Technology Sciences Group Inc.
712 Fifth St., Suite A
Davis, CA 95616
Direct: (530) 601-5064
Fax: (530) 757-1299
E-Mail: jmoore@TSGUSA.com

Jacob S. Moore
Regulatory Consultant

September 1, 2016

USDA/AMS/NOP, Standards Division
1400 Independence Ave. SW
Room 2648-So., Ag Stop 0268
Washington, DC 20250-0268

Attention: **Lisa Brines, PhD**
National List Manager

RE: **National Organic Program Petition for Classification of Natamycin as an Allowed Nonsynthetic Substance**

Dear Dr. Brines:

Technology Sciences Group Inc., on behalf of DSM Food Specialties B.V., submits the enclosed petition for classification of natamycin as an allowed nonsynthetic substance. Natamycin is a naturally-occurring compound produced by fermentation of *Streptomyces natalensis*. As natamycin is known to the National Organic Program and National Organic Standards Board, the petitioner requests that a focused Technical Report be issued to complement the work previously done and resolve the classification status of the petitioned substance.

Please contact me with any questions or concerns.

Jacob S. Moore

TITLE

Petition for Classification of Natamycin as an Allowed Nonsynthetic Substance in Organic Crop Production

AUTHOR

Technology Sciences Group Inc.

DATE

September 1, 2016

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Item A—Indicate which section or sections the petitioned substance will be included on and/or removed from the National List.

Petition for classification of natamycin as an allowed nonsynthetic substance. This petition is at the recommendation of the NOP, as the classification of natamycin is considered unclear.

Item B

1. **Substance Name:**
   Natamycin

2. **Petitioner and Manufacturer Information:**
   DSM Food Specialties B.V.
   Alexander Fleminglaan 1
   2613 AX Delft
   Netherlands
   +31 6 13 48 46 97
   André Keuter
   andre.keuter@dsm.com
   Global Regulatory Affairs Manager

3. **Intended or Current Use**
   Natamycin is a United States Environmental Protection Agency (US EPA)-registered biochemical pesticide for use as a fungistat in enclosed mushroom production facilities and for use as a post-harvest treatment on food commodities to control fungal diseases.

4. **Intended Activities and Application Rate**
   Natamycin is currently approved by the US EPA for use in enclosed mushroom production facilities on mushrooms growth media to prevent the germination of fungal spores and on pineapples, citrus, pome and stone fruit crop groups, cherries, avocado, kiwi, mango, and pomegranate, to control fungal diseases. A summary of the uses and application rates are presented in Table 1.

Commodity	Diseases	Method of Application	Use Rate (ai = Natamycin)
Mushrooms in enclosed production facilities	Dry Bubble (*Verticillium fungicola)*	Surface drench	0.36 – 0.73 oz. ai / 1000 sq. ft.
Citrus	Green or blue rot (*Penicillium spp.*) Sour rot *(Geotricum citri-aurantii, Geotricum candidum)* Gray mold *(Botrytis cinerea)* Mucor rot (*Mucor spp.*)	In-line dip/drench In-line aqueous or fruit coating spray	6.6 – 13.3 oz. ai / 100 gallons
Pome Fruit	Blue mold (*Penicillium expansum)* Gray mold *(Botrytis cinerea)* Mucor rot (*Mucor spp.*) Sphaeropsis rot (*Sphaeropsis pyriputrescens*) Phacidiopycnis rot (*Phacidiopycnis piri*) Speck rot (*Phacidiopycnis washingtonensis*)	Bin/truck drench In-line dip/drench Flooders In-line aqueous or fruit coating spray	6.6 – 13.3 oz. ai / 100 gallons
Stone Fruit	Brown rot (*Monilinia spp.*) Gray mold *(Botrytis cinerea)* Sour rot (*Geotricum spp.*) Rhizopus rot (*Rhizopus spp.*)	In-line dip/drench	6.6 – 13.3 oz. ai / 100 gallons
		In-line aqueous or fruit coating spray	3.3 – 13.3 oz. ai / 200,000 lbs of fruit
Cherries	Brown rot (*Monilinia spp.*) Gray mold (*Botrytis cinerea*)	In-line aqueous or flooder application High volume application	3.3 – 13.3 oz. / 50,000 lbs of fruit
Avocado	Anthracnose (*Colletotrichum spp.)* Stem end rot (*Dothiorella spp.)*	In-line dip/drench	6.6 – 13.3 oz. ai / 100 gallons
		In-line aqueous or fruit coating spray application	6.6 – 13.3 oz. ai / 200,000 lbs of fruit
Kiwi	Botrytis fruit rot (*Botrytis cinerea*)	In-line dip/drench	6.6 – 13.3 oz. ai / 100 gallons
		In-line aqueous or fruit coating spray application	6.6 – 13.3 oz. ai / 200,000 lbs of fruit

Commodity	Diseases	Method of Application	Use Rate (ai = Natamycin)
Mango	Anthracnose (*Colletotrichum spp.)* Stem end rot (*Dothiorella spp.)*	In-line dip/drench	6.6 – 13.3 oz. ai / 100 gallons
		In-line aqueous or fruit coating spray application	6.6 – 13.3 oz. ai / 200,000 lbs of fruit
Pomegranate	Botrytis fruit rot (*Botrytis cinerea*) Blue mold *(Penicillium expansum)*	In-line dip/drench	6.6 – 13.3 oz. ai / 100 gallons
Pineapple	*Fusarium oxysporum* *Penicillium funiculosum* *Rhizophus stolonife* *Aspergillus niger* *Thielaviopsis paradoxa*	Dip/pour/cascade	0.18 – 1.44 oz. ai / gallon of water and aqueous dilution of wax

Table 1. Summary of EPA-approved uses of natamycin.

5. **The source of the substance and a detailed description of its manufacturing or processing procedures from the basic components to the final product.**
Natamycin is a nonsynthetic pesticide active ingredient produced by fermentation of a naturally-occurring soil microorganism, *Streptomyces natalensis*. As discussed in Item B.13, natamycin meets the NOP criteria for classification as a nonsynthetic. The manufacturing process of commercially-produced natamycin follows these general steps.

Step 1 – Fermentation
Controlled fermentation of the *Streptomyces spp.* results in the production of natamycin in a crystal form. Natamycin exists naturally within the fermentation broth in crystal form due to its low water solubility.

Step 2 - Autolysis
The fermentation biomass is lysed under heat to release the natamycin crystals.

Step 3- Harvest & Centrifuge Concentration
The fermentation broth containing natamycin crystals is then centrifuged to remove the biomass; a solvent that is later removed is added to maintain microbiological stability.

Step 4 – Recovery
Acid-base extraction is then used to purify and remove the solid natamycin crystals from the broth. Solvent and excess water still present are then removed by pressing the natamycin crystals. This extraction step does not chemically change or alter natamycin from how it occurs naturally.

Step 5 - Packaging
Finally, the natamycin crystals are dried, milled for consistent particle size, and packaged. The resulting product is highly pure (nominally 91% w/w).

Impurities present in the final product include water of hydration, several naturally-occurring natamycin-related by-products co-extracted with the natamycin, residual solvent, and several natamycin related degradates individually < 0.1% w/w of the final product.[1] Natamycin is extracted as a trihydrate, in a crystal lattice identical to that present in the fermentation.

The final substance is analyzed for natamycin content and to ensure lack of contamination in order to meet minimum specification requirements for use in pesticide, food, and pharmaceutical applications, as summarized in Table 2.

[1] Per 40 CFR §158.320(d), the US EPA does not require identification of impurities <0.1 % w/w when they are not toxicologically significant.

Parameter	Specification
Appearance	Powder
Odor	Neutral
Color	White
Natamycin assay	88.0 – 93.0 %
Water Content	6.5 – 8.0 %
pH	5.5 – 7.5
Heavy metals	< 5 ppm
Arsenic	< 1 ppm
Mercury	< 1 ppm

Table 2. Minimum specification for petitioner's natamycin used in commercial applications.

At the end of the extraction process, the natamycin has not been transformed into a different substance via chemical change. Natamycin clearly and without question fits in the NOP definition and NOP guidance for classification as an allowed nonsynthetic substance. Natamycin has not been altered into a form that does not exist in nature, and any synthetic materials used during the manufacturing process have been removed from the technical grade active ingredient such that they have no technical or functional effect in the final product.

6. Ancillary Substances

Not Applicable. No ancillary substances are present in the technical grade active ingredient (TGAI).

7. Previous Reviews

Natamycin was previously evaluated and approved for use as an organic crop input by the Organic Materials Review Institute (OMRI) in accordance with the National Organic Program's (NOP) Draft Guidance for Classification of Materials 5033 (NOP, 2013). OMRI determined that natamycin is a nonsynthetic material.[2]

In addition, natamycin has been reviewed and is currently approved for use by OMRI Canada, and is allowed for use in organic crop production in Canada. A copy of the Canadian organic certification provided by OMRI Canada is included in Attachment 1.

The NOSB previously reviewed and rejected, in March 2007, a petition by a third party for natamycin for use as a preservative in processed food (NOSB, 2007). New information is presented in this petition addressing natamycin as an allowed nonsynthetic substance in accordance with NOP Draft Guidance 5033, Classification of Materials, which was not available at the time of the former petition from the third party. In the March 2007 NOSB Recommendation the ingredient was termed a synthetic; however, it was unclear why this determination was made,

[2] Organic Materials Review Institute: Out of Scope and Beyond Resolution, Issue #3: Natamycin. http://www.omri.org/suppliers/OMRIscope.

as the technical report issued on August 11, 2006, and the NOP draft guidance 5033, section 4.6 indicate that natamycin should be classified as nonsynthetic. As discussed in Items B.5 and B.13, at the end of the extraction process, the material has not been transformed into a different substance via chemical change, the material has not been altered into a form that does not exist in nature, and any synthetic materials used to separate, isolate, or extract the substance have been removed from the final substance such that they have no technical or function effect in the final product.

8. Regulatory Authority

A Technical Grade Active Ingredient (TGAI), and two formulated end-use products (EP) with the active ingredient natamycin are approved by the US EPA for use as a fungistat in enclosed mushroom production houses and as a post-harvest treatment on several commodities to control fungal diseases.

Brand Name / EPA Registration Number	Formulation Type	Percent Natamycin (w/w)
Natamycin TGAI (87485-1)	TGAI/Manufacturing-use Product	91.02 %
Zivion M (Natamycin L) (87485-2)	EP	10.34 %
Zivion P (87485-3)	EP	4 %

Table 3. Natamycin US EPA registrations.

At present the end-use product is registered in the following states:

<u>Zivion M (Natamycin L) (87485-2)</u>
California
Colorado
Delaware
Florida
Maryland
Nevada
Oklahoma
Oregon
Pennsylvania
Texas
Utah
Washington

Natamycin is exempt from the requirement of a tolerance when used in or on mushrooms, pineapples, citrus, pome, stone fruit crop groups, avocado, kiwi, mango, and pomegranates (40 CFR §180.1315).

9. Chemical Abstract Service (CAS) Number and Product Labels

<u>CAS No.:</u> 7681-93-8
EPA stamped acceptable labels are included in Attachment 2.

10. Physical and Chemical Properties

Natamycin is a polyene macrolide produced by aerobic fermentation of *Streptomyces natalensis.* A summary of the physical and chemical properties is presented in Table 4 and a summary of safety information is presented in Item B. 11.

Property	Description of Result
IUPAC Name	(1R,3S,5R,7R,8E,12R,14E,16E,18E,20E,22R,24S,25R,26S)-22-[(3-amino-3,6-dideoxy-D-mannopyranosyl)oxy]-1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28-trioxatricyclo[22.3.1.05,7]octacosa-8,14,16,18,20-pentaene-25-carboxylic acid
Chemical formula	$C_{33}H_{47}NO_{13}$
Molecular Weight	665.7 g/mol
Color	Colorless
Physical State	Powder
Odor	Odorless
Stability to normal and elevated temperatures	Stable at 54°C for 14 days, and at least for 2 years below 25 °C protected from light, stored in a dry place
pH	6.5 (1% aqueous solution)
Density	Loose bulk density: 0.3 g/mL Tapped bulk density: 0.59 g/mL
Octanol/water partition coefficient	Log K_{ow} = -3.67
Water Solubility	30-50 ppm @ 20-25°C

Table 4. Summary of physical and chemical properties for Natamycin.

Figure 1. Chemical Structure of Natamycin

11. Safety Information

The safety of natamycin has been extensively evaluated by Food Safety authorities around the world, including the United States Food and Drug Administration (US FDA) in 2001, 2004, 2014, and 2015, European Food Safety Authority (EFSA) in 2009, World Health Organization Joint Expert Committee on Food Additives (WHO/JEFCA) in 2002 and specifically for crop applications

by the US EPA in 2012, 2014, and 2016, the Health Canada Pest Management Regulatory Agency (PMRA) in 2012, and the California Department of Pesticide Regulation (CDPR) in 2013. The regulatory decision issued by the US EPA summarizes the risk of natamycin when used as a biochemical pesticide. The EPA determined that "no unreasonable adverse effects to the U.S. population in general, and to infants and children in particular, will result from the use of natamycin as a pesticide […]." Natamycin is currently approved for use indoors, and data on nontarget organisms or environmental fate were not required by the US EPA. (US EPA, 2016). A summary of the acute toxicity endpoints and EPA-assigned toxicity categories are presented in Table 5. Pesticides with EPA Toxicity Category III or IV acute studies are required to have a "CAUTION" signal word displayed prominently on the label.[3]

Acute Toxicity:

Study Type	Study Result	EPA Toxicity Category
Acute Oral Toxicity	LD_{50} > 2000 mg/kg	III
Acute Dermal Toxicity	LD_{50} > 5050 mg/kg	IV
Acute Inhalation Toxicity	LC_{50} > 2.39 mg/L	IV
Primary Eye Irritation	No corneal or positive irritation effects at 24-hr post instillation	IV
Primary Dermal Irritation	PII = 0.1	IV
Skin Sensitization	LLNA SI < 3	Not a sensitizer

Table 5. Acute toxicity summary for natamycin.

Mutagenicity:

The US EPA reviewed two mutagenicity studies using natamycin and determined that the studies were sufficient to confirm that "there are no expected dietary or non-occupational risks of mutagenicity with regard to food use of natamycin" (US EPA, 2016). A summary of the mutagenicity studies is presented in Table 6.

[3] Per 40 CFR §156.64, Category I = Danger, Category II = Warning, Category III = Caution, Category IV = No Signal Word required, Caution optional.

Study Type	Summary of Study
Bacterial Reverse Gene Mutation	Doses of test substance up to cytotoxicity, with and without metabolic S9 activation, found no incidences of a 2-fold or greater increase in the number of revertants compared to the control. Natamycin is considered to be non-mutagenic under the conditions of the study.
In vitro Mammalian Cell Chromosome Aberration	Natamycin did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations in the presence or absence of S9. Natamycin is considered to be non-mutagenic and does not cause chromosome aberrations under the conditions of the study.

Table 6. Summary of natamycin mutagenicity studies submitted to US EPA.

Subchronic/Developmental Toxicity:

The US EPA evaluated a subchronic oral toxicity study using natamycin and established a NOAEL (No Observed Adverse Effect Level) of 2,000 mg natamycin/kg bw/day, which corresponded with the highest concentration tested in the assay. In a developmental toxicity study using natamycin, the test substance showed no effects on key developmental parameters up to the highest concentration tested, 50 mg natamycin/kg bw/day.

Based on the results of these studies, the EPA determined that levels of exposure to the most sensitive population subgroup, children one to two years old, was several orders of magnitude lower than the observed NOAEL. Further, natamycin showed low acute toxicity and is not a developmental toxicant or mutagen. Therefore, the EPA concluded that there is a "reasonable certainty that no harm will result to the U.S. population, including infants and children, from aggregate exposure to the residues of natamycin when it is used as labeled and in accordance with good agricultural practices" (US EPA, 2016).

Ecological Hazards and Environmental Fate

Based on the currently approved use patterns, exposure to non-target organisms is not expected. All currently approved uses are indoors only, and risk to non-target organisms or the environment will be minimal (US EPA, 2016).

A Safety Data Sheet (SDS) for natamycin is included in Attachment 3.

12. Research Information

The National Organic Standards Board has previously expressed concerns over the inconsistency between the prohibition of antibiotics in livestock and the use of antibiotics as organic crop protection products (NOSB, 2011). Concern over whether natamycin is an antibiotic or not was also expressed during the NOSB meeting that discussed the original petition to list natamycin as an allowed organic preservative (NOSB, 2007).

Natamycin is a fungistat, inhibiting growth of molds and yeasts (US EPA, 2016). It does not affect bacteria and is unrelated to antibiotics used to treat mammalian bacterial infections. The discussion is semantic, with the underlying concern in the organic community being the overall increased resistance of microbial organisms to antimicrobial agents, including human and livestock pathogens. As discussed in the EFSA Report on natamycin, polyene macrolides like natamycin act by binding to fungal sterols, mainly ergosterol, a critical compound in fungal cell membranes. Natamycin does not act on bacteria because their membranes lack ergosterols. Due to natamycin's unique mechanism of action, the EFSA Panel concluded that there was no concern for the induction of antimicrobial resistance, which confirmed the same conclusion of WHO/JECFA in 2002 (EFSA, 2009) (WHO/JECFA, 2002).

Fungal infections are an increasing public-health concern and effective antimicrobial agents are limited. Due to the limited agents available and concern for resistance development, research has been dedicated to identifying causes of resistance to common antimicrobial compounds. Concerns have been expressed over polyene substances similar to natamycin, including amphotericin B ((Kanafani & Perfect, 2008). Other studies have implied that due to its similarity to other polyene macrolides, natamycin may exert a polyene resistance selective pressure, theoretically increasing the potential for resistance to the entire group of polyene macrolides (Dalhoff & Levy, 2015). The petitioner has responded to these concerns in a published letter, included in Attachment 4.

Natamycin, among the polyene macrolides, is unique in that it blocks fungal growth by binding to ergosterol without permeating the membrane. Researches have studied this mechanism of action to better understand why natamycin acts differently compared to other members of the polyene macrolide family (te Welscher, et al., 2007) (te Welscher, et al., 2010) (te Welscher, van Leeuwen, de Kruijff, Dijksterhuis, & Breukink, 2012).

Given the growing concern of antibacterial resistance, general antimicrobial resistance, and the use of natamycin in the marketplace, multiple journal articles have studied the long-term effect of natamycin on target fungi. In these comprehensive studies, looking at decades of use, no change in composition or sensitivity of target fungi to natamycin has been observed (De Boer E. S.-H., 1977) (De Boer E. L.-H., 1979) (Hoekstra, 1998).

DSM Food Specialties B.V. organized an independent literature review on natamycin resistance. The literature review was performed by TNO, the Netherlands Organization for Applied Scientific Research in 2012. The search covered the literature published in the period 2000 until 2012 (TNO, 2012). The literature review was further extended by DSM for the period 2012 – 2015 (Streekstra, 2015). The literature is still in accordance with the conclusions drawn previously by the WHO/JECFA and EFSA that the potential occurrence of resistant variants – if

these occur at all - is no reason for concern, as these variants grow poorly, and are unable to compete in the environment (WHO/JECFA, 2002) (EFSA, 2009).

13. Petition Justification Statement

Natamycin was originally petitioned for listing as an allowed nonsynthetic nonagricultural substance in or on processed products labeled as “organic” or “made with organic” (7 CFR §205.605). The NOSB Committee Recommendation, in March 2007, was to reject the petition for inclusion on the National List.

This petition is to confirm the classification of natamycin as an allowed nonsynthetic substance for use as a biochemical pesticide in organic crop production. A biochemical pesticide is defined by the EPA as “naturally-occurring substance or structurally-similar and functionally identical to a naturally-occurring substance” (40 CFR §158.2000(a)(1)(i)). In parallel to this definition, the USDA defines a nonsynthetic (natural) substance as one “derived from mineral, plant, or animal matter and does not undergo a synthetic process as defined in section 6502(21) of the Act (7 U.S.C. 6502(21)).”

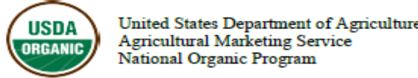
Until the release of NOP 5033 Classification of Materials in 2013, the procedure used to classify materials as synthetic or nonsynthetic under the USDA organic regulations was not clearly delineated. For a material like natamycin, the manufacturing process must be re-evaluated under the guidelines established in NOP 5033 to confirm its status as nonsynthetic. NOP 5033 paragraph 4.6, *Extraction of Nonorganic Materials*, lays out the three criteria for classification of an extracted material as nonsynthetic.

- At the end of the extraction process, the material has not been transformed into a different substance via chemical change;
- The material has not been altered into a form that does not occur in nature; and
- Any synthetic materials used to separate, isolate, or extract the substance have been removed from the final substance (e.g., via evaporation, distillation, precipitation, or other means) such that they have no technical or functional effect in the final product.

Natamycin is a natural compound extracted from a fermentation growth of a soil microorganism, *Streptomyces natalensis.* The petitioner uses an extraction process to remove and purify the naturally-occurring natamycin crystals from the fermentation medium. However, at the end of the process, the isolated material has not been transformed into a different substance, or into a form that does not occur in nature. As noted in the Technical Evaluation Report commissioned by the NOSB for natamycin, “a chemical process is used to extract the natamycin from the fermentation medium. However, the extraction steps do not alter the identity of the natamycin produced by the microbial culture” (ICF International, 2006).

Best efforts are made to remove and/or minimize residual synthetic materials used in the extraction process, and they have no technical or function effect in the final product.

Alongside the NOP 5033 *Classification of Materials*, the USDA released a “Draft Guidance: Decision Tree for Classification of Materials as Synthetic or Nonsynthetic,” NOP 5033-1.

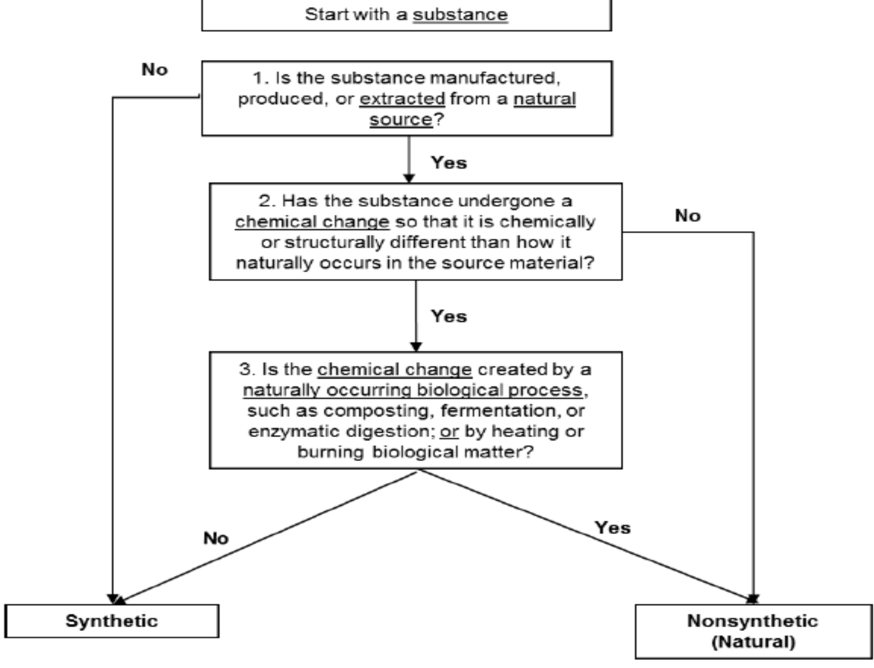

United States Department of Agriculture
Agricultural Marketing Service
National Organic Program

1400 Independence Avenue SW.
Room 2646-South Building
Washington, DC 20250

NOP 5033-1
Effective Date: TBD
Page 1 of 3

Draft Guidance
Decision Tree for Classification of Materials as Synthetic or Nonsynthetic

Underlined terms defined on page 2



File Name: *NOP 5033-1 Decision Tree for Classification Syn/NS 03 26 13*   Authorized Distribution: Public

Proceeding from the beginning of the decision tree, natamycin, a substance with CAS Registry No. 7681-93-8:

1. Is the substance extracted from a natural source?

   Natamycin is withdrawn from a *Streptomyces spp.* fermentation broth with use of solvents, acid-base extraction, and/or mechanical or physical methods as discussed in Item B.5.

2. Has the substance undergone a chemical change so that it is chemically or structurally different than how it naturally occurs in the source material?

   Natamycin is naturally present in the source material as a crystal structure due to its low water solubility. The extracted natamycin is structurally and chemically identical to that present in the source material. No chemical change has taken place; therefore, the substance is considered nonsynthetic (natural).

Natamycin, a naturally-occurring fungicide, would provide organic growers with a valuable tool to manage fruit decay. Growers who embrace organic agriculture have a diverse set of tools to create a sustainable crop. Best management practices are essential to organic agriculture, including targeted use of inputs when necessary. Natamycin-based products provide an efficacious alternative to the limited organic fungicides currently on the market. Natamycin is proven to be low risk to human health and the environment. The impact of use is minimal while the benefits are increased yield and health of the commodity, and therefore increased availability of affordable organic produce to consumers. As consumer interest in organic agriculture continues to grow and the market matures, it is critical that organic growers have access to innovative, natural products that are in line with the spirit and mission of the organic community.

Conclusion

Natamycin, a naturally-occurring polyene macrolide, is produced with use of a fermentation of *Streptomyces natalensis*. The rules promulgated by the National Organic Program and discussed above demonstrate that natamycin is a nonsynthetic substance allowed for use in organic crop production.

References

Dalhoff, A. A., & Levy, S. B. (2015). Does use of the polyene natamycin as a food preservative joepardise the clinical efficacy of amphotericin B? A word of concern. *International Journal of Antimicrobial Agents*, 564-567.

De Boer, E. L.-H. (1979). Sensitivity to natamycin of fungi in factories producing dry sausage. *Fleischwirtschaft*, 1868-1869.

De Boer, E. S.-H. (1977). Sensitivity to natamycin (pimaricin) of fungi isolated in cheese warehouses. *Jounral of Food Protection*, 533-536.

EFSA. (2009). *Scientific Opinion on the use of natamycin (E 235) as a food additive.* European Food Safety Authority.

Hoekstra, E. V. (1998). Survery of the fungal flora in Dutch cheese factories and warehouses. *Journal of Food Microbiology*, 13-22.

ICF International. (2006). *Technical Evalulation Report - Natamycin.* National Organic Standards Board.

Kanafani, Z. A., & Perfect, J. R. (2008). Resistance to Antifungal Agents: Mechanisms and Clinical Impact. *Clinical Infectious Diseases*, 120-128.

NOP. (2013). *National Organic Program Draft Guidance: Classification of Materials.* Washington, DC: United States Department of Agriculture | Agricultural Marketing Service.

NOSB. (2007). National Organic Standards Board Meeting, March 29, 2007., (pp. 107 - 112). Washington, DC.

NOSB. (2011). *2012 Sunset Recommendation - Steptomycin.* National Organic Standards Board.

Streekstra, H. (2015). *Susceptibility of fungal isolates to natamycin and other polyene antifungals - Overview based on TNO report 2012 R10746, an update of more recently published MIC values, with special reference to the occurence of resistance and cross-resistance.* Netherlands: DSM report RA-DLF-00070484.

te Welscher, Y. M., Jones, L., van Leeuwen, M. R., Dijksterhuis, J., de Kruijff, B., Eitzen, G., & Breukink, E. (2010). Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol. *Antimicrobial Agents and Chemotherapy, 54*, 2618 - 2625.

te Welscher, Y. M., ten Napel, H. H., Balague, M. M., Souza, C. M., Riezman, H., de Kruijff, B., & Breukink, E. (2007). Natamycin Blocks Fungal Growth by Binding Specifically to Ergosterol without Permeabilizng the Membrane. *Journal of Biological Chemistry*, 6393-6401.

te Welscher, Y. M., van Leeuwen, M. R., de Kruijff, B., Dijksterhuis, J., & Breukink, E. (2012). Polyene antibiotic that inhibits membrane transport proteins. *Proceedings of the National Academy of Sciences*. Retrieved from www.pnas.org/cgi/doi/10.1073/pnas.1203375109

TNO. (2012). *Literature study of antifungal resistance in general and natamycin resistance in particular.* TNO R10746.

US EPA. (2016). *Federal Food, Drug, and Cosmetic (FFDCA) Considerations for Natamycin.* Washington: U.S. Envrionmental Protection Agency, Office of Pesticide Programs.

WHO/JECFA. (2002). Evaluation of certain food additives and Contaminants, 57th Report of the Joint FAO/WHO Expert Committee on Food Additives. *WHO Technical Report Series*, pp. 25-43.

Attachment 1 – Organic Materials Review Institute Canada Certificate

OMRI Listed®

The following product may be used in certified organic production or food processing and handling in accordance with the Canadian Organic Standards.

Product

Zivion M Agricultural Suspension

Company

DSM Food Specialties B.V.

Andre Keuter

Internal Post Code 600-0250
P.O.B. 1
Delft 2600 MA The Netherlands

Status	Category	Issue date
Allowed with Restrictions	COR: Fungicides	28-May-2015

Product number	Class	Expiration date
dfs-5615	Crop Pest, Weed, and Disease Control	01-Jun-2017

Restrictions

May be used as a pesticide if the requirements of CAN/CGSB-32.310 section 5.6.2 are met, which require the use of organic management practices and mechanical techniques.

Peggy Miars

Executive Director

Product review is conducted according to the policies in the current *OMRI Policy Manual*© and based on the standards in the applicable *OMRI Standards Manual*©. To verify the current status of this or any OMRI Listed product, view the most current version of the *OMRI Canada Products List*© at OMRI.org. OMRI listing is not equivalent to organic certification and is not a product endorsement. It cannot be construed as such. Final decisions on the acceptability of a product for use in a certified organic system are the responsibility of a CFIA accredited Certification Body. It is the operator's responsibility to properly use the product, including following any restrictions.

Organic Materials Review Institute
P.O. Box 11558, Eugene, OR 97440-3758, USA
541.343.7600 · fax 541.343.8971 · info@omri.org · www.omri.org

Attachment 2 – EPA Stamped Acceptable Labels for Natamycin as a Pesticide

NATAMYCIN TGAI

For Agricultural Use Only

For formulation into end-use fungistat products used in enclosed mushroom production facilities and for post-harvest use on pineapple, the citrus, pome, and stone fruit crop groups, avocado, kiwi, mango and pomegranates.

ACCEPTED
07/22/2016
Under the Federal Insecticide, Fungicide and Rodenticide Act as amended, for the pesticide registered under EPA Reg. No. 87485-1

ACTIVE INGREDIENT:

Natamycin	91.02%
OTHER INGREDIENTS	8.98%
Total	100.00%

CAUTION

KEEP OUT OF REACH OF CHILDREN

See side/back panel for precautionary statements.

Manufactured by:

DSM Food Specialties B.V.
PO Box 1, 2600 MA, Delft
The Netherlands
+31 (15) 279 34 74

Questions? - Phone: 1-574-232-5000
Fax: 1-574-232-2468

EPA Registration Number 87485-1
EPA Est. No. 87485-FRA-001
Batch/Lot Code: ________

Net Weight 55 pounds

Precautionary Statements

Hazards to Humans and Domestic Animals

CAUTION — Harmful if swallowed. Causes moderate eye irritation. Avoid contact with eyes. Wear protective eyewear. Wash thoroughly with soap and water after handling and before eating, drinking, chewing gum, using tobacco or using the toilet. Remove and wash contaminated clothing before reuse.

First Aid	
IF SWALLOWED	• Call a poison control center or doctor immediately for treatment advice. • Have a person sip a glass of water if able to swallow. • Do not induce vomiting unless told to by a poison, control center or doctor. • Do not give anything by mouth to an unconscious person.
IF IN EYE	• Hold eye open and rinse slowly and gently with water for 15-20 minutes. • Remove contact lenses, if present, after 5 minutes, then continue rinsing eye. • Call a poison control center or doctor for treatment advice
Have the product container or label with you when calling a poison control center or doctor, or going for treatment. For emergency information call: Poison Control Center Emergency Number, **1-800-222-1222,** 24 hours per day, 7 days a week.	

Environmental Hazards

Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit, and the permitting authority has been notified in writing prior to discharge: Do not discharge effluent containing this product to sewer system without previously notifying the local sewage treatment plant authority. For guidance contact your State Water Board or Regional Office of the EPA.

Physical or Chemical Hazards

Prevent forming of dust clouds. Finely dispersed particles can be ignited causing a dust explosion.

Directions for Use

It is a violation of Federal law to use this product in a manner inconsistent with its labeling.

Only for formulation into EPA registered pesticides. Formulators using this product are responsible for providing data required by EPA for registration of their products.

This product may be used to formulate products for any additional uses not listed on the MP label if the formulator, user group, or grower has complied with U.S. EPA data submission requirements regarding the support of such use(s).

Storage and Disposal

Do not contaminate water, food, or feed by storage and disposal.

Pesticide Storage: Store only in original container in a cool, dry, dark area. When product is not in use, tightly seal container. When transferring, minimize exposure to light.

Pesticide Disposal: To avoid waste, use all material in this container by application according to label directions. **If** wastes cannot be avoided, offer remaining product to a waste disposal facility or pesticide disposal program (often such programs are run by state or local governments or by industry).

Container Disposal: Nonrefillable container. Do not reuse or refill this container.

- Outer packaging: Offer for recycling, if available.
- Liners: Completely empty liner by shaking and tapping sides and bottoms to loosen clinging particles. Empty residue into formulation equipment. Then dispose of liner in a sanitary landfill or by incineration if allowed by State and local authorities.

Warranty Statement

The directions for use of this product are believed to be adequate and must be followed carefully. It is impossible to eliminate all risks inherently associated with the use of this product. Crop injury, ineffectiveness, or other unintended consequences may result due to such factors as weather conditions, presence or absence of other materials, or the manner of use or application, all of which are beyond the control of DSM Food Specialties B.V., the manufacturer or the seller.

To the extent consistent with applicable law, the products sold to you are furnished "as is" by DSM Food Specialties B.V. To the extent consistent with applicable law, the manufacturer and the seller are subject only to the manufacturer's warranties, if any, which appear on the label of the product sold to you. To the extent consistent with applicable law and except as warranted by this label, DSM Food Specialties B.V., the manufacturer, or the seller makes no warranties, guarantees, or representations of any kind to the buyer or the user, either express or implied, or by usage of trade, statutory or otherwise, with regard to the product sold or use of the product, including, but not limited to, merchantability, fitness for a particular purpose or use, or eligibility of the product for any particular trade usage. To the extent consistent with applicable law, buyer's or user's exclusive remedy, and DSM Food Specialties B.V., the manufacturer's or the seller's total liability shall be limited to damages not exceeding the cost of the . product. No agent or employee of DSM Food Specialties B.V., or the seller is authorized to amend the terms of this warranty disclaimer or the product's label or to make a presentation or recommendation different from or inconsistent with the label of this product.

To the extent consistent with applicable law, DSM Food Specialties B.V., the manufacturer, or the seller shall not be liable for consequential, special, or indirect damages resulting from the use, handling, application, storage, or disposal of this product or for damages in the nature of penalties.

MASTER LABEL

Natamycin L

Alternate Brand Names: Zivion™ M, BIOSPECTRA 100SC[1], Nature's Shield 100SC

Sublabel A: Agricultural Use-Mushrooms
Sublabel B: Postharvest Use

EPA Registration Number: 87485-2

Note: Text in parentheses is optional.

ACCEPTED
08/19/2016
Under the Federal Insecticide, Fungicide and Rodenticide Act as amended, for the pesticide registered under
EPA Reg. No. 87485-2

Sublabel A: Agricultural Use-Mushrooms

Natamycin L

Alternate Brand Names: Zivion™ M

Liquid Suspension Formulation - For Agricultural Use Only
For use to control Dry Bubble Disease caused by *Verticillium fungicola* in enclosed mushroom production facilities

ACTIVE INGREDIENT:
Natamycin 10.34%
OTHER INGREDIENTS: 89.66%
Total: 100.00%

CAUTION
KEEP OUT OF REACH OF CHILDREN
See side/back panel for precautionary statements.

FIRST AID	
IF SWALLOWED • Call a poison control center or doctor immediately for treatment advice. • Have a person sip a glass of water if able to swallow • Do not induce vomiting unless told to do so by a poison control center or doctor. • Do not give anything by mouth to an unconscious person.	IF IN EYE • Hold eye open and rinse slowly and gently with water for 15 – 20 minutes. • Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. • Call a poison control center or doctor for treatment advice.
Have the product container or label with you when calling a poison control center or doctor, or going for treatment. For emergency information call: Poison Control Center Emergency Number, I-800-222-1222, 24 hours a day, 7 days a week.	

Manufactured by:
DSM Food Specialties B.V.
PO Box 1
2600 MA Delft
The Netherlands
+31 (0)15 279 34 74

EPA Registration Number: 87485-2
EPA Est. No.: 87485-FRA-01

Batch/Lot Code:
Production Date:

Questions?
Phone: 1-574-232-5000
Fax: 1-574-232-2468
Net Contents: 0.26 Gallon (1 liter)
This product contains 0.93 pounds of Natamycin per gallon

Natamycin L
Liquid Suspension Formulation- For Agricultural Use Only

For use to control Dry Bubble Disease cause by *Verticillium fungicola* in enclosed mushroom room production facilities

PRECAUTIONARY STATEMENTS HAZARDS TO HUMANS (AND DOMESTIC ANIMALS)

CAUTION
Harmful if swallowed. Causes moderate eye irritation. Avoid contact with eyes. Wear protective eyewear. Wash thoroughly with soap and water after handling and before eating, drinking, and chewing gum, using tobacco or using the toilet. Remove and wash contaminated clothing before reuse.

Handler Personal Protective Equipment (PPE)
Mixers, Loaders, Applicators and all others handlers must wear:
- Protective eyewear
- Long sleeved shirt
- Long pants
- Socks plus shoes

Statement for Contaminated PPE
Follow the manufacturer's instructions for cleaning/maintaining PPE.
If no such instructions for washables, use detergent and hot water.
Keep and wash PPE separately from other laundry.

User Safety Recommendations
- Users should remove clothing/PPE immediately if pesticide gets inside. Then wash thoroughly and put on clean clothing.
- Users should remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash thoroughly and change into clean clothing.

Environmental Hazards
Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit, and the permitting authority has been notified in writing prior to discharge. Do not discharge effluent containing this product to sewer system without previously notifying the local sewage treatment plant authority. For guidance contact your State Water Board or Regional Office of the EPA.

DIRECTIONS FOR USE
It is a violation of Federal law to use this product in a manner inconsistent with its labeling.
Do not apply this product in a way that will contact workers or other persons, either directly or through drift. Only protected handlers may in the area during application. For any requirement specific to your State and Tribe, consult the State/Tribal agency responsible for pesticide regulation.

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AGRICULTURAL USE REQUIREMENTS

Use this product only in accordance with its labeling and with the Worker Protection Standard, 40 CFR part 170. This standard contains requirements for the protection of agricultural workers on farms, forests, nurseries, and greenhouses, and handlers of agricultural pesticides. It contains requirements for training, decontamination, notification, and emergency assistance. It also contains specific instructions and exceptions pertaining to the statements on this label about personal protective equipment (PPE), notification to workers, and restricted-entry interval. The requirements in this box apply to uses of this product that are covered by the Worker Protection Standard.

Do not enter or allow worker entry into treated areas during the restricted entry interval (REI) of 4 hours.

PPE required for early entry to treated areas (that is permitted under the Worker Protection Standard and that involves contact with anything that has been treated, such as plants, soil, or water), is:
- Protective eyewear
- Long-sleeved shirt and long pants
- Shoes plus socks

Notify workers of the application by warning them orally and by posting warning signs at entrances to treated areas.

Do not enter or allow entry into treated areas during the restricted entry interval (REI)of 4 hours.

GENERAL INFORMATION

Natamycin L is a stable aqueous suspension of Natamycin crystals, a natural fermentation product of *Streptomyces natalensis*. The active ingredient, Natamycin, is a fungistat that acts as a barrier to entry of fungal disease by preventing germination of fungal spores and inhibiting beginning growth of hypha. **Natamycin has no effect against other types of microorganism**, nor against fungal mycelium once growth begins. This product is formulated for use in enclosed mushroom production facilities for the control of Dry Bubble Disease caused by *Verticillium fungicola* in mushroom production beds.

MIXING INSTRUCTIONS:

Measure the amount of Natamycin L to be applied based on the area to be treated (see rates and timing on next page). Add Natamycin L while stirring to the volume of water to be applied, or to a smaller volume that is then added to more water to make the expected final volume. Continuously stir the treatments solution unless it is to be applied immediately.

APPLICATION INSTRUCTIONS:

Do not apply this product by chemigation. Apply the final dilution by hand or through automatic watering systems. See additional application instructions below.

For use in mushroom production.

Target Pest	**Dry Bubble Disease *(Verticillium fungicola)***
Application Time	May be applied: 1) Immediately after casing 2) After flushing 3) Between first and second breaks 4) Between second and third breaks
Application Rate After Casing	Apply Natamycin L at the rate of 3.1 to 6.3 fl. oz per 1000 square feet as a surface drench once at any time after the casing layer has been applied and before flushing, using sufficient water to ensure an even application.
Application Rate After Flushing	Apply Natamycin L at the rate of 3.1 to 6.3 fl. oz per 1000 square feet as a surface drench once at any time after flushing up until 6 hours (6-hour PHI) before picking begins, using sufficient water to ensure an even application.
Application Rate When Applied Between Breaks	Apply Natamycin L at the rate of 3.1 to 6.3 fl. oz per 1000 square feet as a surface drench at any time between each set of breaks, up until 6 hours (6-hour PHI) before picking begins, using sufficient water to ensure an even application. Do not apply to mushrooms remaining on the beds that will be harvested within 6 hours after application. Do not apply to mushrooms remaining more than one time between each set of breaks.
Maximum Number of Applications	4 applications maximum
Pre-Harvest Interval	DO NOT apply within 6 hours of harvest.
Disposal of Compost & Casing	All spent medium must be steamed within the mushroom house at the end of each production run for no less than 12 hours at a temperature of 150°F (65°C) or greater before disposal outdoors.
Additional Comments	Natamycin L is to be used preventatively for control of Dry Bubble Disease (*Verticillium fungicola*). Make application prior to or in early states of disease development. Integrate Natamycin L into an overall disease management strategy. Follow practices known to reduce disease development. Consult local agricultural authorities for specific IPM strategies, developed for specific mushroom crops and locations.
For Answers to Grower Questions, Call	Phone: 1-574-232-5000 Fax: 1-574-232-2468

STORAGE AND DISPOSAL

Do not contaminate water, food, or feed by storage or disposal.

PESTICIDE STORAGE:
Store in original container in a cool, dry, dark place.

PESTICIDE DISPOSAL:
To avoid waste, use all material in this container by application according to label directions. If wastes cannot be avoided, offer remaining product to a waste disposal facility or pesticide disposal program (often such programs are run by state or local governments or by industry).

CONTAINER DISPOSAL:
For containers less than or equal to 5 gallons:
Nonrefillable container. Do not reuse or refill this container. Triple rinse container promptly after emptying. Triple rinse as follows: Empty the remaining contents into application equipment or a mix tank and drain for 10 seconds after the flow begins to drop. Fill the container ¼ full with water and recap. Shake for 10 seconds. Pour rinsate into application equipment or a mix tank or store rinsate for later use or disposal. Drain for 10 seconds after the flow begins to drop. Repeat this procedure two more times. Offer for recycling if available or dispose of in a sanitary landfill or by other procedures approved by state and local authorities.

For containers greater than 5 gallons:
Nonrefillable container. Do not reuse or refill this container. Offer for recycling if available. Triple rinse container promptly after emptying. Triple rinse as follows: Empty the remaining contents into application equipment or a mix tank. Fill the container ¼ with water. Replace and tighten closures. Tip container on its side and roll it back and forth, ensuring at least one complete resolution, for 30 seconds. Stand the container on its end and tip it back and forth several times. Turn the container over onto its other end and tip it back and forth several times. Empty rinsate into application equipment or a mix tank or store rinsate for later use or disposal. Repeat this procedure two more times. Offer for recycling if available or dispose of in a sanitary landfill or by other procedures approved by state and local authorities.

WARRANTY STATEMENT

The directions for use of this product are believed to be adequate and must be followed, carefully, it is impossible to eliminate all risks inherently associated with the use of this product. Crop injury, ineffectiveness, or other unintended consequences may result due to such factors as weather conditions, presence of absence of other materials, or the manner of use or application, all of which are beyond the control of DSM Food Specialties B.V., the manufacturer, or the seller.

