibiotic was introduced into the meat by delivering it in the exploding head of the harpoon used. This method of application was replaced by pumping the antibiotic into the abdominal and chest cavity during the period the whale was being inflated.

The use of chlortetracyclines in fish preservation was approved in the United States in 1959. Approval included a tolerance of 5 ppm for residues in or on fish, scallops, and shrimp for retardation of spoilage of these sea foods when

each is in fresh, uncooked, unfrozen form. This approval was withdrawn by the FDA in August, 1967 in light of the criteria established by the Committee on Veterinary Medical and Non-Medical Uses of Antibiotics, May 1966. The FDA concluded there was no serious problem in fish handling that would justify this food additive use of chlortetracycline and cited possible public health problems involved if its use continued.

The approval for the use of tetracyclines for the preservation of fish was rather widespread but its use in many countries has been subsequently rescinded in view of the reports of recent advisory committees which have studied such use of antibiotics in foods. In the United Kingdom, the use of tetracyclines in fish was approved in 1960, and a permissible residue level of 5 ppm of the tetracycline in fish flesh was established. In July 1972, the U.K. Food Additive and Contaminant Committee reviewed this use of tetracyclines in fish and recommended its discontinuance. The U.K. Committee indicated that this use of tetracycline did not significantly retard the early stage of fish spoilage, and frequently only extended the storage life by 3 days. In addition, its use had been very slight in the United Kingdom, in view of the increasing use of modern fishing trawlers with freezing facilities. In other countries, such as Japan, and reportedly in USSR, the use of tetracycline in fish still persists. The FAO/WHO Expert Committee on Food Additives recommended against the use of tetracyclines as intentional food additives.

In Meats.—Several workers have shown the value of the use of the tetracyclines in meats. Methods of application of the antibiotic have included surface sprays, infusion into the tissue, both ante- and postmortem. In the case of antemortem treatment, distribution of the antibiotic is accomplished by the action of blood and lymph. Tissue levels of as low as 1 ppm of oxytetracycline were shown to delay internal spoilage, processed meats such as hamburger and sausage having a materially extended shelf-life with a tetracycline treatment. Fresh pork sausage, for example, showed a 50–100% longer shelf-life as a result of adding 3–5 ppm of oxytetracycline.

In Poultry.—In poultry processing, chilled chickens remained salable for only approximately one week at commercial storage temperature. This lead to the investigation of the use of tetracyclines to increase storage life. The addition of 10 ppm to the slush ice reportedly increased the storage time 50–100%.

The FDA approved such use of oxytetracycline and chlortetracycline in the processing of poultry in 1960. As in the case of chlortetracycline with treated fish, the FDA reversed its approval for such direct food addition of the tetracyclines to poultry and rescinded the tolerance for a residue of 7 ppm of the oxytetracycline and chlortetracycline in the edible tissue of uncooked poultry. (This 7 ppm residue was reportedly destroyed during normal cooking.) The revocation of approval and tolerance for tetracyclines was made effective at the same time the FDA rescinded approval for the use of chlortetracycline in fish.

The basis for rescinding both was the same: The FDA no longer felt that such processing aids were essential; and the use of tetracyclines could result in the selection of naturally resistant organisms, and the emergence of resistant strains, as well as an increase in the mold and yeast development.

Subtilin

Subtilin has been the subject of considerable investigation as a direct food additive aid in canning of foods in light of its activity against spores of *Clostridium botulinum*. This investigational food additive activity was terminated when it was discovered that the combination of subtilin and mild heat was not always effective in killing spores of *Clostridium botulinum*.

Streptomycin

In the present climate of the restricted regulatory attitude towards the use of antibiotics as direct food additives, as recommended by the several scientific advisory groups studying the problem, many of the previous direct food additive uses of antibiotics have been revised or rescinded. In the case of pome fruits, however, this was not the case. The U.S. Secretary of Agriculture advised that certain commercial varieties of apples or pears are highly susceptible and are severely damaged by fire blight; he indicated that streptomycin was the only nonphytotoxic chemical giving effective control.

It was therefore concluded that the serious fire-blight problem justified the use of streptomycin on pome fruits and a tolerance of 0.25 ppm (negligible residue) in or on pome fruit was established when streptomycin was used for controlling fire blight. Streptomycin had been widely used for 15 yr in the control of fire blight on the basis of no residues being present at harvest as determined by the analytical procedure available which was sensitive to 0.25 ppm. The residue tolerance of 0.25 ppm was established in 1968 as a "negligible residue" and was determined by the sensitivity of the assay employed.