To the extent consistent with applicable law, the products sold to you are furnished "as is" by DSM Food Specialties B.V. To the extent consistent with applicable law, the manufacturer and the seller are subject only to the manufacture's warranties, if any, which appear on the label of the product sold to you. To the extent consistent with applicable law, except as warranted by this label, DSM Food Specialties B.V., the manufacturer, or the seller makes no warranties, guarantees, or representations of any kind to the buyer or the user, either express or implied, or by usage of trade, statutory or otherwise, with regard to the product sold or use of the product, including, but not limited to, merchantability, fitness for a particular purpose or use, or eligibility

of the product for any particular trade usage. To the extent consistent with applicable law, buyer's or user's exclusive remedy, and DSM Food Specialties B.V., the manufacturer's or the seller's total liability shall be limited to damages not exceeding the cost of the product. No agent or employee of DSM Food Specialties B.V., or the seller is authorized to amend the terms of this warranty disclaimer or the product's label or to make a presentation or recommendation different from or inconsistent with the label of this product.

To the extent consistent with applicable law, DSM Food Specialties B.V., the manufacturer, or the seller shall not be liable for consequential, special, or indirect damages resulting from the use, handling, application, storage, or disposal of this product or for damages in the nature of penalties.

Sublabel B: Postharvest Use

Natamycin L

Alternate Brand Names: Nature's Shield 100SC, BIOSPECTRA 100SC[1]
For use to control several postharvest diseases on citrus, pome, and stone fruit (crop groups), (cherries), avocado, kiwi, mango and pomegranate

ACTIVE INGREDIENT:
Natamycin* 10.34%
OTHER INGREDIENTS: 89.66%
Total: 100.00%
*CAS No. 7681-93-8
Contains 0.93 lbs Natamycin per gallon.

CAUTION
KEEP OUT OF REACH OF CHILDREN
See side/back panel for precautionary statements.

FIRST AID	
IF SWALLOWED • Call a poison control center or doctor immediately for treatment advice. • Have a person sip a glass of water if able to swallow • Do not induce vomiting unless told to do so by a poison control center or doctor. • Do not give anything by mouth to an unconscious person.	IF IN EYE • Hold eye open and rinse slowly and gently with water for 15 – 20 minutes. • Remove contact lenses, if present, after the first 5 minutes, then continue rinsing eye. • Call a poison control center or doctor for treatment advice.
Have the product container or label with you when calling a poison control center or doctor, or going for treatment. (For emergency information call: Poison Control Center Emergency Number, I-800-222-1222, 24 hours a day, 7 days a week.)	
(HOT LINE NUMBER Medical Emergency Phone Number 888-271-4649 (PROSAR) Transportation Emergency Phone Number 800-424-9300 (CHEMTREC))	

Manufactured by:
DSM Food Specialties B.V.
PO Box 1
2600 MA Delft
The Netherlands
+31 (0)15 279 34 74
Questions?
Phone: 1-574-232-5000
Fax: 1-574-232-2468
Net Contents:

(Marketed by:
Pace International LLC
5661 Branch Road
Wapato, WA 98951
800.936.6750
www.paceint.com)

EPA Registration Number: 87485-2
EPA Est. No.: (87485-FRA-0I) (xxxxx-xxx-xx)
Batch/Lot Code:
Production Date:

(Optional Logo) DSM BRIGHT SCIENCE. BRIGHTER LIVING.

PRECAUTIONARY STATEMENTS
HAZARDS TO HUMANS AND DOMESTIC ANIMALS

CAUTION: Harmful if swallowed. Causes moderate eye irritation. Avoid contact with eyes. Wear protective eyewear. Wash thoroughly with soap and water after handling and before eating, drinking, and chewing gum, using tobacco or using the toilet. Remove and wash contaminated clothing before reuse.

Personal Protective Equipment (PPE)

Mixers, Loaders, Applicators and all others handlers must wear:

- Protective eyewear
- Long sleeved shirt
- Long pants
- Socks plus shoes
- Water proof gloves

Follow the manufacturer's instructions for cleaning/maintaining PPE. If no such instructions for washables, use detergent and hot water. Keep and wash PPE separately from other laundry.

User Safety Recommendations

- Users should remove clothing/PPE immediately if pesticide gets inside. Then wash thoroughly and put on clean clothing.
- Users should remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash thoroughly and change into clean clothing.

ENVIRONMENTAL HAZARDS

Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit, and the permitting authority has been notified in writing prior to discharge. Do not discharge effluent containing this product to sewer system without previously notifying the local sewage treatment plant authority. For guidance contact your State Water Board or Regional Office of the EPA.

DIRECTIONS FOR USE

It is a violation of Federal law to use this product in a manner inconsistent with its labeling. Do not apply this product in a way that will contact workers or other persons, either directly or through drift. Only protected handlers may in the area during application. For any requirement specific to your State and Tribe, consult the State/Tribal agency responsible for pesticide regulation.

GENERAL INFORMATION

Natamycin L is a stable aqueous suspension containing Natamycin, a natural fermentation product of *Streptomyces natalensis*. Natamycin is classified as a macrolide polyene antifungal compound and has a unique mode of action binding to ergosterol which is present in the cell membranes of yeasts and fungi preventing germination of fungal spores and inhibiting beginning growth of hypha. Natamycin has no effect against other types of microorganism or against fungal mycelium once growth begins.

FUNGICIDE RESISTANCE MANAGEMENT
Use of Natamycin L should be integrated into an overall disease management program within each packinghouse. Fungal pathogens can develop resistance to products with the same mode of action when used repeatedly. Because resistance development cannot be predicted, use of postharvest resistance management strategies should be practiced. These may include rotating and/or tank mixing with product having different modes of action. Responsible resistance management practices are necessary to ensure the long-term effectiveness for decay control.

MIXING INSTRUCTIONS
Fill tank with half of the required amount of water or water wax and start mechanical agitation. Add the required amount of Natamycin L and then add the remaining volume of water or water wax. Maintain agitation after mixing and do not allow treating solution to stand overnight or for prolonged periods. For in-line injection systems better distribution will be achieved by injecting a larger volume of a more dilute solution per unit of time.

The physical compatibility of Natamycin L with all tank mix partners and wax types has not been fully investigated. This product cannot be mixed with any product containing a label prohibition against such mixing. Follow the more restrictive labeling requirements of any tank mix partner. No label dosage rates for the individual products may be exceeded.

Prior to tank mixing with Natamycin L, conduct a jar test with the volumes and rates typically used for postharvest disease control. Add proportionate amounts of the products to a small container of water or water wax in the following order: Wettable powders and water-dispersible granules first, followed by liquid flowables, and emulsifiable concentrates last. After thorough mixing, let stand for at least 15 minutes. Separation, globules, sludge, flakes or other precipitates are indicative of physical incompatibility. Physical compatibility is indicated if the combination remains mixed or can be remixed readily.

THE CROP SAFETY OF ALL POTENTIAL PESTICIDE TANK MIXES WITH NATAMYCIN L INCLUDING ANTI-SCALD AGENTS AND WAXES, HAS NOT BEEN TESTED ON ALL CROPS AND VARIETIES. BEFORE APPLYING ANY TANK MIXTURE, CONFIRM SAFETY TO THE TARGET CROP BEFORE USE.

CROP USE DIRECTIONS

CITRUS: Calamondin (*Citrus mitis, Citrofortunella mitis*), Citrus citron (*Citrus medica*), Citrus hybrids (*Citrus spp.*) (includes chironja, tangelo, tangor), Grapefruit (*Citrus paradise*), Kumquat (*Fortunella spp.*), Lemon (*Citrus jambhiri, Citrus limon*), Lime (*Citrus arantiifolia*), Mandarin (tangerine) (*Citrus reticulate*), Orange, sour (*Citrus aurantium*), Orange, sweet (*Citrus sinensis*), Pummelo (*Citrus grandis, Citrus maxima*), Satsuma mandarin (*Citrus unshiu*), and all cultivars and hybrids.

Use Natamycin L as a postharvest dip, drench, flood, or spray for the control of certain postharvest fruit rots caused by:

- Green or Blue mold (*Penicillium spp.*)
- Sour rot (*Geotricum citri-aurantii, Geotricum candidum*)
- Gray mold (*Botrytis cinerea*)
- Mucor rot (*Mucor spp.*)

Application Method	Disease	Rate fl.oz.	Directions
In-Line Dip/drench	Green mold Blue mold Sour rot Gray mold Mucor rot	57-114 fl. oz./ 100 gals	• Ensure proper coverage of fruit. • Mix 57-114 fl. oz. in 100 gals of water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion • For in-line dip or drench applications, treat fruits for 10 seconds and allow the fruit to drain.
In-line Aqueous or Fruit Coating Spray Application		57-114 fl. oz./ 100 gals	• Ensure proper coverage of fruit. • Mix the fungicide solution in appropriate water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion.
• Clean citrus fruits treated by aqueous or wax line spray applications prior to application.			

POME FRUIT: Apple (*Malus domestica*), Crabapple (*Malus spp.*), Loquat (*Eriobotrya japonica*), Mayhaw (*Crataegus azarolus*), Pear (*Pyrus communis*), Oriental pear (*Pyrus spp.*), Quince (*Chaeonomeles spp.*) and cultivars, varieties and/or hybrids of these.

Use Natamycin L as a postharvest dip, drench, flood, or spray for the control of certain postharvest fruit rots caused by:

- Blue mold (*Penicillium expansum)*
- Gray mold (*Botrytis cinerea*)
- Mucor rot (*Mucor piriformis*)
- Sphaeropsis rot (*Sphaeropsis pyriputrescens*)
- Phacidiopycnis rot (*Phacidiopycnis piri*)
- Speck rot (*Phacidiopycnis washingtonensis*)

Application Method	Disease	Rate fl.oz.	Directions
Bin/Truck Drench or In-Line Dip/Drench or Flooders	Blue mold Gray mold Mucor rot Sphaeropsis rot Phacidiopycnis rot Speck rot	57-114 fl. oz./ 100 gals	• Ensure proper coverage of fruit. • Mix in 57-114 fl. oz. in 100 gals of water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion • For in-line dip or drench applications, treat fruits for 10 seconds and allow the fruit to drain.
In-line Aqueous or Fruit Coating Spray Application		57-114 fl. oz./ 100 gals	• Ensure proper coverage of fruit. • Mix the fungicide solution in appropriate water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion. • Use T-jet, CDA or similar application system.

STONE FRUIT: Apricot (*Prunus armeniaca*), Nectarine (*Prunus persica)*, Peach (*Prunus persica*), Plum, Chickasaw (*Prunus agustifolia*); Plum, Damson (*Prunus domestica spp. insititia*);

Plum, Japanese (*Prunus salicina*); Plumcot (*Prunus armeniaca* x *P. domestica*); Prune (fresh)(*Prunus domestica, Prunus spp.*); as well as all other cultivars and hybrids of these.

Use Natamycin L as a postharvest dip, drench, flood, or spray for the control of certain postharvest fruit rots caused by:

- Brown rot (*Monilinia spp.*)
- Gray mold (*Botrytis cinerea*)
- Sour rot (*Geotricum spp.*)
- Rhizopus rot (*Rhizopus spp.*)

Application Method	Disease	Rate fl.oz.	Directions
In-line Dip/Drench	Brown rot Gray mold Sour rot Rhizopus rot	57-114 fl. oz./ 100 gals	• Ensure proper coverage of fruit. • Mix 57-114 fl. oz. in 100 gals of water, wax/emulsion, or aqueous dilution of wax/oil emulsion. • Dip for 10 seconds and allow the fruit to drain.
In-line Aqueous or Fruit Coating Spray Application		28-114 fl. oz./ 200,000 lbs of fruit	• Ensure proper coverage of fruit. • Mix the fungicide solution in appropriate water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion. • Use T-jet, CDA or similar application system.

CHERRIES: Cherry, sweet (*Prunus avium*); Cherry, tart (*Prunus cerasis*); and all other cultivars and hybrids of these.

Use Natamycin L as a post-harvest dip, drench, flood, or spray for the control of certain postharvest fruit rots caused by:

- Brown rot (*Monilinia spp.*)
- Gray mold (*Botrytis cinerea*)

Application Method	Disease	Rate fl.oz.	Directions
In-line Aqueous or Flooder Application High volume application	Brown rot Gray mold	28-114 fl. oz./ 50,000 lbs of fruit	• Ensure proper coverage of fruit. • Mix 28-114 fl. oz. in 100 gals of water, wax/emulsion, or aqueous dilution of wax/oil emulsion. • Use flooder, T-jet or similar application system

AVOCADO

Use Natamycin L as a postharvest dip, drench, flood, or spray for the control of certain postharvest fruit rots caused by:

- Anthracnose (*Colletotrichum spp.)*
- Stem end rot (*Dothiorella spp.)*

Application Method	Disease	Rate fl.oz.	Directions
In-Line Dip /Drench	Anthracnose Stem end rot	57-114 fl. oz./ 100 gals	• Ensure proper coverage of fruit • Mix 57-114 fl. oz. in 100 gals of water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion • For in-line dip or drench applications, treat fruits for 10 seconds and allow the fruit to drain.
In-line Aqueous or Fruit Coating Spray Application		57-114 fl. oz./200,000 lbs of fruit	• Ensure proper coverage of fruit. • Mix the fungicide solution in appropriate water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion.

KIWI

Use Natamycin L as a postharvest dip, drench, flood, or spray for the control of certain postharvest fruit rots caused by:

- Botrytis fruit rot (*Botrytis cinerea*)

Application Method	Disease	Rate fl.oz.	Directions
In-Line Dip /Drench	Botrytis fruit rot	57-114 fl. oz. /100 gals	• Ensure proper coverage of fruit • Mix 57-114 fl. oz. in 100 gals of water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion • For in-line dip or drench applications, treat fruits for 10 seconds and allow the fruit to drain.
In-line Aqueous or Fruit Coating Spray Application		57-114 fl. oz./200,000 lbs of fruit	• Ensure proper coverage of fruit. • Mix the fungicide solution in appropriate water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion.

MANGO

Use Natamycin L as a postharvest dip, drench, flood, or spray for the control of certain postharvest fruit rots caused by:

- Anthracnose (*Colletotrichum spp.)*
- Stem end rot (*Dothiorella spp.)*

Application Method	Disease	Rate fl.oz.	Directions
In-Line Dip /Drench	Anthracnose Stem end rot	57-114 fl. oz./ 100 gals	• Ensure proper coverage of fruit • Mix 57-114 fl. oz. in 100 gals of water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion • For in-line dip or drench applications, treat fruits for 10 seconds and allow the fruit to drain.
In-line Aqueous or Fruit Coating Spray Application		57-114 fl. oz./200,000 lbs of fruit	• Ensure proper coverage of fruit. • Mix the fungicide solution in appropriate water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion.

POMEGRANATE

Use Natamycin L as a postharvest dip, drench, flood, or spray for the control of certain postharvest fruit rots caused by:

- Botrytis fruit rot (*Botrytis cinerea*)
- Blue mold *(Penicillium expansum)*

Application Method	Disease	Rate fl.oz.	Directions
In-Line Dip /Drench	Botrytis fruit rot Blue mold	57-114 fl. oz. /100 gals	• Ensure proper coverage of fruit • Mix 57-114 fl. oz. in 100 gals of water, wax/oil emulsion, or aqueous dilution of wax oil/emulsion • For in-line dip or drench applications, treat fruits for 10 seconds and allow the fruit to drain.

STORAGE AND DISPOSAL

Do not contaminate water, food, or feed by storage or disposal.

PESTICIDE STORAGE:
Store in original container only in a cool, dry, dark place.

PESTICIDE DISPOSAL:
To avoid waste, use all material in this container by application according to label directions. If wastes cannot be avoided, offer remaining product to a waste disposal facility or pesticide disposal program (often such programs are run by state or local governments or by industry).

CONTAINER HANDLING:
For containers less than or equal to 5 gallons:
Nonrefillable container. Do not reuse or refill this container. Triple rinse container promptly after emptying. Triple rinse as follows: Empty the remaining contents into application equipment or a mix tank and drain for 10 seconds after the flow begins to drop. Fill the container ¼ full with water and recap. Shake for 10 seconds. Pour rinsate into application equipment or a mix tank or store rinsate for later use or disposal. Drain for 10 seconds after the flow begins to drop. Repeat this procedure two more times. Offer for recycling if available or dispose of in a sanitary landfill or by other procedures approved by state and local authorities.

For containers greater than 5 gallons:
Nonrefillable container. Do not reuse or refill this container. Offer for recycling if available. Triple rinse container promptly after emptying. Triple rinse as follows: Empty the remaining contents into application equipment or a mix tank. Fill the container ¼ with water. Replace and tighten closures. Tip container on its side and roll it back and forth, ensuring at least one complete resolution, for 30 seconds. Stand the container on its end and tip it back and forth several times. Turn the container over onto its other end and tip it back and forth several times. Empty rinsate into application equipment or a mix tank or store rinsate for later use or disposal. Repeat this procedure two more times. Offer for recycling if available or dispose of in a sanitary landfill or by other procedures approved by state and local authorities.

(WARRANTY STATEMENT)

(The directions for use of this product are believed to be adequate and must be followed, carefully, it is impossible to eliminate all risks inherently associated with the use of this product. Crop injury, ineffectiveness, or other unintended consequences may result due to such factors as weather conditions, presence of absence of other materials, or the manner of use or application, all of which are beyond the control of DSM Food Specialties B.V., the manufacturer, or the seller.

To the extent consistent with applicable law, the products sold to you are furnished "as is" by DSM Food Specialties B.V. To the extent consistent with applicable law, the manufacturer and the seller are subject only to the manufacture's warranties, if any, which appear on the label of the product sold to you. To the extent consistent with applicable law, except as warranted by this label, DSM Food Specialties B.V., the manufacturer, or the seller makes no warranties, guarantees, or representations of any kind to the buyer or the user, either express or implied, or by usage of trade, statutory or otherwise, with regard to the product sold or use of the product, including, but not limited to, merchantability, fitness for a particular purpose or use, or eligibility

of the product for any particular trade usage. To the extent consistent with applicable law, buyer's or user's exclusive remedy, and DSM Food Specialties B.V., the manufacturer's or the seller's total liability shall be limited to damages not exceeding the cost of the product. No agent or employee of DSM Food Specialties B.V., or the seller is authorized to amend the terms of this warranty disclaimer or the product's label or to make a presentation or recommendation different from or inconsistent with the label of this product.

To the extent consistent with applicable law, DSM Food Specialties B.V., the manufacturer, or the seller shall not be liable for consequential, special, or indirect damages resulting from the use, handling, application, storage, or disposal of this product or for damages in the nature of penalties.)

(Optional Logo)

(NOTICE TO BUYER
IMPORTANT: Read the information below before using this product. If the terms are not acceptable, you should return the unopened product container immediately for a complete refund.

LIMITED WARRANTY, TERMS OF SALE, AND LIMITATION OF LIABILITY
Upon purchase of this product, purchaser and user agree to the following terms:
Warranty: Pace International, LLC (the Company) warrants that this product conforms to the chemical description on the label in all material respects. To the extent consistent with applicable law, except as expressly stated in the forgoing statement, the Company makes no further warranties, expressly disclaims any implied warranties of merchantability or fitness for a particular purpose, and any representation or warranty.
Terms of Sale: The Company's directions for use of this product must be followed carefully. It is impossible to eliminate all risks inherently associated with use of this product. Crop injury, ineffectiveness or other unintended consequences may result because of such factors as weather conditions, presence of other materials, and the manner or use or application (including failure to adhere to label directions), all of which are beyond the Company's control. To the extent consistent with applicable law, all such risks are assumed by the user.
Limitation of Liability: To the extent consistent with applicable law, the exclusive remedy against the Company for any cause of action relating to the handling or use of this product is a claim for damages, and in no event shall damages or any other recovery of any kind exceed the price of the product which caused the alleged loss, damage, injury, or other claim. To the extent consistent with applicable law, under no circumstances shall the Company be liable for any special, indirect, incidental, or consequential damages of any kind, including loss of profits or income, and any such claims are hereby waived.
To the extent consistent with applicable law, the Company and the seller offer this product, and the purchaser and user accept this product, subject to the foregoing warranty, terms of sale, and limitation of liability, which may be varied or modified only by an agreement in writing signed on behalf of the Company by an authorized representative.)

Optional Label Claims
Postharvest Fungicide
Postharvest Fungicide for Citrus, Pome, and Stone Fruit (Crop Groups), Cherries, Avocado, Kiwi, Mango
and Pomegranates
A flowable suspension concentrate
Postharvest Fungicide for Drench and Dip Treatment

Zivion™ P

For post-harvest use on pineapple to control fungal diseases *Fusarium oxysporum, Penicillium funiculosum, Rhizophus stolonifer, Aspergillus niger* and *Thielaviopsis paradoxa* during transport and storage

ACCEPTED
DEC 0 1 2014
Under the Federal Insecticide, Fungicide, and Rodenticide Act, as amended, for the pesticide registered under EPA Reg. No. 87485-3

ACTIVE INGREDIENT:
Natamycin .... 4 %
OTHER INGREDIENTS............................... 96 %
Total ....100 %

CAUTION

KEEP OUT OF REACH OF CHILDREN
See side/back panel for precautionary statements.

First Aid	
IF SWALLOWED • Call a poison control center or doctor immediately for treatment advice. • Have a person sip a glass of water if able to swallow. • Do not induce vomiting unless told to by a poison control center or doctor. • Do not give anything by mouth to an unconscious person.	IF IN EYE • Hold eye open and rinse slowly and gently with water for 15-20 minutes. • Remove contact lenses, if present, after 5 minutes, then continue rinsing eye. • Call a poison control center or doctor for treatment advice.
Have the product container or label with you when calling a poison control center or doctor, or going for treatment. For emergency information call: Poison Control Center Emergency Number, 1-800-222-1222, 24 hours a day, 7 days a week.	

Manufactured by:

DSM Food Specialties Superdex S.A.S.
Z.A. de Labarthete
Route de Magnas
32380 Saint Clar
France

EPA Registration Number 87485-xx
EPA Est. No. 87485-FRA-01
Batch/Lot code:________

Net Contents: ______

This product contains 0.36 lbs. of Natamycin per gallon.

Questions?
Phone: 1-574-232-5000
Fax: 1-574-232-2468

Zivion™ P

Liquid Suspension Formulation – For Agricultural Use Only

For use to control fungal diseases *Fusarium oxysporum, Penicillium funiculosum, Rhizophus stolonifer, Aspergillus niger* and *Thielaviopsis paradoxa* during transport and storage of pineapples

PRECAUTIONARY STATEMENTS
HAZARDS TO HUMANS (AND DOMESTIC ANIMALS)

CAUTION
Harmful if swallowed. Causes moderate eye irritation. Avoid contact with eyes. Wear protective eyewear. Wash thoroughly with soap and water after handling and before eating, drinking, and chewing gum, using tobacco or using the toilet. Remove and wash contaminated clothing before reuse.

Handler Personal Protective Equipment (PPE)
Mixers, Loaders, Applicators and all other handlers must wear protective eyewear, long sleeved shirt and long pants, shoes plus socks and gloves.

Statements for Contaminated PPE
Follow the manufacturer's instructions for cleaning/maintaining PPE. If no such instructions for washables, use detergent and hot water. Keep and wash PPE separately from other laundry.

User Safety Recommendations

- Users should remove clothing/PPE immediately if pesticide gets inside. Then wash thoroughly and put on clean clothing.
- Users should remove PPE immediately after handling this product. Wash the outside of gloves before removing. As soon as possible, wash thoroughly and change into clean clothing.

Environmental Hazards
Do not discharge effluent containing this product into lakes, streams, ponds, estuaries, oceans or other waters unless in accordance with the requirements of a National Pollutant Discharge Elimination System (NPDES) permit, and the permitting authority has been notified in writing prior to discharge. Do not discharge effluent containing this product to sewer system without previously notifying the local sewage treatment plant authority. For guidance contact your State Water Board or Regional Office of the EPA.

DIRECTIONS FOR USE
It is a violation of Federal law to use this product in a manner inconsistent with its labeling.

For indoor use in packinghouses (packing sheds). Do not apply this product in a way that will contact workers or other persons, either directly or through drift. Only protected handlers may be in the area during application. For any requirement specific to your State and Tribe, consult the State/Tribal agency responsible for pesticide regulation.

General Information
Zivion™ P is a stable aqueous suspension of Natamycin crystals, a natural fermentation product of *Streptomyces natalensis*. The active ingredient, Natamycin, is a fungistat that acts as a barrier to entry of fungal disease by preventing germination of fungal spores and inhibiting beginning growth of hypha. **Natamycin has no effect against other types of microorganism**, nor against fungal mycelium once growth begins. This product is formulated for use post-harvest for the control of fungal diseases in pineapple transport and storage.

Mixing Instructions:
Measure the amount of Zivion™ P to be applied based on the volume to be used. Add Zivion™ P while stirring to the volume of water and aqueous dilution of wax to be applied, or to a smaller volume that is then added to more water and aqueous dilution of wax to make the expected final volume. Continuously stir the treatment solution unless it is to be applied immediately.

Application Instructions:
Apply the final dilution by dipping, pouring equipment or through automatic cascading systems: after air drying fruit spray peduncle with 0.034 fl. oz. of treatment solution;

For use to control fungal diseases in pineapple transport and storage

Target Pest	*Fusarium oxysporum, Penicillium funiculosum, Rhizophus stolonifer, Aspergillus niger, Thielaviopsis paradoxa*
Application Time	May be applied: Post-harvest
Application rate	4 to 32 fl oz product per gallon of water and aqueous dilution of wax, depending on pest pressure
Maximum number of application	1
Application method	Commercial types of application (e.g. dip/ pouring / cascade)

STORAGE AND DISPOSAL
Do not contaminate water, food or feed by storage and disposal

PESTICIDE STORAGE:
Store in original container in a cool, dry, dark place.

PESTICIDE DISPOSAL:
To avoid waste, use all material in this container by application according to label directions. If wastes cannot be avoided, offer remaining product to a waste disposal facility or pesticide disposal program (often such programs are run by state or local governments or by industry).

CONTAINER DISPOSAL:
For containers less than or equal to 5 gallons:
Nonrefillable container. Do not reuse or refill this container. Offer for recycling if available. Triple rinse container promptly after emptying. Triple rinse as follows: Empty the remaining contents into application equipment or a mix tank and drain for 10 seconds after the flow begins to drip. Fill the container ¼ full with water and recap. Shake for 10 seconds. Pour rinsate into application equipment or a mix tank or store rinsate for later use or disposal. Drain for 10 seconds after the flow begins to drip. Repeat this procedure two more times. Offer for recycling if available or dispose of in a sanitary landfill or by other procedures approved by state and local authorities.

For containers greater than 5 gallons:
Nonrefillable container. Do not reuse or refill this container. Offer for recycling if available. Triple rinse container promptly after emptying. Triple rinse as follows: Empty the remaining contents into application equipment or a mix tank. Fill the container ¼ full with water. Replace and tighten closures. Tip container on its side and roll it back and forth, ensuring at least one complete revolution, for 30 seconds. Stand the container on its end and tip it back and forth several times. Turn the container over onto its other end and tip it back and forth several times. Empty rinsate into application equipment or a mix tank or store rinsate for later use or disposal. Repeat this procedure two more times. Offer for recycling if available or dispose of in a sanitary landfill or by other procedures approved by state and local authorities.

WARRANTY STATEMENT

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Attachment 3 – Natamycin Safety Data Sheet (SDS)

SAFETY DATA SHEET

Natamycin TGAI

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1 Product identifier

Product name	:	Natamycin TGAI
Internal code	:	WW49231
Chemical product name	:	Natamycin
Chemical formula	:	C33-H47-N-O13
EC number	:	231-683-5
REACH Registration number	:	Not available.

1.2 Relevant identified uses of the substance or mixture and uses advised against

Recommended use : This product is used as active Ingredient for antimycotic (bio)pesticide formulations

1.3 Details of the supplier of the safety data sheet

Supplier : DSM Food Specialties B.V.
P.O. Box 1
2600 MA Delft
The Netherlands
Phone: +31 (0) 15 279 4001
Telefax: +31 (0) 15 279 4020

e-mail address of person responsible for this SDS : Info.Worldwise@dsm.com

1.4 Emergency telephone number

Emergency telephone number : **+31 (0)15 2792380**

SECTION 2: Hazards identification

2.1 Classification of the substance or mixture

Product definition : Substance

Classification according to Regulation (EC) No. 1272/2008 [CLP/GHS]

Not classified.

The product is not classified as hazardous according to Regulation (EC) 1272/2008 as amended.

Classification according to Directive 67/548/EEC [DSD]

Not classified.

2.2 Label elements

Hazard pictograms	:	
Signal word	:	No signal word.
Hazard statements	:	No known significant effects or critical hazards.
Supplemental label elements	:	Not applicable.

Precautionary statements

Prevention	:	Not applicable.
Response	:	Not applicable.
Storage	:	Not applicable.
Disposal	:	Not applicable.

2.3 Other hazards

Substance meets the criteria for PBT according to Regulation (EC) No. 1907/2006, Annex XIII	:	No. P: Not available. B: Not available. T: No.
Substance meets the criteria for vPvB according to Regulation (EC) No. 1907/2006, Annex XIII	:	Not available.

Other hazards which do not result in classification : Fine dust clouds may form explosive mixtures with air. Handling and/or processing of this material may generate a dust which can cause mechanical irritation of the eyes, skin, nose and throat.

SECTION 3: Composition/information on ingredients

3.1 Substances / 3.2 Mixtures : Substance

Product/ingredient name	Identifiers	%	Classification	
			67/548/EEC	Regulation (EC) No. 1272/2008 [CLP]
natamycin	EC: 231-683-5 CAS: 7681-93-8	100	Not classified. **See Section 16 for the full text of the R-phrases declared above.**	Not classified. **See Section 16 for the full text of the H statements declared above.**

There are no additional ingredients present which, within the current knowledge of the supplier and in the concentrations applicable, are classified as hazardous to health or the environment, are PBTs or vPvBs or have been assigned a workplace exposure limit and hence require reporting in this section.

Occupational exposure limits, if available, are listed in Section 8.

SECTION 4: First aid measures

4.1 Description of first aid measures

Eye contact : Immediately flush eyes with plenty of water, occasionally lifting the upper and lower eyelids. Check for and remove any contact lenses. Get medical attention if irritation occurs.

Inhalation : Remove victim to fresh air and keep at rest in a position comfortable for breathing. Get medical attention if symptoms occur. In case of inhalation of decomposition products in a fire, symptoms may be delayed. The exposed person may need to be kept under medical surveillance for 48 hours.

Skin contact : Flush contaminated skin with plenty of water. Remove contaminated clothing and shoes. Get medical attention if symptoms occur.

Ingestion : Wash out mouth with water. Remove victim to fresh air and keep at rest in a position comfortable for breathing. Do not induce vomiting unless directed to do so by medical personnel. Get medical attention if symptoms occur.

Protection of first-aiders : No action shall be taken involving any personal risk or without suitable training.

4.2 Most important symptoms and effects, both acute and delayed

__Potential acute health effects__

Eye contact : Exposure to airborne concentrations above statutory or recommended exposure limits may cause irritation of the eyes.

Inhalation : Exposure to airborne concentrations above statutory or recommended exposure limits may cause irritation of the nose, throat and lungs. Exposure to decomposition products may cause a health hazard. Serious effects may be delayed following exposure.

Skin contact : No significant irritation expected other than possible mechanical irritation.

Ingestion : No known significant effects or critical hazards.

__Over-exposure signs/symptoms__

Eye contact : Adverse symptoms may include the following:
irritation
redness

Inhalation : Adverse symptoms may include the following:
respiratory tract irritation
coughing

Skin contact : No specific data. No significant irritation expected other than possible mechanical irritation.

Ingestion : No specific data.

4.3 Indication of any immediate medical attention and special treatment needed

Notes to physician : In case of inhalation of decomposition products in a fire, symptoms may be delayed. The exposed person may need to be kept under medical surveillance for 48 hours.

Specific treatments : No specific treatment.

SECTION 5: Firefighting measures

5.1 Extinguishing media

Small fire

Suitable	:	Use dry chemical or CO_2.
Not suitable	:	Do not use water jet.

Large fire

Suitable	:	Use extinguishing media suitable for surrounding materials.
Not suitable	:	Do not use water jet.

5.2 Special hazards arising from the substance or mixture

Hazards from the substance or mixture	:	Fine dust clouds may form explosive mixtures with air.
Hazardous combustion products	:	In case of fire, may produce hazardous decomposition products such as carbon monoxide, carbon dioxide, nitrogen oxides (NO, NO_2 etc.), ammonia (NH_3), amines.

5.3 Advice for firefighters

Special protective actions for fire-fighters	:	No special measures required.
Special protective equipment for fire-fighters	:	Fire-fighters should wear appropriate protective equipment and self-contained breathing apparatus (SCBA) with a full face-piece operated in positive pressure mode. Clothing for fire-fighters (including helmets, protective boots and gloves) conforming to European standard EN 469 will provide a basic level of protection for chemical incidents.

SECTION 6: Accidental release measures

6.1 Personal precautions, protective equipment and emergency procedures

For non-emergency personnel	:	No action shall be taken involving any personal risk or without suitable training. Evacuate surrounding areas. Keep unnecessary and unprotected personnel from entering. Do not touch or walk through spilt material. Shut off all ignition sources. No flares, smoking or flames in hazard area. Avoid breathing dust. Put on appropriate personal protective equipment.
For emergency responders	:	If specialised clothing is required to deal with the spillage, take note of any information in Section 8 on suitable and unsuitable materials. See also the information in "For non-emergency personnel".

6.2 Environmental precautions	:	Avoid dispersal of spilt material and runoff and contact with soil, waterways, drains and sewers. Inform the relevant authorities if the product has caused environmental pollution (sewers, waterways, soil or air).

6.3 Methods and material for containment and cleaning up

Small spill	:	Move containers from spill area. Vacuum or sweep up material and place in a designated, labelled waste container. Use spark-proof tools and explosion-proof equipment. Dispose of via a licensed waste disposal contractor.
Large spill	:	Move containers from spill area. Approach the release from upwind. Prevent entry into sewers, water courses, basements or confined areas. Vacuum or sweep up material and place in a designated, labelled waste container. Avoid creating dusty conditions and prevent wind dispersal. Use spark-proof tools and explosion-proof equipment. Dispose of via a licensed waste disposal contractor. Note: see Section 1 for emergency contact information and Section 13 for waste disposal.

6.4 Reference to other sections	:	See Section 1 for emergency contact information. See Section 8 for information on appropriate personal protective equipment. See Section 13 for additional waste treatment information.

SECTION 7: Handling and storage

The information in this section contains generic advice and guidance. The list of Identified Uses in Section 1 should be consulted for any available use-specific information provided in the Exposure Scenario(s).

7.1 Precautions for safe handling

Protective measures : Put on appropriate personal protective equipment (see Section 8). Eating, drinking and smoking should be prohibited in areas where this material is handled, stored and processed. Workers should wash hands and face before eating, drinking and smoking. Remove contaminated clothing and protective equipment before entering eating areas. Do not ingest. Avoid contact with eyes, skin and clothing. Avoid breathing dust. Avoid the creation of dust when handling and avoid all possible sources of ignition (spark or flame). Prevent dust accumulation. Use only with adequate ventilation. Wear appropriate respirator when ventilation is inadequate. Keep in the original container or an approved alternative made from a compatible material, kept tightly closed when not in use. Electrical equipment and lighting should be protected to appropriate standards to prevent dust coming into contact with hot surfaces, sparks or other ignition sources. Take precautionary measures against electrostatic discharges. To avoid fire or explosion, dissipate static electricity during transfer by earthing and bonding containers and equipment before transferring material. Empty containers retain product residue and can be hazardous. Do not reuse container.

Advice on general occupational hygiene : Eating, drinking and smoking should be prohibited in areas where this material is handled, stored and processed. Workers should wash hands and face before eating, drinking and smoking. Remove contaminated clothing and protective equipment before entering eating areas. See also Section 8 for additional information on hygiene measures.

7.2 Conditions for safe storage, including any incompatibilities : Do not store above the following temperature: 20°C (68°F). Store in accordance with local regulations. Store in a segregated and approved area. Store in a dry, cool and well-ventilated area, away from incompatible materials (see Section 10). Eliminate all ignition sources. Separate from oxidizing materials. Keep container tightly closed and sealed until ready for use. Containers that have been opened must be carefully resealed and kept upright to prevent leakage. Do not store in unlabelled containers. Use appropriate containment to avoid environmental contamination. Store in original container, protected from direct sunlight.

Do not store above the following temperature: 20 °C.

Keep container tightly closed in a cool place. Keep container dry. Protect from (sun)light. Keep only in the original container.

Packaging materials

Suitable : Polyethylene.

7.3 Specific end use(s)

Recommendations : Not available.

Industrial sector specific solutions : Not available.

SECTION 8: Exposure controls/personal protection

The information in this section contains generic advice and guidance. Information is provided based on typical anticipated uses of the product. Additional measures might be required for bulk handling or other uses that could significantly increase worker or exposure or environmental releases.

8.1 Control parameters

Occupational exposure limits

No exposure limit value known.

Recommended monitoring procedures : If this product contains ingredients with exposure limits, personal, workplace atmosphere or biological monitoring may be required to determine the effectiveness of the ventilation or other control measures and/or the necessity to use respiratory protective equipment. Reference should be made to monitoring standards, such as the following: European Standard EN 689 (Workplace atmospheres - Guidance for the assessment of exposure by inhalation to chemical agents for comparison with limit values and measurement strategy) European Standard EN 14042 (Workplace atmospheres - Guide for the application and use of procedures for the assessment of exposure to chemical and biological agents) European Standard EN 482 (Workplace atmospheres - General requirements for the performance of procedures for the measurement of chemical agents) Reference to national guidance documents for methods for the determination of hazardous substances will also be required.

DNELs/DMELs

No DNELs/DMELs available.

PNECs

No PNECs available

8.2 Exposure controls

Appropriate engineering controls	: Use only with adequate ventilation. If user operations generate dust, fumes, gas, vapour or mist, use process enclosures, local exhaust ventilation or other engineering controls to keep worker exposure to airborne contaminants below any recommended or statutory limits. The engineering controls also need to keep gas, vapour or dust concentrations below any lower explosive limits. Use explosion-proof ventilation equipment.

Individual protection measures

Hygiene measures	: Wash hands, forearms and face thoroughly after handling chemical products, before eating, smoking and using the lavatory and at the end of the working period. Appropriate techniques should be used to remove potentially contaminated clothing. Wash contaminated clothing before reusing. Ensure that eyewash stations and safety showers are close to the workstation location.
Eye/face protection	: Safety glasses with side shields.
Hand protection	: Chemical-resistant, impervious gloves complying with an approved standard should be worn at all times when handling chemical products if a risk assessment indicates this is necessary. 4 - 8 hours (breakthrough time): Practical experience has shown that gloves of polychloroprene (neoprene), nitril rubber, butyl rubber, fluor rubber (Viton) and polyvinyl chloride (PVC) offer sufficient protection against (undissolved) solids.
Skin and body	: Working clothes.
Respiratory protection	: Wear dust protection mask P2.
Environmental exposure controls	: Emissions from ventilation or work process equipment should be checked to ensure they comply with the requirements of environmental protection legislation. In some cases, fume scrubbers, filters or engineering modifications to the process equipment will be necessary to reduce emissions to acceptable levels.

Advice on personal protection is applicable for high exposure levels. Select proper personal protection based on a risk assessment of the actual exposure situation.

SECTION 9: Physical and chemical properties

9.1 Information on basic physical and chemical properties

Physical state	: Solid. [Crystalline powder]
Colour	: White to off-white.
Odour	: Neutral.
Odour threshold	: Not available.
pH	: 5 to 7.5 (Concentration 0.1%)
Melting point/freezing point	: Decomposes.
Initial boiling point and boiling range	: Not available.
Softening range	: Not available.
Flash point	: Not applicable.
Evaporation rate	: Not available.
Flammability (solid, gas)	: Not available.
Upper/lower flammability or explosive limits	: Not available.
Vapour pressure	: Not available.
Vapour density	: Not available.
Relative density	: Not available.
Density (g/cm^3)	: Not available.
Bulk density	: ≈3500 kg/m^3
Solubility	: Very slightly soluble in the following materials: cold water and methanol.
Solubility in water	: 0.041 g/100 ml (21°C)
Solubility at room temperature	: 0.41 g/l
Partition coefficient: n-octanol/water	: -3.67
Auto-ignition temperature	: Not applicable.
Decomposition temperature	: 280 to 300°C
Viscosity	: Not available.
Explosive properties	: Not available.
Oxidising properties	: None.

9.2 Other information

Conductivity	: 0.131 pS/m
Molecular weight	: 665.74 g/mole
Minimum ignition energy	: 1 to 3 mJ

Dust explosion class : St1 - moderately explosive.

Remarks : Solubility methanol; 0.971 g/100 ml

SECTION 10: Stability and reactivity

10.1 Reactivity : No specific test data related to reactivity available for this product or its ingredients.

10.2 Chemical stability : The product is stable.

10.3 Possibility of hazardous reactions : Under normal conditions of storage and use, hazardous reactions will not occur.

10.4 Conditions to avoid : Avoid the creation of dust when handling and avoid all possible sources of ignition (spark or flame). Take precautionary measures against electrostatic discharges. To avoid fire or explosion, dissipate static electricity during transfer by earthing and bonding containers and equipment before transferring material. Prevent dust accumulation.

10.5 Incompatible materials : Reactive or incompatible with the following materials:
oxidizing materials

10.6 Hazardous decomposition products : Carbon oxides (CO, CO_2) , nitrogen oxides (NO, NO_2 etc.) .

SECTION 11: Toxicological information

11.1 Information on toxicological effects

Acute toxicity

Product/ingredient name	Result	Species	Dose	Exposure
natamycin	LC0 Inhalation Dusts and mists LD50 Dermal LD50 Oral	Rat Rat - Male, Female Rat	2.39 mg/l >5050 mg/kg 2730 mg/kg	4 hours - -

Conclusion/Summary :

Irritation/Corrosion

Product/ingredient name	Result	Species	Score	Exposure	Observation
natamycin	Skin - Oedema Skin - Erythema/ Eschar Eyes - Cornea opacity	Rabbit Rabbit Rabbit	0 0.25 0.75	4 hours 4 hours 72 hours	72 hours 72 hours 72 hours

Conclusion/Summary

Eyes : No significant irritation expected other than possible mechanical irritation.

Skin : No significant irritation expected other than possible mechanical irritation.

Respiratory : Not available.

Sensitisation

Product/ingredient name	Route of exposure	Species	Result
natamycin	skin	Mouse	Not sensitizing

Conclusion/Summary

Skin : Non-sensitiser to skin.

Respiratory : Not available.

Mutagenicity

Product/ingredient name	Test	Experiment	Result
natamycin	OECD 471 Bacterial Reverse Mutation Test	Experiment: In vitro Subject: Bacteria Cell: Germ	Negative

Conclusion/Summary : No mutagenic effect.

Carcinogenicity

Conclusion/Summary : Not carcinogenic in animal experiments.

Reproductive toxicity

Conclusion/Summary : Not available.

Teratogenicity

Product/ingredient name	Result	Species	Dose	Exposure
natamycin	Negative - Oral	Rabbit	50 mg/kg NOEL	-

Conclusion/Summary : No teratogenic effects in animal experiments.

Specific target organ toxicity (single exposure)

Not available.

Specific target organ toxicity (repeated exposure)

Not available.

Aspiration hazard

Not available.

Potential acute health effects

Eye contact : Exposure to airborne concentrations above statutory or recommended exposure limits may cause irritation of the eyes.

Inhalation : Exposure to airborne concentrations above statutory or recommended exposure limits may cause irritation of the nose, throat and lungs. Exposure to decomposition products may cause a health hazard. Serious effects may be delayed following exposure.

Skin contact : No significant irritation expected other than possible mechanical irritation.

Ingestion : No known significant effects or critical hazards.

Symptoms related to the physical, chemical and toxicological characteristics

Eye contact : Adverse symptoms may include the following:
irritation
redness

Inhalation : Adverse symptoms may include the following:
respiratory tract irritation
coughing

Skin contact : No specific data.

Ingestion : No specific data.

Potential chronic health effects

Product/ingredient name	Result	Species	Dose	Exposure
natamycin	Chronic NOAEL Oral	Rat	25 mg/kg	2 years

Conclusion/Summary :

General : Repeated or prolonged inhalation of dust may lead to chronic respiratory irritation.

Carcinogenicity : No known significant effects or critical hazards.

Mutagenicity : No known significant effects or critical hazards.

Teratogenicity : No known significant effects or critical hazards.

Developmental effects : No known significant effects or critical hazards.

Fertility effects : No known significant effects or critical hazards.

SECTION 12: Ecological information

12.1 Toxicity

Conclusion/Summary : Not available.

12.2 Persistence and degradability

Conclusion/Summary : Not available.

Product/ingredient name	Aquatic half-life	Photolysis	Biodegradability
natamycin	-	-	Readily

12.3 Bioaccumulative potential

Product/ingredient name	$LogP_{ow}$	BCF	Potential
natamycin	-3.67	-	low

12.4 Mobility in soil

Soil/water partition coefficient (K_{OC}) : Not available.