Unintentional Food Additives

Antibiotics may enter human food channels from unintentional carryover in meat, eggs, and milk from the agricultural and veterinary use of antibiotics which have been added to animal feeds for the purposes of stimulating the animal's growth and for the prevention and treatment of animal diseases. Additionally, they may enter human food as unintentional residues from oral or parenteral medication of animals for disease treatment.

In the case of all antibiotics used in animals, either a zero tolerance or a "negligible residue" in the tissue has been established. The negligible residue is based upon assay sensitivity. The general philosophy of the regulations and the specific allowances are detailed in the U.S. Code of Federal Regulations, Title 21, Food and Drugs, Part 135-G: .1 General Considerations; .4 Bacitracin; .6 Oleandomycin; .8 Chlortetracycline; .10 Hygromycin; .11 Streptomycin; .12 Penicillin; .13 Novobiocin; .14 Oxytetracycline; .15 Tylosin; .17 Nystatin; .18 Dihydrostreptomycin; .24 Spectinomycin; .25 Neomycin; .35 Erythromycin; .54 Carbomycin; .65 Lincomycin; .66 Polymixin B; .72 Tetracycline. It is published by the Office of the Federal Register and available from the U.S. Government Printing Office, Washington, D.C.

These U.S. regulations also establish specific tolerances for chlortetracycline and oxytetracycline in certain uncooked edible tissue. The permissible tolerance for oxytetracycline for the uncooked tissue of chicken and turkey is 3 ppm for the kidney and 1 ppm for the muscle, liver, fat, and skin. In the case of oxytetracycline, the permissible residue in the uncooked tissue of chicken and turkey is 4 ppm in the kidney, and 1 ppm for muscle, liver, skin, and fat.

In uncooked swine tissue, allowable chlortetracycline residues are 4 ppm in the kidney, 2 ppm in the liver, 1 ppm in muscle, and 0.2 ppm in the fat. In calves, allowable chlortetracycline residues are 4 ppm in the kidney and 1 ppm in muscle and fat. In beef and nonlactating dairy cows, the residues allowed are 0.1 ppm in muscle, kidney, and liver.

These specific tolerances for oxytetracycline and chlortetracycline have been granted after extensive toxicological testing establishing safety and on proof that these residues permitted in the uncooked tissues are destroyed in normal cooking.

Examples of established negative residues are: bacitracin at 0.5 ppm in uncooked edible tissue of cattle, swine, chickens, turkeys, pheasants, quail, milk, and eggs; oleandomycin at 0.15 ppm in uncooked edible tissue of chickens, turkeys, and swine; penicillin at 0.05 ppm in uncooked edible tissue of cattle; oxytetracycline at 0.1 ppm in uncooked edible tissue of salmonids and catfish.

In granting approval for the use of antibiotics in animal feeds or in animal medication, the FDA rigorously establishes, on the basis of assay data at variable withdrawal periods, the required legal withdrawal period for the antibiotic between the time of last addition and the animal's slaughter. Occasionally, these required withdrawal periods are not always respected in practice and tissue residues are ocassionally found in the uncooked edible foodstuff.

It is the requirement of USDA to monitor the possible tissue residues in edible foodstuffs; accordingly, USDA maintains such a surveillance program.

Recently, it has been established that, besides assaying tissue for residues, it is possible to effectively monitor withdrawal periods. By assaying urine samples it is possible to determine if proper withdrawal periods have been followed. In those instances where illegal residues are found, the product is removed from food channels.

There is a continuing review of the approval of antibiotics used in livestock production, and a National Academy of Sciences—National Research Council review of efficacy for existing products was recently completed which resulted in modification of some of the claims and requirements for additional documentation.

Considerable attention had been addressed to a review of combinations of antibiotics and drugs for animal use and the indication was that additional documentation must be generated to support their use. Eighteen hundred previously approved combinations, including at least one antibiotic were on file; however, in response to the FDA's requirement of additional supporting data required to satisfy the efficacy documentation, over 1000 products have been dropped as the sponsor indicated he did not intend to supply the required information. On approximately 750 remaining combination products, a commitment was made by the sponsoring organization to supply the required data to support the continued approval of the product.