Mobility : Not available.

12.5 Results of PBT and vPvB assessment

PBT : No.
P: Not available. B: Not available. T: No.

vPvB : Not available.
vP: Not available. vB: Not available.

12.6 Other adverse effects : No known significant effects or critical hazards.

SECTION 13: Disposal considerations

The information in this section contains generic advice and guidance. The list of Identified Uses in Section 1 should be consulted for any available use-specific information provided in the Exposure Scenario(s).

13.1 Waste treatment methods

Product

Methods of disposal : The generation of waste should be avoided or minimised wherever possible. Disposal of this product, solutions and any by-products should at all times comply with the requirements of environmental protection and waste disposal legislation and any regional local authority requirements. Dispose of surplus and non-recyclable products via a licensed waste disposal contractor. Waste should not be disposed of untreated to the sewer unless fully compliant with the requirements of all authorities with jurisdiction. Waste packaging should be recycled. Incineration or landfill should only be considered when recycling is not feasible. This material and its container must be disposed of in a safe way. Empty containers or liners may retain some product residues. Avoid dispersal of spilt material and runoff and contact with soil, waterways, drains and sewers.

Hazardous waste : Within the present knowledge of the supplier, this product is not regarded as hazardous waste, as defined by EU Directive 91/689/EEC.

Packaging

Methods of disposal : The generation of waste should be avoided or minimised wherever possible. Waste packaging should be recycled. Incineration or landfill should only be considered when recycling is not feasible.

Special precautions : This material and its container must be disposed of in a safe way. Empty containers or liners may retain some product residues. Avoid dispersal of spilt material and runoff and contact with soil, waterways, drains and sewers.

SECTION 14: Transport information

	ADR/RID	ADN	IMDG	IATA
14.1 UN number	Not regulated.	Not regulated.	Not regulated.	Not regulated.
14.2 UN proper shipping name	-	-	-	-
14.3 Transport hazard class(es)	-	-	-	-
14.4 Packing group	-	-	-	-
14.5 Environmental hazards	No.	No.	No.	No.
Additional information	-	-	-	-

14.6 Special precautions for user : **Transport within user's premises:** always transport in closed containers that are upright and secure. Ensure that persons transporting the product know what to do in the event of an accident or spillage.

14.7 Transport in bulk according to Annex II of MARPOL 73/78 and the IBC Code : Not available.

SECTION 15: Regulatory information

15.1 Safety, health and environmental regulations/legislation specific for the substance or mixture

EU Regulation (EC) No. 1907/2006 (REACH)

Annex XIV - List of substances subject to authorisation

Annex XIV

None of the components are listed.

Substances of very high concern

None of the components are listed.

Annex XVII - Restrictions on the manufacture, placing on the market and use of certain dangerous substances, mixtures and articles

Not applicable.

National regulations

International regulations

Chemical Weapon Convention List Schedules I, II & III Chemicals

Ingredient name	List name	Status
Not listed.		

Montreal Protocol (Annexes A, B, C, E)

Ingredient name	List name	Status
Not listed.		

Stockholm Convention on Persistent Organic Pollutants

Ingredient name	List name	Status
Not listed.		

Rotterdam Convention on Prior Inform Consent (PIC)

Ingredient name	List name	Status
Not listed.		

UNECE Aarhus Protocol on POPs and Heavy Metals

Ingredient name	List name	Status
Not listed.		

15.2 Chemical Safety Assessment : No Chemical Safety Assessment has been carried out.

SECTION 16: Other information

Procedure used to derive the classification according to Regulation (EC) No. 1272/2008 [CLP/GHS]

Classification	Justification
Not classified.	

Full text of abbreviated H statements : Not applicable.

Full text of classifications [CLP/GHS] : Not applicable.

Full text of abbreviated R phrases : Not applicable.

Full text of classifications [DSD/DPD] : Not applicable.

Alterations compared to the previous version : Alterations compared to the previous version are marked with a little (blue) triangle.

Abbreviations and acronyms : ATE = Acute Toxicity Estimate
CLP = Classification, Labelling and Packaging Regulation [Regulation (EC) No. 1272/2008]
DMEL = Derived Minimal Effect Level
DNEL = Derived No Effect Level
EUH statement = CLP-specific Hazard statement
PBT = Persistent, Bioaccumulative and Toxic
PNEC = Predicted No Effect Concentration
RRN = REACH Registration Number
vPvB = Very Persistent and Very Bioaccumulative

Sources of key data : Literature data and/or investigation reports are available through the manufacturer.

Internal code : WW49231

Training advice : Before handling this substance/preparation, the personnel involved should be instructed by means of this safety data sheet.

Notice to reader

The information contained in the Safety Data Sheet is based on our data available on the date of publication. The information is intended to aid the user in controlling the handling risks; it is not to be construed as a warranty or specification of the product quality. The information may not be or may not altogether be applicable to combinations of the product with other substances or to particular applications.
The user is responsible for ensuring that appropriate precautions are taken and for satisfying themselves that the data are suitable and sufficient for the product's intended purpose. In case of any unclarity we advise consulting the supplier or an expert.

History

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Version : 2

Attachment 4 – Petitioner Response to Dalhoff & Levy, 2015

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Letter to the Editor

Reaction to Dalhoff and Levy: 'Does use of the polyene natamycin as a food preservative jeopardise the clinical efficacy of amphotericin B? A word of concern'

Sir,

Our attention has been drawn to the paper 'Does use of the polyene natamycin as a food preservative jeopardise the clinical efficacy of amphotericin B? A word of concern' by A.A.H. Dalhoff and S.B. Levy, published in the June issue of your journal [1]. As scientists employed by a company that produces natamycin as a food preservative, the question raised in the paper is of direct interest to us. We are fully committed to the safety of our product, and its use, and would like to respond to the concerns raised.

The authors state that almost three decades of use of natamycin to prevent mould growth on cheese and sausages has not led to changes in natamycin sensitivity. However, they argue that the use of natamycin in yogurt and beverages is different, as the effect on the human intestinal flora must be considered because of higher dose levels and higher exposure. We are of the opinion that the authors make very selective use of scientific (and patent) sources—sources that are only distantly relevant to the subject—to raise their concern. In fact, we would like to argue that the sources actually point in the opposite direction.

Their calculation of maximum exposure relies on the assumption that high levels of natamycin (500 mg/L) could hypothetically be used in beverages. This ignores the fact that the maximum dosage is regulated. The exact level may vary per country and per application, but is in any case below 20 mg/L. The authors' argument relies on a single source—a patent application that describes the use of a specific natamycin–cyclodextrin inclusion complex to overcome the low solubility of natamycin in water (ca. 50 mg/L). The source is not representative since such formulations are not used in practice, if only because they are not required to achieve the desired effect. One may conclude, as indeed the authors do, that within current regulatory approvals and known usage, the intake of natamycin is well below the ADI.

The ADI, the acceptable daily intake without adverse effects on humans over a lifetime exposure, does not specifically relate to the intestinal flora. The authors state that *Candida albicans* may be killed by as little as 3 ppm and that resistance development has been observed when natamycin was given orally for the treatment of candidosis [2]. However, this is in contrast to the conclusion drawn by the original authors: Gehring et al. [2] conclude that the treatment (using a high dosage of 4× 100 mg of natamycin per day) was ineffective in eliminating *Candida* and that no marked changes in minimum inhibitory concentrations (MICs) occurred. Moreover, this study was not a treatment of intestinal candidosis, but employed non-selected test subjects, of whom ca. 50% were positive for *Candida* in their stool samples.

This is followed by the statement that strains isolated from women with vaginal candidosis also showed decreased susceptibility following natamycin treatment, referenced to [3]. First, this study employed fresh clinical isolates, and there is no indication that these were from patients who had been treated with natamycin, or with any other drug. Second, the observation and the numbers ascribed to this reference cannot be found in the original paper—the paper merely shows that all *Trichomonas* isolates were insensitive to natamycin, whereas all but one *Candida* isolate were sensitive (mean MIC of 6.4 mg/L).

It is then stated that amphotericin resistance in *Candida* exists. We would like to remark that authors reviewing this subject usually state that clinical resistance is rare, especially in view of the long history of clinical use of amphotericin [4,5]. The authors' statement that polyene-insensitive *Candida* strains have acquired this due to polyene treatment is not supported by a referenced source.

In the end the authors conclude that the use of natamycin as a preservative in the production of semi-solid food such as yogurt is safe since the daily intake of natamycin is much lower than the ADI level. Their main concern is that special formulations, such as a cyclodextrin–natamycin inclusion complex designed to increase natamycin availability in aqueous systems, could alter the risk profile. Of course new technologies should be evaluated, already from the perspective of dose, and this is common practice in the food industry. In the context of current practice, we feel that the authors raise concern that is not warranted and not supported by their sources.

Funding: None.
Competing interests: None declared.
Ethical approval: Not required.

References

[1] Dalhoff AAH, Levy SB. Does use of the polyene natamycin as a food preservative jeopardise the clinical efficacy of amphotericin B? A word of concern. Int J Antimicrob Agents 2015;45:564–7.
[2] Gehring W, Späte W, Gehse M, Gloor M, Braun KJ. Results of a combination treatment with natamycin and butylscopolamine in cases of intestinal *Candida* colonization. Mycoses 1990;33:140–5.
[3] Lövgren T, Salmela I. In vitro sensitivity of *Trichomonas vaginalis* and *Candida albicans* to chemotherapeutic agents. Acta Pathol Microbiol Scand B 1978;86B:155–8.
[4] Gray KC, Palacios DS, Dailey I, Endo MM, Uno BE, Wilcock BC, et al. Amphotericin primarily kills yeast by simply binding ergosterol. Proc Natl Acad Sci U S A 2012;109:2234–9.
[5] Shapiro RS, Robbins N, Cowen LE. Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiol Mol Biol Rev 2011;75:213–67.

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Hugo Streekstra
André Keuter
Lonneke Wilms*
DSM Food Specialties B.V., Postal Point 600-250, Alexander Fleminglaan 1, 2613 AX Delft, The Netherlands

* Corresponding author. Tel.: +31 15 279 2209/+31 683 642 539.
E-mail address: Lonneke.Wilms@dsm.com (L. Wilms)

2 July 2015

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DSM answer on FDA question during evaluation of GRAS Notice No 578, 2015.
FDA had no further questions and provided DSM their final 'no question'-letter without any reference to Dalhoff et al.

FDA Question 1

During the course of our safety review we received an outside inquiry concerning the use of natamycin in beverages and possible development of resistance to polyenes in Candida species. The inquirer provided a published article (attached). Points of discussion on the use of natamycin in the article include pharmacology, dietary exposure, resistance development, and horizontal gene transfer. In addition, there are a number of open questions listed at the end. Because this publication may have an impact on the safety conclusion as well as the general recognition aspect of the intended use of natamycin, these questions need to be adequately addressed.
Comprehensive response to the concerns discussed in the article as indicated above.

DSM Response

Dalhoff et al. (2015) raise the concern that the use of natamycin as a food preservative could affect the clinical efficacy of amphotericin B. This concern is not supported by any published research that indicates that there is an actual problem. Instead, the authors resort to combining a limited set of selectively chosen literature sources, dealing with a wide range of only marginally related research topics, partly misrepresenting the data, ignoring the interpretations of the original authors, and constructing a hypothetical worst-case situation that ignores regulatory constraints. An official reaction by DSM submitted to the journal and a reply of the authors will be published as Letters to the Editor (both in press and attached).

Their review summarizes the evidence based on the probable use of a novel water-soluble natamycin formulations for the preservation of beverages as mentioned in a patent which may exert a resistance selective pressure on the gastrointestinal fungal flora, hypothetically jeopardizing the efficacy of polyenes as a class of life-saving antifungal human agents.

DSM showed in their submitted GRAS Notice on beverage applications (GRAS GRN 000578) that there is no limitation in efficacy because of the low water solubility. The natamycin level of 5 ppm was scientifically supported by efficacy studies and proved to be sufficient for the prevention of the growth of food spoiling yeasts and molds. The rationale for the maximum dosage of 5 ppm in the mentioned beverages is to provide sufficient preservative action until the end of shelf life.

Therefore, there is no necessity for a higher soluble natamycin formulation. Furthermore, in our opinion, the current set of toxicological data mentioned in both GRAS Notices (GRN 517 and GRN 578), and reviewed by JECFA in 2002 and EFSA in 2009 do not cover the safety of a natamycin–cyclodextrine complex. Consequently, a natamycin-cyclodextrine complex is not in the scope of the GRAS Notices submitted by DSM for the use in yogurt and in beverages.

However, DSM has chosen to respond via a letter to the editor (in press). A more elaborated discussion of the paper is given below.

The authors ignore the fact that the high dosage level of natamycin (500 mg/l) mentioned in the referenced patent exceed existing regulatory limits 10- 25 times, which will not be accepted by Food Safety Authorities.

EFSA (2009) confirmed that products formed in the stomach in acid conditions are likely to be similar to degradation products as described by Brik (1976). Approximately 50% natamycin is broken down in 1 hour

in simulated gastric juice, and losses from the stomach of 33-43% and 0-31% occurred in fasted and non-fasted rats respectively (Morgenstern et al., 1975).
Therefore the human gastrointestinal flora may be exposed to trace quantities of ingested natamycin residues. The intestinal microflora is predominated by bacterial species, whereas yeast and fungal species are only ca. 0.001% of the total flora. Several studies in experimental animals indicate that natamycin and any potential degradation products do not express antibiotic activity in the colon (JECFA, 2002; EFSA, 2009). Because natamycin is not absorbed from the intestines (Blankwater et al., 1979), it has no importance as a systemic antifungal agent.

The authors refer twice to the article of Brik (1981) for the degradation of natamycin in an acidic medium. The second time it is used as evidence that degradation products of natamycin retained still some activity. However, this is a misinterpretation of table 10 on page 548 of the referenced article. The values of the bio-assay column are related to the percentage of natamycin left in the acidic medium and has no relation whatsoever with the activity of degradation products.

Furthermore, Koontz et al. (2003) concluded that the antifungal activity of the cyclodextrin complexes appears to be nearly equivalent to that of natamycin, which rejects fully the theoretical assumption of the authors that an increased solubility is followed by increased antifungal activity.
Dalhoff et al refer to Koontz et al (2003) for acidic degradation products and their activity, however, these are not mentioned by Koontz in this paper.

The ADI, the acceptable daily intake without adverse effects on humans over a lifetime exposure, does not specifically relate to the intestinal flora. The authors state that *Candida albicans* may be killed by as little as 3 ppm and that resistance development has been observed when natamycin was given orally for the treatment of candidosis (Gehring et al., 1990). However, this is in contrast to the conclusion drawn by the original authors: Gehring et al. (1990) conclude that the treatment (using a high dosage of 4× 100 mg of natamycin per day) was ineffective in eliminating *Candida* and that no marked changes in minimum inhibitory concentrations (MICs) occurred. Moreover, this study was not a treatment of intestinal of intestinal candidosis, but employed non-selected test subjects, of whom ca. 50% were positive for Candida in their stool samples.

This is followed by the statement that strains isolated from women with vaginal candidosis also showed decreased susceptibility following natamycin treatment, referenced to Lövgren et al., 1978. First, this study employed fresh clinical isolates, and there is no indication that these were from patients who had been treated with natamycin, or with any other drug. Second, the observation and the numbers ascribed to this reference cannot be found in the original paper—the paper merely shows that all *Trichomonas* isolates were insensitive to natamycin, whereas all but one *Candida* isolate were sensitive (mean MIC of 6.4 mg/L).

<u>Horizontal gene transfer (HGT) creating a threat to human health</u>
There has been no example with polyene resistance being coupled to a single gene – the authors themselves discuss that polyene insensitivity is associated with lowered ergosterol levels, and this is very well documented (see lit TNO review and additional literature search). Lowered ergosterol levels are not caused by HGT. HGT in fungi is acknowledged in recent literature but still natamycin resistance or cross-resistance is rare.

<u>Resistance development of the resident flora to polyenes</u>
An additional literature search covering the period 2012 till 2015 was executed by DSM to extend the search executed by TNO in 2012. This TNO report was already referenced in the GRAS Notice 000517 related to yogurt application and the current GRAS Notice 000578 related to beverage application.

Conclusions

- Special formulations, such as a cyclodextrin–natamycin inclusion complex designed to increase natamycin availability in aqueous systems are not part of the scope of GRAS Notification GRN 000578;

- These special formulations could alter the risk profile. Therefore, new technologies should be evaluated from the perspective of dose and safety, and this is common practice in the food industry;

- The human gastrointestinal flora may be exposed to trace quantities of ingested natamycin residues;

- Natamycin and any potential degradation products do not express antibiotic activity in the colon;

- Historically, resistance is rare, despite of decades of use of Amphotericin B in the clinic, and natamycin in food applications;

- The practical use of polyene antifungals has not led to the build-up of resistant populations.

Literature

Brik H., Natamycin, In: Florey K., editor, Analytical profiles of drug substances, vol. 10, New York, NY: Academic Press, Inc; 1981, p. 513-61;

Dalhoff A.A.H., Levy S.B. Does use of the polyene natamycin as a food preservative jeopardise the clinical efficacy of amphotericin B? A word of concern. Int. J. Antimicrob. Agents, 2015; 45: p. 564–7;

EFSA, Scientific Opinion on the use of natamycin (E 235) as a food additive, 2009

Gehring W., Späte W., Gehse M., Gloor M., Braun K.J., Results of a combination treatment with natamycin and butylscopolamine in cases of intestinal *Candida* colonization. Mycoses 1990; 33: p. 140–5;

JECFA, Natamycin. Evaluation of certain food additives, Report of the Joint FAO/WHO Expert Committee on Food Additives (57th meeting), 2002, WHO Food Additives Series 48, p. 49-76;

Koontz J.L., Marcy J.E., Formation of natamycin: cyclodextrin inclusion complexes and their characterization. J. Agric. Food Chem., 2003; 51: p. 7106–10;

Lövgren T., Salmela I., In vitro sensitivity of *Trichomonas vaginalis* and *Candida albicans* to chemotherapeutic agents. Acta Pathol Microbiol Scand B,1978; 86B: p. 155–8;

Morgenstern A.P., Muskens G.J.A.M., Further data on the toxicity of the decomposition products of pimaricin, 1975, Unpublished report submitted to WHO by DSM (formerly Gist-brocades NV) Delft; (as reviewed and cited by EFSA 2009)

APPENDIX – REFERENCES

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Review

Does use of the polyene natamycin as a food preservative jeopardise the clinical efficacy of amphotericin B? A word of concern

Axel A.H. Dalhoff[a,*], Stuart B. Levy[b]

[a] University Medical Center Schleswig-Holstein, Institute for Infection Medicine, Brunswiker Str. 4, D-24105 Kiel, Germany
[b] Center for Adaptation Genetics and Drug Resistance, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA

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ABSTRACT

Natamycin is a poorly soluble, polyene macrolide antifungal agent used in the food industry for the surface treatment of cheese and sausages. This use is not of safety concern. However, highly soluble natamycin–cyclodextrin inclusion complexes have been developed for the protection of beverages. This practice leads to high drug exposures exceeding the safety level. Apart from the definition of an acceptable daily dietary exposure to natamycin, its effect on the faecal flora as a reservoir for resistance has to be examined. Consumption of food to which natamycin has been added and mixed homogeneously, such as yoghurt, and in particular the addition of cyclodextrin inclusion complexes to beverages and wine generates high faecal natamycin concentrations resulting in high drug exposures of faecal *Candida* spp. Development of natamycin resistance has been observed in *Candida* spp. colonising the intestinal tract of patients following natamycin treatment of fungal infections. Horizontal gene transfer among different *Candida* spp. and within *Aspergillus fumigatus* spreads resistance. Therefore, it cannot be denied that use of natamycin for preservation of yoghurt and beverages may foster development of resistance to polyenes in *Candida* spp.

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1. Introduction

Natamycin is a member of the polyene macrolide class of anti-infectives (in the following 'polyene'). Natamycin shows in vitro activity against yeasts and filamentous fungi such as *Candida* spp., *Aspergillus* spp., *Cephalosporium* spp., *Fusarium* spp. and *Penicillium* spp. but is inactive against Gram-positive and Gram-negative aerobic and anaerobic bacteria [1]. In addition, it is useful in the treatment of human disease and in the protection of foods.

Natamycin is given to humans for the topical treatment of fungal eye, mouth, skin and vaginal infections. Other polyene antifungals applied in human medicine are amphotericin B and nystatin. Whilst nystatin is useful for the prevention or treatment of minor fungal infections such as oropharyngeal or vaginal *Candida* infections, amphotericin B is used to treat serious life-threatening fungal infections.

The food industry employs natamycin for the preservation of cheese, sausages, yoghurt and, in some countries, juices and wine. Natamycin is preferable to many other preservatives as it is free from odour and colour so that it causes no taste aversion and therefore does not adversely affect consumer acceptance. It resists dissolving in water so it is not easily removed by washing, thus maintaining its activity as a preservative. Natamycin is extremely sensitive to ultraviolet (UV) light [2]. Cheese products are exposed to light in the retail dairy industry, thus natamycin treatment on the products is likely degraded by the time of purchase by the consumer [3]. Even if the surface of these products may not be removed or if natamycin may not be inactivated by UV light, the estimated dietary exposure to natamycin is ten times lower than the acceptable daily intake (ADI) level defined by the Joint Food and Agriculture Organization of the United Nations and World Health Organization Expert Committee on Food Additives (JECFA). Therefore, natamycin for the surface treatment of cheese and sausages can be regarded as safe for human use [4].

However, recent concern is being raised regarding the use of natamycin as an additive to beverages and yoghurt, as gastrointestinal *Candida* spp. may be exposed to high drug concentrations, hypothetically exerting a resistance selective pressure. The risks associated with the use of anti-infectives by the food industry are discussed controversially, but antibiotic use in veterinary medicine and for growth promotion and disease prevention in agriculture, aquaculture and horticulture is also a major contributing factor to resistance development [5].

* Corresponding author. Tel.: +49 202 265 5236; fax: +49 202 265 5297.
E-mail address: adalhoff@t-online.de (A.A.H. Dalhoff).

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0924-8579/© 2015 The Authors. Published by Elsevier B.V. on behalf of International Society of Chemotherapy. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Addition of natamycin to food is different from the use of anti-infectives in veterinary medicine. In the latter instance, antibiotic resistance genes of microbial origin may be transferred from treated animals to human beings via the food chain and/or consumption of contaminated food, whilst the use of natamycin as a food additive may expose the human intestinal flora directly to the selective pressure. This review summarises the evidence that preservation of yoghurt with natamycin and the probable use of novel water-soluble natamycin formulations for the preservation of beverages may exert a resistance selective pressure on the gastrointestinal fungal flora, hypothetically jeopardising the efficacy of polyenes as a class of life-saving antifungal human agents.

2. Pharmacology of natamycin

Apart from its low water solubility and instability to UV light, natamycin decomposes at the extremes of pH. Degradation of natamycin in an acidic medium, as occurs in beverage products, yields mycosamine and three degradation products all containing an intact lactone ring; the degradation products are considered to be inactive [6,7]. In principle, these characteristics may be advantageous for the use of natamycin in the production of solid food. However, the limited water solubility of natamycin is doubly disadvantageous; first, the dissolved fraction only diffuses to the site of action, so that low solubility is a limiting factor in the antifungal activity of natamycin [2]; and second, natamycin is not sufficiently soluble in beverages to function as a beverage preservative in and of itself [8]. The highest achievable natamycin concentration in beverages is 52 mg/L, a concentration four-fold less than the amount needed to prohibit outgrowth of fungi for a period of 16 weeks, the limit of product shelf-life [8]. Therefore, natamycin has been formulated as a cyclodextrin inclusion complex. Cyclodextrins act as host molecules to form inclusion complexes with guest molecules. The cyclodextrin molecule can at least partially shield the guest molecule from degradation caused by light, oxidation, heat, and acidic or alkaline conditions [9]. Cyclodextrins not only insulate labile compounds from a potentially corrosive environment, they also increase solubility of poorly soluble agents [8,10]. Inclusion of natamycin into cyclodextrin permits the preparation of a complex containing 500 mg/L of solubilised natamycin and protects natamycin from inactivation in an acidic environment as prevailing in beverages. Consequently, the natamycin–cyclodextrin complex remained stable for 16 weeks of storage in the dark [8,10].

It is important to note that inclusion of natamycin into cyclodextrin left the antifungally active moiety of the natamycin molecule, i.e. the mycosamine group, intact and freely accessible. Ergosterol binding via the mycosamine group represents the first and decisive reaction required for antifungal action; the lactone ring of polyenes inserts into the lipid bilayer of fungi [1,11,12]. In addition to ergosterol binding, natamycin impairs membrane fusion via perturbation of ergosterol-dependent priming reactions that precede membrane fusion.

Considering the increased solubility of natamycin–cyclodextrin complexes and chemical structures being relevant for its mode of action, theoretically increased solubility is followed by increased antifungal activity, and decomposition to mycosamine- and/or lactone ring-containing products should yield degradation products still being antifungally active. Both theories have been confirmed. Natamycin–cyclodextrin complexes are approximately twice as active as natamycin itself and degradation products have been characterised that have retained 75%, 46% and 14% of the activity of the intact molecule [6,10].

3. Dietary exposure to natamycin following consumption of natamycin-containing beverages or yoghurt

Given that natamycin is 'very poorly absorbed' from the gastrointestinal tract, and based on several toxicological studies in animals and a clinical study performed in humans, JECFA established in 1968 an acceptable daily intake (ADI) of 0.3 mg/kg body weight per day (i.e. 18 mg per adult weighing 60 kg), which was confirmed in 2002 by JECFA [4,13]. It is essential to indicate that the classification of natamycin as being 'very poorly absorbed' is based on a review article published by Brik [6], who referred to a study performed by Lynch et al. [14] published in 1961. Natamycin serum concentrations were quantitated biologically. Eight patients were dosed with 125–500 mg of natamycin per day. Eleven samples were withdrawn at non-specified time points. The frequency of dosing and allocation of patients to dose groups was not specified. Serum concentrations below the limit of detectability of 3.8 mg/L were recorded under these experimental conditions. The use of biological methods for drug quantitation was adequate and standard 50 years ago, but the design and analytical methods of the study are suboptimal based on present capabilities.

It is also important to note that the JECFA decision is based on studies that have been performed with the almost insoluble natamycin and not with the soluble natamycin–cyclodextrin inclusion complex. Data on the pharmacokinetics and toxicology of natamycin–cyclodextrin inclusion complexes have not yet been published. The increased solubility of cyclodextrin inclusion complexes of a drug can increase its dissolution rate, so that increased oral bioavailability is achieved for agents that are otherwise non-absorbable [15]. For example, the bioavailability of itraconazole and its metabolite hydroxyl-itraconazole could be enhanced by 30–37% following oral administration of cyclodextrin inclusion complexes compared with the conventional capsule [16]. Thus, natamycin could hypothetically be absorbed from the gastrointestinal tract following oral administration of a soluble natamycin–cyclodextrin inclusion complex; absorbed natamycin could hypothetically expose organ systems relevant for toxicological examinations to higher drug concentrations than studied previously. Even if this scenario is speculative, the hypothesis merits further study.

The solubility of natamycin in apple juice is 20 mg/L at 25 °C and pH 3.4 and 10 mg/L at 4 °C and pH 3.4 [8]. Consumption of 1 L of apple juice containing either 10 mg or 20 mg natamycin by an adult human consumer weighing 60 kg results in a dietary exposure corresponding to 50% and 111% of the ADI level. However, a use level of 8.33 mg/kg body weight/day would result from the consumption of the maximally soluble fraction of natamycin–cyclodextrin inclusion complex of 500 mg/L, so that the ADI level will be exceeded 27.8-fold.

Yoghurt products containing 5–10 mg natamycin/kg are commercially available in South Africa, Canada and China and may be available in the USA in due course. Consumption of such preparations may result in an estimated 2-day daily intake of natamycin from background and proposed uses for the total US population of 0.61–1.22 mg/day [17], which is clearly below the ADI level.

But more importantly, faecal *Candida* spp. will be exposed to relatively high natamycin concentrations, which may hypothetically trigger the development of polyene resistance. Assuming first a daily production of faeces of 150 g by an adult Caucasian, and assuming second that the entire amount of natamycin consumed with beverages or yoghurt is deposited in the faeces, faecal concentrations of natamycin may range from 3.33 mg/kg following consumption of 100 g of yoghurt containing 5 ppm to 3333.0 mg/kg following consumption of 1 L of beverage containing the maximally soluble concentration of 500 mg of natamycin. Assuming third that natamycin is not bound to faecal matter and is thus antifungally

active, even the lowest faecal concentration of natamycin exceeds the minimum inhibitory concentrations (MICs) for *Candida albicans* and *Saccharomyces cerevisiae* ranging from 1.1 mg/L to 2.6 mg/L [6,10], so that faecal natamycin concentrations may exert a selective pressure on the resident flora. Thus, there is concern that natamycin may have a propensity for drug resistance selection.

4. Natamycin resistance development in environmental fungi

Natamycin has been used for almost three decades for the preservation of cheese and sausages. Surveys in cheese warehouses and in dry sausage factories where natamycin had been used for up to 9 years showed no change in the composition or sensitivity of the contaminating fungal flora [18–21].

In vitro exposure of strains isolated from cheese warehouses to increasing natamycin concentrations revealed that after 25–30 transfers none of the strains had become less sensitive to natamycin [18].

Based on these data [18–21] and the fact that natamycin is not active against bacteria, the European Food Safety Authority (EFSA) panel concluded that there was no concern for the induction of antimicrobial resistance. However, the panel did not assess the effect of natamycin on the resident human flora.

5. Resistance development of the resident flora to polyenes

The human resident microflora acts as a barrier against colonisation by potentially pathogenic micro-organisms [22–27]. Emergence of resistance among the resident microflora and distribution of resistance genes by transfer of DNA in the microbial community can contribute to an increased load of resistant, potentially pathogenic micro-organisms. Another effect of a disturbed normal microflora is a reduction in colonisation resistance, leading to overgrowth of already present or exogenous micro-organisms [22–27].

Since *C. albicans* lacks any apparent environmental reservoir, it generally grows in association with a mammalian host, where it is a very effective coloniser. *Candida albicans* is commonly found as a component of the normal flora of humans, residing in the gastrointestinal and genitourinary tracts and on the skin [28–30]. Colonising organisms are thought to be benign, but they may cause life-threatening infections in an immunocompromised host such as the elderly, transplant recipients, or patients with cancer or human immunodeficiency virus (HIV). Resistance development is of particular concern in these patients. Only agents of the polyene class remained effective as fungal resistance to azoles, candins and 5-fluorocytosine and multidrug resistance increased worldwide, so that the authors of a recent review article conclude that 'the rapid development of antifungal resistance, the toxicity and the variability in available formulations of some agents, and the increase in the frequency of non-albicans *Candida* spp. infections support the need for more effective and less toxic treatment strategies' [31]. This statement underscores the high clinical relevance to maintain the efficacy of the polyenes.

It is therefore important to analyse the effect of any antimicrobial agent on the resident flora. In the context of the use of natamycin as a food antimicrobial, it has to be determined whether polyenes in general or natamycin in particular may deteriorate the susceptibility pattern of colonising *Candida* spp. Development of resistance to natamycin has been observed. Natamycin has been given orally for the treatment of intestinal candidosis at a daily dose of 400 mg for 10 days to 356 patients [32]. The drug susceptibility pattern of *Candida* spp. isolated before, during and after therapy with natamycin changed significantly. Strains with an MIC of 1.25 mg/L were isolated from 56% of these patients prior to, 33% during, 51% at 5 days after and 60% at 3 months after therapy [32]. A significant reduction in susceptibility to natamycin was observed during exposure to this agent; the susceptibility pattern returned to baseline levels during the post-exposure phase. Thus, the loss of susceptibility of *Candida* spp. is a drug exposure-related effect. Strains isolated from women with vaginal candidosis also showed decreased susceptibility following natamycin treatment. The MICs of natamycin for *Candida* spp. increased from 2.9–31 mg/L for strains isolated from untreated women to 9.8–64 mg/L for strains from treated women [33].

Amphotericin resistance has been detected in *Candida* spp. causing invasive diseases [34–36]. A recent analysis of *Aspergillus* spp. isolated from patients with haematological malignancies revealed that 25% of the strains studied were characterised by MICs above the epidemiological cut-off value of 4 mg/L. Thus, the findings strongly suggest that these isolates have acquired resistance to amphotericin B [37].

These reports demonstrate that, although rare, polyene resistance amongst human clinical isolates of pathogenic *Candida* spp. as well as amongst *Candida* spp. colonising the human intestinal tract have acquired polyene resistance. Loss of natamycin susceptibility upon oral administration of natamycin is of particular concern as this finding demonstrates that short-term exposure of *Candida* spp. residing in the gut can cause resistance development.

6. Horizontal gene transfer (HGT) creating a threat to human health

HGT is defined as the exchange of genes either within one species or between different species. HGT is a major force in microbial evolution and a great source of genetic innovation in prokaryotes [38]. In comparison with prokaryotes, HGT is thought to occur much less frequently in eukaryotes. The phenomenon of HGT in fungi is exciting in so far as fungi are the most recalcitrant of all micro-organisms to transfer genes, possessing robust cell walls and having lost phagotrophic capacities. None the less, interfungal and intrafungal species HGT has been described. HGT has also proved to be a factor in the evolution of eukaryotic genomes, enabling fungi to colonise additional environments or to spread resistance (reviewed in [39]). Thus, HGT among different *Candida* spp. and within *Aspergillus fumigatus* has contributed to pathoadaptive responses such as biofilm formation and also to the spread of polyene and azole resistance. Consequently, it cannot be excluded that consumption of natamycin-containing beverages or yoghurt may in theory prime intestinal yeasts to acquire polyene resistance. This resistance may spread horizontally and vertically among colonising *Candida* spp. and may disseminate to infectious sites, putting the patient, in particular the immunocompromised patient, at risk.

7. Open questions

Natamycin was discovered and developed more than 50 years ago. Studies were performed to characterise the toxicological and pharmacokinetic profile and mirror the methods applied and the study designs used in those times. Furthermore, natamycin has been developed for surface treatment of solid food and topical administration in human medicine. Consequently, a broadened use of natamycin in food production, such as addition to beverages or yoghurt, and the development of a new soluble formulation has never been considered before. In contrast to the use of natamycin for surface treatment of solid food, its use in liquid and semi-solid food inevitably exposes the faecal flora to natamycin. This effect raises concerns that need to be addressed.

- Assessment of the pharmacokinetics of natamycin and in particular its cyclodextrin inclusion complex.
- Quantitation of total and free faecal natamycin concentrations at different times following drug ingestion.
- Effect of natamycin and its cyclodextrin inclusion complex on the resident human microflora for relevant periods of time.
- Monitor polyene resistance development among the human resident fungal flora.
- Re-assessment of the antifungal activity of natamycin degradation products.

8. Conclusions

Use of natamycin by the food industry for the surface treatment of solid food is considered to be safe. If at all measurable, natamycin concentrations are much lower than the ADI level. Likewise, use of natamycin as a preservative in the production of semi-solid food such as yoghurt is safe in so far as the daily intake of natamycin is much lower than the ADI level. However, natamycin concentrations in the faeces may exert a polyene resistance selective pressure, so that faecal *Candida* spp. may harbour and spread polyene resistance, hypothetically putting elderly and immunocompromised patients at risk. This risk will be enhanced by the consumption of soluble natamycin–cyclodextrin inclusion complex-containing beverages or wine as these products contain high natamycin concentrations with higher potential to exert probably a strong selective pressure on the emergence of resistance in faecal *Candida* spp. Importantly, the risks associated with the use of natamycin in semi-solid food or the use of natamycin–cyclodextrin inclusion complexes cannot be assessed until questions regarding broader issues, such as the questions raised above, are addressed.

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Competing interests

None declared.

Ethical approval

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References

[1] Raab WP. Natamycin (Pimaricin): its properties and possibilities in medicine. Stuttgart, Germany: Georg Thieme; 1972.
[2] Stark J. Permitted preservatives—natamycin. In: Robinson RK, Batt CA, Patel PD, editors. Encyclopedia of food microbiology, vol. 3. San Diego, CA: Academic Press; 2000. p. 1776–81.
[3] Thoma K, Kübler N. New results in the photostability of antimycotics. In: Albini A, Fasani E, editors. Drugs: photochemistry and photostability. Cambridge, UK: The Royal Society of Chemistry; 1998. p. 116–33.
[4] JECFA. Specifications for the identity and purity of food additives and their toxicological evaluations: some antibiotics. In: Twelfth Report of the Joint FAO/WHO Expert Committee on Food Additives, July 1–8, 1968. Geneva, Switzerland: JECFA; 1968. WHO Technical Report Series No. 430/FAO Nutrition Meetings Report Series No. 45.
[5] Laxminarayan R, Duse A, Wattal C, Zaidi AK, Wertheim HF, Sumpradit N, et al. Antibiotic resistance—the need for global solutions. Lancet Infect Dis 2013;13:1057–98.
[6] Brik H. Natamycin. In: Florey K, editor. Analytical profiles of drug substances, vol. 10. New York, NY: Academic Press, Inc; 1981. p. 513–61.
[7] Brik H. New high-molecular decomposition products of natamycin (pimaricin) with intact lactone-ring. J Antibiot (Tokyo) 1976;29:632–7.
[8] Smith RT. Beverage preservative system containing pimaricin–cyclodextrin complex. United States patent no. 8,425,968 B2. Date of patent April 23, 2013.
[9] Loftsson T, Brewster ME. Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. J Pharm Sci 1996;85:1017–25.
[10] Koontz JL, Marcy JE. Formation of natamycin:cyclodextrin inclusion complexes and their characterization. J Agric Food Chem 2003;51:7106–10.
[11] te Welscher YM, ten Napel HH, Balagué MM, Souza CM, Riezman H, de Kruijff B, et al. Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. J Biol Chem 2008;283:6393–401.
[12] te Welscher YM, Jones L, van Leeuwen MR, Dijksterhius J, de Kruijff B, Eitzen G, et al. Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol. Antimicrob Agents Chemother 2010;54:2618–25.
[13] JECFA. Natamycin evaluation of certain food additives. Report of the joint FAO/WHO expert committee on food additives (57th meeting), vol. 48. JECFA; 2002. p. 49–76. WHO Food Additives Series 2002.
[14] Lynch HJ, Furcolow ML, Yates JL, Tosh FE, Larsh HW. Toxicity and absorption studies in humans of newer antifungal agents. Antimicrob Agents Annu 1961:551–7.
[15] Brewster ME, Loftsson T. Cyclodextrins as pharmaceutical solubilizers. Adv Drug Delivery Rev 2007;59:645–66.
[16] Barone JA, Moskovitz BL, Guarnieri J, Hassell AE, Colaizzi JL, Birman RH, et al. Enhanced bioavailability of itraconazole in hydroxypropyl-β-cyclodextrin solution versus capsules in healthy volunteers. Antimicrob Agents Chemother 1998;42:1862–5.
[17] Office of Food Additive Safety (HFS-200), Center for Food Safety and Applied Nutrition (CFSAN), Food and Drug Administration, by DSM Food Specialties B.V. GRAS notification for the use of natamycin in yogurt. Submitted to: Office of Food Additive Safety (HFS-200), Center for Food Safety and Applied Nutrition (CFSAN), Food and Drug Administration, by DSM Food Specialties B.V.; 2015. ⟨http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-foods-gen/documents/document/ucm404947.pdf⟩ (accessed 12 March 2015).
[18] de Boer E, Stolk-Horsthuis M. Sensitivity to natamycin (pimaricin) of fungi isolated in cheese warehouses. J Food Prot 1977;40:533–6.
[19] de Boer E, Labots H, Stolk-Horsthuis M, Visser JN. Sensitivity to natamycin of fungi in factories producing dry sausage. Fleischwirtschaft 1979;59:1868–9.
[20] Hoekstra ES, Van der Horst MI. Survey of the fungal flora in Dutch cheese factories and warehouses. J Food Mycol 1998;1:13–22.
[21] Food preservation with chemicals.Jay JM, editor. Modern food microbiology. 5th ed. New York, NY: Chapman & Hall; 1996. p. 273–303.
[22] Sullivan A, Edlund C, Nord CE. Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect Dis 2001;1:101–14.
[23] Simon GL, Gorbach SL. The human intestinal microflora. Dig Dis Sci 1986;31(9 Suppl):147S–62S.
[24] Savage DC. Microbial biota of the human intestine: a tribute to some pioneering scientists. Curr Issues Intest Microbiol 2001;2:1–15.
[25] Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science 2005;308:1635–8.
[26] Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 2006;124:837–48.
[27] Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. Nat Rev Genet 2012;13:260–70.
[28] Odds FC. Candida infections: an overview. Crit Rev Microbiol 1987;15:1–5.
[29] Soll DR, Galask R, Schmid J, Hanna C, Mac K, Morrow B. Genetic dissimilarity of commensal strains of *Candida* spp. carried in different anatomical locations of the same healthy women. J Clin Microbiol 1991;29:1702–10.
[30] Rosenbach A, Dignard D, Pierce JV, Whiteway M, Kumamoto CA. Adaptations of *Candida albicans* for growth in the mammalian intestinal tract. Eukaryot Cell 2010;9:1075–86.
[31] Spampinato C, Leonardi D. Candida infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. Biomed Res Int 2013;2013:204237.
[32] Gehring W, Späte W, Gehse M, Gloor M, Braun KJ. Results of a combination treatment with natamycin and butylscopolamine in cases of intestinal *Candida* colonization. Mycoses 1990;33:140–5.
[33] Lövgren T, Salmela I. In vitro sensitivity of *Trichomonas vaginalis* and *Candida albicans* to chemotherapeutic agents. Acta Pathol Microbiol Scand Sect B 1978;86B:155–8.
[34] Gheith S, Saghrouni F, Bannour W, Youssef YB, Khelif A, Normand AC, et al. In vitro susceptibility to amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin of *Aspergillus* spp. isolated from patients with haematological malignancies in Tunisia. Springer Plus 2014;3:19.
[35] Rodloff C, Koch D, Schaumann R. Epidemiology and antifungal resistance in invasive candidiasis. Eur J Med Res 2011;16:187–95.
[36] Pahwa N, Kumar R, Nirkhiwale S, Bandi A. Species distribution and drug susceptibility of *Candida* in clinical isolates from a tertiary care centre at Indore. Indian J Med Microbiol 2014;32:44–8.
[37] de Cássia Orlandi Sardi J, de Souza Pitangui N, Gullo FP; Maria José Soares Mendes Giannini AMFA. A mini review of *Candida* species in hospital infection: epidemiology, virulence factor and drugs resistance and prophylaxis. Trop Med Surg 2013;1:141.
[38] Francino MP, editor. Horizontal gene transfer in microorganisms. Wymondham, UK: Caister Academic Press; 2012.
[39] Anderson JB. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. Nat Rev Microbiol 2005;3:547–56.

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Letter to the Editor

Response to the reaction to Dalhoff and Levy: 'Does use of the polyene natamycin as a food preservative jeopardise the clinical efficacy of amphotericin B? A word of concern'

Sir,

The reasons to express our views on the use of natamycin by the food-producing industry were two-fold: first, maintenance of polyene efficacy in an environment of rapid development of antifungal resistance; and second, the risk assessment and the pharmacokinetic/pharmacodynamic characterisation of natamycin is based on studies that mirror the methodology used in previous years, leaving many questions associated with new formulations and a broadened use of natamycin unanswered. Whilst the second aspect has not been disputed by the authors of the comment, they doubt whether our concern is well-based and, in particular, whether natamycin may exert a pharmacological effect on the resident flora.

It is generally accepted that disturbance of the human resident flora is a major factor contributing to the development of resistance. Therefore, we addressed the question of whether the use of natamycin in either beverages or yoghurt may interfere with the gastrointestinal flora and may bear the potential to exert a selective pressure. Our considerations are based on a maximum/minimum scenario. The maximum concentration could not only hypothetically be used in beverages but is needed to prohibit outgrowth of fungi for a period of 16 weeks, the limit of product shelf-life [1].

Even within current regulatory approvals and known usage of natamycin being well below the ADI (acceptable daily intake) level, the minimum faecal natamycin concentration may exceed its minimum inhibitory concentrations (MICs) for *Candida* spp. Exposure of humans to natamycin may indeed affect the susceptibility pattern of faecal *Candida* spp.; it is correct that Gehring et al. [2] concluded that 'no induction of resistance against natamycin worth mentioning takes place'. But our argument was that during short-term exposure of intestinal *Candida* spp. their susceptibility deteriorated compared with pre-exposure isolates, while it improved again during the post-exposure phase, demonstrating a direct and mono-causal drug effect. Furthermore, it does not matter whether natamycin has been administered to patients for treatment of a fungal disease or to healthy volunteers to assess the impact on the resident flora, it only matters that the intestinal flora has been exposed to natamycin.

The second study we quoted in support of this finding [3] is an in vitro study comparing the susceptibility pattern of *Candida albicans* (and also *Trichomonas vaginalis*) isolated from vaginitis patients with previously reported values. A natamycin-producing company also referred in their GRAS notification on the use of natamycin in yoghurt [4] to this study and interpreted the data in exactly the same way as we did: '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 (3). 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' [4]. This second study confirms that the natamycin susceptibility of *Candida* spp. decreased during and because of exposure to natamycin.

Finally, the colleagues criticise our statement that amphotericin B resistance exists and that this statement is not supported by referenced sources. However, we quoted four recent publications in support of this statement and could have quoted more if space would have allowed to do so.

Therefore, we are convinced that natamycin has to be used cautiously by the food industry and that exposure of the human resident flora should be minimised extensively in order to maintain the life-saving potential of polyenes.

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Competing interests

None declared.

Ethical approval

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References

[1] Smith RT. Beverage preservative system containing pimaricin–cyclodextrin complex. United States Patent No. 8,425,968 B2. Date of patent April 23, 2013.
[2] Gehring W, Späte W, Gehse M, Gloor M, Braun KJ. Results of a combination treatment with natamycin and butylscopolamine in cases of intestinal *Candida* colonization. Mycoses 1990;33:140–5.
[3] Lövgren T, Salmela I. In vitro sensitivity of *Trichomonas vaginalis* and *Candida albicans* to chemotherapeutic agents. Acta Pathol Microbiol Scand B 1978;86B:155–8.
[4] DSM Food Specialities B.V. GRAS notification use of natamycin in yoghurt. Submitted on May 6, 2014 to: Office of Food Additive Safety (HFS-200), Center for Food Safety and Applied Nutrition (CFSAN), Food and Drug Administration GRAS Notice (GRN) No. 517. http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm [accessed 04.08.15].

Axel Dalhoff*
University Medical Center Schleswig-Holstein, Institute for Infection Medicine, Brunswiker Str. 4, D-24105 Kiel, Germany

* Tel.: +49 202 2655236; fax: +49 202 2655297.
E-mail address: adalhoff@t-online.de

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SCIENTIFIC OPINION

Scientific Opinion on the use of natamycin (E 235) as a food additive[1]

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Following a request from the European Commission to EFSA, the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to provide a scientific opinion on the safety in use of natamycin (E 235) as a food additive, and on the issue of antimicrobial resistance to natamycin. Natamycin is a fungicide of the polyene macrolide group. According to Directive 95/2/EC, natamycin may be used for the surface treatment of semi-hard and semi-soft cheese and dry, cured sausage at a maximum level of 1 mg/dm^2 in the outer 5 mm of the surface. The SCF in 1979 considered that the database was adequate to conclude that natamycin does not give rise to safety concern, but inadequate to establish an ADI. JECFA assigned an ADI of 0.3 mg/kg bw/day (1968, 1976, 2002). The Panel considered that the available data are not sufficiently robust for the purpose of deriving an ADI because of the limitations of the present database on natamycin (design of the animal studies, limited number of animals, lack of a carcinogenicity study) and in view of the inadequate description of the human data. The highest potential exposure to natamycin was below 0.1 mg/kg bw/day for children at the 97.5th percentile. Given that natamycin is very poorly absorbed, the Panel considers that this conservative estimate would provide an adequate margin of safety from the effect level seen from the long-term animal studies and the human study used by JECFA to establish an ADI. The Panel considered that the proposed use levels of natamycin are not of safety concern if it is only used for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages. The Panel concluded that there was no concern for the induction of antimicrobial resistance.

KEY WORDS

Natamycin, pimaricin, antibiotics, E 235, CAS 7681-93-8, antibiotic resistance.

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1 On request from the European Commission, Question No EFSA-Q-2006-009, adopted on 26 November 2009.
2 Panel members: F. Aguilar, U.R. Charrondiere, B. Dusemund, P. Galtier, J. Gilbert, D.M. Gott, S. Grilli, R. Guertler, J. Koenig, C. Lambré, J-C. Larsen, J-C. Leblanc, A. Mortensen, D. Parent-Massin, I. Pratt, I.M.C.M. Rietjens, I. Stankovic, P. Tobback, T. Verguieva, R.A. Woutersen. Correspondence: unit-ANS@efsa.europa.eu
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SUMMARY

Following a request from the European Commission to the European Food Safety Authority, the Scientific Panel on Food Additives and Nutrient Sources added to Food (ANS) was asked to provide a scientific opinion on the safety in use of natamycin (E 235) as a food additive. In addition, EFSA should address the issue of antimicrobial resistance to natamycin.

Natamycin (pimaricin) is a fungicide of the polyene macrolide group. According to Directive 95/2/EC, natamycin may be used for the surface treatment of semi-hard and semi-soft cheese and dry, cured sausage at a maximum level of 1 mg/dm^2 in the outer 5 mm of the surface, corresponding to 20 mg/kg.

The Scientific Committee for Food (SCF) in 1979 did not establish an Acceptable Daily Intake (ADI) but considered that in relation to the uses of natamycin on cheese and sausages, the database was adequate and did not give rise to safety concern.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed the safety of pimaricin (natamycin) in 1968, 1976 and 2002 and assigned an ADI of 0.3 mg/kg body weight (bw)/day.

Information available on the metabolism of natamycin suggests that natamycin is not absorbed to a significant extent from the gastrointestinal tract and is rapidly excreted in faeces either unchanged or as degradation products.

In toxicological studies, the effects observed in animals were a decrease in food intake with a decrease in the rate of body weight gain, gastrointestinal irritation and diarrhoea. Dogs are the most sensitive species to these effects.

Three subchronic toxicity studies with natamycin are available, two in the rat and one in the dog. In the first study, no modifications of haematological and biochemical parameters and organ weights were noted. In the second rat study, decreases of mean food intake and mean body weight have been observed. The No-Observed-Adverse-Effect Level (NOAEL) is considered to be 45 mg/kg bw/day. In the third study, dogs were exposed for 3 months to natamycin. Transient diarrhoea and slight body weight loss have been observed. The NOAEL is considered to be 12 mg/kg bw/day.

Two long-term studies are available, a 2-year chronic toxicity study in the rat and a 2-year chronic toxicity study in the dog. In the rat study, decrease of food intake and reduced growth rate were seen only at the highest dose group. The data showed that the numbers and types of tumours were not significantly different in any of the natamycin-treated groups compared with the untreated control animals. The NOAEL of this study is considered to be 22.4 mg/kg bw/day. In the dog study, the highest dietary concentration induced obesity among the animals. Dietary levels of 6.25 mg/kg bw/day, or less did not affect body weight gain. The NOAEL of this study is considered to be 6.25 mg/kg bw/day.

Natamycin bears a structural alert for genotoxicity since the molecule contains an epoxide ring. However, in the light that:

- the induction of chromosomal aberrations observed in a recent study was accompanied by cytotoxicity,
- there are *in vitro* studies on mutagenicity in bacteria and mammalian cells and on chromosomal aberrations in mammalian cells which were performed in compliance with GLP and were negative,
- no substance-related neoplastic effects were observed in the long term studies,

the Panel considered that the available data do not raise concern with respect to genotoxicity of natamycin.

In a three-generation study of reproductive toxicity in the rat, at the highest dose, an increased number of fetuses born dead, and a decreased number of animals born alive surviving at 21 days in F1 generation, was described. The NOAEL of this study amounts to 50 mg/kg bw/day.

A developmental toxicity study has been performed in female rats from the second litter of the F1 generation of the three-generation reproductive toxicity study. 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. The NOAEL of this study amounts to 50 mg/kg bw/day. In a rabbit developmental study on mated female Dutch belted rabbits, the maternal mortality rates were 0, 5, 9 and 19% in the 4 treatment groups (0, 5, 15 or 50 mg/kg bw/day), respectively. A significant increase in extra sternebrae was noted in groups treated at 15 and 50 mg/kg bw/day, but was considered as normal variation by the Panel. The NOAEL of this study is considered to be 15 mg/kg bw/day due to maternal toxicity at the higher dose level.

A clinical study in humans performed in 1960 showed that natamycin, used for systemic mycoses, induced nausea, vomiting and diarrhoea. Anorexia, nausea, vomiting and flatulence were observed at different doses in different patients. The Panel considered that this study is too limited to derive a NOAEL.

In 1968, JECFA established an ADI of 0.3 mg/kg bw/day based on these human data. The level causing no toxicological effects in man was estimated to be 200 mg/per/day, equivalent to 3 mg/kg bw/day. Given that this dose was derived from human data, an uncertainty factor equal to 10 has been used to calculate the ADI. In 2002 JECFA confirmed this ADI.

Because of the limitations in the present database on natamycin (design of the animal studies, limited number of animals, lack of a carcinogenicity study) and in view of the inadequate description of the human data, the Panel considered that an ADI could not be established from these data.

The highest potential exposure to natamycin was at the 97.5th percentile below 0.1 mg/kg bw/day for children and below 0.05 mg/kg bw/day for adults, derived from the high level consumption of cheese (assuming solely a rind treatment with natamycin) and dried, cured sausages.

Given that natamycin is very poorly absorbed, the Panel considers that this conservative estimate would provide an adequate margin of safety from the effect level seen from the long-term studies in animals and the human study used by JECFA to establish an ADI. The Panel considered that the proposed use levels of natamycin are not of safety concern if it is only used for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages.

The Panel noted that natamycin is used in the food industry as an antifungal preservative in cheeses and sausages. Natamycin is a polyene antibiotic. The mechanism of action for polyene antibiotics is binding to sterols (principally ergosterol) in the fungal cell membrane. Bacteria are insensitive to polyene antibiotics because their membrane lacks sterols. Furthermore, induction of natamycin-resistant mutants in yeast is reported to be difficult.The Panel concluded that there was no concern for the induction of antimicrobial resistance.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

Natamycin is authorised for food preservation in the European Union by Directive 95/2/EC[4] on food additives other than colours and sweeteners. Natamycin is permitted for the surface treatment of hard, semi-hard and semi soft cheese and dried, cured sausages. Specific purity criteria for natamycin are laid down in Directive 2008/84/EC[5].

The Scientific Committee on Food (SCF) in 1979 evaluated the safety of natamycin and considered its use acceptable for the surface treatment of the rind of whole pressed cheese and for casings of certain sausages (SCF, 1979). At that time the SCF recommended that the residues of natamycin in food at the time of sale, expressed in terms of surface area of the casing or rind, should not exceed 1 mg/dm^2 and that they should not be present at a depth of greater than 5 mm in the food.

The Scientific Committee on Food adopted an opinion on antimicrobial resistance in 28 May 1999. On the basis of this opinion, the Commission adopted on 20 June 2001 a communication on a Community strategy against antimicrobial resistance. Action 9 listed in the Communication is to review the use of two antimicrobial agents in food.

The two substances mentioned are nisin (E 234) and natamycin (E 235).

Therefore, in addition to the toxicological review of natamycin, the issue of antimicrobial resistance should also be addressed.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1) (a) of Regulation (EC) N° 178/2002[6], the European Commission asks the European Food Safety Authority to provide a scientific opinion on the safety in use of natamycin. In addition, EFSA should address the issue of antimicrobial resistance to natamycin.

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[4] European Parliament and Council Directive 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners. OJ No L 61, 18. 3. 1995, p. 1.

[5] Commission Directive 2008/84/EC of 27 August laying down specific purity criteria on food additives other than colours and sweeteners

[6] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.

ASSESSMENT

1. Introduction

The present opinion deals with the safety of natamycin when used for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages requiring maturation before marketing.

2. Technical data

2.1. Identity of the substance

Natamycin (pimaricin) is a fungicide of the polyene macrolide group. It has a molecular mass of 665.725 g/mol. The CAS Registry Number of natamycin is 7681-93-8 and the molecular formula is $C_{33}H_{47}NO_{13}$. The primary structure of natamycin consists of a large lactone ring of 25 carbon atoms (Figure 1). The lactone ring is linked to a mycosamine moiety, m-amino-sugar, by a glycosidic linkage. Natamycin is classified as a polyene macrolide antibiotic and specifically as a tetraene antibiotic because of its four conjugated double bonds. The mycosamine moiety (3-amino-3,6-dideoxy-D- mannose) of natamycin at the C15 position is a six-membered pyranose ring. Natamycin forms a cylindrical structure due to the alignment of the hydroxyl groups of its amphipathic chain towards each other (Figure 1). The exterior of the cylinder is completely non-polar.

Figure 1: Natamycin

The solubility of natamycin is 20-50 mg/L in water. Natamycin is soluble in glacial acetic acid, methylpyrrolidone, dimethylformamide, dimethylsulfoxide, glycerol and propylene glycol. Natamycin is insoluble in higher alcohols, ethers, esters, aromatic or aliphatic hydrocarbons, chlorinated hydrocarbons, ketones, dioxane, cyclohexanol and various oils (Raab, 1972).

Mycosamine is a major product of hydrolysis of natamycin (Figure 2).

Figure 2: Mycosamine

The petitioner indicates that mycosamine and traces of 13-hydroxy-2,4,6,8,10-tetradeca pentaen-l-al (Figure 3) have been identified in pharmaceutical or industrial natamycin preparations.

Figure 3: 13-hydroxy-2,4,6,8,10-tetradeca pentaen-l-al

Evidence for the existence of decomposition products of natamycin with an intact lactone ring was obtained when an attempt was made to degrade natamycin at a low pH. In an aqueous 5% weight/volume suspension at pH 1.5, natamycin lost its biological activity completely after having been kept in the dark for 2 months at room temperature, or for 2 weeks at 40°C. From the reaction mixture, the aglycon apo-natamycin (Figure 4) was isolated as a light yellow amorphous substance in a rather high yield. In the degradation reaction, two moles of natamycin gave rise to one mole of apo-natamycin and one mole of the mycosamine

Apo-natamycin contains one natamycin- and one natamycinolide-moiety with each of the epoxy group (at C4 – C5) hydrolysed (Brik, 1976).

(R) = mycosamine moiety

Figure 4: Apo-natamycin

According to Brik (1976), more drastic acid degradation of natamycin eliminates the aminosugar, with formation of the dimer of the hypothetical aglycone of natamycin natamycinolide (in Figure 4, with R=OH). In this dimer, the epoxy groups are also hydrolysed.

2.2. Specifications

Specifications have been defined in Directive 2008/84/EC on purity criteria on food additives others than colours and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006) (Table 1).

Natamycin is a fungicide of the polyene macrolide group, and is produced by natural strains of *Streptomyces natalensis* or of *Streptococcus lactis*.

Table 1: Specifications for natamycin according to Commission Directive 2008/84/EC and JECFA (JECFA, 2006)

Purity	Commission Directive 2008/84/EC	JECFA (2006)
Loss on drying	Not more than 8% (over P_2O_5, in vacuum at 60°C to constant weight	Not more than 8% (60°C, over P_2O_5, pressure less than 5 mmHg)
Sulphated ash	Not more than 0.5%	Not more than 0.5%
Arsenic	Not more than 3 mg/kg	-
Lead	Not more than 5 mg/kg	Not more than 2 mg/kg
Mercury	Not more than 1 mg/kg	-
Heavy metals (as Pb)	Not more than 10 mg/kg	-
Microbiological criteria	Not more than 100cfu/g	-

2.3. Manufacturing process

Natamycin is produced by submerged aerobic fermentation by *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. Dried natamycin recovered from the fermentation broth is white to cream-coloured and has little or no odour or taste; in the crystalline form it is very stable. Optimisation of nutrients in the fermentation media for natamycin production by *S. natalensis* in submerged batch culture has been performed (Farid *et al*., 2000). *S. natalensis* is absent from the final product. During the extraction procedure the natamycin is dissolved and filtered through a membrane. The membrane is not permeable to the organism and the concentration of the solvent is high enough to kill the organism.

2.4. Mode of action and antimicrobial resistance

Natamycin is used in the food industry as an antifungal preservative in cheeses and sausages. The preservative is effective at concentrations between 1 and 10 mg/kg (Thomas and Delver-Broughton, 2003; Stark, 2004). In general, yeasts are more sensitive than moulds.

The antifungal activities of natamycin and other polyenes (i.e. amphotericin B) are due to their binding to the cell membrane sterol, ergosterol, which is the principal sterol in fungal membranes. Natamycin has a large lactone ring with a rigid lipophilic chain containing conjugated double bonds and a flexible

hydrophilic portion bearing several hydroxyl groups. It is probable that the hydrophobic region complexes with ergosterol in the membrane forming a polar pore through which small ions such as K^+, H^+, amino acids and other metabolites can pass freely, disrupting the cell's ionic control and killing the cell (Hamilton-Miller, 1974; Deacon, 1997).

Bacteria are insensitive to these antibiotics because their membranes lack sterols and are therefore naturally resistant to natamycin. Reported Minimum Inhibitory Concentrations (MICs) of natamycin for bacteria are higher than 250 mg/L. Animal cell membranes have cholesterol as their major membrane sterol, for which natamycin has a much lower specificity than ergosterol.

Induction of natamycin-resistant mutants in fungi is reported to be difficult (Athar and 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. *Candida* strains resistant to nystatin contain less ergosterol than sensitive ones (Athar and Winner, 1971; Safe *et al.,* 1977). It is generally accepted that there is a potential risk of development of resistance among fungal flora as a consequence of prolonged, repeated application of natamycin. However, such studies indicate that the level of resistance would be low. *C. albicans* resistance to natamycin has been induced after 25 passages in media with increasing concentrations of natamycin. This resistance developed gradually, and the MIC increased from 2.5-12 to 12-50 mg/L. JECFA in 1976 reported that the selection of natamycin-resistant strains *in vitro* has not induced cross-resistance to other polyenes (JECFA, 1976).

Surveys in cheese warehouses and in dry sausage factories where a 50% natamycin preparation had been used for up to 10 years showed no change in the composition or the sensitivity of the contaminating fungal flora (de Boer and Stolk-Horsthuis, 1977; de Boer *et al.,* 1979; Hoekstra and Van der Horst, 1998). De Boer and Stolk-Horsthuis (1977) attempted to induce tolerance in strains of fungi by transferring each culture 25-31 times in media containing concentrations of natamycin equal to and greater than the MIC. Following multiple transfers, the MIC increased in only 8 of 26 strains by a maximum of 4 mg/L. The overall lack of increased resistance was due to the lethal (fungicidal) mode of action, and the compound's instability.

The human gastrointestinal flora may be exposed to trace quantities of ingested natamycin residues. The intestinal microflora is predominated by bacterial species, whereas yeast and fungal species are only ca. 0.001% of the total flora. Several studies in experimental animals indicate that natamycin and any potential degradation products do not express antibiotic activity in the colon. There is no experimental evidence of fungi acquiring resistance to natamycin. As bacteria are not affected by polyenes, and fungi are found in low quantities in the intestinal tract, the consequences of exposure to ingested traces of natamycin could be considered as minimal.

2.5. Therapeutic applications in humans and animals

The antifungal properties of natamycin were originally used in the development of products for the treatment of topical fungal disorders. Historically natamycin has been used for treatment of infections of the eye, hair, mucous membranes, nails, and skin involving organisms of the genera *Candida* (candidiasis), *Epidermophyton*, *Microsporum* and *Trichophyton* (tinea; ringworm).

Although originally introduced in a number of countries, natamycin-containing drugs for common fungal infections have become nearly obsolete. However, the transition to newer treatment modalities is not complete worldwide. In some countries, natamycin is still in use. Nonetheless, global sales of natamycin for pharmaceutical use, as recorded by IMS Health (MIDAS), showed a decline of 39% between 1997 and 2000 (JECFA, 2002). The drug encyclopaedia Martindale shows no new additions to its uses section for natamycin from 1972 to 2002 (Martindale 1972, 1977, 1989, 2002).

The only significant remaining human therapeutic use for natamycin is in the treatment of fungal keratitis. A review of the scientific literature since 2002 continues to support this conclusion.

In Europe, a veterinary medicine containing natamycin is available. Although natamycin is still used, newer modalities, e.g. the antimycotic azole agents, are preferred.

2.6. Methods of analysis in foods

Methods of analysis in foods were based on organic solvent extraction followed by UV detection or further HPLC separation with UV detection. The detection limits can reach 0.05 to 0.25 mg/kg (de Ruig *et al.,* 1987; Riedl *et al.*, 1984; Fletouris *et al.,* 1995; Tuinstra and Traag, 1982; Luf and Brandl, 1986; Maruyama *et al.,* 1988). An enzyme immunoassay with anti-natamycin antibody from rabbit was developed (Maertlbauer *et al.,* 1990). Cross-reaction with related antimicrobials (amphotericin B and nystatin) was <0.001% (equivalent to 0.1 mg/kg) and the detection limit was reported to be 0.005 mg/dm^2, with a recovery of 76 to 84%.

2.7. Stability, reaction and fate on food

Natamycin shows good stability in foods provided that pH is in the range from 5 to 9 (Raab, 1972). It is less stable in foods outside this pH range (Stark, 2004). Natamycin is sensitive to inactivation by oxidants such as peroxides, chlorine and heavy metals (Raab, 1972).

2.8. Case of need and proposed uses

Natamycin is proposed for use for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages requiring maturation before marketing.

Natamycin is approved under Directive 95/2/EC (Annex III Part C) for the surface treatment of semi-hard and semi-soft cheese and dry, cured sausages at a maximum level of 1 mg/dm^2 surface (not present at a depth of 5 mm).

2.9. Existing authorisations and evaluations

According to Directive 95/2/EC, natamycin may be used for the surface treatment of semi-hard and semi-soft cheese and dry, cured sausage at a maximum level of 1 mg/dm^2 in the outer 5 mm of the surface, corresponding to 20 mg/kg. According to the definitions in the Codex General Standard for Cheese (CODEX Stan A-6-1978, rev1-1999, amended 2003), the term ‘cheese surface’ is used for the outside layer of cheese or parts of cheese, even in the sliced, shredded or grated form. The term includes the outside of whole cheese, disregarding whether a rind had been formed or not (CODEX STAN, 2003).

The SCF reviewed natamycin in 1979 and concluded as follows:

“1. Natamycin has a limited but important use in human medicine and is therefore not acceptable as a food additive for general use in and on foodstuffs.

2. Its use for the surface treatment of the rind of whole pressed cheese (semi-hard) ripened under aerobic conditions e.g. Gouda and Edam, and on the casings of certain sausages requiring maturation before marketing is acceptable, provided that:

- *the substance is applied only to the final product*
- *the residues of natamycin in food at a time of sale, expressed in relation to the surface area of the casing or rind, do not exceed 1 mg/dm² and that they will not be present at a depth greater than 5 mm.*

3. The use of natamycin on the casings of these foods shall be clearly indicated by suitable labelling.

4. The position should be reviewed if there is any significant increase in the range of therapeutic uses."

However, the SCF in 1979 did not establish an ADI but considered that in relation to the uses of natamycin on cheese and sausages, the database was adequate and did not give rise to safety concern. Neither natamycin nor its principal degradation products are absorbed from the digestive system.

JECFA has reviewed the safety of natamycin (pimaricin) in 1968, 1976 and 2002 and assigned an ADI of 0.3 mg/kg bw/day. The review concluded that "*New information was available on the effects of breakdown products and the development of microbial resistance to the antimycotic if it is used for food preservation. While the Committee expressed a general concern about the use of therapeutic agents in food, it agreed that the data on natamycin showed that problems were unlikely to arise from microbial resistance*".

This was confirmed by JECFA in 2002, as more recent publications had not conflicted with earlier studies.

On the issue of resistance to antibiotics, JECFA (2006) noted that 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 the selection of natamycin-resistant fungi is not an issue.

2.10. Exposure

The petitioner provided exposure estimates based on an assessment made by the JECFA in 2002. These calculations were based on the consumption of natamycin in 'a wider range of cheeses and meats' and partly at higher use levels than currently approved in the EU. The mean potential dietary exposure was 0.014 mg/kg body weight (bw) per day for UK consumers, and 0.015 and 0.01 mg/kg bw/day for children and people aged more than 10 years in Germany. For high consumers (97.5th percentile) these estimates were 0.041, 0.051 and 0.031 mg/kg bw/day, respectively.

The Panel noted that JECFA re-evaluated the exposure to natamycin at its sixty-seventh meeting in 2006 (JECFA, 2007). Refined estimates of dietary exposure were also based on individual consumption surveys from the UK and Germany, with a focus on children aged 1.5-4.5 years and 4-10 years, respectively (Gregory *et al.*, 1995, 2000; Heseker *et al.,* 1994). Children generally have higher food intake than adults, when expressed on a body weight basis, and therefore represent the group with the highest potential exposure to natamycin per kg body weight. The high level exposure estimates (97.5th percentile) for consumers only were presented separately for cheese (assumed use level 40

mg/kg) and cured meat comminuted such as salamis and other dried sausages (use level 20 mg/kg). The Panel used this information to make an estimated exposure using the present EU authorised use levels, corresponding to 20 mg/kg in both cheese and dry, cured sausages.

As shown in Table 2, the estimated high level exposure to natamycin from cheese was 0.04 mg/kg bw/day in the UK and 0.03 mg/kg bw/day in Germany for children, and 0.02 mg/kg bw/day in the UK and 0.025 mg/kg bw/day in Germany, for adults. The estimated high level exposure to natamycin from dry, cured sausages was 0.04 and 0.03 mg/kg bw in children, and 0.006 and 0.02 mg/kg bw in adults in UK and Germany, respectively.

The highest potential exposure to natamycin was at the 97.5th percentile below 0.1 mg/kg bw/day for children and below 0.05 mg/kg bw/day for adults, derived from the high level consumption of cheese (assuming solely a rind treatment with natamycin) and dried, cured sausages. If cheese were treated with natamycin after grating or shredding, the surface on which the treatment is applied would increase significantly. For instance, assuming a surface of 42 cm^2/cm^3 and grated to pieces of 1 cm x 0.1 cm x 0.1 cm with a density of 1g/cm^3, the theoretical maximal concentration level in the rated cheese would be 420 mg/kg.

Table 2: Estimated dietary exposure to natamycin, based on individual food consumption data

Country	Food category	Use level (mg/kg)	Children[1] at the 97.5th percentile		Adults at the 97.5th percentile	
			Food consumption (g/day)	Dietary exposure (mg/kg bw/day)	Food consumption (g/day)	Dietary exposure (mg/kg bw/day)[2]
UK	Cheese	20[3]	28	0.04	62	0.02
Germany	Cheese	20	40	0.03	74	0.025
UK	Cured meat comminuted[4]	20	30	0.04	19	0.006
Germany	Cured meat comminuted	20	43	0.03	64	0.02

[1]UK: 1.5-4.5 years (body weight of 15 kg); Germany: 4-10 years (body weight of 25 kg)
[2] based on a body weight of 60 kg
[3] all cheeses other than cream cheese are included, as well as cheeses used in recipes
[4] salamis and other dried sausages

3. Biological and toxicological data

3.1. Absorption, distribution, metabolism and excretion

3.1.1. Animals

After an oral 50 mg/kg bw dosage of ^{14}C-natamycin to rats, virtually no radioactivity could be demonstrated outside the gastrointestinal tract by whole-body autoradiography. It may be assumed that the label is distributed uniformly over the large ring system which is made by ^{14}C-acetate units. At 1 hour, radioactivity was solely concentrated in the oesophagus, stomach and small intestine. At 2 hours, there was some radioactivity in the caecum as well. At 4 hours, it reached the colon. At 8 hours, it concentrated in the intestine, but the stomach still contained radioactive material. At 24 hours, the radioactivity in the gastrointestinal tract was found to have decreased considerably, the largest

concentration was noted in the caecum and colon. Radioactivity was still detected in the stomach and not in the small intestine.

After oral administration, the majority of the radiolabel was eliminated in the faeces within 24 hours. Traces of radioactivity in the liver, kidneys and fatty tissue were only visible following extremely long exposures of the autoradiographic plates (150 days), which was indicative of an extremely low absorption of natamycin from the gastrointestinal tract (Blankwater and Hespe, 1979).

In dogs, after oral administration of ^{14}C-natamycin, the radioactivity is mainly found in the faeces. It may be assumed that the label is distributed uniformly over the large ring system which is made by ^{14}C-acetate units. Less than a few percentages of the dose applied are found in the urine. No essential differences appeared whether the ^{14}C-natamycin was administered via a capsule, as a suspension or via cheese. If the presence of the radioactivity in the urine is the result of absorption, this can be considered to be very low. The authors considered that the low level of radioactivity found in the urine could be caused, partly or in total, by contamination with the radioactivity eliminated via the faeces (Hespe and Meier, 1980). Following intravenous administration of ^{14}C-natamycin in the dog, radioactivity was predominantly excreted via bile. The authors concluded based on both the oral and intravenous data that a maximum of 5% of the radioactivity was absorbed.

Products formed in stomach in acid conditions are likely to be similar to degradation products as described by Brick (1976). Approximately 50% natamycin is broken down in 1 hour in simulated gastric juice, and losses from the stomach of 33-43% and 0-31% occurred in fasted and non-fasted rats respectively (Morgenstern and Muskens, 1976).

3.1.2. Humans

Little information is available on the absorption, distribution, excretion, or metabolism of natamycin in humans. Less than1 mg natamycin/L (LOD) could be detected in the blood following the ingestion of 500 mg by human subjects (Anonymous, 1968).

3.2. Toxicological data

3.2.1. Acute oral toxicity

The LD_{50} values of natamycin after oral administration are reported to be greater than 1400 mg/kg bw for mouse (Ottens, 1965), 2700 and 4700 mg/kg bw in male and female rats and 1400 mg/kg bw in the rabbit (Levinskas *et al.*, 1966). The LD_{50} for female guinea-pigs is reported to be 450 mg/kg bw (Struyk *et al.,* 1958).

3.2.2. Short-term and subchronic toxicity

Three subchronic toxicity studies are available, two in the rat and one in the dog.

In the first rat study, in which rats (15 males and 15 females per group) were fed natamycin at levels of 0 or 500 mg/kg diet, equivalent to 45 mg/kg bw/day, for 94 to 96 days, there were no significant differences in haematological parameters, organ histology and mean body weight gain of animals

receiving 500 mg/kg natamycin in the diet compared to their respective controls (Hutchison *et al.*, 1966).

The second rat study was carried out using dose levels of 0, 125, 500, 2000 or 8000 mg/kg diet, (equivalent to 0, 10, 45, 190 and 750 mg/kg bw/day for 94 to 96 days, 20 male and 20 female Carworth Farms rats per group). Haematological findings and organ weights were within normal limits, and no gross or microscopic lesions were found that could be attributed to natamycin. After 3 months on test, food consumption of males and females of the highest dose group was approximately 23% and 17% less respectively than that of rats receiving a control diet, while at the 190 mg/kg bw/day level, mean food intake was decreased about 5% for both sexes. Males and females at the highest dose level (750 mg/kg /bw/day), had mean body weights averaging 54% and 67% of their respective controls. At the 190 mg/kg bw/day level, animals averaged about 85% of the mean body weight of their controls (Levinskas *et al.*, 1966).

The Panel concludes that the No-Observed-Adverse-Effect Level (NOAEL) of this study was 45 mg/kg bw/day.

Natamycin was administered to Beagle dogs, (2 male and 2 female per group) in doses of 0, 12, and 25 mg/kg bw/day for 3 months. Clinical findings reported were a transient diarrhoea, recorded mainly in the high-dose group, which lasted in one male for 39 days, and in two females for 8 or 10 days, respectively, and a slight body weight loss in the high dose group. A transient diarrhoea was considered by the authors of the study to be the result of a local bowel irritation (van Eeken *et al.*, 1984).

The Panel noticed that the NOAEL of this study was 12 mg/kg bw/day.

3.2.3. Genotoxicity

The mutagenic potential of natamycin (a 50% suspension of natamycin in water), some of its degradation products (i.e. apo-natamycin, natamycinolidediol and mycosamine hydrochloride), and nitrite with or without a 50% suspension of natamycin in water, have been evaluated in *Bacillus subtilis*, *Salmonella typhimurium* (TA1535, TA1538, TA98 and TA100) and *Escherichia coli* (WP2 trp- and his mutant WP2 uvrA^{-}) without exogenous metabolic activation, except for the 50% suspension of natamycin in water. No statistical analyses were reported. The author reported that no positive responses were observed in the spot tests in any of the 3 test systems, except a slight positive response observed with nitrite alone and with the 50% suspension of natamycin in water and nitrite. The authors concluded that the slight positive effect of nitrite is not enhanced by the 50% suspension of natamycin in water (Khoudokormoff, 1977).

The Panel noted that the study's protocol would not match the current standards.

Bone-marrow preparations of 5 male and 5 female rats selected at random from litters produced by the F0, F1 and F2 from the three-generation study in Wistar rat (dosed 0, 5, 15, 50 or 100 mg natamycin/kg bw/day for 11 weeks) were examined. Animals were given colchicine 3 to 4 hours before sacrifice in order to induce metaphase arrest. 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).

According to a report of the European Agency for the Evaluation of Medicinal Products (EMEA) (1998), the mutagenicity of natamycin has been tested in a set of GLP-compliant studies. A bacterial mutation assay in *S. typhimurium* strains TA1535, TA1537, TA1538, TA98, TA100 with and without metabolic activation (S9 mixed from Arochlor 1254-induced liver preparations), a mouse lymphoma

mutation assay at the TK locus with and without metabolic activation and a chromosomal aberration assay with Chinese hamster ovary (CHO) cells *in vitro* have been carried out. In none of the experiments was there any observed evidence that natamycin had mutagenic potential.

In 2009, Rencüzoğullari *et al.*, investigated the effects of natamycin on chromosome aberrations (CAs), sister chromatid exchanges (SCEs), and micronucleus (MN) formation in human lymphocytes. The human lymphocytes were treated with 13, 18, 23 and 28 µg/mL of natamycin for 24 and 48 hours. According to the authors, natamycin increased the SCE frequency at the highest concentration for 48 hours only; however, it increased the structural CA and MN frequency at all concentrations when compared to control and at all concentrations, except the lowest concentration (13 µg/mL), when compared to solvent control. Natamycin showed a cytotoxic effect as indicated by decrease in the replication index, mitotic index, and nuclear division index (NDI), especially at the highest concentrations for two treatment periods. The Panel considered the results from the SCE and MN assays negative, since the effects observed were very weak. The results of the CA assay were of limited relevance, since these effects were accompanied by cytotoxicity.

3.2.4. Chronic toxicity and carcinogenicity

Two long-term toxicity studies are available, a 2-year chronic toxicity study in the rat and a 2-year chronic toxicity study in the dog.

The rat study (35 male and 35 female Carworth Farms rats per group), was carried out at dietary levels of 0, 125, 250, 500 or 1000 mg/kg diet, equivalent to 4.5, 11.0, 22.4 and 46.3 mg/kg bw/day in males, and 7.58, 15.4, 30.4 and 63.7 mg/kg bw/day in females. Natamycin had no effect on the survival of the rats. Decreased food intake and reduced growth rate were seen only at the highest dose group. After 6 months on test, females fed 500 mg/kg of natamycin had a significant increase in hemoglobin value. Since there were no significant differences in mean hemoglobin concentration at other times or among animals fed higher level of natamycin, this difference was considered to be of no consequence. Means haematocrits at all times did not differ significantly from corresponding control values. Total and differential leukocyte counts at each period did not indicate any deviations from normal values. The data showed that the numbers and types of tumours, which were mainly mammary gland adenocarcinoma, pituitary chromophobe adenoma and uterine and vaginal polyps, were not significantly different in any of the natamycin-treated groups compared with the untreated control animals (Levinskas *et al.*, 1963, 1966).

The Panel concludes that the NOAEL of this study amounts to 22.4 mg/kg bw/day, given the decrease of food intake and the reduced growth rate at the highest level.

The Beagle dog study (3 males and 3 females per group) was carried out at dietary levels of 0, 125, 250 or 500 mg/kg diet. Body weights increased steadily from the start of the experiment until the 15th month of the trial. The daily dose was then reduced by one-sixth because of excessive obesity among the animals at the highest dose; there was a marked reduction in body weight of all dogs. After dose reduction, two males and one female were unable to maintain an adequate body weight. Dietary levels of 250 mg/kg or less, did not affect body weight gain or its maintenance. Periodic determinations of haematologic and clinical chemistry values did not reveal any alterations which could be ascribed to feeding of natamycin. Males fed 125 and 250 mg natamycin/kg diet had mean liver weights at autopsy which were significantly lower than the mean liver weights of the controls. Since mean liver weight of both sexes fed 500 mg/kg diet did not differ significantly from the corresponding value for their respective controls, and since there is no indication of a dose-response effect, the authors concluded that feeding of natamycin did not affect liver-to-body weight ratios (Levinskas *et al.,* 1966).

The Panel concludes that the NOAEL of this study was 250 mg/kg diet, equivalent to 6.25 mg/kg bw/day.

3.2.5. Reproductive and developmental toxicity

3.2.5.1. Studies of reproductive toxicity

A study of reproductive toxicity has been performed in the rat, at dietary levels of 0 or 1000 mg/kg diet. 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 were considered treatment-related by the authors (Levinskas *et al.*, 1963; 1966).

The Panel concludes that this study is too limited to derive a NOAEL.

In a three-generation study of reproductive toxicity in the rat (0, 5, 15, 50 or 100 mg/kg bw/day for 11 weeks, 10 males and 20 females per group, Wistar), animals exposed to 100 mg/kg bw/day had an increased number of fetuses born dead, and a decrease in the number of animals born alive surviving at 21 days in F1 generation. Pup weight at 21 days was also depressed for the second generation. Fertility, gestation, viability and lactation indices were within normal limits for both litters of all three generations. Based on growth and reproduction data, the highest dose level of 100 mg/kg bw/day of natamycin in the diet of rats is considered an effect level. Natamycin dietary doses of 5, 15 and 50 mg/kg bw/day had no effect on growth, reproduction and on gross and microscopy pathology (Cox *et al.*, 1973).

The Panel concludes that the NOAEL of this study amounts to 50 mg/kg bw/day.

3.2.5.2. Developmental studies

A developmental toxicity study has been performed in female Wistar rats from the second litter of the F1 generation of the three-generation study of reproductive toxicity. They were reared to maturity and mated with untreated control males. The 20 pregnant females/group were given the same dose as their parents (0, 5, 15 or 50 mg/ kg bw/day of natamycin according to their original group) by intragastric intubation on days 6 to 18 of gestation, and were killed and examined on day 20. 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 (Cox *et al.,* 1973).

The Panel concludes that the NOAEL of this study amounts to 50 mg/kg bw/day.

In a rabbit developmental study, a 50% suspension of natamycin in water was administered by gavage to mated female Dutch belted rabbits (0, 5, 15 or 50 mg/kg bw/day on days 6 to 18 of gestation, 20-26 females per group). The maternal mortality rates were 0% (0/20), 5% (1/20), 9% (2/22), and 19% (5/26) in the 4 groups, respectively. The cause of death was not indicated in the report. There were no significant differences in pregnancy, implantation, number of live fetuses, number of dead fetuses or number of resorptions per dam between any test group and the control. Fetuses were evaluated for skeletal anomalies. The abnormalities noted in fetuses whose dams received natamycin at 5, 15 and 50 mg/kg bw/day consisted of skeletal anomalies generally regarded as spontaneous variations rather than malformations. A significant increase in extra sternebrae was noted in groups treated at 15 and 50 mg/kg bw/day in 5 litters out of 7, and 3 litters out of 14 respectively.

The Panel considered as indicated by the petitioner, that extra sternebrae is a common variant in developmental toxicity studies, particularly in the presence of maternal toxicity as in this study, and is not considered to be indicative of a teratogenic effect of natamycin, since there were no other significant skeletal effects that could be ascribed to treatment (Knickerbocker and Re, 1978, 1979).

The Panel considered that the incidence of mortality at the level of 5 and 15 mg/kg bw/day could be expected in a normal rabbit colony and therefore considered that only the dose level of 50 mg/kg bw/day provided conclusive evidence of toxicity. The Panel derived a NOAEL of 15 mg/kg bw/day based on maternal toxicity.

3.2.6. Human data

In a study by Newcomer *et al.* (1960), natamycin has been administrated orally to 10 patients suffering mycosis. The doses given to the patients varied from 25 to 1000 mg/person/day for 20 to 180 days. The treatment caused anorexia, nausea and vomiting at doses of 200 mg/person/day and above. At a level of 50 mg/person/day flatulence was described. Only one of the 10 patients, individually administered 25-75 mg/person/day during 70 days did not report any adverse effect of treatment. In three cases, the treatment was stopped because of toxicity. One patient tolerated a level of 400 mg/day without gastro-intestinal troubles, but could not exceed this dose. According to the authors, one patient reported anorexia, nausea and vomiting at a level of 50 mg/day. JECFA allocated the ADI of 0.3 mg/kg bw/day from this study in 1968, considering that the level causing no toxicological effects in man was 200 mg/per/day, equivalent to 3 mg/kg bw/day, with an uncertainty factor of 10.

The Panel considers that this study is too limited to derive a NOAEL.

Natamycin has been used for over 40 years to treat vaginal candidiasis, including during early pregnancy. An early study found no effect of treatment on congenital abnormalities (Patel, 1973). A large case-control study in Hungary covering births from 1980-1996 (Czeizel *et al.,* 2003) included 22843 pregnancies resulting in a congenital abnormality and 38151 pregnancies with normal outcomes. Among these, there were 62 cases and 98 controls that had been treated with natamycin during pregnancy by intravaginal tablet of 25 mg/day, once or twice per day, for a minimum of at least 2 days. There was no increase in fetal abnormalities following maternal treatment with natamycin at any time during pregnancy (Odds Ratio 1.1, 95% Confidence Interval 0.8-1.5), nor was there any increase when the data for treatment during the susceptible period of the second or third month of pregnancy was analysed (Odds Ratio 0.9, 95% Confidence Interval 0.4-1.8).

The Panel notes that these data do not raise any concern, but that they cannot be used for the risk assessment of natamycin as a food additive.

4. Discussion

The information on the toxicokinetics of natamycin suggests that natamycin is not absorbed to any significant extent from the gastrointestinal tract and is excreted in the faeces. After oral administration of ^{14}C-natamycin to rats, virtually no radioactivity could be demonstrated outside the gastrointestinal tract by whole-body autoradiography, and most of the radiolabel was eliminated in the faeces within 24 hours. Products formed in acidic conditions of the stomach are likely to be the same as degradation products obtained in acidic conditions *in vitro* (Brik, 1976).

Three subchronic toxicity studies with natamycin are available, two in the rat and one in the dog. In the first rat study, no modifications of haematological, biochemical parameters and organ weight were noted. In the second rat study, decreases of mean food intake and mean body weight have been observed. The NOAEL is considered to be 45 mg/kg bw/day. In the third study, dogs were exposed for 3 months to natamycin. Transient diarrhoea and slight body weight loss have been observed. The NOAEL is considered to be 12 mg/kg bw/day.

Two long-term studies are available, a 2-year chronic toxicity study in the rat and a 2-year chronic toxicity study in the dog. In the rat study, decrease of food intake and reduced growth rate were seen only at the highest dose group. The data showed that the numbers and types of tumours were not significantly different in any of the natamycin-treated groups compared with the untreated control animals. The NOAEL of this study is considered to be 22.4 mg/kg bw/day. In the dog study, the highest dietary concentration induced obesity among the animals. Dietary levels of 6.25 mg/kg bw/day or less, did not affect body weight gain. The NOAEL of this study is considered to be 6.25 mg/kg bw/day.

In a three-generation study of reproductive toxicity in the rat, at the highest dose an increased number of fetuses born dead, and a decreased number of animals born alive surviving at 21 days in F1 generation was described. The NOAEL of this study amounts to 50 mg/kg bw/day.

A developmental toxicity study has been performed in female rats from the second litter of the F1 generation of the three-generation study of reproductive toxicity. 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. The NOAEL of this study amounts to 50 mg/kg bw/day. In a rabbit developmental study on mated female Dutch belted rabbits, the maternal mortality rates were 0, 5, 9, and 19% in the 4 treatment groups (0, 5, 15 or 50 mg/kg bw/day), respectively. A significant increase in extra sternebrae was noted in groups treated at 15 and 50 mg/kg bw/day, but considered as normal variation by the Panel.

The NOAEL of this study is considered to be 15 mg/kg bw/day due to maternal toxicity at the higher dose level.

Natamycin bears a structural alert for genotoxicity since the molecule contains an epoxide ring. However, in the light that:

- the induction of chromosomal aberrations observed in a recent study was accompanied by cytotoxicity,
- there are *in vitro* studies on mutagenicity in bacteria and mammalian cells and on chromosomal aberrations in mammalian cells which were performed in compliance with GLP and were negative,
- no substance-related neoplastic effects were observed in the long term studies,

the Panel considered that the available data do not raise concern with respect to genotoxicity of natamycin.

A clinical study in humans performed in 1960 showed that natamycin, used for systemic mycoses, induced nausea, vomiting and diarrhoea. Anorexia, nausea, vomiting and flatulence were observed at different doses in different patients. The Panel considered this study too limited to derive a NOAEL.