The widespread use of antibiotics for growth promotion and disease control has been common practice in the United States for many years and to a somewhat smaller extent than in many other countries. In general, the large-scale use of antibiotics is related to the more concentrated animal production practices commonly found in the United States. At the present time there are 16 antibiotics which may be administered to animals through the feed; 12 of these are widely used. These include chlortetracycline, oxytetracycline,

penicillin, bacitracin, streptomycin, tylosin, neomycin, tetracycline, erythromycin, oleandomycin, hygromycin, novobiocin, lincomycin, spectinomycin, nystatin, and griseofulvin.

The range of allowable antibiotic application to feed varies from 0.6 gm per ton with procaine to 1000 gm per ton with tylosin. The usual levels for growth promotion are 1 to 50 gm per ton; for disease prevention levels are 50 to 200 gm per ton, and for treatment, 100 to 500 gm per ton.

Most of these antibiotics also find use in human clinical medicine. When all routes of administration are considered as well as all microbial agents, there is a total of 30 microbial agents presently used for improvement of growth rate or disease control and 23 of these are also used in human clinical medicine.

In the United States, virtually all chickens and turkeys receive antibiotics for growth promotion and/or disease prevention and treatment. Low levels for growth, for example, may be 0.6–4.0 gm of penicillin per ton or 4–6 gm of bacitracin per ton of feed for chickens. In the case of turkeys, 3–15 gm of penicillin or 10–50 gm of bacitracin per ton may be used for growth promotion. It is quite common to orally administer fairly high levels of tetracyclines or tetracyclines plus neomycin, or tylosin, etc., through the feed or water as a prophylactic or therapeutic treatment for poultry during periods of stress or anticipated disease outbreak. This short-term high level prophylactic treatment represents a significant portion of the current use of antibiotics.

In the case of swine, approximately 90% of the animals in the United States receive antibiotics for growth promotion and/or the prevention of disease. If baby pigs receive artificial sow's milk, such milk contains a high level of a tetracycline in most instances; also, the baby pig usually receives fairly high levels of tetracycline in the creep feed. It is common, for example, for weaned pigs to receive 250 gm per ton of a tetracycline-penicillin-sulfamethazine combination or a 200-gm combination of tylosin and sulfamethazine per ton of feed until the pig reaches a weight of 75 lb. Lower levels, usually of single antibiotics, are fed from 75 lb to the time the animal almost reaches market weight.

Milk fed calves, for example, usually receive a high level of tetracycline in their milk replacers. Feeder calves, when transported or exposed to stress, commonly receive an injection of 1 million units of penicillin and 1 gm of streptomycin or an injection of 500 mg of oxytetracycline. This is usually followed by antibiotic additions to the feed. Beef cattle, commonly receive a high level regimen of a tetracycline, or a tetracycline and a sulfa for 10 days upon shipment or receipt in a feed lot, and subsequently receive approximately 75 mg per head per day of tetracycline until marketing period for control of liver abscess and improvement in performance.

In the case of antibiotics used for animal medication, specific directions for use and prescribed withdrawal periods are required whether the product is distributed through a veterinarian only or whether a product is available to the farmer or rancher. For example, in mastitis products, which are used for intramammary application, discarding of the milk is required for a period of 48–96 hr depending upon the excretion pattern of the antibiotic in the vehicle employed. The FDA also assists in minimizing undesirable milk residues by limiting the amount of penicillin that may be contained in an intramammary treatment form to 100,000 units per infusion, a dosage limitation not existing in most other countries.

Previously in the United States, penicillin doses for intramammary infusion of several million units had been employed. Such high levels contributed materially to undesired carryover of penicillin in the milk without materially improving the therapeutic efficacy of the medication. Through this restriction and better medication practices and surveillance, penicillin residues now are found in only 0.25–0.50% of the milk in the United States, whereas 15 yr ago, as high as 12–14% of the milk samples were contaminated with undesirable residues of penicillin.

These residues obviously have undesirable public health implications. Additionally, it has been shown that metabolites of penicillin, having at least as great a sensitizing action as penicillin itself, were formed but not detected by the normal penicillin assay; and they may persist even slightly longer than the penicillin itself.

In 1971, FDA set forth provisions stipulating the allowable use of certain penicillin, streptomycin, dihydrostreptomycin and neomycin products in intramammary infusions, as well as other veterinary drugs allowable in these combinations.