In 1968, JECFA established an ADI of 0.3 mg/kg bw/day based on these human data. The level causing no toxicological effects in man was estimated to be 200 mg/per/day, equivalent to 3 mg/kg bw/day. Given that this dose was derived from human data, an uncertainty factor equal to 10 has been used to calculate the ADI. In 2002, JECFA considered that the results of the developmental study

performed by Knickerbocker and Re (1978, 1979) 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, JECFA, in agreement with the general consensus on the significance of extra sternebrae, considered that there was evidence that the extra sternebrae observed in fetuses at the intermediate (15 mg/kg bw/day) and high (50 mg/kg bw/day) doses of natamycin were variations rather than malformations. In view of the known sensitivity of the rabbit to gastrointestinal disturbance from antibiotics and the evidence of maternal toxicity in this study, JECFA confirmed in 2002 the ADI of 0.3 mg/kg bw/day.

Because of the limitations in the present database on natamycin (design of the animal studies, limited number of animals, lack of a carcinogenicity study) and in view of the inadequate description of the human data, the Panel considered that an ADI could not be established from these data.

The highest potential exposure to natamycin was at the 97.5th percentile below 0.1 mg/kg bw/day for children and below 0.05 mg/kg bw/day for adults, derived from the high level consumption of cheese (assuming solely a rind treatment with natamycin) and dried, cured sausages. If cheese were treated with natamycin after grating or shredding, the surface on which the treatment is applied would increase significantly. For instance, assuming a surface of 42 cm^2/cm^3 and grated to pieces of 1 cm x 0.1 cm x 0.1 cm with a density of 1g/cm^3, the theoretical maximal concentration level in the rated cheese would be 420 mg/kg.

The Panel noted that natamycin is used in the food industry as an antifungal preservative in cheeses and sausages. Natamycin is a polyene antibiotic. The mechanism of action for polyene antibiotics is binding to sterols (principally ergosterol) in the fungal cell membrane. Bacteria are insensitive to polyene antibiotics because their membrane lacks sterols. Furthermore, induction of natamycin-resistant mutants in yeast is reported to be difficult.

CONCLUSIONS

The Panel considered that the available data are not sufficiently robust for the purpose of deriving an ADI because of the limitations of the database on natamycin (design of the animal studies, limited number of animals, lack of carcinogenicity study) and in view of the inadequate description of the human data.

The highest potential exposure to natamycin was at the 97.5th percentile below 0.1 mg/kg bw/day for children and below 0.05 mg/kg bw/day for adults, derived from the high level consumption of cheese (assuming solely a rind treatment with natamycin) and dried, cured sausages.

Given that natamycin is very poorly absorbed, the Panel considers that this conservative estimate would provide an adequate margin of safety from the effect level seen from the long-term studies in animals and the human study used by JECFA to establish an ADI. The Panel considered that the proposed use levels of natamycin are not of safety concern if it is only used for the surface treatment of the rind of semi-hard and semi-soft cheese and on the casings of certain sausages.

The Panel concluded that there was no concern for the induction of antimicrobial resistance.

DOCUMENTATION PROVIDED TO EFSA

1. A Review of the Safety of Natamycin. December 2005. Submitted by Cantox Health Sciences International.

REFERENCES

Anonymous, 1968. Absorption of pimaricin following oral administration. Unpublished report submitted to WHO by the Royal Netherlands Fermentation Industries Ldt., Delft.

Athar MA and Winner HI, 1971. The development of resistance by *Candida* species to polyene antibiotics *in vitro*. J. Med. Microbiol. 4, 505–517.

Blankwater YJ and 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.

Brick H, 1976. New molecular decomposition products of Natamycin (Pimaricin) with intact lactone-ring. J. Antibiotics 29, 632-637.

CODEX STAN, 2003. Codex General Standard for Cheese (CODEX Stan A-6-1978, rev1-1999, amended 2003). Appendix.

Cox GE, Bailey DE and Morgareidge K, 1973. Multigeneration Reproduction Studies in Rats with Delvocid Brand of Pimaricin. Unpublished report No.1-1052 submitted to WHO by Food and Drug Research Laboratories Inc.

Czeizel AE, Kazy Z, Vargh P, 2003. A case-control teratological study of vaginal natamycin treatment during pregnancy. Reprod. Toxicol. 17 (4), 387-91.

Deacon JW (ed.), 1997. Prevention and control of fungal growth. In: Modern Mycology, 3rd Ed., Oxford: Blackwell Science, pp. 289–290.

de Boer E and 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 and Visser JN, 1979. Sensitivity to natamycin of fungi in factories producing dry sausage. Fleischwirtschaft 59, 1868–1869.

de Ruig WG, van Oostrom JJ and Leenheer K, 1987. Spectrometric and liquid chromatographic determination of natamycin in cheese and cheese rind. JAOAC 70, 944-948.

EMEA, 1998. Natamycin. Committee for Veterinary Products. EMEA/MRL/342/98-FINAL. Available at: http://www.emea.europa.eu/pdfs/vet/mrls/034298en.pdf

Farid MA, El Enshasy HA, El Diwany AI, El Sayed EA, 2000. Optimisation of the cultivation medium for natamycin production by *Streptomyces natalensis*. Journal of Basic Microbiology 40 (3), 157-166.

Fletouris DJ, Botsoglou NA, Mantis AJ, 1995. Rapid spectrophotometric method for analysing natamycin in cheese and cheese rind. JAOAC 78 (4), 1024-1029.

Gregory JR, Collin DL, Davies PSW, Hugues JM, Clarke PC, 1995. National diet and nutrition survey: children aged 1½ to 4½ years. Vol. 1: Report of the diet and nutrition survey. HMSO, London, UK.

Gregory J, Lowe S, Bates CJ, Prentice A, Jackson LV, Smithers G, Wenlock R, Farron M, 2000. National Diet and Nutrition Survey: Young People Aged 4 to 18 years. Vol. 1. Report of the Diet and Nutrition Survey. London: The Stationery Office.

Hamilton-Miller JMT, 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.

Heseker H, Adolf T, Eberhardt W, Hartmann S, Kubler W, Schneider R, 1994. Lebensmittel und Nahrstoffaufnahme Erwachsener in der Bundesrepublik Deutschland. VERA Schriftenreihe, Band III. Wiss. Fachverlag Dr. Fleck, Niederkleen.

Hespe W and Meier AM, 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 ES and Van der Horst MI, 1998. Survey of the fungal flora in Dutch cheese factories and warehouses. J. Food Mycol 1, 13–22.

Hutchison EB, Ribelin WE and Levinskas GJ, 1966. Report on acid-degraded pimaricin: Ninety-eight day repeated feeding to rats. Unpublished report submitted to WHO by American Cyanamid Co., Central Medical Department.

JECFA, 1968. Specifications for the identity and purity of food additives and their toxicological evaluations: some antibiotics. Twelfth Report of the Joint FAO/WHO Expert Committee on Food Additives, July 1-8, 1968, Geneva, Switzerland. WHO Technical Report Series N°430/FAO Nutrition Meetings Report Series N° 45.

JECFA, 1976. Evaluation of certain food additives. Twentieth Report of the Joint FAO/WHO Expert Committee on Food Additives, April 21-29, 1976, Rome, Italy. WHO Technical Report Series N°599/FAO Food and Nutrition Series N°1.

JECFA, 2002. Natamycin. Evaluation of certain food additives. Report of the Joint FAO/WHO Expert Committee on Food Additives, (57th meeting). WHO Food Additives Series 48, pp. 49-76.

JECFA, 2006. Combined compendium of food additive specifications - all specifications monographs from the 1st to the 65th meeting (1956-2005). FAO JECFA Monographs Series, No. 1 Volume 1-3, 2006.

JECFA, 2007. Evaluation of certain food additives. Report of the Joint FAO/WHO Expert Committee on Food Additives (67th meeting). WHO technical report N° 940 pp. 25-29.

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.

Knickerbocker M and Re TA, 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 and Re TA, 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.

Levinskas GJ, Shaffer CB, Bushey C, Kunde ML, Stackhouse DW and Vidone LB, Javier B, Monell E, 1963. Two-year feeding to rats. Unpublished report from the Central Medical Department. Submitted to WHO by American Cyanamid Co.

Levinskas GJ, Ribelin WE and Shaffer CB, 1966. Acute and chronic toxicity of pimaricin. Toxicol. Appl. Pharmacol. 8, 97-109.

Luf W and Brandl E, 1986. Simultaneous determination of sorbic acid and natamycin in and on cheese surfaces by means of high performance liquid chromatography (HPLC). Meijeritiet. Aikak. 44**,** 56-60. [Dairy Sci. Abst. (1987), 49, 460].

Maertlbauer E, Ali H, Dietrich R and Terplan G, 1990. Enzyme immunoassay for the detection of natamycin in cheese rind. Arch. Lebensmittelhyg. 41**,** 112-115 [Chem. Abst., 114, 553].

Martindale, 1972. Natamycin. In: The Extra Pharmacopoeia: Incorporating Squire's Companion*,* 26th Ed. Pharmaceutical Press, London, England, pp. 776-777.

Martindale, 1977. Natamycin. In: The Extra Pharmacopoeia: Incorporating Squire's Companion, 27th Ed. Pharmaceutical Press, London, England, 648-649.

Martindale, 1989. Natamycin. In: Martindale: The Extra Pharmacopoeia, 29th Ed. Pharmaceutical Press, London, England, 431-432.

Martindale, 2002. Natamycin. In: Martindale: The Complete Drug Reference, 33rd Ed. Pharmaceutical Press, London, England, p. 392.

Maruyama T, Watanabe Y, Tobano H, Matsuura N and Asai Y, 1988. An HPLC method for determination of natamycin in natural cheese. Shokuhin Eiseigaku Zasshi 29**,** 413-418.

Morgenstern AP and Muskens GJAM, 1976. Further data on the toxicity of the decomposition products of pimaricin. Unpublished report Gist-Brocades NV, Delft, 4 pages.

Newcomer VD, Sternberg TH, Wright ET, Reisner RM, McNall EG and Sorensin LJ, 1960. The treatment of systemic diseases with orally administered pimaricin: Preliminary report. Ann. N.Y. Acad. Sci., 89, 240-246.

Ottens H, 1965. Toxicity of the decomposition products of pimaricin. Unpublished report submitted to WHO by Royal Netherlands Fermentation Industries, Delft.

Patel VR, 1973. Natamycin in the treatment of vaginal candidiasis in pregnancy. Practitioner. 210, 701-703, as cited by Schardein In: Chemically Induced Birth Defects, Third edition, Marcel Dekker, New York, 2000.

Raab W, 1972. Natamycin (Pimaracin). Its Properties and Possibilities in Medicine. Georg Thieme Publishers, Stuttgart, Germany.

Rencüzoğullari E, Azirak S, Canimoglu S, Parlak S, Buyukleyla M. 2009 Effects of natamycin on sister chromatid exchanges, chromosome aberrations and micronucleus in human lymphocytes. Drug Chemical Toxicology, 32, 47-52.

Riedl R, Luf W and Brandl E, 1984. Spectrophotometric estimation of natamycin in cheese. Z. Lebensm. Unters. Forsch. 179, 394-398 [Dairy Sci. Abst. (1986), 48, 263].

Safe LM, Safe SH, Subden RE and Morris DC, 1977. Sterol content and polyene antibiotic resistance in isolates of *Candida krusei*, *Candida parakrusei* and *Candida tropicalis*. Can. J. Microbiol. 23, 398-401.

SCF, 1979. E235 Natamycin. In Report of the Scientific Committee for Food, Ninth Series, October 1979. Available from: http://ec.europa.eu/food/fs/sc/scf/reports/scf_reports_09.pdf

SCF, 1999. Opinion of the Scientific Steering Committee on antimicrobial resistance. 28 May 1999. Available at: http://ec.europa.eu/food/fs/sc/ssc/out50_en.pdf.

Stark J, 2004. Permitted preservatives - natamycin. In Robinson RK, Batt, CA and Patel P (eds.). Encyclopaedia of Food Microbiology, Academic Press, New York, pp. 1776-1781.

Struyk AP, Hoette I, Drost G, Waisvisz JM, van Eek T and Hoogerheide JC, 1958. Pimaricin, a new antifungal antibiotic. In: Antibiotics Annual 1957-1958 (H. Welch and F. Marti-Ibanez, eds.), pp. 878-885. Medical Encyclopedia Inc., New York.

Thomas LV and Delves-Broughton J, 2003. Natamycin. In: Encyclopedia of Food Sciences and Nutrition. Eds. B Caballero, L Trugo and P Finglas, pp. 4109-4115. Elsevier Science Ltd.

Tuinstra LGMT and Traag WA, 1982. Liquid chromatographic determination of natamycin in cheese at residue levels. JAOAC 65, 820-822.

van Eeken CJ, Birtwhistle RDR and Aboulwafa-wan Velthoven MJE, 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.

GLOSSARY [AND/OR] ABBREVIATIONS

ADI	Acceptable Daily Intake
ANS	Scientific Panel on Food Additives and Nutrient Sources added to Food
bw	body weight
CA	Chromosome Aberrations
CAS	Chemical Abstract Service
cfu	colony-forming units
EFSA	European Food Safety Authority
EMEA	European Medicines Agency
FAO/WHO	Food and Agriculture Organization/World Health Organization
GLP	Good Laboratory Practice
HPLC	High-Performance Liquid Chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	Limit Of Detection
MN	Micronucleus
NDI	Nuclear Division Index
NOAEL	No-Observed-Adverse-Effect Level
SCE	Sister Chromatid Exchanges
SCF	Scientific Committee for Food
UV	Ultra-Violet

Natamycin

Handling/Processing

1

2 **Identification of Petitioned Substance**

3 **Chemical Name (IUPAC):**	22 **Trade Names:**
4 22-[(3-amino-3,6-dideoxy-β-D-mannopyranosyl)-	Delvocid
5 oxy]-1,3,26-trihydroxy-	Delvolan
6 12-methyl-10-oxo-6,11,28-	Mycophyt
7 trioxatricyclo[22.3.1.0^{5,7}] octacosa-8,14,16,18,20-	Myprozine
8 pentaene-25-	Natamax
9 carboxylic acid	Natacyn
10	
11 **Other Names:**	**CAS Number:**
12 Antibiotic A-5283	7681-93-8
13 CL 12625	
14 Delvopos	**Other Codes:**
15 Natafucin	235 (INS Number)
16 Pimafucin	231-683-5 (EINECS Number)
17 Pimaricin	
18 Pimaricine	
19 Synogil	
20 Tennecetin	

21

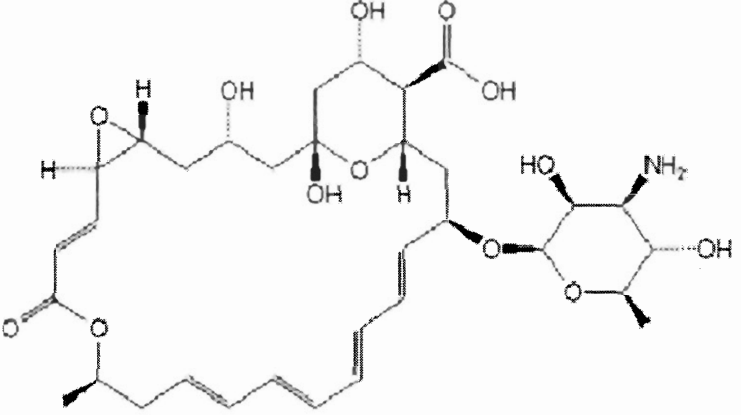
23 **Characterization of Petitioned Substance**

24

25 **Composition of the Substance:**

26 Natamycin is a naturally occurring antimicrobial compound that is generally produced by the bacterium
27 *Streptomyces natalensis*. The chemical structure of natamycin is illustrated in Figure 1.

28



29

30 **Figure 1. Chemical Structure of Natamycin (ChemIDplus 2006)**

31

32 **Properties of the Substance:**

33 Natamycin is a white, tasteless, and odorless powder. It is practically insoluble in water except at extreme
34 pH levels. Natamycin is soluble in glacial acetic acid and demethylformamide (Thomas and Delves-
35 Broughton 2003).

36

37 **Specific Uses of the Substance:**
38 Natamycin is being petitioned for use as a post-baking surface treatment of yeast-leavened and non-yeast-
39 leavened baked goods to prevent or delay mold growth and to maintain the wholesomeness of packaged
40 baked goods.
41
42 In general, the food industry uses natamycin as a preservative to inhibit fungal growth on cheese; it also is
43 used for other foods like meat and sausages (Mattia et al. 2002).
44
45 Natamycin is also used as a medicinal product in both humans and animals (Mattia et al. 2002). In
46 humans, natamycin is currently used as an antifungal medicine to treat fungal infections of the eye
47 (Drugs.com 2006). It was previously used in the treatment of fungal infections of the skin and mucous
48 membranes (Mattia et al. 2002). Although no literature was found detailing why natamycin is no longer
49 used in this way, one source stated that natamycin does not appear to be absorbed to a significant extent
50 from mucous membranes, skin, or from the GI tract (AHFS Drug Information 2004). In animals, natamycin
51 is used to treat ringworm in horses and cattle by applying it topically to the skin or mucous membranes
52 (Mattia et al. 2002).
53
54 Natamycin can be used as a pesticide. According to the World Health Organization (WHO), natamycin is a
55 Class III (i.e., slightly toxic) pesticide that is primarily used as a fungicide (WHO 2005).
56
57 **Approved Legal Uses of the Substance:**
58 Under 21 CFR 172.155, natamycin is an additive permitted by the U.S. Food and Drug Administration
59 (FDA) for direct addition to food intended for human consumption. Specifically, it is approved as a
60 preservative for the inhibition mold and yeast in cheese; levels may not exceed 20 milligrams per kilogram
61 (20 ppm) in the finished product (FDA 2001).
62
63 Although the petitioner states that natamycin is a "Generally Recognized as Safe" (GRAS) food additive, it
64 is not listed as such by FDA (FDA 2006a) nor has a GRAS notification been submitted to FDA. The
65 petitioner also refers to self-affirmation of GRAS status by the manufacturer of natamycin, Danisco USA,
66 Inc. A manufacturer may self-affirm that a compound is GRAS by performing all necessary research,
67 including review by qualified experts (FDA 2004b). The manufacturer does not need to get approval from
68 FDA or notify FDA of the results of its investigation, as long as the qualified experts agree that the
69 compound is safe (Ziker 2002). However, the manufacturer may choose to notify FDA of the results of its
70 investigation under the voluntary GRAS notification program.[1] The petitioner provides a letter from the
71 manufacturer describing its self-affirmation process. The letter does not contain sufficient information to
72 determine whether the manufacturer followed the proper procedures for the self-affirmation of GRAS
73 status; there is no mention of an expert panel or its findings.
74
75 According to FDA's "Electronic Orange Book," natamycin is approved as the active ingredient for use in
76 Natacyn, an ophthalmic suspension used to treat fungal blepharitis, conjunctivitis, and keratitis caused by
77 susceptible organisms including *Fusarium solani* keratitis (FDA 2006b).[2] Although it also is effective in the
78 treatment of oral, cutaneous, or vaginal candidiasis, this use is not currently included in the labeling
79 approved by the FDA (AHFS Drug Information 2004).
80
81 In 2004, FDA announced that it was amending its regulations for food additives permitted in feed and
82 drinking water of animals to allow the use of natamycin in broiler chicken feeds (FDA 2004a). Under the

[1] The GRAS notification program is a voluntary procedure operating under a proposed rule published by FDA on April 17, 1997 (62 FR 18936).
[2] FDA's "Electronic Orange Book" is a database available on-line that is based on FDA's annual publication of the "Orange Book Annual Edition," which identifies drug products approved on the basis of safety and effectiveness by the FDA under the Federal Food, Drug, and Cosmetic Act. The electronic version enables searching of the approved drug list by active ingredient, proprietary name, applicant holder or applicant number. Data are updated concurrently with the publication of the annual edition or cumulative supplements (FDA 2006c).

83 amendment, up to 11 parts per million (ppm) of natamycin may be added to broiler chicken feed to retard
84 the growth of *Aspergillus parasiticus* in the feed for up to 14 days.
85
86 **Action of the Substance:**
87 The mode of action for natamycin is to disrupt the cell membrane by binding to sterols (e.g., ergosterol[3])
88 and increase the permeability of the fungal cell membrane, which eventually leads to cell death (Myers
89 2006).
90
91 **Status**
92
93 **International:**
94 Natamycin is used in the European Union, the United States, much of South America and Eastern Europe,
95 as well as several Middle Eastern countries (Deift 2002). It is listed as an allowed preservative for the
96 European Union. According to WHO Food Additives Series, natamycin is proposed in the Codex draft
97 General Standard for Food Additives (GSFA) for use in the following: cheese at 40 mg/kg; cured and
98 dried non-heat treated processed meats, poultry, and game products at 6 mg/kg; and cured and dried non-
99 heat comminuted meat, poultry, and game products at 20 mg/kg (Mattia et al. 2002). According to the
100 Codex Alimentarius (2001), 2 mg/dm^2 natamycin can be added to the surface during cheese rind
101 treatment, as long as it is not present at a depth of 5 mm. The Codex standard also states that 20 mg/kg of
102 natamycin can be added to the surface of sliced, cut, shredded, and grated cheese as a preservative during
103 the kneading and stretching process. The Food Standards Australia and New Zealand has been petitioned
104 to approve natamycin for the following uses (FSANZ 2004):
105
106 • breads and bakery products;
107 • fruit and vegetable preparations, including pulp;
108 • dairy and fat based desserts, dips, and snacks; and
109 • sauces and toppings (including mayonnaises and salad dressings).
110
111 The WHO Food Additives Series (Mattia et al. 2002) on natamycin reports that the acceptable daily intake
112 (ADI) is 0-0.3 mg/kg body weight. The expected daily intake (EDI) for internationally approved uses of
113 natamycin in cheese and meats is not expected to exceed the ADI.
114
115 Natamycin is not specifically listed for the petitioned use (i.e., as a post-baking surface treatment of yeast-
116 leavened and non-yeast-leavened baked goods to prevent or delay mold growth and to maintain the
117 wholesomeness of packaged baked goods) or other uses in the following international organic standards:
118
119 • Canadian General Standards Board
120 • CODEX Alimentarius Commission
121 • European Economic Community (EEC) Council Regulation 2092/91
122 • International Federation of Organic Agriculture Movements
123 • Japan Agricultural Standard for Organic Production
124
125 **Evaluation Questions for Substances to be used in Organic Handling**
126
127 **Evaluation Question #1: Is the petitioned substance formulated or manufactured by a chemical process?**
128 **(From 7 U.S.C. § 6502 (21).)**
129
130 Natamycin can be isolated from *Streptomyces natalensis* or related *Streptomyces* bacteria by a submerged
131 aerobic fermentation process. It is isolated after several days of fermentation either by broth extraction or
132 extraction of the mycelium (Mattia et al. 2002). When produced for commercial applications, however, the
133 petitioner describes a method by Borden et al. (US Patent No. 5942611, 1999) that recovers natamycin by

[3] Ergosterol is a part of fungal cell membranes and is similar in function to cholesterol in animal cells (Wikipedia 2006).

adjusting the pH of the fermentation broth to above 10. The agent used to bring the fermentation broth above a pH of 10 is not specified in the petition; however, the Borden et al. patent states that the following alkaline compounds are all appropriate: sodium carbonate, potassium carbonate, sodium hydroxide, potassium hydroxide, or a combination of these. Isopropanol (or other water-miscible solvent) is then added to dissolve natamycin in the broth. Insoluble solids are removed from the broth and the pH is lowered so that natamycin is precipitated.

Thus, although natamycin is produced by a naturally occurring biological process, a chemical process is used to extract the natamycin from the fermentation medium. However, the extraction steps do not alter the identity of the natamycin produced by the microbial culture.

Evaluation Question #2: Is the petitioned substance formulated or manufactured by a process that chemically changes the substance extracted from naturally occurring plant, animal, or mineral sources? (From 7 U.S.C. § 6502 (21).)

No information was found that indicates that the extraction steps alter the identity of the natamycin produced by the microbial culture.

Evaluation Question #3: Is the petitioned substance created by naturally occurring biological processes? (From 7 U.S.C. § 6502 (21).)

Natamycin can be isolated from the bacterium *Streptomyces natalensis* (Suloff 1999).

Evaluation Question #4: Is there a natural source of the petitioned substance? (From 7 CFR § 205.600 (b) (1).)

As noted in Evaluation Question #1, natamycin can be isolated from the bacterium *Streptomyces natalensis* (Suloff 1999). Natamycin was first isolated from this bacterium in 1955 from a soil sample taken from South Africa (Suloff 1999). Later in 1959, natamycin was isolated from was isolated from *Streptomyces gilvosporus* (Suloff 1999). Natamycin can also be isolated from related *Streptomyces* bacteria. Although natamycin can be isolated from *Streptomyces natalensis* and related *Streptomyces* bacteria that are naturally occurring, there is no information indicating that natural sources produce natamycin in quantities sufficient for commercial uses.

Evaluation Question #5: Is there an organic agricultural product that could be substituted for the petitioned substance? (From 7 CFR § 205.600 (b) (1).)

No other organic agricultural products were identified that could be substituted for the petitioned substance.

Evaluation Question #6: Are there adverse effects on the environment from the petitioned substance's manufacture, use, or disposal? (From 7 CFR § 205.600 (b) (2).)

There is no information available from EPA or FDA to suggest that adverse environmental effects result from the manufacture, use, or disposal of natamycin. When used as a preservative for cheese or in broiler chicken feeds, natamycin does not individually or cumulatively have a significant effect on the environment (FDA 2001, 2004a).

The petitioner intends to obtain natamycin from a supplier that produces the compound in Denmark. According to the petitioner, this facility disposes of no waste to the environment since the extracted fermentation broth is either (1) disposed of in the waste water treatment facility at the factory or (2) used in gas production by a local utility to generate electricity.

In addition, the petitioner states that more than 99 percent of the isopropanol used during manufacture is recovered via distillation and is subsequently reused. Consequently, very little isopropanol would be lost

188 to the environment. However, a small amount of isopropanol would enter the wastewater treatment
189 facility where, according to the petitioner, it would be readily biodegraded. Isopropanol is readily
190 biodegraded in aerobic aqueous systems; the range of half-lives for aerobic degradation using a sewage
191 sludge inocula is <1 day to 48 days (HSDB 2006). Isopropanol is also biodegraded under anaerobic aqueous
192 conditions (HSDB 2006).
193
194 **Evaluation Question #7: Does the petitioned substance have an adverse effect on human health as**
195 **defined by applicable Federal regulations? (From 7 CFR § 205.600 (b) (3).)**
196
197 The WHO Food Additives Series (Mattia et al. 2002) on natamycin reports the results of two studies where
198 humans showed an adverse effect to oral doses of natamycin. In one study, oral daily doses of 300-400 mg
199 of natamycin caused nausea, vomiting, and diarrhea; there were no changes in peripheral blood cells. In
200 the second study, a group of 10 patients with systemic mycoses received oral daily doses of 50-1000 mg for
201 13-180 days. At 600-1000 mg, patients experienced nausea, vomiting, and diarrhea. Based on these
202 findings, the ADI of natamycin was determined to be 0-0.3 mg/kg body weight, or up to 2.1 mg for a 70-kg
203 adult.
204
205 The WHO Food Additives Series (Mattia et al. 2002) reported that no allergic sensitization was observed
206 among 111 patients that were treated with natamycin for a variety of conditions. In another study, 102
207 patients with various forms of eczema that underwent repeated exposure to natamycin did not show any
208 allergic sensitization to it. In 73 workers employed for an average of five years in the manufacture of
209 natamycin, there was no history of any allergic reactions; no allergic reactions occurred when 71 of the
210 workers were tested.
211
212 Oral animal toxicity testing also has been conducted for natamycin and is summarized in Mattia et al.
213 (2002). These results indicate that natamycin has low toxicity, with the only adverse effect reported in a
214 short-term toxicity study in dogs being diarrhea. This effect occurred most frequently in high-dose animals
215 (equivalent to 25 mg/kg body weight per day); however, as only two dogs were tested, the usefulness of
216 this study is limited.
217
218 **Evaluation Question #8: Is the nutritional quality of the food maintained when the petitioned**
219 **substance is used? (From 7 CFR § 205.600 (b) (3).)**
220
221 No information was identified that states or implies that natamycin changes the nutritional quality of food.
222 Producers of Natamax (a mold and yeast inhibitor made with natamycin) claim that natamycin does not
223 interfere with the nutritional value, taste, or odor of the food product.[4]
224
225 **Evaluation Question #9: Is the petitioned substance to be used primarily as a preservative? (From 7**
226 **CFR § 205.600 (b) (4).)**
227
228 All information provided in the petition indicates that natamycin would be used as a food preservative.
229 Specifically, the petitioner intends to treat the surface of yeast-leavened and non-yeast-leavened baked
230 goods after baking to prevent or delay mold growth.
231
232 **Evaluation Question #10: Is the petitioned substance to be used primarily to recreate or improve**
233 **flavors, colors, textures, or nutritive values lost in processing (except when required by law, e.g.,**
234 **vitamin D in milk)? (From 7 CFR § 205.600 (b) (4).)**
235
236 Natamycin is petitioned for use as a preservative. It is not intended to recreate or improve flavors, colors,
237 textures, or nutritive values lost in processing.
238
239 **Evaluation Question #11: Is the petitioned substance generally recognized as safe (GRAS) when used**
240 **according to FDA's good manufacturing practices? (From 7 CFR § 205.600 (b) (5).)**

[4] Further information is available at http://www.kelleysupply.com/ext/ext_promo.html.

241
242 As noted previously (see "Approved Legal Uses of the Substance"), although the petitioner states that
243 natamycin is a GRAS food additive, it is not listed as such by FDA (FDA 2006a) nor has a GRAS
244 notification been submitted to FDA. The petitioner also refers to self-affirmation of GRAS status by the
245 manufacturer of natamycin, Danisco USA, Inc. In this regard, the petitioner provides a letter from the
246 manufacturer describing its self-affirmation process. However, the letter does not contain sufficient
247 information to determine whether the manufacturer followed the proper procedures for the self-
248 affirmation of GRAS status; there is no mention of an expert panel or its findings.
249
250 **Evaluation Question #12: Does the petitioned substance contain residues of heavy metals or other**
251 **contaminants in excess of FDA tolerances? (From 7 CFR § 205.600 (b) (5).)**
252
253 No information was found that indicates that natamycin contains residues of heavy metals or other
254 contaminants in excess of FDA tolerances. Table 1 illustrates an analysis of natamycin from Penglai
255 Chemical, Inc. that conforms to the Food Chemical Codex (FCC) IV, which is an activity of the Food and
256 Nutrition Board of the Institute of Medicine and is supported by FDA.
257
258 **Table 1. Analysis of Natamycin** (source: http://www.penglaichem.com/OLDPAGE/Natamycin.htm)
259

Item	Standard Requirement	Result
Appearance	White to yellow crystalline powder	Conform
Purity	≥50%	51.2%
Moisture	6.0-9.0%	6.7%
PH	5.0-7.5	6.0
Specific Rotation	+2760 ~ +2800	+2780
Ash	≤0.5%	0.2%
Heavy metals	< 20 ppm	< 10 ppm
Pb	< 20 ppm	< 5 ppm
Arsenic (As)	< 3 ppm	< 3 ppm
Hg	< 1 ppm	< 1 ppm
Microbiological Count	< 10 colony forming units (cfu)/g	< 10 cfu/g
Pathogen	Absent	Absent
E. coli	Negative/in 25g	Negative/in 25g
Salmonella	Negative/in 25g	Negative/in 25g

260
261 **References**
262
263 AHFS Drug Information. 2004. American Society of Health-System Pharmacists, Inc.: Bethesda, Maryland.
264 http://www.medicinescomplete.com/mc/ahfs/2005/a382219.htm (membership needed to access link).
265
266 Borden, G.W.,J.M. Maher, C. Sklavounos. 1999. Process for natamycin recovery. United States Patent
267 5942611. (as cited from the petitioner). Further information available at:
268 http://www.freepatentsonline.com/5942611.html?highlight=5942611,borden.
269
270 ChemIDplus. 2006. Natamycin. http://chem.sis.nlm.nih.gov/chemidplus/.
271
272 Codex Alimentarius. 2001. Codex Group Standard for Unripened Cheese Including Fresh Cheese. CODEX
273 STAN 221-2001. http://www.codexalimentarius.net/ download/standards/363/CXS_221e.pdf.
274
275 Deift, N.L. 2002. Natamycin Approved for Use on Fermented Sausages and Cheese in Switzerland.
276 http://www.dsm.com/en_US/html/dfs/news_items/natamycin_approved_swiss.htm.
277

278 Drugs.com (Drug Information Online). 2006. Physician's Desk Reference (PDR) Drug information for
279 Natacyn Antifungal Ophthalmic Suspension.
280 http://www.drugs.com/pdr/natacyn_antifungal_ophthalmic_suspension.html.
281
282 FDA (U.S. Food and Drug Administration). 2001. Food Additives Permitted for Direct Addition to Food for
283 Human Consumption; Natamycin (Pimaricin). Federal Register 66(46): 13846-13847.
284
285 FDA. 2004a. Food additives permitted in feed and drinking water of animals; natamycin. U.S. Food and
286 Drug Administration, Fed. Reg. 69(71): 19320-19321. Available at:
287 http://a257.g.akamaitech.net/7/257/2422/14mar20010800/edocket.access.gpo.gov/2004/04-8249.htm.
288
289 FDA. 2004b. "Guidance for Industry: Frequently Asked Questions About GRAS," U.S. Food and Drug
290 Administration, Center for Food Safety and Applied Nutrition (CFSAN), December 2004, Hypertext
291 updated March 15, 2006. Accessed at: http://www.cfsan.fda.gov/~dms/grasguid.html#Q1
292
293 FDA. 2006a. EAFUS: A Food Additive Database. http://vm.cfsan.fda.gov/~dms/eafus.html.
294
295 FDA. 2006b. Electronic Orange Book. Last revised May, 2006.
296 http://www.fda.gov/cder/ob/docs/queryai.htm.
297
298 FDA. 2006c. Approved Drug Products with Therapeutic Equivalence Evaluations
299 Orange Book. Last revised July, 2006. http://www.fda.gov/cder/orange/default.htm.
300
301 FSANZ (Food Standard Australia and New Zealand). 2004. Initial Assessment Report. Application A542.
302 Natamycin – Extension of Use as a Food Additive.
303 http://www.foodstandards.gov.au/_srcfiles/A542_Natamycin_IAR_FINAL.pdf.
304
305 HSDB (Hazardous Substances Data Bank). 2006. Isopropanol. CASRN: 67-63-0.
306 http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB
307
308 Mattia, A., C. Cerniglia, J. Baines. 2002. WHO Food Additives Series: 48. Safety Evaluation of Certain Food
309 Additives and Contaminants. Natamycin (Pimaricin).
310 http://www.inchem.org/documents/jecfa/jecmono/v48je06.htm.
311
312 Myers, R.S. 2006. VI. Antifungal Agents. In Immunizing and Antimicrobial Agents.
313 http://courses.washington.edu/medch401/pdf_text/401_06_VI_Antifungal.pdf.
314
315 Suloff, E.C. 1999. Comparative Study of Semisynthetic Derivative of Natamycin and the Parent Antibiotic
316 on the Spoilage of Shredded Cheddar Cheese. .
317 http://scholar.lib.vt.edu/theses/available/etd-120399-111506/unrestricted/thesisetd.pdf.
318
319 Thomas L.V.J. Delves-Broughton. 2003. Natamycin. In Encyclopedia of Food Sciences and Nutrition. B.
320 Caballero, L. Trugo, P. Finglas (eds.). pp 4109-4115. Elsevier Science Ltd.
321
322 Wikipedia. 2006. Ergosterol. http://en.wikipedia.org/wiki/Ergosterol.
323
324 WHO (World Health Organization). 2005. The WHO Recommended Classification of Pesticides by Hazard
325 and Guidelines to Classification: 2004.
326 http://www.inchem.org/documents/pds/pdsother/class.pdf.
327
328 Ziker, D. 2002. Regulating Functional Foods: Pre- and Post-market Strategy. Duke Law and Technology
329 Review. http://www.law.duke.edu/journals/dltr/articles/2002dltr0024.html
330

George M. Eliopoulos, Section Editor

Resistance to Antifungal Agents: Mechanisms and Clinical Impact

Zeina A. Kanafani[1] and John R. Perfect[2]

[1]American University of Beirut Medical Center, Beirut, Lebanon; and [2]Duke University Medical Center, Durham, North Carolina

Despite advances in preventive, diagnostic, and therapeutic interventions, invasive fungal infections cause significant morbidity and mortality in immunocompromised patients. The burden of antifungal resistance in such high-risk patients is becoming a major concern. A better understanding of the mechanisms and clinical impact of antifungal resistance is essential to the prompt and efficient treatment of patients with invasive mycoses and to improving the outcome of such infections. Although recent guidelines have attempted to standardize antifungal susceptibility testing, limitations still exist as a result of the incomplete correlation between in vitro susceptibility and clinical response to treatment. Four major mechanisms of resistance to azoles have been identified, all of which rely on altered gene expression. Mechanisms responsible for polyene and echinocandin resistance are less well understood. In addition to discussing the molecular mechanisms of antifungal resistance, this article elaborates on the concept of clinical resistance, which is critical to the understanding of treatment failure in patients with invasive fungal infections.

Invasive fungal infections constitute a significant burden in patients with impaired immunity [1, 2]. The spectrum of fungal pathogens causing infections in immunocompromised hosts is growing [3]. However, the available therapeutic options are limited, particularly for pathogens that are resistant to ⩾1 class of antifungals (table 1) [4–24].

We review the molecular mechanisms that underlie in vitro resistance of yeasts and molds to various classes of antifungals. We also discuss the causes and implications of clinical antifungal resistance and offer directives regarding the optimal approach to treatment failure in fungal infections.

DEFINITIONS

Microbiological resistance refers to nonsusceptibility of a fungus to an antifungal agent by in vitro susceptibility testing, in which the MIC of the drug exceeds the susceptibility breakpoint for that organism. Microbiological resistance can be primary (intrinsic) or secondary (acquired). Primary resistance is found naturally among certain fungi without prior exposure to the drug and emphasizes the importance of identification of fungal species from clinical specimens. Examples include resistance of *Candida krusei* to fluconazole and of *Cryptococcus neoformans* to echinocandins. Secondary resistance develops among previously susceptible strains after exposure to the antifungal agent and is usually dependent on altered gene expression. The development of fluconazole resistance among *Candida albicans* and *C. neoformans* strains illustrates this type of resistance [25, 26].

Clinical resistance is defined as the failure to eradicate a fungal infection despite the administration of an antifungal agent with in vitro activity against the organism. Such failures can be attributed to a combination of factors related to the host, the antifungal agent, or the pathogen. Although clinical resistance cannot always be predicted, it highlights the importance of individualizing treatment strategies on the basis of the clinical situation.

ANTIFUNGAL SUSCEPTIBILITY TESTING

Until recently, the techniques for antifungal susceptibility testing were not standardized. The Clinical and Laboratory Standards Institute published reference methods for susceptibility testing of yeasts [27] and filamentous fungi [28]. These guidelines have created a standard for comparison of clinical data. The European Committee on Antibiotic Susceptibility Testing

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Reprints or correspondence: Dr. Zeina A. Kanafani, Div. of Infectious Diseases, American University of Beirut Medical Center, Cairo St., PO Box 113-6044, Hamra 110 32090, Beirut, Lebanon (zk10@aub.edu.lb).

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Table 1. Antifungal drug susceptibility of selected drug-resistant fungi.

Species	MIC range, mg/mL (no. of isolates)				CAS, minimal effective concentration range in mg/mL (no. of isolates)
	ICZ	VCZ	PCZ	AMB	
Aspergillus lentulus	0.5–1 (8)	1–2 (8)	NA	1–2 (8)	2–16 (8)
Aspergillus ustus	1 to >8 (10)	4–8 (10)	2 (1)	0.25–8 (10)	2–8 (8)
Aspergillus terreus	0.03–8 (63)	0.25–4 (63)	0.06–0.25 (8)	0.12–16 (63)	0.06–0.5 (13)
Scedosporium apiospermum	0.03–2 (30)	≤0.03 to 0.5 (30)	0.125–1 (13)	0.5 to >16 (30)	0.25–4 (11)
Scedosporium prolificans	2 to >32 (55)	0.5–8 (55)	2 to >8 (55)	1 to >16 (55)	4–8 (2)
Fusarium solani	≥8 (15)	1–4 (10)	>8 (6)	0.25–8 (15)	≥8 (29)
Paecilomyces lilacinus	1 to >8 (3)	0.2–1 (3)	0.12–0.5 (3)	>8 (3)	3 to >100 (5)
Scopulariopsis brevicaulis	>8 (25)	>8 (25)	>8 (25)	8 to >16 (25)	4 to >16 (25)
Zygomycetes	0.03–32 (51)	2–64 (51)	0.06–2 (36)	0.03–2 (51)	>16 (15)
Trichosporon asahii	0.03–8 (15)	0.015–8 (15)	0.12–1 (5)	0.5–16 (15)	>16 (9)
Geotrichum capitatum	0.03–0.5 (23)	0.03–0.5 (23)	NA	0.06–0.25 (23)	0.5 (1)
Cladophialophora bantiana	≤0.03 to 0.25 (10)	≤0.03 to 1 (10)	<0.03 to 0.06 (5)	0.25–0.5 (10)	2–8 (5)

NOTE. Data are compiled from [4–24]. AMB, amphotericin B; CAS, caspofungin; ICZ, itraconazole; NA, not available; PCZ, posaconazole; VCZ, voriconazole.

has also published guidelines for testing *Candida* and *Aspergillus* isolates [29–31]. However, clinicians are still faced with the challenge of how to interpret the results of in vitro antifungal susceptibility testing. MIC values do not always directly associate with response to antifungal therapy [32]. Although in vitro resistance predicts treatment failure in patients with HIV infection with oropharyngeal or esophageal candidiasis [33], no such correlation has been replicated in other settings [34]. The discordance between in vivo and in vitro data is illustrated by the "90–60 rule," which maintains that infections due to susceptible strains respond to appropriate therapy in ~90% of cases, whereas infections due to resistant strains respond in ~60% of cases [32].

Another limitation of MIC determination is that MIC levels are not always the most optimal measure of resistance. Some reports have suggested that the minimum effective concentration, defined as the drug concentration at which morphological alterations of hyphal cells are detected, might be a more appropriate end point than MIC for testing the susceptibility of filamentous fungi to echinocandins [35]. Furthermore, with mold infections, antifungal exposure detects activity against conidia rather than activity against the more clinically relevant hyphal structures.

RESISTANCE TO AZOLE COMPOUNDS

Mechanism of Action of Azoles

Azoles exert their action by inhibiting the C14α demethylation of lanosterol in fungi, which interferes with the synthesis of ergosterol in the fungal cell membrane. Azoles differ in their affinities to their target, which may account for differences in their spectrum of activity (i.e., in the primary resistance profiles of various fungi) [36]. In addition, variations in the structure of azoles are thought to be responsible for the cross-resistance patterns among *Candida* species [37–41]. For example, although complete cross-resistance between the triazoles has been observed with *Candida glabrata*, no such pattern exists with *C. krusei* [42].

Epidemiology of Azole Resistance

Prior to the introduction of antiretroviral therapy, there was an increase in the prevalence of fluconazole-resistant *C. albicans* among HIV-infected patients with oropharyngeal or esophageal candidiasis. Widespread use of itraconazole and fluconazole is thought to have been the major driver of azole resistance [43]. Up to one-third of patients with advanced AIDS in one study harbored fluconazole-resistant *C. albicans* in their oral cavities [44]. Azole-resistant *C. albicans* is less common among patients with other diseases, such as vaginal candidiasis [45] and candidemia [46]. In general, the rates of azole resistance among the most commonly encountered invasive *Candida* species remain low, with reported rates of 1.0%–2.1% in *C. albicans,* 0.4%–4.2% in *Candida parapsilosis,* and 1.4%–6.6% in *Candida tropicalis* [47, 48]. A clear exception is *C. glabrata,* which is second to *C. albicans* in causing systemic fungal infections in the United States [47]. According to data from the ARTEMIS Global Antifungal Surveillance Program, the incidence of fluconazole resistance in *C. glabrata* increased from 7% in 2001 to 12% in 2004 [49]. In addition to the changing trends in antifungal susceptibility, there has been a recent shift towards more infections in the immunocompromised host being caused by *Candida* species other than *C. albicans* [50, 51]. Several studies have initially incriminated the environmental pressure

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imposed by exposure to fluconazole [52, 53]. However, such a temporal association has not been consistently demonstrated [54, 55]. Other factors, such as exposure to antibacterial agents, immunosuppressive therapy, and the underlying medical condition of the host, might prove to be better predictors of the distribution of *Candida* species than fluconazole use [56, 57].

Mechanisms of Azole Resistance

Four major mechanisms of resistance to azoles have been described in *Candida* species. More than 1 mechanism can be functioning in any given fungal strain with additive effects.

Decreased drug concentration. The development of active efflux pumps results in decreased drug concentrations at the site of action. Efflux pumps are encoded in *Candida* species by 2 gene families of transporters: the *CDR* genes of the ATP-binding cassette super family, and the *MDR* genes of the major facilitators class [58, 59]. Up regulation of *CDR1, CDR2,* and *MDR1* has been demonstrated in azole-resistant *C. albicans* [60, 61]. Other transporter genes have been detected in other *Candida* species, such as *CgCDR1* and *PDH1* in *C. glabrata* and *CdCDR1* and *CdMDR1* in *Candida dubliniensis* [62]. Whereas *CDR* gene up-regulation confers resistance to almost all azoles, *MDR*-encoded efflux pumps have a narrower spectrum specific for fluconazole.

Target site alteration. It has been demonstrated that mutations in *ERG11*, the gene encoding for the target enzyme lanosterol C14α-demethylase, prevents binding of azoles to the enzymatic site [63]. Furthermore, intrinsic resistance to fluconazole in *C. krusei* isolates has been attributed to decreased affinity of ERG11p to the drug [64]. In excess of 80 amino acid substitutions in ERG11p have been detected [65]. Different mutations can coexist in the same gene with additive effects.

Up-regulation of target enzyme. Some *Candida* isolates with reduced susceptibility to azoles have higher intracellular concentrations of ERG11p than do azole-susceptible strains [66]. The antifungal agent is, therefore, overwhelmed, and routine therapeutic concentrations can no longer effectively inhibit ergosterol synthesis. Target enzyme up-regulation can be achieved through gene amplification, increased transcription rate, or decreased degradation of the gene product. However, this mechanism is thought to contribute little to the overall resistance burden in *Candida* species, because only modest increases in enzyme levels have been described.

Development of bypass pathways. Exposure to azole compounds results in depletion of ergosterol from the fungal membrane and accumulation of the toxic product 14α-methyl-3,6-diol, leading to growth arrest. Mutation of the *ERG3* gene prevents the formation of 14α-methyl-3,6-diol from 14α-methylfecosterol [67]. Replacement of ergosterol with the latter product leads to functional membranes and negates the action of azoles on the ergosterol biosynthetic pathway. *Candida* strains with *ERG3* mutation are also resistant to polyenes, because their cell membranes are devoid of ergosterol.

Although uncommon (with the exception of fluconazole), resistance to azole compounds among *Aspergillus* species is well-recognized. The first resistance mechanism described is through reduced intracellular concentration of itraconazole caused by expression of efflux pumps [68]. The second and more prevalent mechanism relies on modification of the 14α-sterol demethylase enzyme, which is encoded by the *cyp51A* and *cyp51B* genes [69, 70]. In particular, amino acid substitutions at the M220 position are associated with a resistance phenotype with elevated MICs to all azoles, whereas substitutions at G54 result in cross-resistance to itraconazole and posaconazole. Recently, a new mechanism of azole resistance in *Aspergillus fumigatus* has been described, where mutations in the promoter region of *cyp51A* lead to overexpression of the protein product [71]. Continued surveillance for azole resistance and the magnitude of cross-resistance between azoles among *Aspergillus* species is warranted, especially with the increasing use of new-generation azoles for the prevention and treatment of invasive aspergillosis.

It should be additionally noted that the activity of azoles against emerging fungal pathogens, such as zygomycetes and *Fusarium* and *Scedosporium* species, is variable. Although fluconazole consistently lacks activity against these organisms, new-generation azoles possess variable activity, highlighting the importance of relying on susceptibility testing to guide directed antifungal therapy.

RESISTANCE TO POLYENES

Mechanism of action of polyenes. Polyenes (amphotericin B deoxycholate and its lipid-associated formulations) act by inserting into the fungal membrane in close association with ergosterol. The subsequent formation of porin channels leads to loss of transmembrane potential and impaired cellular function.

Epidemiology of polyene resistance. Although resistance to amphotericin B among *Candida* strains remains rare [72], there have been recent reports of increasing MICs to amphotericin B among *C. krusei* and *C. glabrata* isolates [73]. In addition, intrinsic polyene resistance is frequently noted in *Candida lusitaniae* [74] and *Trichosporon beigelii* [75]. However, identification of polyene-resistant isolates has been difficult to reproduce [76, 77]. Filamentous fungi are more likely than yeasts to have reduced susceptibility to polyenes. Among *Aspergillus* species, *Aspergillus terreus* is generally resistant to amphotericin B [78]. Polyene resistance is increasingly encountered in other *Aspergillus* species, such as *Aspergillus flavus* and even *A. fumigatus,* which traditionally exhibits the highest susceptibility to amphotericin B [78, 79]. According to the SENTRY program, the prevalence of polyene resistance among *Aspergillus* species

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has increased remarkably, with only 11.5% of *A. fumigatus* isolates inhibited at ⩽1 μg/mL [79]. However, large longitudinal studies are lacking. In addition, rare *Aspergillus* species, such as *Aspergillus ustus* or *Aspergillus lentulus,* which are relatively resistant to most antifungal agents, have been reported to cause invasive disease [17, 80]. Other molds, such as *Scedosporium apiospermum, Scedosporium prolificans,* and *Fusarium* species, are typically resistant to amphotericin B [78].

Mechanisms of polyene resistance. Resistance breakpoints for polyenes have not been determined. Most clinicians use an MIC of ⩾1.0 μg/mL to indicate resistance to amphotericin B. Defects in the *ERG3* gene involved in erogosterol biosynthesis lead to accumulation of other sterols in the fungal membrance. Consequently, polyene-resistant *Candida* and *Cryptococcus* isolates have relatively low ergosterol content, compared with that of polyene-susceptible isolates [81]. Resistance to amphotericin B may also be mediated by increased catalase activity, with decreasing susceptibility to oxidative damage [82].

RESISTANCE TO ECHINOCANDINS

Echinocandins inhibit the synthesis of β-1,3-D glucan, which is integral to the structure and function of the fungal cell wall. The formation of a defective cell wall leads to cell rupture in yeasts and aberrant hyphal growth in molds. Echinocandins are highly effective against *Candida* and *Aspergillus* species, but they have no activity against zygomycetes or against *Cryptococcus, Trichosporon, Scedosporium,* and *Fusarium* species [12]. Among the *Candida* species, *C. parapsilosis* and *Candida guilliermondii* isolates have higher MIC values than do *C. albicans* isolates, although the clinical significance of this reduced in vitro susceptibility has been debated [83]. Nonetheless, breakthrough infections with *C. parapsilosis* have been reported in patients receiving echinocandins for other indications [84]. Of note, optimal detection of echinocandin resistance requires variation from the Clinical and Laboratory Standards Institute methodology [85].

The mechanisms of echinocandin resistance are still being investigated. In *Candida* species, secondary resistance is associated with point mutations in the *Fks1* gene of the β-1,3-D-glucan synhase complex [86]. Within *Fks1* lies a highly conserved region where several mutations have been identified, mostly at the Ser645 position. On the other hand, the mechanism of resistance in *C. neoformans* is not completely understood. Possibilities include an echinocandin-resistant β-1,3-D-glucan synthase target, efflux pumps, and degradation pathways [87].

It has been observed that echinocandin-susceptible *Candida* and *Aspergillus* isolates have the ability to grow in vitro at concentrations exceeding the MICs of caspofungin [88]. This paradoxical phenomenon, which is referred to as the "eagle effect," is strain-dependent and has been related to up-regulation of chitin synthesis in the fungal cell wall [89]. However, its in vivo consequences have not been fully determined, and the highest treatment doses of echinocandins have not yet shown this phenomenon in humans [90].

The burden of echinocandin resistance is still poorly appreciated. There have been recent reports of echinocandin resistance in patients with *Candida* infections (due to *C. albicans, C. glabrata, C. krusei,* and *C. parapsilosis*) [91–94]. Infections ranged from esophageal candidiasis to prosthetic valve endocarditis. In all of the described cases, resistance to echinocandins developed during therapy and was associated with treatment failure. Resistance mechanisms other than *Fks1* mutations were involved in some cases [94].

RESISTANCE TO FLUCYTOSINE

Flucytosine is a base pyrimidine analog that inhibits cellular DNA and RNA synthesis. Some yeast strains are intrinsically resistant to flucytosine because of impaired cellular uptake secondary to a mutation in cytosine permease. On the other hand, acquired resistance results from defects in flucytosine metabolism through mutations in cytosine deaminase or uracil phosphoribosyl transferase. The prevalence of primary resistance to flucytosine remains low (1%–2% among *Candida* isolates [53] and <2% among *C. neoformans* isolates [95]). However, the speed at which these yeasts can develop resistance to flucytosine has prompted clinicians to use flucytosine only in combination with other antifungal agents, mainly amphotericin B [96].

CLINICAL RESISTANCE

Recent therapeutic trials in invasive mycoses have found that overall (empirical and directed) treatment success rates have ranged from 32% to 74% [83, 97, 98], indicating that in vitro susceptibility testing alone is not sufficient to predict clinical success. The majority of patients with invasive mycoses experience treatment failure because of clinical resistance, which is a concept critical to the outcome of a fungal infection. Clinical resistance can occur under several circumstances (table 2).

First, an incorrect diagnosis can be categorized into 2 areas: (1) with the use of empirical and preemptive strategies, treatment failure may relate to another diagnosis or multiple pathogens; (2) in patients receiving cytotoxic therapy, monoclonal antibodies, antiretroviral therapy, and other immune modulating therapies, the immune reconstitution inflammatory syndrome can dominate the clinical response to antifungal therapy. Because it is associated with prominent signs and symptoms of inflammation, immune reconstitution inflammatory syndrome can be confused with failure to control fungal growth [99].

Second, the net state of immunosuppression is often so negative that antifungals cannot overcome the severe immunodeficiencies [100]. For instance, marrow failure and prolonged

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Table 2. Principal factors determining antifungal clinical resistance.

Factor	Implication
Wrong diagnosis	Weak diagnostics and/or IRIS
Net state of immunosuppression	Improvement in immunity of host is essential
High burden of fungus at initiation of treatment	Earlier treatment intervention improves outcome
Strain acquisition of increased virulence	Probably less of a problem than host factors but can be measured
Pharmacokinetics and/or pharmacodynamics	Drug toxicity, drug–drug interaction, drug levels
Site of infection	Drug penetration, tissue necrosis, foreign body
Length of treatment and/or compliance	Precision is not certain; patient and clinician may lose focus on long-term drug administration
Underlying disease	Final arbitrator in most invasive mycoses

NOTE. IRIS, immune reconstitution inflammatory syndrome.

neutropenia are difficult to overcome in the treatment of invasive aspergillosis, and specific immune modulators have not been convincingly shown to improve host immunity [101, 102].

Third, recent data support that early treatment of fungal infection with a lower burden of organisms reduces the number of treatment failures [103–105]. In some patients, the burden of the fungus is too great for antifungals to help recovery.

Fourth, some fungal strains have been found to possess more virulent characteristics than other strains, and infection with such strains might lead to worse prognosis. For example, an outbreak of *Cryptococcus gattii* infections in Vancouver, Canada, was linked to the creation of a more virulent cryptococcal strain through a recombination event in nature [106]. Most patients were immunocompetent individuals who resided in or had recently traveled to Vancouver [107]. In contrast, infections with the relatively less virulent *C. parapsilosis* can be often successfully treated with echinocandins despite reduced in vitro susceptibility [108].

Fifth, when using a polypharmacy approach to care, toxicities from polyenes (nephrotoxcitiy) and azoles (hepatitis) can be a cause of treatment failure [83, 97]. Furthermore, drug-drug interactions can contribute to morbidity and mortality [109, 110]. High flucytosine levels have always been a cause for concern regarding toxicity. As for azole compounds, the increasing use of serum drug levels has begun to emphasize the correlation between direct drug exposure and outcome [111–114]. For instance, a drug-resistant infection could result from poor drug absorption or genetic differences in the metabolism of antifungals. Therefore, it is important to be cognizant of the importance of adequate dosing, especially in the less-studied pediatric population.

Sixth, the site of infection can have a major influence on drug resistance. For example, the good penetration of fluconazole into the CSF (>70% of serum concentration [115]) makes it a better choice for treating meningitis than itraconazole (the rate of recrudescence among patients with cryptococcal meningitis after initial response to itraconazole is 20% [116]). Voriconazole, with its antifungal activity and CNS penetration, has become a first-line choice for certain CNS mold infections [117]. When the site of infection is necrotic with poor blood supply, a debulking surgery is essential to overcome antifungal treatment resistance [118]. Finally, the ability of fungi to form biofilms on foreign bodies is a primary reason for clinical failure. The echinocandins and lipid formulations of amphotericin B can impact fungal growth in biofilms better than amphotericin B and azoles [119]. However, numerous trials involving candidemia have shown that treatment failure and mortality are high among patients with catheter-related infections without removal of the catheter [120–122].

Seventh, a suboptimal length of treatment often predicts failure. Rigorously studied appropriate lengths of therapy are lacking for most mycoses. Moreover, compliance is a potential problem for success with prolonged courses of therapy.

Finally, the biggest obstruction to success is the underlying disease, which is the barometer that measures clinical success and failure. It is necessary to either prevent fungal infections in high-risk patients or successfully control the underlying disease when mycoses occur.

CONCLUSIONS

Antifungal drug resistance is a prominent feature in the management of invasive mycoses, and its epidemiological characteristics continue to evolve. Fortunately, unlike bacteria, there are no described drug resistance plasmids or transposons to amplify antifungal resistance. A principal factor in patients with serious underlying diseases is clinical resistance. This term covers less mechanistic understandings but can lead to an unfavorable outcome, and clinicians must approach patients who are experiencing treatment failure with the 8 factors of clinical resistance in mind.

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References

1. Pagano L, Caira M, Candoni A, et al. The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. Haematologica **2006**; 91:1068–75.
2. Sanz Alonso MA, Jarque Ramos I, Salavert Lleti M, Peman J. Epidemiology of invasive fungal infections due to *Aspergillus* spp. and Zygomycetes. Clin Microbiol Infect **2006**; 12(Suppl 7):2–6.
3. Maschmeyer G. The changing epidemiology of invasive fungal infections: new threats. Int J Antimicrob Agents **2006**; 27(Suppl 1):3–6.
4. Araujo R, Pina-Vaz C, Rodrigues AG. Susceptibility of environmental versus clinical strains of pathogenic *Aspergillus.* Int J Antimicrob Agents **2007**; 29:108–11.
5. Balajee SA, Nickle D, Varga J, Marr KA. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. Eukaryot Cell **2006**; 5:1705–12.
6. Christakis G, Perlorentzou S, Aslanidou M, Megalakaki A, Velegraki A. Fatal *Blastoschizomyces capitatus* sepsis in a neutropenic patient with acute myeloid leukemia: first documented case from Greece. Mycoses **2005**; 48:216–20.
7. Dannaoui E, Meletiadis J, Mouton JW, Meis JF, Verweij PE. In vitro susceptibilities of zygomycetes to conventional and new antifungals. J Antimicrob Chemother **2003**; 51:45–52.
8. Del Poeta M, Schell WA, Perfect JR. In vitro antifungal activity of pneumocandin L-743,872 against a variety of clinically important molds. Antimicrob Agents Chemother **1997**; 41:1835–6.
9. Di Bonaventura G, Pompilio A, Picciani C, Iezzi M, D'Antonio D, Piccolomini R. Biofilm formation by the emerging fungal pathogen *Trichosporon asahii*: development, architecture, and antifungal resistance. Antimicrob Agents Chemother **2006**; 50:3269–76.
10. Espinel-Ingroff A. Comparison of in vitro activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743,872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. J Clin Microbiol **1998**; 36:2950–6.
11. Espinel-Ingroff A, Chaturvedi V, Fothergill A, Rinaldi MG. Optimal testing conditions for determining MICs and minimum fungicidal concentrations of new and established antifungal agents for uncommon molds: NCCLS collaborative study. J Clin Microbiol **2002**; 40: 3776–81.
12. Espinel-Ingroff A. In vitro antifungal activities of anidulafungin and micafungin, licensed agents and the investigational triazole posaconazole as determined by NCCLS methods for 12,052 fungal isolates: review of the literature. Rev Iberoam Micol **2003**; 20:121–36.
13. Girmenia C, Pizzarelli G, D'Antonio D, Cristini F, Martino P. In vitro susceptibility testing of *Geotrichum capitatum*: comparison of the E-test, disk diffusion, and sensititre colorimetric methods with the NCCLS M27-A2 broth microdilution reference method. Antimicrob Agents Chemother **2003**; 47:3985–8.
14. Gomez-Lopez A, Garcia-Effron G, Mellado E, Monzon A, Rodriguez-Tudela JL, Cuenca-Estrella M. In vitro activities of three licensed antifungal agents against spanish clinical isolates of *Aspergillus* spp. Antimicrob Agents Chemother **2003**; 47:3085–8.
15. Johnson EM, Szekely A, Warnock DW. In-vitro activity of voriconazole, itraconazole and amphotericin B against filamentous fungi. J Antimicrob Chemother **1998**; 42:741–5.
16. Meletiadis J, Meis JF, Mouton JW, Rodriquez-Tudela JL, Donnelly JP, Verweij PE. In vitro activities of new and conventional antifungal agents against clinical *Scedosporium* isolates. Antimicrob Agents Chemother **2002**; 46:62–8.
17. Panackal AA, Imhof A, Hanley EW, Marr KA. *Aspergillus ustus* infections among transplant recipients. Emerg Infect Dis **2006**; 12:403–8.
18. Pfaller MA, Marco F, Messer SA, Jones RN. In vitro activity of two echinocandin derivatives, LY303366 and MK-0991 (L-743,792), against clinical isolates of *Aspergillus, Fusarium, Rhizopus,* and other filamentous fungi. Diagn Microbiol Infect Dis **1998**; 30:251–5.
19. Pfaller MA, Messer SA, Hollis RJ, Jones RN. Antifungal activities of posaconazole, ravuconazole, and voriconazole compared to those of itraconazole and amphotericin B against 239 clinical isolates of *Aspergillus* spp. and other filamentous fungi: report from SENTRY Antimicrobial Surveillance Program, 2000. Antimicrob Agents Chemother **2002**; 46:1032–7.
20. Rodriguez-Tudela JL, Diaz-Guerra TM, Mellado E, et al. Susceptibility patterns and molecular identification of *Trichosporon* species. Antimicrob Agents Chemother **2005**; 49:4026–34.
21. Safdar A. Progressive cutaneous hyalohyphomycosis due to *Paecilomyces lilacinus:* rapid response to treatment with caspofungin and itraconazole. Clin Infect Dis **2002**; 34:1415–7.
22. Singh J, Rimek D, Kappe R. In vitro susceptibility of 15 strains of zygomycetes to nine antifungal agents as determined by the NCCLS M38-A microdilution method. Mycoses **2005**; 48:246–50.
23. Yildiran ST, Mutlu FM, Saracli MA, et al. Fungal endophthalmitis caused by *Aspergillus ustus* in a patient following cataract surgery. Med Mycol **2006**; 44:665–9.
24. Zeng J, Kamei K, Zheng Y, Nishimura K. Susceptibility of *Pseudallescheria boydii* and *Scedosporium apiospermum* to new antifungal agents. Nippon Ishinkin Gakkai Zasshi **2004**; 45:101–4.
25. Alves SH, Lopes JO, Costa JM, Klock C. Development of secondary resistance to fluconazole in *Cryptococcus neoformans* isolated from a patient with AIDS. Rev Inst Med Trop Sao Paulo **1997**; 39:359–61.
26. Marichal P, Koymans L, Willemsens S, et al. Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans.* Microbiology **1999**; 145(Pt 10):2701–13.
27. NCCLS. Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard. NCCLS document M27-A2. Wayne, PA: NCCLS, **2002**.
28. NCCLS. Reference method for broth dilution antifungal susceptibility testing of conidial-forming filamentous fungi: approved standard. NCCLS M38–A. Wayne, PA: NCCLS, **2002**.
29. NCCLS. Reference method for antifungal disk diffusion susceptibility testing of yeasts: approved guideline. NCCLS document M44–A. Wayne, PA: NCCLS, **2004**.
30. Chryssanthou E, Cuenca-Estrella M. Comparison of the EUCAST-AFST broth dilution method with the CLSI reference broth dilution method (M38–A) for susceptibility testing of posaconazole and voriconazole against *Aspergillus* spp. Clin Microbiol Infect **2006**; 12: 901–4.
31. Espinel-Ingroff A, Barchiesi F, Cuenca-Estrella M, et al. International and multicenter comparison of EUCAST and CLSI M27-A2 broth microdilution methods for testing susceptibilities of *Candida* spp. to fluconazole, itraconazole, posaconazole, and voriconazole. J Clin Microbiol **2005**; 43:3884–9.
32. Rex JH, Pfaller MA. Has antifungal susceptibility testing come of age? Clin Infect Dis **2002**; 35:982–9.
33. Walsh TJ, Gonzalez CE, Piscitelli S, et al. Correlation between in vitro and in vivo antifungal activities in experimental fluconazole-resistant oropharyngeal and esophageal candidiasis. J Clin Microbiol **2000**; 38: 2369–73.
34. Lionakis MS, Lewis RE, Chamilos G, Kontoyiannis DP. *Aspergillus* susceptibility testing in patients with cancer and invasive aspergillosis: difficulties in establishing correlation between in vitro susceptibility data and the outcome of initial amphotericin B therapy. Pharmacotherapy **2005**; 25:1174–80.
35. Kurtz MB, Heath IB, Marrinan J, Dreikorn S, Onishi J, Douglas C. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-beta-D-glucan synthase. Antimicrob Agents Chemother **1994**; 38:1480–9.
36. Xiao L, Madison V, Chau AS, Loebenberg D, Palermo RE, McNicholas PM. Three-dimensional models of wild-type and mutated forms of cytochrome P450 14alpha-sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding. Antimicrob Agents Chemother **2004**; 48:568–74.
37. Cuenca-Estrella M, Gomez-Lopez A, Mellado E, Buitrago MJ, Monzon A, Rodriguez-Tudela JL. Head-to-head comparison of the activ-

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ities of currently available antifungal agents against 3,378 Spanish clinical isolates of yeasts and filamentous fungi. Antimicrob Agents Chemother **2006**; 50:917–21.

38. Nguyen MH, Yu CY. Voriconazole against fluconazole-susceptible and resistant *Candida* isolates: in-vitro efficacy compared with that of itraconazole and ketoconazole. J Antimicrob Chemother **1998**; 42: 253–6.
39. Pfaller MA, Messer SA, Boyken L, et al. In vitro activities of voriconazole, posaconazole, and fluconazole against 4,169 clinical isolates of *Candida* spp. and *Cryptococcus neoformans* collected during 2001 and 2002 in the ARTEMIS global antifungal surveillance program. Diagn Microbiol Infect Dis **2004**; 48:201–5.
40. Pfaller MA, Messer SA, Boyken L, et al. Cross-resistance between fluconazole and ravuconazole and the use of fluconazole as a surrogate marker to predict susceptibility and resistance to ravuconazole among 12,796 clinical isolates of *Candida* spp. J Clin Microbiol **2004**; 42: 3137–41.
41. Pfaller MA, Messer SA, Boyken L, et al. Use of fluconazole as a surrogate marker to predict susceptibility and resistance to voriconazole among 13,338 clinical isolates of *Candida* spp. tested by Clinical and Laboratory Standards Institute–recommended broth microdilution methods. J Clin Microbiol **2007**; 45:70–5.
42. Pfaller MA, Diekema DJ. Azole antifungal drug cross-resistance: resistance mechanisms, epidemiology, and clinical significance. J Invasive Fungal Infect 2007; 1:74–92.
43. Goldman M, Cloud GA, Smedema M, et al. Does long-term itraconazole prophylaxis result in in vitro azole resistance in mucosal *Candida albicans* isolates from persons with advanced human immunodeficiency virus infection? The National Institute of Allergy and Infectious Diseases Mycoses study group. Antimicrob Agents Chemother **2000**; 44:1585–7.
44. Law D, Moore CB, Wardle HM, Ganguli LA, Keaney MG, Denning DW. High prevalence of antifungal resistance in *Candida* spp. from patients with AIDS. J Antimicrob Chemother **1994**; 34:659–68.
45. Ribeiro MA, Paula CR, John R, Perfect JR, Cox GM. Phenotypic and genotypic evaluation of fluconazole resistance in vaginal *Candida* strains isolated from HIV-infected women from Brazil. Med Mycol **2005**; 43:647–50.
46. Sanglard D, Odds FC. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis **2002**; 2:73–85.
47. Pfaller MA, Diekema DJ. Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of *Candida.* Clin Microbiol Infect **2004**; 10(Suppl 1):11–23.
48. Pfaller MA, Diekema DJ, Rinaldi MG, et al. Results from the ARTEMIS DISK Global Antifungal Surveillance Study: a 6.5-year analysis of susceptibilities of *Candida* and other yeast species to fluconazole and voriconazole by standardized disk diffusion testing. J Clin Microbiol **2005**; 43:5848–59.
49. Pfaller MA, Diekema DJ, Sheehan DJ. Interpretive breakpoints for fluconazole and *Candida* revisited: a blueprint for the future of antifungal susceptibility testing. Clin Microbiol Rev **2006**; 19:435–47.
50. Hajjeh RA, Sofair AN, Harrison LH, et al. Incidence of bloodstream infections due to *Candida* species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. J Clin Microbiol **2004**; 42:1519–27.
51. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev **2007**; 20:133–63.
52. Abi-Said D, Anaissie E, Uzun O, Raad I, Pinzcowski H, Vartivarian S. The epidemiology of hematogenous candidiasis caused by different *Candida* species. Clin Infect Dis **1997**; 24:1122–8.
53. Price MF, LaRocco MT, Gentry LO. Fluconazole susceptibilities of *Candida* species and distribution of species recovered from blood cultures over a 5-year period. Antimicrob Agents Chemother **1994**; 38:1422–4.
54. Baran J Jr, Muckatira B, Khatib R. Candidemia before and during the fluconazole era: prevalence, type of species and approach to treatment in a tertiary care community hospital. Scand J Infect Dis **2001**; 33:137–9.
55. Blot S, Janssens R, Claeys G, et al. Effect of fluconazole consumption on long-term trends in candidal ecology. J Antimicrob Chemother **2006**; 58:474–7.
56. Lin MY, Carmeli Y, Zumsteg J, et al. Prior antimicrobial therapy and risk for hospital-acquired *Candida glabrata* and *Candida krusei* fungemia: a case-case-control study. Antimicrob Agents Chemother **2005**; 49:4555–60.
57. Malani A, Hmoud J, Chiu L, Carver PL, Bielaczyc A, Kauffman CA. *Candida glabrata* fungemia: experience in a tertiary care center. Clin Infect Dis **2005**; 41:975–81.
58. Albertson GD, Niimi M, Cannon RD, Jenkinson HF. Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. Antimicrob Agents Chemother **1996**; 40:2835–41.
59. Sanglard D, Ischer F, Monod M, Bille J. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. Microbiology **1997**; 143(Pt 2):405–16.
60. Sanglard D, Kuchler K, Ischer F, Pagani JL, Monod M, Bille J. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob Agents Chemother **1995**; 39:2378–86.
61. White TC. Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrob Agents Chemother **1997**; 41:1482–7.
62. Sanglard D, Bille J. Current understanding of the modes of action of and resistance mechanisms to conventional and emerging antifungal agents for treatment of *Candida* infections. In: Calderone RA, ed. *Candida* and candidiasis. Washington, DC: ASM Press, **2002**:349–83.
63. Loffler J, Kelly SL, Hebart H, Schumacher U, Lass-Florl C, Einsele H. Molecular analysis of cyp51 from fluconazole-resistant *Candida albicans* strains. FEMS Microbiol Lett **1997**; 151:263–8.
64. Orozco AS, Higginbotham LM, Hitchcock CA, et al. Mechanism of fluconazole resistance in *Candida krusei.* Antimicrob Agents Chemother **1998**; 42:2645–9.
65. Sanglard D, Ischer F, Koymans L, Bille J. Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob Agents Chemother **1998**; 42:241–53.
66. Lopez-Ribot JL, McAtee RK, Lee LN, et al. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob Agents Chemother **1998**; 42:2932–7.
67. Kelly SL, Lamb DC, Kelly DE, et al. Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol delta5,6-desaturation. FEBS Lett **1997**; 400:80–2.
68. Slaven JW, Anderson MJ, Sanglard D, et al. Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet Biol **2002**; 36:199–206.
69. Mann PA, Parmegiani RM, Wei SQ, et al. Mutations in *Aspergillus fumigatus* resulting in reduced susceptibility to posaconazole appear to be restricted to a single amino acid in the cytochrome P450 14alpha-demethylase. Antimicrob Agents Chemother **2003**; 47:577–81.
70. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. Substitutions at methionine 220 in the 14alpha-sterol demethylase (Cyp51A) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. Antimicrob Agents Chemother **2004**; 48:2747–50.
71. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, et al. A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance

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to azole antifungals involves a combination of cyp51A alterations. Antimicrob Agents Chemother **2007**; 51:1897–904.
72. Tortorano AM, Prigitano A, Biraghi E, Viviani MA. The European Confederation of Medical Mycology (ECMM) survey of candidaemia in Italy: in vitro susceptibility of 375 *Candida albicans* isolates and biofilm production. J Antimicrob Chemother **2005**; 56:777–9.
73. Pfaller MA, Messer SA, Boyken L, Tendolkar S, Hollis RJ, Diekema DJ. Geographic variation in the susceptibilities of invasive isolates of *Candida glabrata* to seven systemically active antifungal agents: a global assessment from the ARTEMIS Antifungal Surveillance Program conducted in 2001 and 2002. J Clin Microbiol **2004**; 42:3142–6.
74. Pfaller MA, Messer SA, Hollis RJ. Strain delineation and antifungal susceptibilities of epidemiologically related and unrelated isolates of *Candida lusitaniae*. Diagn Microbiol Infect Dis **1994**; 20:127–33.
75. Walsh TJ, Melcher GP, Rinaldi MG, et al. *Trichosporon beigelii,* an emerging pathogen resistant to amphotericin B. J Clin Microbiol **1990**; 28:1616–22.
76. Rex JH, Cooper CR Jr, Merz WG, Galgiani JN, Anaissie EJ. Detection of amphotericin B–resistant *Candida* isolates in a broth-based system. Antimicrob Agents Chemother **1995**; 39:906–9.
77. Wanger A, Mills K, Nelson PW, Rex JH. Comparison of Etest and National Committee for Clinical Laboratory Standards broth macrodilution method for antifungal susceptibility testing: enhanced ability to detect amphotericin B–resistant *Candida* isolates. Antimicrob Agents Chemother **1995**; 39:2520–2.
78. Sabatelli F, Patel R, Mann PA, et al. In vitro activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. Antimicrob Agents Chemother **2006**; 50:2009–15.
79. Messer SA, Jones RN, Fritsche TR. International surveillance of *Candida* spp. and *Aspergillus* spp.: report from the SENTRY Antimicrobial Surveillance Program (2003). J Clin Microbiol **2006**; 44:1782–7.
80. Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot Cell **2005**; 4:625–32.
81. Dick JD, Merz WG, Saral R. Incidence of polyene-resistant yeasts recovered from clinical specimens. Antimicrob Agents Chemother **1980**; 18:158–63.
82. Sokol-Anderson ML, Brajtburg J, Medoff G. Amphotericin B–induced oxidative damage and killing of *Candida albicans*. J Infect Dis **1986**; 154:76–83.
83. Mora-Duarte J, Betts R, Rotstein C, et al. Comparison of caspofungin and amphotericin B for invasive candidiasis. N Engl J Med **2002**; 347: 2020–9.
84. Cheung C, Guo Y, Gialanella P, Feldmesser M. Development of candidemia on caspofungin therapy: a case report. Infection **2006**; 34: 345–8.
85. Odds FC, Motyl M, Andrade R, et al. Interlaboratory comparison of results of susceptibility testing with caspofungin against *Candida* and *Aspergillus* species. J Clin Microbiol **2004**; 42:3475–82.
86. Balashov SV, Park S, Perlin DS. Assessing resistance to the echinocandin antifungal drug caspofungin in *Candida albicans* by profiling mutations in FKS1. Antimicrob Agents Chemother **2006**; 50:2058–63.
87. Feldmesser M, Kress Y, Mednick A, Casadevall A. The effect of the echinocandin analogue caspofungin on cell wall glucan synthesis by *Cryptococcus neoformans*. J Infect Dis **2000**; 182:1791–5.
88. Stevens DA, Espiritu M, Parmar R. Paradoxical effect of caspofungin: reduced activity against *Candida albicans* at high drug concentrations. Antimicrob Agents Chemother **2004**; 48:3407–11.
89. Stevens DA, Ichinomiya M, Koshi Y, Horiuchi H. Escape of *Candida* from caspofungin inhibition at concentrations above the MIC (paradoxical effect) accomplished by increased cell wall chitin; evidence for beta-1,6-glucan synthesis inhibition by caspofungin. Antimicrob Agents Chemother **2006**; 50:3160–1.
90. Clemons KV, Espiritu M, Parmar R, Stevens DA. Assessment of the paradoxical effect of caspofungin in therapy of candidiasis. Antimicrob Agents Chemother **2006**; 50:1293–7.
91. Hakki M, Staab JF, Marr KA. Emergence of a *Candida krusei* isolate with reduced susceptibility to caspofungin during therapy. Antimicrob Agents Chemother **2006**; 50:2522–4.
92. Hernandez S, Lopez-Ribot JL, Najvar LK, McCarthy DI, Bocanegra R, Graybill JR. Caspofungin resistance in *Candida albicans:* correlating clinical outcome with laboratory susceptibility testing of three isogenic isolates serially obtained from a patient with progressive *Candida esophagitis*. Antimicrob Agents Chemother **2004**; 48:1382–3.
93. Laverdiere M, Lalonde RG, Baril JG, Sheppard DC, Park S, Perlin DS. Progressive loss of echinocandin activity following prolonged use for treatment of *Candida albicans* oesophagitis. J Antimicrob Chemother **2006**; 57:705–8.
94. Moudgal V, Little T, Boikov D, Vazquez JA. Multiechinocandin- and multiazole-resistant *Candida parapsilosis* isolates serially obtained during therapy for prosthetic valve endocarditis. Antimicrob Agents Chemother **2005**; 49:767–9.
95. Brandt ME, Pfaller MA, Hajjeh RA, et al. Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates in the United States: 1992 to 1994 and 1996 to 1998. Antimicrob Agents Chemother **2001**; 45:3065–9.
96. Hospenthal DR, Bennett JE. Flucytosine monotherapy for cryptococcosis. Clin Infect Dis **1998**; 27:260–4.
97. Herbrecht R, Denning DW, Patterson TF, et al. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med **2002**; 347:408–15.
98. van der Horst CM, Saag MS, Cloud GA, et al. Treatment of cryptococcal meningitis associated with the acquired immunodeficiency syndrome. National Institute of Allergy and Infectious Diseases Mycoses Study Group and AIDS Clinical Trials Group. N Engl J Med **1997**; 337:15–21.
99. Singh N, Perfect JR. Immune reconstitution syndrome associated with opportunistic mycoses: risk factors, pathophysiological basis and approach to management. Lancet Infect Dis **2007**; 7:395–401.
100. Kontoyiannis DP, Rubin RH. Infection in the organ transplant recipient: an overview. Infect Dis Clin North Am **1995**; 9:811–22.
101. Nemunaitis J, Shannon-Dorcy K, Appelbaum FR, et al. Long-term follow-up of patients with invasive fungal disease who received adjunctive therapy with recombinant human macrophage colony-stimulating factor. Blood **1993**; 82:1422–7.
102. Pappas PG, Bustamante B, Ticona E, et al. Recombinant interferon-gamma 1b as adjunctive therapy for AIDS-related acute cryptococcal meningitis. J Infect Dis **2004**; 189:2185–91.
103. Garey KW, Rege M, Pai MP, et al. Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. Clin Infect Dis **2006**; 43:25–31.
104. Morrell M, Fraser VJ, Kollef MH. Delaying the empiric treatment of candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. Antimicrob Agents Chemother **2005**; 49:3640–5.
105. Upton A, Kirby KA, Carpenter P, Boeckh M, Marr KA. Invasive aspergillosis following hematopoietic cell transplantation: outcomes and prognostic factors associated with mortality. Clin Infect Dis **2007**; 44:531–40.
106. Fraser JA, Giles SS, Wenink EC, et al. Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. Nature **2005**; 437:1360–4.
107. Hoang LM, Maguire JA, Doyle P, Fyfe M, Roscoe DL. *Cryptococcus neoformans* infections at Vancouver Hospital and Health Sciences Centre (1997–2002): epidemiology, microbiology and histopathology. J Med Microbiol **2004**; 53:935–40.
108. Ostrosky-Zeichner L, Kontoyiannis D, Raffalli J, et al. International, open-label, noncomparative, clinical trial of micafungin alone and in combination for treatment of newly diagnosed and refractory candidemia. Eur J Clin Microbiol Infect Dis **2005**; 24:654–61.
109. Andes D. Pharmacokinetics and pharmacodynamics of antifungals. Infect Dis Clin North Am **2006**; 20:679–97.
110. Shakeri-Nejad K, Stahlmann R. Drug interactions during therapy with

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three major groups of antimicrobial agents. Expert Opin Pharmacother **2006**; 7:639–51.
111. Denning DW, Ribaud P, Milpied N, et al. Efficacy and safety of voriconazole in the treatment of acute invasive aspergillosis. Clin Infect Dis **2002**; 34:563–71.
112. Smith J, Safdar N, Knasinski V, et al. Voriconazole therapeutic drug monitoring. Antimicrob Agents Chemother **2006**; 50:1570–2.
113. Trifilio S, Pennick G, Pi J, et al. Monitoring plasma voriconazole levels may be necessary to avoid subtherapeutic levels in hematopoietic stem cell transplant recipients. Cancer **2007**; 109:1532–5.
114. Walsh TJ, Raad I, Patterson TF, et al. Treatment of invasive aspergillosis with posaconazole in patients who are refractory to or intolerant of conventional therapy: an externally controlled trial. Clin Infect Dis **2007**; 44:2–12.
115. Mian UK, Mayers M, Garg Y, et al. Comparison of fluconazole pharmacokinetics in serum, aqueous humor, vitreous humor, and cerebrospinal fluid following a single dose and at steady state. J Ocul Pharmacol Ther **1998**; 14:459–71.
116. Denning DW, Tucker RM, Hanson LH, Hamilton JR, Stevens DA. Itraconazole therapy for cryptococcal meningitis and cryptococcosis. Arch Intern Med **1989**; 149:2301–8.
117. Lutsar I, Roffey S, Troke P. Voriconazole concentrations in the cerebrospinal fluid and brain tissue of guinea pigs and immunocompromised patients. Clin Infect Dis **2003**; 37:728–32.
118. Caillot D, Casasnovas O, Bernard A, et al. Improved management of invasive pulmonary aspergillosis in neutropenic patients using early thoracic computed tomographic scan and surgery. J Clin Oncol **1997**; 15:139–47.
119. Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob Agents Chemother **2002**; 46:1773–80.
120. Almirante B, Rodriguez D, Park BJ, et al. Epidemiology and predictors of mortality in cases of *Candida* bloodstream infection: results from population-based surveillance, Barcelona, Spain, from 2002 to 2003. J Clin Microbiol **2005**; 43:1829–35.
121. Raad I, Hanna H, Boktour M, et al. Management of central venous catheters in patients with cancer and candidemia. Clin Infect Dis **2004**; 38:1119–27.
122. Tortorano AM, Biraghi E, Astolfi A, et al. European Confederation of Medical Mycology (ECMM) prospective survey of candidaemia: report from one Italian region. J Hosp Infect **2002**; 51:297–304.

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1 recombination. We've got to move forward and discuss net
2 pens and fish meal and so I'm looking for some leadership
3 from you and other people in your situation to help get this
4 rift over with and get everybody at the table and to work out
5 a consensus so we can move the aquaculture industry forward.
6 MR. MESH: I'm more than happy to help. It's no
7 problem. I do -- none of the NGO's or environmental groups
8 have said that, you know, to manage aquaculture for organic
9 shrimp, tilapia is not compatible with their perception .
10 It's more the net pen dilemma and I'm willing to help with
11 that, but, let's not hold up the shrimp, catfish, you know,
12 tilapia leg and get it out in the market.
13 MS. CAROE: Thank you. Any other comments? Bea?
14 MS. JAMES: I appreciate your comments on the
15 natamycin and I look forward to hearing from you again when
16 601 comes up for tetracycline and streptomycin.
17 MR. MESH: Yes, ma'am.
18 MS. CAROE: Anybody else? Rich Theuer. Thank you,
19 Marty.
20 MR. THEUER: I'd like to just answer some of the
21 questions or addresses some of the issues that came up this
22 morning on natamycin and just as a slight digress and get at
23 the point when I received a call two years ago from George
24 Weston Bakeries they said they were interested in natamycin
25 on English Muffins.

1 My first question was, a preservative? You're
2 kidding. And, so, I checked the Merck index and it said it's
3 non-synthetic and therefore 600B4 does not apply maybe and
4 then I checked EFIS and said this stuff is only allowed on
5 cheese. It's not allowed on English Muffins or baked goods
6 or anything. Well, the background is that back in 1995 under
7 the reinventing government where they hit all the
8 regulations, we've tried to find nowadays and can't because
9 they they're not published anymore, the FDA Modernization Act
10 was passed that basically enabled manufacturers of materials
11 to do self-affirmation of GRAS tests and that basically
12 required them to pull together a panel of experts,
13 toxicologists, food scientists to review literature, review
14 applications and to make a self-affirmation of generally
15 recognized as safe status.
16 There is the possibility of providing that GRAS
17 report to FDA and FDA to issue a letter of non -- no problem
18 basically. In fact, there are several materials that are
19 being petitioned FOS. It went through that process because
20 it had not been described before. There is a 21 CFR 172 155
21 reference to natamycin which is the cheese and there is also
22 one for something in chicken feed.
23 So, the manufacturer, in this case, Nabisco USA
24 pulled together its board -- its expert panel. They looked
25 at it and extended the GRAS use to baked goods. And some

1 countries they don't have this procedure and I think in
2 Australia this has been specifically petitioned to the
3 government because they got to do it that way there. In the
4 United States that's not required.
5 And that letter was included with the petition and
6 there was some problem with the TAP review reflecting on it
7 and we got a letter too. It should be in the file where we
8 said there actually was a letter that said it did have the
9 GRAS -- the technical committee review it.
10 Now, the question, it's called an antimitotic which
11 means it kills mold or it keeps mold from growing. And are
12 there other antibiotics on the national list and the answer
13 is yes. There's lactic acid. Now, in the meat industry
14 people are allowed to use lactic acid as a spray on hide
15 carcasses, cold carcasses to reduce E. Coli standard plate
16 count and salmonella. It's allowed in poultry as well as in
17 beef. So, in a sense there is a precedent.
18 It doesn't have a bad name, a funny looking name,
19 but, it's the same thing. And this is why I felt it was
20 "morally good" to petition this in an attempt to get through.
21 I always thought this was mission impossible. But, mold
22 isn't good. It's a penicillium mold. If you let that mold
23 grow you're going to get traces of penicillin and I think
24 more people are sensitive to penicillin and mold than they
25 are to natamycin.

1 And, finally, I'd raise the question. This is a
2 non-synthetic material produced by soil, streptomyces
3 metolensis. It's isolated from the earth, the ground, and
4 it's using glucose type substrates. There's a foreseeable
5 possibility that it could be produced organically and, so, it
6 could be that in three years, five years, ten years there
7 will be an English Muffin with nothing in it except at the
8 end organic natamycin. Now, the question is, is it an
9 antibiotic by some people's definition? It's a preservative
10 obviously.
11 Question is, what happens then? You say you can't
12 do that. So, that's the regulatory history on it. It's
13 still confirmed as GRAS and the letter was included in the
14 petition. Thanks.
15 MS. CAROE: Thank you, Rich. Comments? Bea?
16 MS. JAMES: I just to point out that when mold
17 grows on a product it's kind of an alert to the consumer so
18 that they know that it's there.
19 MR. THEUER: That's correct.
20 MS. JAMES: And that the natamycin will not be
21 known by the consumer and I doubt it will be listed on the
22 ingredient.
23 MR. THEUER: Oh, it must be listed. It is listed
24 now in regular English Muffins. It must be listed on the
25 ingredient declaration. It's not a processing aid in the

1 definition of processing aid. It touches and goes away. At
2 the point it's manufactured, at the point it's in
3 distribution, it's on the muffin. When it dissipates over
4 time, when it goes away, is when the mold happens so it will
5 be labeled.
6 MS. JAMES: And, you know, a lot of consumers don't
7 read ingredient lists but they do look at mold and they
8 recognize not to eat something when there's mold on it and I
9 just want to voice that I believe that most consumers
10 interested in organic products, if they fully understood that
11 that was sprayed on their English Muffin, that it wouldn't be
12 favorable response.
13 MR. THEUER: I hear you. And that's why I thought
14 it was mission impossible.
15 MS. JAMES: I give you credit for trying.
16 MS. CAROE: Tracy.
17 MS. MIEDERMA: When I first saw natamycin being
18 petitioned I was confused because as a mother I have
19 definitely put natamycin drops in my children's ears and
20 immediately associate it with as being a medical antibiotic
21 and, you know, I'm also an organic consumer and now an
22 organic consumer rep on the board and I just, you know, feel
23 that I would have been stunned if I turned over a package of
24 organic English Muffins and saw that an ear drop ingredient
25 listed and so I had to speak from that personal perspective

1 as well.