Considerable controversy has recently arisen regarding the use of antibiotics in feeds for growth promotion and for the treatment of animal diseases. Particular focus has been directed to the real or imagined public health hazard associated with the use of the same drugs in animal feeding and medication that are employed in human medicine. The cited potential hazards relate to the possible indirect toxic effects, including hypersensitivity to allergy should the consumer be sensitized to the drug. The potential hazardous bacteriological and epidemiological effects have received more attention as hazards to human

health in that animals receiving antibiotics are reported to significantly increase their reservoir of Gram negative bacilli capable of causing human disease, and which may be capable of being transmitted to man.

In a recent epidemiological survey on farms in the United States, which had various programs of antibiotic feeding including some which fed antibiotics continuously in the feed and others who employed the antibiotic for therapeutic and prophylactic purposes, the characteristics and frequency of antimicrobial resistance to *E. coli* was studied in relationship to the veterinary and management use of antimicrobial agents. Multiple resistance in the *E. coli* isolates was found to occur in 84.8% of the herd exposed to continuous feeding of antimicrobial agents, compared to 15.7% in the herds not receiving antimicrobials.

Resistance patterns to 3–4 agents were the most commonly observed. The frequency of transfer factors was much higher in multiple-resistant organisms from the herds exposed to antimicrobial medication. The *E. coli* isolated were relatively efficient in fostering and transferring heterologous resistance factors.

This entire complex area of real or imagined public health hazards associated with the use of antibiotics and determination of the actual risk-benefit ratio involved, has received much attention in many countries. In the United Kingdom, the Swann Committee studied the problem and their conclusions have resulted in legislation in the United Kingdom which prohibits the continuous feeding of the tetracyclines, penicillin and sulfas without the specific prescription of an attending veterinarian.

In the United States, the same subject has been the object of an 18-month study by a special FDA Antibiotic Task Force. It was the Task Force majority recommendation that the tetracyclines, streptomycin, dihydrostreptomycin, sulfonamides, and penicillin be prohibited from use for growth-promoting and any subtherapeutic application in animals. They recommended that these antibiotics be reserved for therapy unless they meet the criteria established for safety and efficacy for growth promotion or subtherapeutic use.

The FDA Antibiotic Task Force also recommended that therapeutic application be restricted to short-term treatment and then only by decision of a veterinarian or on the authority of a veterinarian's prescription. They further recommended that those antibiotics which select for bacterial resistance to the antibiotics most critically needed for man and animals be prohibited for use in animal feed. At the present time, this category includes chloramphenicol, semisynthetic penicillin, gentamycin, and kanamycin. They recommended that those antibiotics effective and essential for the therapy of certain animal diseases and which select for R-factor mediated multiple resistance should be available for short-term use, only at therapeutic levels and by a veterinarian or on his prescription.

The FDA Antibiotic Task Force recommendations were based on their conclusions that the use of certain antibiotics and sulfonamide drugs, particularly when used at the growth-promotant and subtherapeutic levels, favored the selection and development of single- and multiple-resistant and R-factor-bearing bacteria in food animals which may serve as a reservoir for antibiotic-resistant pathogens and nonpathogens which could produce human infection.

These observations, together with the fact that the prevalence of multiresistant R-factor in animals has been related to the use of certain antibiotics and sulfonamide drugs, and the finding of resistant organisms on meat and meat products, gave rise to the logical conclusions, although not fully documented, that such medication practices may be hazardous to human health.

In their review of the use of antibiotics, a risk-benefit relationship had to be explored in establishing a balance between the value of antibiotic use in agriculture and the potential hazards associated with such use. In their economic evaluation of the use of antibiotics in animal feeds, the Task Force estimated the following economic value for such use in meat animals. Their breakdown for the year 1970 was as follows:

Broilers	$ 33,419,000
Turkeys	13,920,000
Beef	148,890,000
Veal	14,431,000
Swine	202,489,000
	$413,149,000

These data, however, are not intended as an accurate estimate of the economic impact of restricting antibiotics in feeds as all antibiotic use would not cease; undoubtedly some antibiotics would continue to be available for growth-promotant purposes.

The Task Force also estimated the economic value of the use of antibiotics to the pharmaceutical industry. Their estimates for the year 1968–1969, were as follows:

Broilers	$ 2,172,800
Turkeys	583,923
Hogs	46,400,000
Cattle	14,112,000
Calves	761,600
	$64,030,323

The proper regulated use of antibiotics is presently under active discussion and review. Modified regulations have recently been issued in the United States and are in preparation and partial implementation in the European Common Market and other areas of the world.