2 MS. CAROE: Any other comments or questions from

3 the board? Thank you, Rich.

4 MR. THEUER: Thank you.

5 MS. CAROE: And that concludes public comment for

6 this meeting. We will take a break. It's now just after

7 8:00 in California so 11:15 we will come back. We will be

8 doing voting on policy issues, crops issues, and livestock

9 issues before lunch.

10 (Whereupon, a brief recess was taken)

11 MS. CAROE: Okay. As soon as board members are in

12 their seats we're going to start with policy and Rigo. Oh,

13 wait a second, hold one second. Before we get started with

14 the votes I would entertain the program manager to entertain

15 you. If you want to -- do you want to come up to the podium?

16 MR. BRADLEY: I would.

17 (Discussion off the record)

18 MR. BRADLEY: We had some board members that we

19 have new people on the board and this is something that we do

20 every year. The Secretary of Agriculture, Mike Johanns, is

21 very grateful for the commitment that the organic board, NOSB

22 board members make. It's a huge commitment, as you all know.

23 The regulars at this meeting the 7:30 finish up

24 that we had last night for the public comment is not

25 unprecedented. Was it eight o'clock? Eight thirty. Oh, I

Formal Recommendation by the
National Organic Standards Board (NOSB)
to the National Organic Program (NOP)

Date: **April 29, 2011**

Subject: **Streptomycin Sunset**

Chair: **Tracy Miedema**

The NOSB hereby recommends to the NOP the following:

Rulemaking Action X
Guidance Statement
Other

Statement of the Recommendation (Including Recount of Vote):

The board recommends amending §205.601 Synthetic substances allowed for use in organic crop production.(i) As plant disease control.(10) Streptomycin, for fire blight control in apples and pears only until October 21, 2014, by a vote of 13 yes, 1 no. A motion limiting it to post infection use failed by a vote of 5 yes, 9 no.

The Board expects that members of the industry will collaborate and coordinate efforts in preparing for the eventual removal of this material from the National List, specifically optimizing the use of resistant rootstocks and cultivars, preventive management methods, and the use of alternative, allowed biological and chemical controls whenever warranted.

Rationale Supporting Recommendation (including consistency with OFPA and NOP):

Based on the recommendation of the Crops Committee and testimony submitted to the Board, the Board recommends delisting streptomycin as soon as is reasonable. Testimony indicated that some time is needed for the transition. The Board also asks that the National Organic Program supports the transition to managing fire blight without antibiotics to the extent possible.

NOSB Vote:

§205.601 Synthetic substances allowed for use in organic crop production.
(i) As plant disease control.
(10) Streptomycin, for fire blight control in apples and pears only until October 21, 2014.

Moved: Nick Maravell		Second: Tina Ellor		
Yes: 13	**No: 1**	**Abstain: 0**	**Absent: 0**	**Recusal: 0**

NOSB Backup Vote to Relist:

§205.601 Synthetic substances allowed for use in organic crop production.

(i) As plant disease control.

(10) Streptomycin, for fire blight control in apples and pears.

Moved: John Foster		Second: Colehour Bondera		
Yes: 10	**No: 4**	**Abstain: 0**	**Absent: 0**	**Recusal: 0**

National Organic Standards Board
Crops Committee
2012 Sunset Recommendation
Streptomycin

April 29, 2011

List: §205.601 Synthetic substances allowed for use in organic crop production.
(i) As plant disease control.
(10) Streptomycin, for fire blight control in apples and pears only.

Summary

The Crops Committee requested, but did not receive, prior to adopting its recommendation on streptomycin, an updated Technical Review (TR), noting deficiencies in the previous reviews. The committee had a 2006 TR and a1995 Technical Advisory Panel (TAP) for streptomycin. The committee proceeded based on its own research, pending the receipt of the new TR, which was reviewed when it is received.

The antibiotic streptomycin was first approved in November 1995. Streptomycin and another antibiotic, tetracycline, were each listed with a split vote. The issue of engendering antibiotic resistance in human pathogens and in workers was raised in the 1995 TAP review. The annotation that permitted use for “fire blight control in apples and pears only” was adopted. Streptomycin antibiotics were to be reviewed again in two years, and there was to be a task force to further explore antibiotic use in fruit production.

The 1998 proposed rule would have allowed “antibiotics as pesticides.” There was public opposition to the use of antibiotics as pesticides. When the USDA published the next draft rule in early 2000, it removed the NOSB recommendations allowing streptomycin and tetracycline in order to be consistent with the prohibition of antibiotics in livestock. The two antibiotics were reinstated in the December 2000 final rule in response to comments from growers.

Thus, from the very beginning, there has been controversy over allowing these chemicals to be used in organic agriculture. The board discussion regarding the 2006 sunset included concerns about:

- Promotion of resistance in human pathogens
- Natural substitutes
- Inconsistency with the prohibition of antibiotics in livestock
- Inconsistency with organic principles
- Disagreement with the prophylactic use of antibiotics

- The Centers for Disease Control and Prevention (CDC) opposition to the use of streptomycin and tetracycline in crop production
- Failing to give an incentive for alternatives
- Reaction against organic fruit by consumers
- Possibility that antibiotics might be taken up by fruit trees
- Need for more research
- Restrictions on sales of fruit in Europe
- Disruption of the organic system.

And on the other hand,

- Lack of data showing impact on resistance in human pathogens
- Dependency of growers on the materials

Ultimately, after expressing concern and the wish that someone might petition to remove them sooner than the next sunset, the two antibiotics were renewed with a vote of 7 yes, 4 no, 1 abstention, and 2 absent.

Now the two antibiotics come to board again —streptomycin as a sunset and tetracycline as a petition to remove the annotation, the 2912 expiration date. Although the committee did not have an updated TR on streptomycin, it found that the case against streptomycin has grown stronger and that removal from 601 should be delayed no longer.

The Crops Committee was presented with evidence that streptomycin can contribute to antibiotic resistance in human pathogens when used as pesticides on plants. At the same time, additional products are available for use against fire blight. Serenade Max, Bloomtime Biological FD, BlightBan C9-1 and Blightban A506 are relatively new biological controls. Surround is a kaolin clay product that has had some success in controlling fire blight.

Furthermore, when the committee did receive the new TR, it contained the new information that streptomycin sprays can lead to detectable residues of streptomycin in apples, particularly the cores and skins.

However, most importantly, the majority of the committee believes that the first line of defense is the choice of resistant varieties and rootstocks, a concept that the committee majority believes is a critical organic principle, essential to disease or pest prevention in organic systems. Despite this, the pattern of growth in organic apple and pear varieties in certain areas of the country has been skewed toward those varieties most susceptible to fire blight. In 2010, the leading organic apple varieties grown in Washington state were Fuji, Gala, and Granny Smith and accounted for approximately 54% of organic apple acreage —all highly susceptible to fire blight. (Some other widely-planted varieties are also highly susceptible.) The leading varieties in organic pear production were Bartlett, D'Anjou, and Bosc —80% of organic pear acreage— again among the

most susceptible to fire blight. On the other hand, there are numerous apple and pear varieties that are not susceptible to fire blight.

Given the public health threat associated with antibiotic resistant, the committee majority believes that organic production should not contribute in a small or large way to antibiotic resistance. The options for new antibiotics with efficacy are eluding us as resistance continues to increase.

Similarly, the committee has been told that fire blight resistance to streptomycin in some apple production is found widely. Therefore, the committee found, streptomycin's efficacy and, as a result, essentiality cannot be established.

Prior to the board meeting, the Crops Committee voted to recommend against the continued listing of streptomycin, for fire blight control in apples and pears only.

New public comment was also considered, which resulted in changing this recommendation. The comments included the regional differences in utility of streptomycin and tetracycline. Apple and pear growers commented that they need more time. Consumer representatives commented that consumers expect organic products to be produced without antibiotics.

The Committee expects that members of the industry will collaborate and coordinate efforts in preparing for the eventual removal of this material from the National List, specifically optimizing the use of resistant rootstocks and cultivars, preventive management methods, and the use of alternative, allowed biological and chemical controls whenever warranted.

The Committee also asks that the National Organic Program supports the transition to managing fire blight without antibiotics to the extent possible.

Committee Recommendation

The Crops Committee recommends the continued listing of streptomycin as follows:
§205.601 Synthetic substances allowed for use in organic crop production.
(i) As plant disease control.
(10) Streptomycin, for fire blight control in apples and pears for post-infection use, only until October 21, 2014.

Committee Vote

Motion: Jay Feldman Second: Tina Ellor
Yes: 0 No: 5 Absent: 2 Abstain: 0 Recuse: 0

Report

DSM Food Specialties B.V.
Global Regulatory Affairs
Nutrition Cluster

Alexander Fleminglaan 1
2613 AX Delft
P.O. Box 1
2600 MA Delft
The Netherlands

RA-DLF-00070484, Version 0.1

Date
February 12th, 2015

From
Hugo Streekstra PhD

Susceptibility of fungal isolates to natamycin and other polyene antifungals – Overview based on TNO report 2012 R10746, an update of more recently published MIC values, with special reference to the occurrence of resistance and cross-resistance

Introduction

In 2012, DSM commissioned a literature report on antifungal resistance in general, and natamycin resistance in particular, from the Dutch research institute TNO (TNO, 2012). The main finding was that there have been no recent reports on polyene-induced resistance , although resistant isolates have been described. This resistance is believed to be mainly due to natural insensitivity or clinical resistance (in which the compound cannot reach the concentration required to affect the microorganism, or where the microorganism is in a growth form that is not susceptible), or – in the case of multi-resistant strains – due to selection for resistance by other classes of antifungal compounds, such as azoles.

The present report covers literature published after the literature search for the TNO report (for the search strategy: see Appendix 1). Reported MIC values for natamycin and amphotericin are summarized in the table in Appendix 2, primarily based on the most recent literature, supplemented with older literature sources.

Sensitivity

The table in Appendix 2 gives MIC values for natamycin for filamentous fungi and yeasts, with an emphasis on recent literature. Where available, values for Amphotericin B (AmB) are given as well.

The literature falls into two distinct categories. The majority uses clinical isolates, mainly from eye infections, for which natamycin is one of the possible treatment options. A much smaller category uses laboratory model organisms, for fundamental research on polyene mode of action and potential resistance mechanisms.

The results show that sensitivity towards polyene antifungals differs between fungi. In general, filamentous fungi seem less sensitive to polyenes than yeasts. However within the filamentous fungi, strains of the same species may show quite large differences in sensitivity. From the literature referenced in the Table it can be concluded that a low sensitivity does not seem to be a consequence of earlier contact with polyene drugs, but rather the results of large differences in natural sensitivity within the filamentous fungi.

Resistance and cross-resistance

In accordance with the findings of the TNO report of 2012, no reports have been found of natamycin-induced polyene resistance in the updated literature search. Although polyenes have been utilized in the clinic for decades, drug resistance is rare (Shapiro et al., 2011). On the other hand, it is possible to generate resistant mutants in the laboratory.

The primary reason why polyene antibiotics do not induce resistance during their practical use, is that they do not need to enter the cell for their activity (Ghanoum & Rice, 1999). Polyenes bind to an essential component – ergosterol – in the outer membrane (Gray et al., 2012). The primary mechanism to lower the sensitivity towards polyene antibiotics is lowering the ergosterol content of the membrane (Morace et al., 2014), or sometimes changing the sterol species in the membrane (Sheikh et al., 2013), which compromises the cell's ability to grow and compete.

On the other hand, it has been shown that laboratory-generated mutants with lower ergosterol levels do not necessarily show a lower sensitivity towards AmB (Alcazar-Fuoli & Mellado, 2013). Moreover, it has been shown that resistance to a variety of stresses, such as exposure to fluconazole or oxidative stress, can lead to AmB resistance, which is not necessarily associated with a lowered ergosterol content (Mesa-Arango et al., 2012).

Nevertheless, by exploring the chemical constraints of both polyene and sterol for achieving the inhibition effect, it is now clear that binding to ergosterol is the primary and necessary event in the working mechanism of all polyene antifungals (Gray et al., 2012). This binding to ergosterol in itself causes growth inhibition (Opekarová & Tanner, 2014), but the different polyenes may have additional modes of action, depending on their size and/or amphophilic nature. In the case of natamycin, the known effects are all associated with membrane processes, caused by the lack of functional ergosterol (te Welscher et al., 2008; 2010; 2012). In contrast, AmB seems to exert additional inhibitory effects, such as membrane pore formation, and invoking oxidative stress (Palacios et al., 2011; Mesa-Arango et al., 2012).

In the whole body of literature there is only one paper that clearly shows that laboratory-induced resistance towards polyenes (AmB and nystatin in *Candida albicans*) can lead to cross-resistance towards other polyenes, such as natamycin (Athar & Winner, 1971). This report also describes that the resistant phenotype shows the expected lower ergosterol levels, poor growth on agar plates, and loss of pathogenic potential. After repeated subculturing in the absence of selective force, the resistant phenotype was lost. The authors ascribe this to the poor growth characteristics of the resistant phenotype.

In conclusion, the risk of selecting for (cross-)resistance by the use of polyene antifungals is considered low, because of the following:

- Historically, resistance is rare, in spite of decades of use of AmB in the clinic, and natamycin in food applications;
- The primary target of polyenes (ergosterol) is an essential component of the outer membrane;
- Resistant phenotypes can be constructed or selected for under laboratory conditions;
- Resistant phenotypes have lower ergosterol levels;
- This phenotype could potentially lead to cross-resistance;
- The resistant phenotype is unable to compete with wild-type individuals, and has lost its pathogenic potential;
- The practical use of polyene antifungals has not led to the build-up of resistant populations.

The new literature is still in accordance with the conclusions drawn previously by the WHO/JECFA (2002) that the potential occurrence of resistant variants – if these occur at all - is no reason for concern, as these variants grow poorly, and are unable to compete.

References

1. Reports

EFSA (2009) Scientific opinion on the use of natamycin (E 235) as a food additive. EFSA Journal 7(12), 331-335. doi:10.2903/j.efsa.2009.1412;

TNO (2012) Literature study on antifungal resistance in general and natamycin resistance in particular. TNO report R10746;

WHO/JECFA (2002), Safety evaluation of certain food additives and contaminants; Natamycin (Pimaricin). WHO Food Additives Series, 48.

2. Scientific literature

Alcazar-Fuoli, L., Mellado, E. (2013) Ergosterol biosynthesis in *Aspergillus fumigatus*: its relevance as an antifungal target and role in antifungal drug resistance. *Frontiers in Microbiology*, 3(439), 1-6;

Alfonso E. C. (2008) Genotypic identification of Fusarium species from ocular sources: Comparison to morphologic classification and antifungal sensitivity testing (an AOS thesis). *Transactions of the American Ophthalmological Society*, 106, 227-239;

Athar, M. A., Winner, H. I. (1971). The development of resistance by *Candida* species to polyene antibiotics in vitro. *Journal of Medical Microbiology*, 4(4), 505-517;

Buzzini, P., Turchetti, B., Branda, E., Goretti, M., Amici, M., Lagneau, P.E., Scaccabarozzi, L., Bronzo, V., Moroni, P. (2008) Large-scale screening of the in vitro susceptibility of Prototheca zopfii towards polyene antibiotics. *Medical Mycology*, 46(5), 511-514;

Edelstein, S.L. , Akduman, L., Durham, B.H., Fothergill, A.W., Hsu, H.Y. (2012) Resistant Fusarium keratitis progressing to endophthalmitis. *Eye and Contact Lens*, 38(5), 331-335;

Ghannoum, M. A., & Rice, L. B. (1999). Antifungal agents: Mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clinical Microbiology Reviews*, 12(4), 501-517;

Gray, K.C., Palacios, D.S., Dailey, I., Endo, M.M., Uno, B.E., Wilcock, B.C., Burke, M.D. (2012) Amphotericin primarily kills yeast by simply binding ergosterol. *P.N.A.S. USA*, 109(7), 2234-2239;

Homa, M., Shobana, C.S., Singh, Y.R.B., Manikandan, P., Selvam, K.P., Kredics, L., Narendran, P., Vágvölgyi, C., Galgóczy, L. (2013). *Fusarium* keratitis in South India: causative agents, their antifungal susceptibilities and a rapid identification method for the *Fusarium solani* species complex. *Mycoses*, 56(5), 501-511;

Kurnatowska A, Horwatt, E. (1998) Działanie natamycyny in vitro na szczepy Candida wyodrębnione od pacjentów z kandydozą wieloogniskową (Activity of natamycine in vitro against Candida strains isolated from patients with multifocal candidosis). *Mikol. Lek.*, 5, 9-12;

Lalitha, P., Prajna, N.V., Oldenburg, C.E., Srinivasan, M., Krishnan, T., Mascarenhas, J., Vaitilingam, C.M., McLeod, S.D., Zegans, M.E., Porco, T.C., Acharya, N.R., Lietman, T.M. (2012) Organism, minimum inhibitory concentration, and outcome in a fungal corneal ulcer clinical trial. *Cornea*, 31(6), 662-667;

Lalitha, P., Shapiro, B.L., Loh, A.R., Fothergill, A.W., Prajna, N.V., Srinivasan, M., Oldenburg, C.E., Quigley, D.A., ChidAmBaram, J.D., McLeod, S.D., Acharya, N.R., Lietman, T.M. (2011) Amphotericin B and natamycin are not synergistic in vitro against *Fusarium* and *Aspergillus* spp. isolated from keratitis. *British Journal of Ophthalmology*, 95(5), 744-745;

Lalitha, P., Shapiro, B.L., Srinivasan, M., Prajna, N.V., Acharya, N.R., Fothergill, A.W., Ruiz, J., ChidAmBaram, J.D., Maxey, K.J., Hong, K.C., McLeod, S.D., Lietman, T.M. (2007). Antimicrobial susceptibility of *Fusarium*, *Aspergillus*, and other filamentous fungi isolated from keratitis. *Archives of Ophthalmology*, 125(6), 789-793;

Lalitha, P., Sun, C.Q., Prajna, N.V., Karpagam, R., Geetha, M., O'Brien, K.S., Cevallos, V., McLeod, S.D., Acharya, N.R., Lietman, T.M. (2014). In vitro susceptibility of filamentous fungal isolates from a corneal ulcer clinical trial. *American Journal of Ophthalmology*, 157(2), 318-326;

Lalitha, P., Vijaykumar, R., Prajna, N.V., Fothergill, A.W. (2008) In vitro natamycin susceptibility of ocular isolates of *Fusarium* and *Aspergillus* species: comparison of commercially formulated natamycin eye drops to pharmaceutical-grade powder. *Journal of Clinical Microbiology*, 46(10), 3477-3478;

Mesa-Arango, A.C., Scorzoni, L., Zaragoza, O. (2012) It only takes one to do many jobs: Amphotericin B as antifungal and immunomodulatory drug. *Frontiers in Microbiology*, 3(286), 1-10;

Morace, G., Perdoni, F., Borghi, E. (2014) Antifungal drug resistance in *Candida* species. *Journal of Global Antimicrobial Resistance*, 2, 254-259;

Mukherjee, P.K., Chandra, J., Yu, C., Sun, Y., Pearlman, E., Ghannoum, M.A. (2012) Characterization of *Fusarium* keratitis outbreak isolates: Contribution of biofilms to antimicrobial resistance and pathogenesis. *Investigative Ophthalmology and Visual Science.*, 53(8), 4450-4457;

Opekarová, M., Tanner, W. (2014) Membrane transport inhibition as mode of action of polyene antimycotics: recent data supported by old ones. *Food Technology & Biotechnology*, 52(1), 8-12;

Palacios, D.S., Dailey, I., Siebert, D.M., Wilcock, B.C., Burke. M.D. (2011) Synthesis-enabled functional group deletions reveal key underpinnings of amphotericin B ion channel and antifungal activities. *P.N.A.S. USA*, 108(17), 6733-6738;

Pradhan, L., Sharma, S., Nalamada, S., Sahu, S.K., Das, S., Garg, P. (2011) Natamycin in the treatment of keratomycosis: Correlation of treatment outcome and in vitro susceptibility of fungal isolates. *Indian Journal of Ophthalmology*, 59(6), 511-513;

Reuben, A., Anaissie, E., Nelson, P.E., Hashem, R., Legrand, C., Ho, D.H., Bodey, G.P. (1989) Antifungal susceptibility of 44 clinical isolates of *Fusarium* species determined by using a broth microdilution method. *Antimicrobial Agents & Chemotherapy*, 33(9), 1647-1649;

Rotowa, N.A., Shadomy, H.J., Shadomy, S. (1990) In vitro activities of polyene and imidazole antifungal agents against unusual opportunistic pathogens. *Mycoses*, 33(4), 203-211;

Shapiro, B.L., Lalitha, P., Loh, A.R., Fothergill, A.W., Prajna, N.V., Srinivasan, M., Kabra, A., ChidAmBaram, J., Acharya, N.R., Lietman, T.M. (2010) Susceptibility testing and clinical outcome in fungal keratitis. *British Journal of Ophthalmology*, 94(3), 384-385;

Shapiro, R.S., Robbins, N., Cowen, L.E. (2011) Regulatory circuitry governing fungal development, drug resistance, and disease. *Microbiology & Molecular Biology Reviews*, 75(2), 213-267;

Sheikh, N., Jahagirdar, V., Kothadia, S., Nagoba, B. (2013) Antifungal drug resistance in *Candida* species. *European Journal of General Medicine*, 10(4), 254-258;

Sun, C.Q., Lalitha, P., Prajna, N. V., Karpagam, R., Geetha, M., O'Brien, K.S., Oldenburg, C.E., Ray, K.J., McLeod, S.D., Acharya, N.R., Lietman, T.M. (2014). Association between in vitro susceptibility to natamycin and voriconazole and clinical outcomes in fungal keratitis. *Ophthalmology*, 121(8), 1495-1500;

Stern, G.A. (1978) In vitro antibiotic synergism against ocular fungal isolates. *American Journal of Ophthalmology*, 86, 359-367;

te Welscher, Y.M., ten Napel, H.H., Balagué, M.M., Souza, C.M., Riezman, H., de Kruijff, B., Breukink, E. (2008) Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *Journal of Biological Chemistry*, 283(10), 6393-6401;

te Welscher, Y. M., Jones, L., van Leeuwen, M. R., Dijksterhuis, J., de Kruijff, B., Eitzen, G., Breukink, E. (2010). Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol. *Antimicrobial Agents and Chemotherapy*, 54(6), 2618-2625;

te Welscher, Y. M., van Leeuwen, M. R., de Kruijff, B., Dijksterhuis, J., & Breukink, E. (2012). Polyene antibiotic that inhibits membrane transport proteins. *P.N.A.S. USA*, 109(28), 11156-11159;

Xu, Y., Pang, G., Gao, C., Zhao, D., Zhou, L., Sun, S., & Wang, B. (2009). In vitro comparison of the efficacies of natamycin and silver nitrate against ocular fungi. *Antimicrobial Agents and Chemotherapy*, 53(4), 1636-1638;

Xu, Y., Pang, G., Zhao, D., Gao, C., Zhou, L., Sun, S., & Wang, B. (2010). In vitro activity of thimerosal against ocular pathogenic fungi. *Antimicrobial Agents and Chemotherapy*, 54(1), 536-539;

Zhang, X., Sun, X. , Wang, Z., Zhang, Y., Hou, W. (2012) Keratitis-associated fungi form biofilms with reduced antifungal drug susceptibility. *Investigative Ophthalmology and Visual Science*, 53(12), 7774-7778.

Appendix 1: Search Strategy for Updating the TNO Literature Search of 2012

In 2012, DFS requested the Dutch Research Institute TNO to perform a literature study on the effect of natamycin on fungi, with an emphasis on published literature on natamycin resistance. The literature on the subject was obtained through an independent library search. The results have been published in report TNO 2012 R 10746 '*Literature study on antifungal resistance in general and natamycin resistance in particular*'. More details on the scope of the independent library search are given in this report.
In January 2015 an in-house Patent Information Professional of DSM Intellectual Property has updated the TNO literature search with an open-literature search with a scope similar to the scope of the search disclosed in the 2012 TNO-report.

The first part of the update concerns a search in STN covering the clusters: BioScience, Food, Chemistry, Medicine, Pharmacology. This part of the search covers non-patent-literature published from the year 2012 until present. The search terms used for the literature included:

Natamycin: Natamycin*, Natamicin*, Pimaricin*, Natamax, Delvocid, Pimafucin*
Resistance: Resist*, Tolera*, Sensitiv*, Crossresist*

This search has been performed on January 15, 2015. The results from this search have been screened for occurrence of terms referring to resistance to natamycin or cross-resistance induced by natamycin.
Supplementary searches in Scopus and PubMed have been conducted on January 26, 2015. The scope and timeframe of these searches are similar to scope and timeframe used for the STN search.

For selecting the literature for reviewing, the criteria disclosed in the 2012 TNO-report have been followed:

- Its reference to antimycotics in combination with resistance or failing inhibition.
- Its reference to cross resistance of polyene antimycotics and other antimycotics.
- Nature of antibiotic drug resistance
- Nature of acquisition of antibiotic drug resistance
- Recent review papers on natamycin

Information on the databases contained by the four clusters can be found here:
http://www.stn-international.de/clusters.html

* Indicates position for truncation of search terms. The appropriate truncation symbols (not necessarily asterisks) have been used in the different databases.

Appendix 2: MIC Values for natamycin and Amphotericin B

Genus	Species	Natamycin (mg/ml)				Amphotericin B (mg/ml)				Reference
		MIC avg	MIC_{50}	MIC_{90}	MIC range	MIC avg	MIC_{50}	MIC_{90}	MIC range	
Filamentous fungi										
Acremonium	implicatum				2-4					Zhang et al, 2012
Acremonium	sp.	6.3			4-16					Pradhan et al., 2011
Acremonium	sp.	2.1	2	4	1-4	5.7	32	>32	<0.13->32	Rotowa et al., 1990
Acremonium	sp.	2	2	2	1-2	4.9	4	32	<0.13->32	Rotowa et al., 1990
Acremonium	sp.				4					Shapiro et al., 2010
Alternaria	alternata		4	4						Homa et al., 2013
Alternaria	alternata		4	4	2-8		0.125	4	0.0625-2	Xu et al., 2010
Alternaria	sp.		2		2-2					Lalitha et al., 2014
Alternaria	sp.		2		2-2					Sun et al., 2014
Aspergillus	flavus		32	64	8-64					Lalitha et al., 2008
Aspergillus	flavus		32	32						Lalitha et al., 2012
Aspergillus	flavus		32	64	2-64					Lalitha et al., 2014
Aspergillus	flavus	19			8-32					Pradhan et al., 2011
Aspergillus	flavus		32	64	16-64					Shapiro et al., 2010
Aspergillus	flavus		32	64	8-64					Sun et al., 2014
Aspergillus	flavus		32	32	8-32		2	2	1-32	Xu et al., 2009, 2010
Aspergillus	fumigatus		4	4	1-4					Lalitha et al., 2008
Aspergillus	fumigatus		4	8						Lalitha et al., 2012
Aspergillus	fumigatus		4	64	2-64					Lalitha et al., 2014
Aspergillus	fumigatus	3.9			2-8					Pradhan et al., 2011
Aspergillus	fumigatus				8-8					Shapiro et al., 2010
Aspergillus	fumigatus	3.12			3.12	3.12			3.12	Stern, 1978
Aspergillus	fumigatus		4		2-64					Sun et al., 2014
Aspergillus	fumigatus		4	4	4-32		1	2	0.5-4	Xu et al., 2009, 2010
Aspergillus	niger				1-4					Lalitha et al., 2008

Genus	Species	Natamycin (mg/ml)				Amphotericin B (mg/ml)				Reference
		MIC avg	MIC_{50}	MIC_{90}	MIC range	MIC avg	MIC_{50}	MIC_{90}	MIC range	
Aspergillus	niger		5		2-8					Lalitha et al., 2014
Aspergillus	niger		2							Pradhan et al., 2011
Aspergillus	niger				8-32					Shapiro et al., 2010
Aspergillus	niger	2.34			1.56-3.12	4.3			2.34-6.25	Stern, 1978
Aspergillus	niger		5		2-8					Sun et al., 2014
Aspergillus	niger				0.25-4					Xu et al., 2009
Aspergillus	oryzae		32	32	4-32		1	2	1-2	Xu et al., 2009, 2010
Aspergillus	sp.		32	>32			2	4		Lalitha et al., 2007
Aspergillus	sp.		16	16						Lalitha et al., 2011
Aspergillus	sp.		32	32						Lalitha et al., 2012
Aspergillus	sp.		32	64	1-64					Lalitha et al., 2014
Aspergillus	sp.		32	64	2-64					Sun et al., 2014
Aspergillus	sp.		4	32	0.25-32		1	2	0.125-2	Xu et al., 2009, 2010
Aspergillus	terreus				4-16					Lalitha et al., 2008
Aspergillus	terreus		16		8-16					Lalitha et al., 2014
Aspergillus	terreus		16							Pradhan et al., 2011
Aspergillus	terreus				8-32					Shapiro et al., 2010
Aspergillus	terreus		16		8-16					Sun et al., 2014
Aspergillus	versicolor		8	32	4-32		1	2	0.5-2	Xu et al., 2009, 2010
Bipolaris	sp.		4	4						Lalitha et al., 2012
Bipolaris	sp.	2			2-2					Lalitha et al., 2014
Bipolaris	sp.	2			2					Pradhan et al., 2011
Bipolaris	sp.				32					Shapiro et al., 2010
Bipolaris	sp.	2			2-2					Sun et al., 2014
Cladosporium	sphaerospermum				8-32					Zhang et al, 2012
Cunninghamella	sp.	2.83	2	4	2-4	5.04	8	16	0.25-16	Rotowa et al., 1990
Cunninghamella	sp.	2.83	2	4	2-4	8	16	32	0.25->32	Rotowa et al., 1990

Genus	Species	Natamycin (mg/ml)				Amphotericin B (mg/ml)				Reference
		MIC avg	MIC_{50}	MIC_{90}	MIC range	MIC avg	MIC_{50}	MIC_{90}	MIC range	
Curvularia	sp.		4	256						Lalitha et al., 2012
Curvularia	sp.		2	2	1-16					Lalitha et al., 2014
Curvularia	sp.	1.7			1-2					Pradhan et al., 2011
Curvularia	sp.				4					Shapiro et al., 2010
Curvularia	sp.		2	2	1-2					Sun et al., 2014
Exserohilum	sp.		4	4						Lalitha et al., 2012
Exserohilum	sp.	2			1-2					Lalitha et al., 2014
Exserohilum	sp.	4			4					Pradhan et al., 2011
Exserohilum	sp.	1.5			1-2					Sun et al., 2014
Fusarium	avenaceum		8	8	4-32		2	4	0.5-8	Xu et al., 2009, 2010
Fusarium	dimerum				2-8				4-64	Homa et al., 2013
Fusarium	fujikori				4->64				16->64	Homa et al., 2013
Fusarium	incarnatum-equiseti				4->64				32->64	Homa et al., 2013
Fusarium	moniliforme		4	8	4-8		2	2	1-8	Xu et al., 2009, 2010
Fusarium	oxysporum				8->64				4->64	Homa et al., 2013
Fusarium	oxysporum				4				1	Mukherjee et al, 2012
Fusarium	oxysporum				4-8					Xu et al., 2009
Fusarium	poae				4-8					Xu et al., 2009
Fusarium	solani			4.8						Alfonso, 2008
Fusarium	solani				2->64				0.125->64	Homa et al., 2013
Fusarium	solani				2-4				1	Mukherjee et al, 2012
Fusarium	solani	4.21			3.12-6.25	20.2			3.12-50	Stern, 1978
Fusarium	solani		4	8	4-32		1	2	0.5-16	Xu et al., 2009, 2010
Fusarium	solani				4-16					Zhang et al, 2012
Fusarium	sp.			4.2						Alfonso, 2008
Fusarium	multi-resistant isolate		16						4	Edelstein et al., 2012
Fusarium	sp.		8	16			4	4		Lalitha et al., 2007

Genus	Species	Natamycin (mg/ml)				Amphotericin B (mg/ml)				Reference
		MIC avg	MIC_{50}	MIC_{90}	MIC range	MIC avg	MIC_{50}	MIC_{90}	MIC range	
Fusarium	sp.		4	4	2-8					Lalitha et al., 2008
Fusarium	sp.		16	16						Lalitha et al., 2011
Fusarium	sp.		4	8						Lalitha et al., 2012
Fusarium	sp.		4	8	2-32					Lalitha et al., 2014
Fusarium	sp.	6.7			4-8					Pradhan et al., 2011
Fusarium	sp.		2	4	2-4		1	2	1-2	Reuben et al., 1989
Fusarium	sp.				3.12-6.25				0.25-50	Reuben et al., 1989
Fusarium	sp.	3.56	4	4	0.5->32	0.71	0.5	4	<0.13->32	Rotowa et al., 1990
Fusarium	sp.	3.06	2	4	1->32	1.79	2	8	<0.13->32	Rotowa et al., 1990
Fusarium	sp.		8	16	4-16					Shapiro et al., 2010
Fusarium	sp.		4	8	2-32					Sun et al., 2014
Fusarium	sp.		4	8	4-8		1	2	0.5-2	Xu et al., 2009, 2010
Lasiodiplodia	sp.		4	32						Lalitha et al., 2012
Lasiodiplodia	sp.		2		2-2					Lalitha et al., 2014
Lasiodiplodia	sp.		2		2-2					Sun et al., 2014
Penicillium	lilacinus	9.37			9.37	4.68			4.68	Stern, 1978
Penicillium	sp.	2.34			2.34	4.68			4.67	Stern, 1978
Pseudallescheria	boydii	2.1	2	4	1-4	4.64	2	>32	1->32	Rotowa et al., 1990
Pseudallescheria	boydii	1.64	2	4	1-4	4.42	2	>32	0.5->32	Rotowa et al., 1990
Rhizopus	sp.	9.37			6.25-12.5				2.34->50	Stern, 1978
Scedosporium	apiospermum				2					Pradhan et al., 2011
Scedosporium	sp.		8	8						Lalitha et al., 2012
Scopulariopsis	brevicaulis	3.12			3.12	>50			>50	Stern, 1978
Yeasts										
Candida	albicans					0.48*			0.25-1*	Athar & Winner, 1971
Candida	albicans	2.7				0.23				Gray et al., 2012

Genus	Species	Natamycin (mg/ml)				Amphotericin B (mg/ml)				Reference
		MIC avg	MIC_{50}	MIC_{90}	MIC range	MIC avg	MIC_{50}	MIC_{90}	MIC range	
Candida	albicans	3.27			1.56-4.68	0.36			0.2-0.6	Stern, 1978
Candida	krusei	1.56			1.56	1.56			1.56	Stern, 1978
Candida	parapsilosis	5.2			4.68-6.25	2.35			0.8-3.12	Stern, 1978
Candida	sp.				0.437-43.5					Kuratowska&Horwatt, 1998
Rhodotorula	sp.	2.34			2.34	6.25		6.25		Stern, 1978
Saccharomyces	cerevisiae	1.3				0.46				Gray et al., 2012
Saccharomyces	cerevisiae	1.1								te Welscher et al., 2008
Trichosporon	beigelii	3.03	2	8	2-8	0.32	0.25	0.5	<0.13-0.5	Rotowa et al., 1990
Trichosporon	beigelii	2.64	2	16	1-16	0.22	0.25	0.5	<0.13-0.5	Rotowa et al., 1990
*Numbers in Table II if the source are 1000-fold higher, which must be a mistake in view of its Table VI and common sense										
Algae										
Prototheca	zopfii		3.7	6.3	1-32					Buzzini et al., 2008

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Natamycin Inhibits Vacuole Fusion at the Priming Phase via a Specific Interaction with Ergosterol▽

Yvonne Maria te Welscher,[1]* Lynden Jones,[2] Martin Richard van Leeuwen,[3] Jan Dijksterhuis,[3] Ben de Kruijff,[1] Gary Eitzen,[2] and Eefjan Breukink[1]

Department of Biochemistry of Membranes, Bijvoet Center, Institute of Biomembranes, Utrecht University, Utrecht 3584 CH, Netherlands[1]; Department of Cell Biology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada[2]; and Applied and Industrial Mycology, CBS/Fungal Biodiversity Centre, Uppsalalaan 8, Utrecht 3584 CT, Netherlands[3]

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The antifungal antibiotic natamycin belongs to the family of polyene antibiotics. Its antifungal activity arises via a specific interaction with ergosterol in the plasma membrane (te Welscher et al., J. Biol. Chem. 283:6393–6401, 2008). However, this activity does not involve disruption of the membrane barrier function, a well-known property of other members of the polyene antibiotic family, such as filipin and nystatin. Here we tested the effect of natamycin on vacuole membrane fusion, which is known to be ergosterol dependent. Natamycin blocked the fusion of isolated vacuoles without compromising the barrier function of the vacuolar membrane. Sublethal doses of natamycin perturbed the cellular vacuole morphology, causing the formation of many more small vacuolar structures in yeast cells. Using vacuoles isolated from yeast strains deficient in the ergosterol biosynthesis pathway, we showed that the inhibitory activity of natamycin was dependent on the presence of specific chemical features in the structure of ergosterol that allow the binding of natamycin. We found that natamycin inhibited the priming stage of vacuole fusion. Similar results were obtained with nystatin. These results suggest a novel mode of action of natamycin and perhaps all polyene antibiotics, which involves the impairment of membrane fusion via perturbation of ergosterol-dependent priming reactions that precede membrane fusion, and they may point to an effect of natamycin on ergosterol-dependent protein function in general.

The increase in invasive fungal infections, especially in persons whose immune systems are compromised, is a growing threat to human health. Only a few antifungal agents have proven to be effective, including the polyenes, the fluorocytes, and the azole derivatives, but an increase in resistance has been observed for several members (14). Polyene antibiotic resistance is still a rare occurrence, which makes these antibiotics particularly useful as antifungal agents. In the past, convincing evidence has been presented that this class of antibiotics targets sterols, in particular ergosterol, the abundant and main sterol of fungal membranes. The interaction of these antibiotics with ergosterol leads to changes in the membrane that ultimately cause the destruction of the membrane barrier (3, 10, 11). Natamycin (also called pimaricin) is a very effective member of the polyene antibiotic family, with a large record of applications. Natamycin is produced by *Streptomyces natalensis* and is used for the topical treatment of fungal infections, and it is also widely utilized in the food industry. For many years, people have believed that the polyene antibiotic natamycin would kill fungi by permeabilizing the plasma membrane. Only recently have we discovered that in marked contrast to amphotericin B, filipin, or nystatin, the polyene antibiotic natamycin does not act via membrane permeabilization (32). And yet, its activity is strongly ergosterol dependent and requires a specific sterol structure (32). We aim to elucidate the mode of action of natamycin, and through a detailed understanding of its mechanism, new and improved antifungal formulations may be developed. Because of the specific interaction with ergosterol, natamycin may act via excluding ergosterol from performing important functions in the membrane.

Besides important roles in modulating membrane fluidity, regulatory processes, and domain formation, sterols also have been shown to be important during membrane fusion and fission events (6, 28, 30). Both fusion and fission are similar processes that rely on the central event of a merger or separation of two membranes. This requires a transient reorganization of membrane lipids into highly curved fusion intermediates (7). Both endocytic and exocytic pathways are dependent on the fusion and fission of membranes in which sterols have been shown to be important (12, 29). For example, by deleting different *ERG* genes in *Saccharomyces cerevisiae*, strains with altered sterol compositions are formed (17, 24). These strains show deficiencies in the endocytic process and in plasma membrane fusion (17, 19, 24). This implies that these processes are dependent on ergosterol and have specific structural requirements for the sterols present.

The fusion reaction of isolated vacuoles from yeast can be studied via a content mixing assay and has been used as a model system to examine membrane fusion reactions in general, particularly because it uses many of the same mechanisms as other fusion reactions (15, 37). Ergosterol has been shown to be required for the fusion of vacuoles, indicating the importance of ergosterol in vacuolar fusion in yeast (21, 31). Here we have used this model system to decipher the mode of action of

* Corresponding author. Mailing address: Department of Biochemistry of Membranes, Bijvoet Center, Institute of Biomembranes, Utrecht University, Padualaan 8, Utrecht 3584 CH, Netherlands. Phone: 31 30 2533345. Fax: 31 30 2533969. E-mail: y.m.tewelscher@uu.nl.
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TABLE 1. Strains used in this study

Description	Name	Genotype
Cytosol strain	K91[a]	*pho8*::*URA3 pho4*::*LEU2*
Strain used in vacuole staining	RH448[b]	*MAT***a** *his4 leu2 ura3 lys2 bar1*
WT	KTY1[c]	*pep4*::*kanMX prb1*::*LEU2*
	KTY2[c]	*pho8*::*kanMX*
*erg4*Δ	YWY1-4[d]	KTY1; *erg4*::*HIS3*
	YWY2-4[d]	KTY2; *erg4*::*HIS3*
*erg3*Δ	YWY1-3[d]	KTY1; *erg3*::*HIS3*
	YWY2-3[d]	KTY2; *erg3*::*HIS3*
*erg2*Δ	YWY1-2[d]	KTY1; *erg2*::*HIS3*
	YWY2-2[d]	KTY2; *erg2*::*HIS3*

[a] Obtained from G. Eitzen.
[b] Obtained from H. Riezman.
[c] Based on BY4742 (*his3 leu2 ura3 lys2*) and obtained from G. Eitzen (31).
[d] This study.

natamycin. Natamycin was able to inhibit the vacuolar homotypic fusion. Like the overall inhibitory effect of natamycin on yeast cells, the inhibition on vacuolar fusion was not due to membrane permeabilization. Natamycin acted at an early stage of the fusion process, even before membrane contact. This activity was dependent on the presence of specific chemical features in the structure of ergosterol and may involve an effect on protein functions that are ergosterol dependent.

MATERIALS AND METHODS

Chemicals. The polyene antibiotics nystatin and filipin were dissolved in pure dimethyl sulfoxide (DMSO), and natamycin was dissolved in DMSO-H_2O (85:15 [vol/vol]); all were obtained from Sigma Chemical (St. Louis, MO). The concentrations of the polyene antibiotics were determined spectrophotometrically on a Perkin Elmer UV/visible (UV/Vis) spectrometer (Lambda 18). The molar extinction coefficients and corresponding wavelengths of the polyene antibiotics in methanol were 7.6×10^4 M^{-1} cm^{-1} (318 nm), 6.7×10^4 M^{-1} cm^{-1} (318 nm), and 8.5×10^4 M^{-1} cm^{-1} (356 nm) for natamycin, nystatin, and filipin, respectively.

Poly-L-lysine, *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES), DEAE-dextran, Ficoll (molecular weight, 400,000), *para*-nitrophenylphosphate (*p*NPP), quinacrine, neomycin, ATP, creatine kinase, creatine phosphate, leupeptin, pepstatin, *o*-phenanthroline, Pefabloc SC, and apyrase (VI and VII) were obtained from Sigma Chemical (St. Louis, MO). MDY-64 was purchased from Molecular Probes (Eugene, OR). Antibodies against Sec18p or Vam3p were purified as IgG fractions from rabbit sera as previously described (13). All protein concentrations were measured using Bio-Rad protein assay reagents from Bio-Rad Laboratories (Richmond, CA) using bovine serum albumin as a standard.

Strains, growth conditions, and genetic modifications. Strains used for vacuole staining and isolation are listed in Table 1. Yeast cells were grown at 30°C in 10 g/liter yeast extract, 20 g/liter Bacto peptone, and 20 g/liter dextrose without (YPD) or with (YPDUAT) supplementation with 2 g/liter uracil, 1 g/liter adenine, and 1 g/liter tryptophan. *ERG* gene deletions were performed in strains KTY1 and KTY2 by homologous recombination of PCR products using primers with ~40 nucleotides of homology to the 5′ and 3′ ends of the gene of interest and 20 nucleotides of homology to the pRS 403 vector as the template (Table 2) (4).