In the United States, an ad hoc committee of the National Academy of Sciences—National Research Council has reviewed the FDA Antibiotic Task Force recommendations. On April 30, 1973, the FDA ordered regulations that essentially start to implement some of the antibiotic recommendations, in that all approved subtherapeutic use of the antibiotics and sulfonamides will be revoked unless data are submitted which conclusively resolve their safety and efficacy according to the newer and more rigorous standards which have been recommended. These regulations are outlined in *Statement of Policy and Interpretation Regarding Animal Drugs and Medicated Feeds*, Title 21, Part 135-B, Federal Register, *38*, No. 76, 7811–7814.

In the case of tetracyclines, streptomycin, dihydrostreptomycin, penicillin, and sulfonamides, the effect of subtherapeutic feed use of these drugs on the *Salmonella* reservoir of target animals must be concluded by April 20, 1974.

The importance from a public health standpoint of transferrable drug resistance in *Salmonella* is reviewed, together, with other aspects in the FDA Antibiotic Task Force report and its detailed appendices and in other related literature (Baldwin 1970).

In general, the philosophy of antibiotic use for animals in the United States has been that, in those specific conditions where the antibiotic administration is safe and effective under the prescribed restrictions of application, dosage, withdrawal period, etc., and where adequate directions can be written, a particular antibiotic may be so administered in the feed or other route by the farmer or rancher. In all other cases, veterinary application is required.

In most of the rest of the world, there is a greater tendency towards restricting the use of antibiotics for animals through a veterinarian, or on his prescription, without detailing the same degree of specificity of approved application, level of use, withdrawal period, etc.

In the United States, the FDA Antibiotic Task Force, as well as other advisory groups, recommend that the same antibiotic should preferably not be used for human and animal applications, and an antibiotic should not be used which is cross-resistant with important antibiotics used in humans. The Task Force suggested research for the development of new chemotherapeutic agents for animal applications not used in human medicine. Research is addressed to this ideal goal and the first example of success in this area is the recent approval of a new quinoxaline-*N*-oxide, Carbadox, which has recently been developed and approved in the United States and several countries for use in animals.

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References

BALDWIN, R. A. 1970. The development of transferrable drug resistance in Salmonella and its public health implications. J. Am. Vet. Med. Assoc. *157*, No. 11, 1841–1853.

FAO/WHO. 1969. Twelfth report of Joint FAO/WHO Expert Committee on Food Additives. WHO Tech. Rept. Ser. *430*.

FDA Antibiotic Task Force. (Undated) Report to the Commissioner of FDA on the use of antibiotics in animal feeds and Appendices on economic value of human and animal health hazards. FDA Bur. Vet. Med.

FOWLER, G. G., and McCANN, B. 1971. The growing use of nisin in the dairy industry. Australian J. Dairy Technol. *26*, 44–46.

HUBER, W. C. 1973. Antibiotic tissue residues in animals. Symp. Antibiotics in Animal Feeds, American Society of Microbiologists, Atlantic City, Sept. 28.

MERCER, H. D. *et al.* 1971. Characteristics of antimicrobial resistance of *Escherichia coli* from animals: Relationship to veterinary and management uses of antimicrobial agents. Appl. Microbiol *22*, no. 4, 700–705.

NILSON, K. M., CARRANCEDO, M., and SHAHANI, K. M. 1960. The use of antifungal antibiotics on the retardation of cottage cheese spoilage. J. Dairy Sci. *43*, 842–843.

SHAHANI, K. M., NILSON, K. M., and DOWNS, P. A. 1959. Effect of anti-fungal antibiotics on keeping quality of cottage cheese. Proc. 15th Intern. Dairy Congr. *2*, Sect. 3, 926–932.

STOLL, VON K. 1969. Method for prevention of Johnathan spot. Swiss J. Fruit Wine Growing *16*, 65. (Swiss)

U.S. Dept. Health, Education, and Welfare. 1973. Preslaughter withdrawal times for drugs. For chickens: HEW Publ. *73-6011*, Bur. Vet. Med. Publ. *73-6012*. For turkeys: Publ. *73-6013*. For sheep and goats: Publ. *74-6008*. For cattle and calves: Publ. *74-6006*.

ANTIOXIDANTS

Oxidation is the major problem in the spoilage of fats and fatty foods. Oxidative changes are manifest as changes in flavor, odor, color or, on occasion, texture as viscosity. While the portion of the fat undergoing such changes may be small the resulting off-odors are potent, pervading, and