Vacuole isolation and fusion reactions. Vacuoles were isolated, and their fusion was tested as previously described (15). Standard fusion reaction mixtures contained vacuoles isolated from two strains (3 μg protein each) either with alkaline phosphatase deleted (*pho8*Δ KTY2 parental strains) or with proteinase A and proteinase B deleted (*pep4*Δ *prb1*Δ KTY1 parental strains), 30 μl of PSS buffer [20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)-KOH, pH 6.8, 0.2 M sorbitol, 125 mM KCl, 5 mM $MgCl_2$] supplemented with 10 μM coenzyme A, a protease inhibitor cocktail (6.6 ng/ml leupeptin, 16.6 ng/ml pepstatin, 16.6 μM *o*-phenanthroline, 3.3 μM Pefabloc SC), and an ATP regenerating system (ATP_{reg}; 1 mM Mg-ATP, 0.5 mg/ml creatine kinase, 40 mM creatine phosphate) and 1 mg/ml cytosol (isolated as described previously [18]). Reactions were incubated for 90 min at 27°C and then assayed for alkaline phosphate activity. For this, 470 μl of developer solution (250 mM Tris-Cl, pH 8.5, 0.4% Triton X-100, 10 mM $MgCl_2$, 1.5 mM *p*NPP) was added to the reactions and incubated for 5 min at 30°C. The reaction was stopped by addition of 500 μl 1 M glycine-KOH, pH 11.5, and the absorption at 400 nm was determined. Fusion reactions with the strains containing *erg*Δ that are based on KTY1 and KTY2 (Table 1) were performed in a similar manner, with one exception, that no cytosol was added to these reactions.

Quinacrine uptake assay. Reaction mixtures contained vacuoles (60 μg) freshly isolated from strain KTY2 in 150 μl of PSS buffer. To have an active H^+-pumping system that allows for acidification of the vacuoles, the reactions were supplemented with ATP_{reg}. Quinacrine (200 μM) was added from a 10 mM stock in water, and polyene antibiotics were added in a concentration range from 0 to 400 μM. The reactions were incubated for 20 min at 27°C, after which the reaction mixtures were placed on ice, 1 ml of PSS buffer was added, and the vacuoles were spun down for 4 min, 14,000 rpm at 4°C (26). The pellet was resuspended in 150 μl of 0.4% Triton X-100, and the fluorescence of the quinacrine was determined (excitation at 421 nm/emission at 496 nm) using a QM-4SE spectrofluorometer with a four-position sample holder (Photon Technologies Inc., London, United Kingdom). The percentage of uptake of quinacrine at a given polyene antibiotic concentration was determined by comparing the fluorescence of vacuoles supplemented with ATP_{reg} (100%) to that of vacuoles without ATP_{reg} (0%).

Staging assay. Staging was performed as described previously (9, 26). Standard fusion reactions were started at 27°C as described above. At different time points (0, 10, 20, 30, 45, or 60 min), inhibitors of the specific stages or PS buffer (control; 20 mM PIPES-KOH, pH 6.8, 0.2 M sorbitol) were added. The inhibitor used was 4 μl (250 μg/ml) anti-Sec18 (priming) or 4 μl (150 μg/ml) anti-Vam3 (docking) or placement on ice (fusion) at the indicated times. The inhibitor effects were compared with the effects of the polyene antibiotic natamycin (200 μM), nystatin (200 μM), or filipin (100 μM). After 90 min, the amount of fusion was determined by measuring the alkaline phosphatase activity.

Vacuole staining and observation of live yeast cells. A small single colony of strain RH448 (Table 1) was grown aerobically for 16 h at 30°C in 50 ml YPUADT. Cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.3, and after 1.5 h of growth, the cells were inoculated on poly-L-lysine-coated coverslips as described in reference 35. Concentrations of 0, 0.5, 1.0, and 1.5 μM natamycin were used, and coverslips were incubated for 5 h at 25°C. Yeast cells were stained with 10 μM MDY-64 dissolved in ACES buffer (10 mM ACES, 0.02% Tween 80, pH 6.8) and incubated for 2 min, followed by an ACES buffer wash step. After removal of ACES buffer, the glass cover slides with the immobilized cells were put upside-down on top of a thin layer (<0.5 mm) of 2% agar. Images were acquired by automatic exposure at a magnification of 100×2.0 with a Zeiss Axioplan II microscope equipped with a Plan-ApoChromat 100×/1.4 oil

TABLE 2. Primers used in this study

Primer	Sequence
5′ERG4-KO	5′GATACGGATA TTTACGTAGT GTACATAGAT TAGCATCGCT AGATTGTACT GAGAGTGCAC3′
3′ERG4-KO	5′TG TAAAATAAGT TAATGAAGTG GATAGAAAAA GAAAATAA CTGTGCGGTA TTTCACACCG3′
5′ERG3-KO	5′AAAAAAGATA ATAAGAAAAA TATTCGTCTA GATTTGAGAT AGATTGTACT GAGAGTGCAC3′
3′ERG3-KO	5′TCGACCTCCT TGATGAAATG TTCAACTTCC TTAACTTGAG CTGTGCGGTA TTTCACACCG3′
5′ERG2-KO	5′TCGCTCAATC AAACTAAGAC TAGCCCAGAC CATTATAGCC AGATTGTACT GAGAGTGCAC3′
3′ERG2-KO	5′TAATGGACTA CCGCATGACT GATTTCGTGA GGTCGGGCAG CCTGTGCGGTA TTTCACACCG3′

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FIG. 1. Effect of the polyene antibiotics natamycin, nystatin, and filipin on the fusion of isolated vacuoles. (A) Structures of natamycin (I), filipin (II), and nystatin (III). (B) Vacuoles isolated from yeasts with a wild-type sterol composition (3 μg protein of KTY1 and KTY2 [each]) were incubated with different concentrations of natamycin (●), nystatin (○), and filipin (▼) in a standard fusion reaction. After 90 min, the amount of fusion was determined by measuring the alkaline phosphate activity. The experiment was performed with freshly isolated vacuoles in triplicate, and each measurement was performed in duplicate.

objective and Zeiss filter set 09 (450 to 490, FT510, LP520). Images were acquired by automatic exposure at a magnification of 100 × 2.0 with a Zeiss Axioplan II microscope equipped with a Plan-Apochromat 100×/1.4 oil objective, an additional 2× slider, and Zeiss filter set 09.

RESULTS

Effect of natamycin on fusion of isolated vacuoles. Vacuole fusion can be assayed using a content mixing assay. Vacuoles are isolated from two strains; one strain contains normal vacuole proteases but is deleted for alkaline phosphatase (ALP, the *PHO8* gene product), and the other strain is deleted for vacuolar proteases and hence bears catalytically inactive pro-ALP (Table 1). Neither population of purified vacuoles has phosphatase activity. Vacuole-to-vacuole fusion allows the proteases to gain access to the pro-ALP and convert it to the catalytically active form, which can be assayed by a colorimetric enzyme assay (15, 36). The effect of natamycin on vacuolar fusion was compared to that of two other polyene antibiotics, filipin and nystatin, and the chemical structures are given in Fig. 1A. Different concentrations of these antibiotics were added to standard fusion reactions, and the amount of fusion signal was compared to that for controls with no antibiotics (100% fusion) and incubation on ice (0% fusion) (Fig. 1B). Filipin was most efficient in inhibiting vacuole fusion, with a half-maximal inhibitory concentration (IC_{50}) of 14 μM. This was followed by nystatin, with an IC_{50} of 36 μM. The inhibition profiles of filipin and nystatin are in accordance with the profiles observed by Kato and Wickner (21). Natamycin was also able to inhibit the fusion of vacuoles, with an IC_{50} of 56 μM. The maximal amount of inhibition caused by natamycin (71% ± 2.0%) is lower than those of nystatin (96% ± 0.5%) and filipin (90% ± 7.5%).

Effect of the polyene antibiotics on vacuole permeability. The permeabilization of the vacuolar membrane could potentially explain the observed inhibition on fusion by the polyene antibiotics. Vacuolar fusion requires an intact membrane to maintain a required electrochemical potential as well as the ability to release calcium (9, 27). Although natamycin is unable to permeabilize model membranes or the plasma membrane of yeast cells (32), this does not rule out the possibility that natamycin may permeabilize the vacuolar membrane. To determine the effect of the polyene antibiotics on the permeability of the vacuole, a quinacrine assay was performed (33). Quinacrine is a fluorescent compound, known to accumulate in acidic compartments such as vacuoles. If the pH gradient of the vacuole is compromised (e.g., by permeabilization of the membrane), quinacrine will be unable to accumulate in the vacuole, resulting in a reduced fluorescence. Vacuole acidification is maintained by the vacuolar type H^+-ATPase (V-ATPase), which requires ATP (20). In Fig. 2A, the accumulation of quinacrine in purified vacuoles with or without an active H^+-pumping system, $+ATP_{reg}$ or $-ATP_{reg}$ respectively, is compared. Extended incubation with the ATP-degrading enzyme apyrase results in no uptake of quinacrine due to inhibition of V-ATPase function (Fig. 2A). Both filipin and nystatin used at 100 μM inhibited quinacrine accumulation. The quinacrine uptake is even less that that for vacuoles incubated without ATP_{reg} or added apyrase, indicating that intact vacuoles are still able to accumulate some quinacrine while vacuoles treated with filipin and nystatin do not. This is likely the result of a total loss of the membrane barrier function caused by these polyene antibiotics. Natamycin had no effect on quinacrine accumulation at this concentration. A similar picture emerged when a broader range of polyene concentrations was used. The percentage of uptake of quinacrine in treated vacuoles was determined via normalization to the quinacrine uptake in vacuoles with or without supplementation of ATP_{reg} (Fig. 2B). The results clearly show that both filipin and nystatin were able to cause membrane permeabilization at the same concentrations that inhibit fusion. Natamycin, however, did not disrupt the vacuolar membrane at any of the concentrations used. This is in agreement with the results found previously that natamycin does not permeabilize model membranes nor the yeast plasma membrane (32). These results suggest that the mode of inhibition of vacuolar fusion for natamycin is fundamentally different from that of the other polyenes.

Sterol dependency of inhibition of fusion by polyene antibiotics. Binding of natamycin to membranes is highly dependent on the presence and chemical structure of sterol molecules (32). To test whether the inhibition of vacuolar fusion by natamycin is related to the chemical structure of ergosterol, we studied the effect of natamycin on the vacuole fusion of dif-

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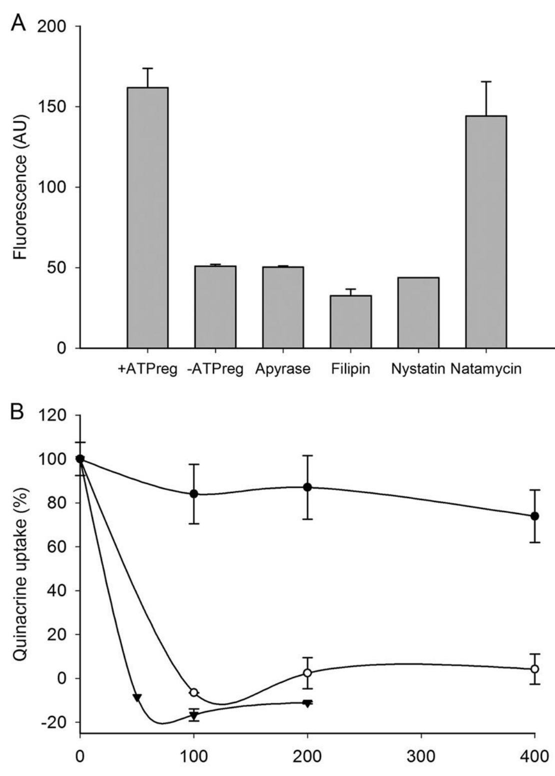

FIG. 2. Quinacrine uptake of isolated vacuoles after incubation with the polyene antibiotics. Vacuoles from the KTY2 strain (60 μg protein) were incubated with quinacrine (200 μM) and different antibiotics. After 20 min of incubation, vacuoles were reisolated, and the accumulation of quinacrine was determined by measuring the amount of fluorescence (excitation at 421 nm/emission at 496 nm). (A) The fluorescent quinacrine uptake of vacuoles incubated with or without ATP_{reg}, apyrase (VI and VII, 5 U each), or polyene antibiotics (100 μM). (B) The percentage of quinacrine uptake of vacuoles incubated with different concentrations of natamycin (●), nystatin (○), or filipin (▼). The experiment was performed with freshly isolated vacuoles in duplicate.

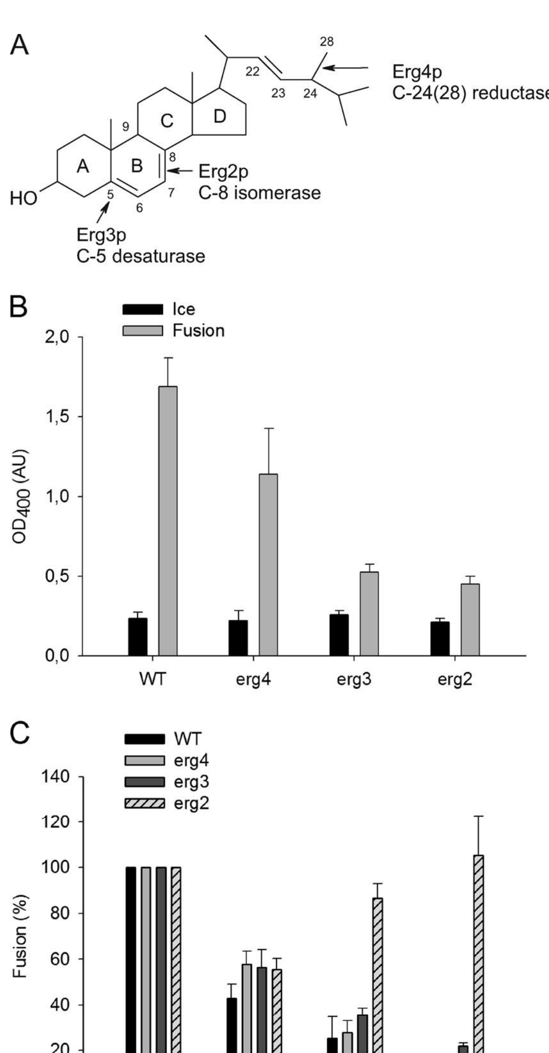

FIG. 3. Ergosterol dependency of the polyene antibiotics for vacuole fusion. (A) The structure of ergosterol together with different proteins in the ergosterol biosynthesis pathway and their functions as indicated (17). (B and C) The effect of the polyene antibiotics on the fusion of vacuoles isolated from yeast strains with different sterol compositions. (B) The fusion of vacuoles isolated from yeasts with different *ERG* deletions and *PHO8* or *PEP4* (3 μg protein each) are compared to a reaction on ice. (C) The fusion of isolated vacuoles without antibiotic corrected for ice was normalized to 100% and compared to the effect on fusion caused by the polyene antibiotics filipin (20 μM), nystatin (100 μM), and natamycin (100 μM). The experiment was performed in triplicate with freshly isolated vacuoles. No cytosol was added to these reactions.

ferent *ERG* mutant strains. This was achieved by deleting the genes of specific sterol biosynthesis proteins in the parental strains KTY1 and KTY2 (Table 1), resulting in different sterol profiles. From these strains, the vacuoles were isolated, and their fusion abilities were tested in the content mixing assay as described above. The biosynthesis proteins and their functions together with the structure of ergosterol are shown in Fig. 3A. Deletion of *ERG4* leads to changes in the tail part of the sterol, while deletion of *ERG3* or *ERG2* causes a loss of double bonds in the B-ring. We found that vacuoles isolated from the *erg4*Δ strain pairs fuse at levels comparable to those of the wild type (WT); however, vacuoles isolated from the *erg3*Δ and *erg2*Δ strain pairs showed a significant reduction in fusion (Fig. 3B). These results confirm that the fusion of vacuoles is dependent on the chemical structure of sterols present in the vacuolar membrane (21). In addition, the isolated vacuole fusion results correlate well with the extent of vacuole fragmentation previously shown by morphological analyses in the intact yeast strains (17, 21). The relatively small but reproducible amount of fusion of the isolated vacuoles from the *erg3*Δ and *erg2*Δ strain pairs allowed us to determine the sterol dependency of the polyene antibiotic fusion inhibiting activity. The results are presented as the percentage of fusion relative to the specific amount of fusion obtained in the absence of polyene antibiotics of that particular *erg* deletion strain pair (Fig. 3C). Filipin did not show any dependence on sterol structure for its inhibition of fusion. This is in accordance with the lack of dependence on sterol structure for its binding to membranes or inhibition of yeast growth (32). Natamycin and nystatin show

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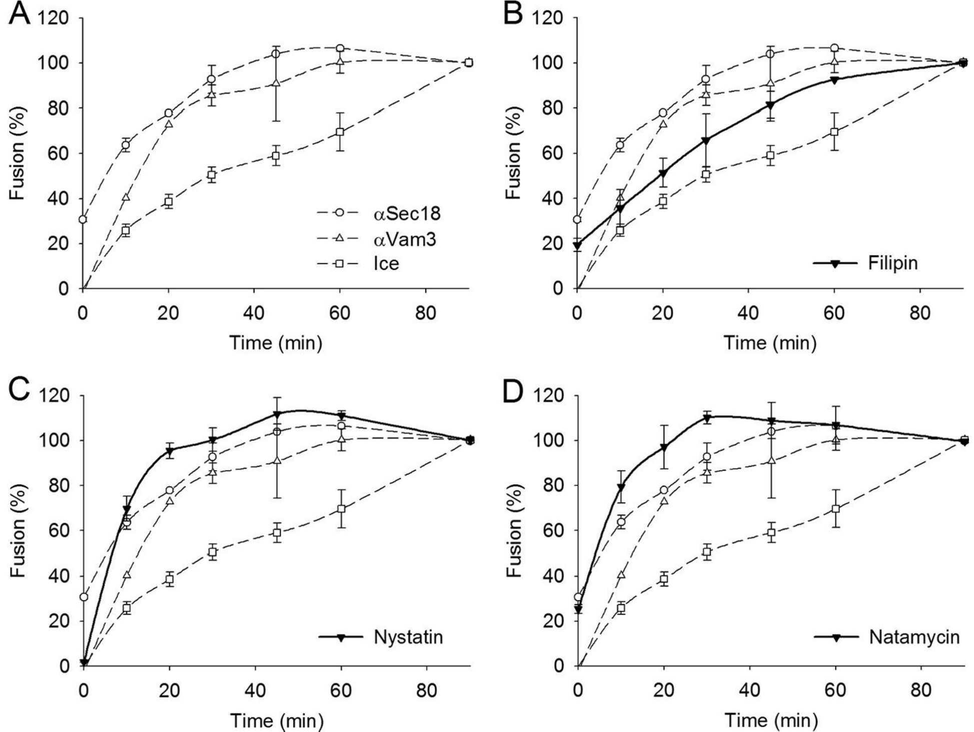

FIG. 4. Inhibitory staging assay of vacuole fusion with the polyene antibiotics filipin, nystatin, and natamycin. Vacuoles isolated from yeasts with a wild-type sterol composition (3 μg protein of KTY1 and KTY2 each) were incubated with different inhibitors for different time periods. (A) Profiles of the staging inhibitors used as controls for priming (anti-Sec18 [○]), docking (anti-Vam3 [△]) and fusion (ice [□]). (B to D) The inhibition patterns of the polyene antibiotics (▼) are given together with the controls for filipin at 100 μM (B), nystatin at 200 μM (C), or natamycin at 200 μM (D), respectively. The experiment was performed in triplicate with freshly isolated vacuoles. See Materials and Methods for details.

similar inhibition patterns. A loss of the double bonds in the B-ring by deletions of *ERG3* (5,6 position) and especially *ERG2* (7,8 position) showed a loss of inhibition caused by natamycin and nystatin (Fig. 3C). Changes in the lipid-embedded tail from deletion of *ERG4* did not have a significant effect on natamycin or nystatin inhibition. Together, these results indicate that the presence of double bonds in the B-ring of the sterol, specifically at the 7,8 position, are essential for the ability of natamycin and nystatin to inhibit vacuole fusion. Similar structural requirements for sterols have been observed for natamycin and nystatin in their membrane binding and inhibition activity toward yeast cells (32). Therefore, the inhibition of vacuole fusion caused by natamycin and nystatin is most likely directly related to their binding of sterols in the membrane.

Stage-specific inhibition of fusion by the polyene antibiotics. To determine how natamycin inhibits fusion, we examined its effect on the different stages in the fusion process (9, 36). Homotypic yeast vacuole fusion occurs in three different stages: priming, docking, and fusion (36). Because different stages are dependent on different proteins, it is possible to examine fusion reactions of isolated vacuoles by using specific inhibitors. For example, the priming and docking stages can be inhibited by antibodies against Sec18p and Vam3p, respectively, proteins that are essential for these steps of the fusion process (9, 13). To inhibit the fusion stage, a vacuole fusion reaction is placed on ice. In this manner, a staging assay can be performed, where the specific inhibition profiles of the polyene antibiotics can be compared to the inhibition profiles of the known inhibitors. Figure 4A shows the inhibition profiles of the controls, anti-Sec18 (priming), anti-Vam3 (docking), and ice (fusion). Vacuole priming occurs within the first 30 min of fusion reactions. Thus, compounds that inhibit this stage of the fusion process show inhibition only within this time span. Indeed, the inhibition profile of anti-Sec18 fits this criterion. Conversely, compounds that inhibit the final stage, membrane bilayer mixing, will show inhibition throughout the whole time span of the reaction. Such an inhibition profile shows the least amount of fusion (Fig. 4A, Ice). Inhibitors of the docking stage will display their activity between the profiles of the priming and the fusion (Fig. 4A, anti-Vam3). For clarity, the inhibition profiles of the polyene antibiotics are compared separately to those of the controls for filipin, nystatin, and natamycin (Fig. 4B to D, respectively).

The inhibition profile of filipin lies in between the docking and fusion profiles, indicating it most likely inhibits between these stages (Fig. 4B). This fits with the membrane-permeabilizing activity of filipin, because a pH gradient is necessary for the docking stage (9, 33). The profile of nystatin inhibition overlaps with that of the control for the priming, anti-Sec18, which indicates it acts on the priming (Fig. 4C). Natamycin also showed an inhibition profile similar to the profile of priming (Fig. 4D). Given its inability to cause membrane permeabilization and the specific interaction of natamycin with ergosterol, this indicates that the effect of natamycin is related to an

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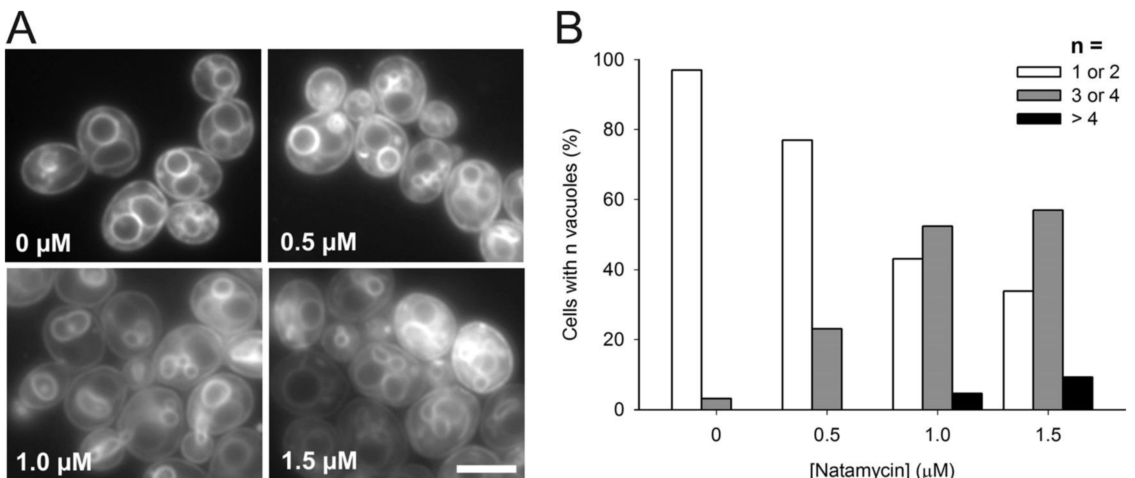

FIG. 5. The effect of natamycin on the cellular morphology of vacuoles in yeast. (A) Yeast cells were incubated with 0, 0.5, 1.0, or 1.5 μM natamycin for 5 h at 25°C. This was followed by staining with MDY-64 and fluorescence microscopy. Bar = 5 μm. (B) The number of vacuoles per yeast cell was determined for a total of 65 cells. White bars represent 1 or 2 vacuole lobes, gray bars 3 or 4 vacuole lobes, and black bars more than 4 vacuole lobes per cell.

ergosterol-dependent function in the priming stage of vacuole fusion.

Effect of natamycin on cellular morphology of vacuoles. To determine if natamycin also has an effect on vacuole fusion in intact yeast cells, cells were incubated with different concentrations of natamycin for 5 h and stained with the vacuolar membrane marker, MDY-64 (Fig. 5). Filipin and nystatin were not included in this assay, because their permeabilizing effect on the yeast membrane may cause a free entry of the dye into the cell (34). Most control cells, untreated with natamycin, have more than one vacuole per cell, and treatment with natamycin resulted in a fragmentation of the vacuoles, already visible after treatment with 0.5 μM, which is about 30% of the MIC of this strain for natamycin (Fig. 5A) (32). A quantification of the number of vacuoles per yeast cell shows that approximately 98% of untreated cells had two vacuoles maximally (Fig. 5B). However, after incubation with natamycin, this number dropped to 35% owing to an increase in the number of vacuoles per cell to a maximum of 6 in 5% of the cases. These results show that natamycin treatment of whole yeast cells results in the fragmentation of the vacuoles.

DISCUSSION

Although the biological consequences of the action of the polyene antifungal compound natamycin are not known, the mode of action is thought to arise via a specific interaction with ergosterol but does not involve membrane permeabilization. In this study, we have demonstrated that natamycin is able to interfere in the process of vacuole fusion in a sterol-dependent manner. This inhibition also did not involve membrane permeabilization and seemed to take place early in the fusion mechanism, even before any membrane contact had occurred.

Ergosterol is known to be important during fusion and fission processes, including vacuole fusion (12, 19, 31). To determine if natamycin was able to act on these processes via its specific interaction with ergosterol, the effects of this antibiotic on the fusion of isolated yeast vacuoles were studied using a content mixing assay (15, 37). Indeed, natamycin was shown to inhibit the fusion process of isolated vacuoles. In addition, this inhibition was not related to a permeabilizing effect, similar to natamycin's inability to permeabilize model membranes or yeast cells (32). The sterol structure dependency of the vacuolar fusion inhibition by natamycin was almost identical to the sterol structure dependency for its activity toward yeast cells and its binding to sterols in model membranes (32). All were dependent on the presence of sterols containing double bonds in the B-ring, most importantly at the 7,8 position (32). Therefore, we conclude that natamycin inhibits vacuolar fusion through the specific interaction with ergosterol.

Treatment of yeast cells with natamycin led to a fragmented vacuolar morphology that is characteristic of a defect in vacuole fusion (1, 2). A similar vacuolar morphology has been observed in conidia of *Penicillium discolor* upon natamycin treatment (M. R. van Leeuwen and J. Dijksterhuis, unpublished observations). We therefore conclude that natamycin is able to inhibit vacuole fusion both in purified vacuoles and in intact yeast cells. Besides this inhibition of vacuolar fusion, natamycin may act on more ergosterol-dependent membrane fusion and fission processes through its interaction with ergosterol (23, 37). Indeed, natamycin has been shown to inhibit the early stages of endocytosis in the fungus *P. discolor* (34), an ergosterol-dependent fission process (17, 24). This suggests that the basis of the toxicity of natamycin could be the inhibition of fusion and fission processes. To act on vacuole fusion in an intact yeast cell most likely requires natamycin to enter this cell. This could be either via permeation across the plasma membrane or in an early stage via endocytosis. Currently we have no information on whether natamycin enters the cell and if so via which mechanism this occurs.

The polyene antibiotics nystatin and filipin were shown to be more efficient in inhibiting the fusion of isolated vacuoles. These differences are probably directly related to the relative affinity of the polyenes for ergosterol and their differences in

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membrane-permeabilizing activity. Nystatin and natamycin had binding affinities similar to those of ergosterol (32), yet nystatin is more efficient in its inhibition of vacuole fusion. This is best explained by the ability of nystatin to permeabilize the vacuole membrane, thereby increasing its efficacy of vacuole fusion inhibition. Filipin displayed the highest affinity for ergosterol, and it severely damages the membrane barrier (10, 32). Altogether, this likely explains why filipin was the most efficient inhibitor of vacuole fusion in our assays.

What would be the mechanism behind the inhibition of fusion caused by natamycin? We have observed that through the specific interaction with ergosterol, natamycin was able to act on the early priming stage of fusion. During this phase, no actual contact between the vacuolar membranes has taken place (25), making it unlikely that natamycin will act on lipid reorganization. The priming phase consists solely of the rearrangements of different protein complexes (for reviews, see references 25 and 36). Thus, the most straightforward conclusion is that natamycin is able to disturb these rearrangements as a result of its binding to ergosterol, and this suggests a more general mode of action, namely, to disturb ergosterol-dependent protein functions.

This immediately poses the question of whether the other members of the family of polyene antibiotics, which all bind to ergosterol (3, 8, 11, 32), are also able to act on the priming stage through their interaction with ergosterol. Indeed, we have shown that nystatin is able to act on the priming stage, as was observed previously as well, and the same is true for amphotericin B (21). The effect of filipin is less clear, because we found it to act in between the docking and fusion stages, while in a different study filipin was shown to act on the priming stage (21). The differences in results are likely best explained by different assay conditions. These findings points to a dual mode of action for some members of the polyene antibiotic family, where all members have the basic ability to act through the inhibition of ergosterol-dependent protein functions, while the additional ability is to permeabilize the membrane. This relates to a freeze fracture electron microscopy study, where natamycin, nystatin, and filipin all produced distinct morphological effects on the fungal membrane, indicating the different end results according to the mechanisms involved in polyene-sterol interactions (22). Because natamycin has only the basic ability to bind ergosterol, it is the ideal candidate for studying the basic mode of action of the polyenes. In addition, this makes natamycin an interesting tool for cell biology when analyzing ergosterol-dependent protein functions.

Interestingly, there is another naturally produced family of antibiotics where several members are known to have a dual mode of action. This is the antibacterial lantibiotic family, a group of small antimicrobial peptides, a large part of which are known to bind the bacterial cell wall component lipid II and through this interaction block cell wall synthesis (5). In the group of lantibiotics that are able to bind lipid II, several members are long enough to span the lipid bilayer and have an additional ability to form pores (5, 16). This striking parallel shows how nature has repeatedly used dual modes of action for membrane-active antibiotics, and this might be applicable to other families of membrane active antibiotics as well.

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REFERENCES

1. **Baars, T. L., S. Petri, C. Peters, and A. Mayer.** 2007. Role of the V-ATPase in regulation of the vacuolar fission-fusion equilibrium. Mol. Biol. Cell **18:** 3873–3882.
2. **Banta, L. M., J. S. Robinson, D. J. Klionsky, and S. D. Emr.** 1988. Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. J. Cell Biol. **107:**1369–1383.
3. **Bolard, J.** 1986. How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochim. Biophys. Acta **864:**257–304.
4. **Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li, P. Hieter, and J. D. Boeke.** 1998. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:**115–132.
5. **Breukink, E., I. Wiedemann, C. van Kraaij, O. P. Kuipers, H. Sahl, and B. de Kruijff.** 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science **286:**2361–2364.
6. **Brown, D. A., and E. London.** 1998. Functions of lipid rafts in biological membranes. Annu. Rev. Cell Dev. Biol. **14:**111–136.
7. **Burger, K. N.** 2000. Greasing membrane fusion and fission machineries. Traffic **1:**605–613.
8. **Clejan, S., and R. Bittman.** 1985. Rates of amphotericin B and filipin association with sterols. A study of changes in sterol structure and phospholipid composition of vesicles. J. Biol. Chem. **260:**2884–2889.
9. **Conradt, B., A. Haas, and W. Wickner.** 1994. Determination of four biochemically distinct, sequential stages during vacuole inheritance in vitro. J. Cell Biol. **126:**99–110.
10. **de Kruijff, B., and R. A. Demel.** 1974. Polyene antibiotic-sterol interactions in membranes of Acholeplasma laidlawii cells and lecithin liposomes. 3. Molecular structure of the polyene antibiotic-cholesterol complexes. Biochim. Biophys. Acta **339:**57–70.
11. **de Kruijff, B., W. J. Gerritsen, A. Oerlemans, P. W. van Dijck, R. A. Demel, and L. L. van Deenen.** 1974. Polyene antibiotic-sterol interactions in membranes of Acholeplasma laidlawii cells and lecithin liposomes. II. Temperature dependence of the polyene antibiotic-sterol complex formation. Biochim. Biophys. Acta **339:**44–56.
12. **D'Hondt, K., A. Heese-Peck, and H. Riezman.** 2000. Protein and lipid requirements for endocytosis. Annu. Rev. Genet. **34:**255–295.
13. **Eitzen, G., N. Thorngren, and W. Wickner.** 2001. Rho1p and Cdc42p act after Ypt7p to regulate vacuole docking. EMBO J. **20:**5650–5656.
14. **Ghannoum, M. A., and L. B. Rice.** 1999. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin. Microbiol. Rev. **12:**501–517.
15. **Haas, A.** 1995. A quantitative assay to measure homotypic vacuole fusion in vitro. Methods Cell Sci. **17:**283–294.
16. **Hasper, H. E., N. E. Kramer, J. L. Smith, J. D. Hillman, C. Zachariah, O. P. Kuipers, B. de Kruijff, and E. Breukink.** 2006. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. Science **313:**1636–1637.
17. **Heese-Peck, A., H. Pichler, B. Zanolari, R. Watanabe, G. Daum, and H. Riezman.** 2002. Multiple functions of sterols in yeast endocytosis. Mol. Biol. Cell **13:**2664–2680.
18. **Isgandarova, S., L. Jones, D. Forsberg, A. Loncar, J. Dawson, K. Tedrick, and G. Eitzen.** 2007. Stimulation of actin polymerization by vacuoles via Cdc42p-dependent signaling. J. Biol. Chem. **282:**30466–30475.
19. **Jin, H., J. M. McCaffery, and E. Grote.** 2008. Ergosterol promotes pheromone signaling and plasma membrane fusion in mating yeast. J. Cell Biol. **180:**813–826.
20. **Kane, P. M.** 2006. The where, when, and how of organelle acidification by the yeast vacuolar H+-ATPase. Microbiol. Mol. Biol. Rev. **70:**177–191.
21. **Kato, M., and W. Wickner.** 2001. Ergosterol is required for the Sec18/ATP-dependent priming step of homotypic vacuole fusion. EMBO J. **20:**4035–4040.
22. **Kitajima, Y., T. Sekiya, and Y. Nozawa.** 1976. Freeze-fracture ultrastructural alterations induced by filipin, pimaricin, nystatin and amphotericin B in the plasma membranes of Epidermophyton, Saccharomyces and red complex-induced membrane lesions. Biochim. Biophys. Acta **455:**452–465.
23. **Mayer, A.** 2002. Membrane fusion in eukaryotic cells. Annu. Rev. Cell Dev. Biol. **18:**289–314.
24. **Munn, A. L., A. Heese-Peck, B. J. Stevenson, H. Pichler, and H. Riezman.**

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1999. Specific sterols required for the internalization step of endocytosis in yeast. Mol. Biol. Cell **10:**3943–3957.
25. **Ostrowicz, C. W., C. T. Meiringer, and C. Ungermann.** 2008. Yeast vacuole fusion: a model system for eukaryotic endomembrane dynamics. Autophagy **4:**5–19.
26. **Peters, C., M. J. Bayer, S. Buhler, J. S. Andersen, M. Mann, and A. Mayer.** 2001. Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. Nature **409:**581–588.
27. **Peters, C., and A. Mayer.** 1998. Ca2+/calmodulin signals the completion of docking and triggers a late step of vacuole fusion. Nature **396:**575–580.
28. **Rodriguez, R. J., C. Low, C. D. Bottema, and L. W. Parks.** 1985. Multiple functions for sterols in Saccharomyces cerevisiae. Biochim. Biophys. Acta **837:**336–343.
29. **Salaun, C., D. J. James, and L. H. Chamberlain.** 2004. Lipid rafts and the regulation of exocytosis. Traffic **5:**255–264.
30. **Simons, K., and E. Ikonen.** 1997. Functional rafts in cell membranes. Nature **387:**569–572.
31. **Tedrick, K., T. Trischuk, R. Lehner, and G. Eitzen.** 2004. Enhanced membrane fusion in sterol-enriched vacuoles bypasses the Vrp1p requirement. Mol. Biol. Cell **15:**4609–4621.
32. **te Welscher, Y. M., H. H. ten Napel, M. Masia Balague, C. M. Souza, H. Riezman, B. de Kruijff, and E. Breukink.** 2008. Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. J. Biol. Chem. **283:**6393–6401.
33. **Ungermann, C., W. Wickner, and Z. Xu.** 1999. Vacuole acidification is required for trans-SNARE pairing, LMA1 release, and homotypic fusion. Proc. Natl. Acad. Sci. U. S. A. **96:**11194–11199.
34. **Van Leeuwen, M. R., E. A. Golovina, and J. Dijksterhuis.** 2009. The polyene antimycotics nystatin and filipin disrupt the plasma membrane, whereas natamycin inhibits endocytosis in germinating conidia of Penicillium discolor. J. Appl. Microbiol. **106:**1908–1918.
35. **Van Leeuwen, M. R., W. Smant, W. de Boer, and J. Dijksterhuis.** 2008. Filipin is a reliable in situ marker of ergosterol in the plasma membrane of germinating conidia (spores) of Penicillium discolor and stains intensively at the site of germ tube formation. J. Microbiol. Methods **74:**64–73.
36. **Wickner, W.** 2002. Yeast vacuoles and membrane fusion pathways. EMBO J. **21:**1241–1247.
37. **Wickner, W., and A. Haas.** 2000. Yeast homotypic vacuole fusion: a window on organelle trafficking mechanisms. Annu. Rev. Biochem. **69:**247–275.

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Natamycin Blocks Fungal Growth by Binding Specifically to Ergosterol without Permeabilizing the Membrane*[S]

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Yvonne M. te Welscher[‡1], Hendrik H. ten Napel[‡], Miriam Masià Balagué[‡], Cleiton M. Souza[§], Howard Riezman[§], Ben de Kruijff[‡], and Eefjan Breukink[‡]

From the [‡]Department Biochemistry of Membranes, Bijvoet Center, Institute of Biomembranes, Utrecht University, Utrecht 3584 CH, The Netherlands and the [§]Department of Biochemistry, University of Genève, CH-1211 Genève 4, Switzerland

Natamycin is a polyene antibiotic that is commonly used as an antifungal agent because of its broad spectrum of activity and the lack of development of resistance. Other polyene antibiotics, like nystatin and filipin are known to interact with sterols, with some specificity for ergosterol thereby causing leakage of essential components and cell death. The mode of action of natamycin is unknown and is investigated in this study using different *in vitro* and *in vivo* approaches. Isothermal titration calorimetry and direct binding studies revealed that natamycin binds specifically to ergosterol present in model membranes. Yeast sterol biosynthetic mutants revealed the importance of the double bonds in the B-ring of ergosterol for the natamycin-ergosterol interaction and the consecutive block of fungal growth. Surprisingly, in strong contrast to nystatin and filipin, natamycin did not change the permeability of the yeast plasma membrane under conditions that growth was blocked. Also, in ergosterol containing model membranes, natamycin did not cause a change in bilayer permeability. This demonstrates that natamycin acts via a novel mode of action and blocks fungal growth by binding specifically to ergosterol.

Fungal infections have recently become a growing threat to human health, especially in persons whose immune systems are compromised (for example, by human immunodeficiency virus and cancer chemotherapy). Only a few effective antifungal agents are currently in use; these include the polyenes, the fluorocytes, and the azole derivatives. One important problem is the increase of drug resistance, particularly against azole antimyotics and fluorocytosine (1). Resistance against polyene antibiotics is still a rare event, which makes these antibiotics particularly interesting as antifungal agents. The polyene antibiotics have a ring structure in which a conjugated double bond system is located opposite to a number of hydroxyl functions. Often a mycosamine group is present in combination with a carboxyl moiety, rendering the molecule amphoteric (Fig. 1). In the past convincing evidence has been presented that several members of this class of antibiotics target sterols and in particular ergosterol, the abundant and main sterol of fungal membranes (2, 3). Different types of polyene antibiotics were shown to have different modes of action despite that they share a common target. The larger polyenes like amphotericin B and nystatin form pores together with ergosterol in the plasma membrane that collapse vital ion gradients, thereby killing the cells. The smaller uncharged filipin also destroys the membrane barrier, but by a completely different mechanism. Filipin forms large complexes with sterols between the leaflets of the lipid bilayer, resulting in loss of the barrier function (2). Natamycin (also called pimaricin) is a very effective member of the polyene antibiotic family with a large standing record of applications. It is produced by *Streptomyces natalensis* and used against fungal infections, but it is also widely utilized in the food industry to prevent mold contamination of cheese and other nonsterile foods (*e.g.* cured meats) (4). Surprisingly, the mechanism of action of this antifungal agent is still unknown and it is even unknown whether it targets ergosterol in the fungal membrane. It is relatively small while it contains a tetraene compared with a pentaene in filipin, which is already considered as a small polyene antibiotic (Fig. 1). It contains a mycosamine group that renders it amphoteric, which is a feature that is also present in nystatin. Whereas natamycin has similar features of both filipin (small) and nystatin (amphoteric), it is difficult to predict its mechanism of action.

We wanted to gain more insight into the mode of action of natamycin, which could in turn help to develop new or improved antifungal formulations or result in novel strategies to prevent fungal spoilage. To determine the interaction of natamycin with membranes in relation to its sterol composition, we tested in a comparative manner using filipin and nystatin as references, the interaction of natamycin with phosphatidylcholine model membranes of varying sterol composition using isothermal titration calorimetry (ITC)[2] and other binding studies. In addition, the ability of natamycin to permeabilize these model membranes was studied.

Parallel to the studies performed on model membranes, the effect of natamycin on yeast growth, the binding of the antibi-

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[1] To whom correspondence should be addressed: Padualaan 8, Utrecht 3584 CH, The Netherlands. Tel.: 31-30-2534157; Fax: 31-30-2533969; E-mail: y.m.tewelscher@uu.nl.

[2] The abbreviations used are: ITC, isothermal titration calorimetry; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; CFDA-SE, 5-(and -6)-carboxyfluorescein diacetate, succinimidyl ester; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt; DDAO, *N*,*N*-dimethyldodecylamine-*N*-oxide; MIC, minimum inhibitory concentration; CF, carboxyfluorescein; LUVs, large unilamellar vesicles; MES, 4-morpholineethanesulfonic acid.

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otic with intact yeast cells, and the plasma membrane integrity were determined. These studies were performed using strains that carry specific mutations in the ergosterol biosynthetic pathway (ergΔ) or that were reprogrammed to contain cholesterol as the main sterol (5). We could demonstrate that, differently from any other polyene antibiotic of which the mode of action is known, natamycin blocks fungal growth by binding specifically to ergosterol, but without permeabilizing the membrane.

EXPERIMENTAL PROCEDURES

Chemicals—1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and cholesterol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Ergosterol was purchased from Larodan AB (Sweden). DOPC or sterols were dissolved in chloroform to a stock concentration of 20 mM. The phospholipid concentration of DOPC was determined by phosphate analysis according to Rouser *et al.* (6). The polyene antibiotics nystatin and filipin were dissolved in Me_2SO, whereas natamycin was dissolved in 85:15 Me_2SO/H_2O (v/v); all were obtained from Sigma. All antibiotic solutions were prepared freshly before the start of an experiment and the concentrations of the polyene antibiotics were determined by UV absorption on a PerkinElmer UV-visible spectrometer (Lambda 18). The molar extinction coefficients of the polyene antibiotics were determined in methanol to be 7.6×10^4 M^{-1} cm^{-1} (318 nm), 6.7×10^4 M^{-1} cm^{-1} (318 nm), and 8.5×10^4 M^{-1} cm^{-1} (356 nm) for natamycin, nystatin, and filipin, respectively. The molar extinction coefficient of ergosterol was measured in methanol to be 0.97×10^4 M^{-1} cm^{-1} (262 nm).

The ionophore nigericin (dissolved in ethanol), ampicillin sodium salt, and the amino acids adenine, uracil, and L-tryptophan were obtained from Sigma. 5-(and -6)-Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) (dissolved in Me_2SO) and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) were both purchased from Invitrogen. *N,N*-Dimethyldodecylamine-N-oxide (DDAO) was bought from Fluka Biochimica (Buchs). All other chemicals used were of analytical or reagent grade.

Strains and Growth Conditions—For all experiments, medium was inoculated directly from plates with colonies that were not older than 2 weeks. Unless otherwise mentioned, cells were grown overnight at 30 °C in rich medium (10 g/liter yeast extract, 20 g/liter Bacto-peptone, and 20 g/liter dextrose with 1 g/liter adenine, 2 g/liter uracil, and 1 g/liter tryptophan (YPUADT)) supplemented with 0.1 mg/ml ampicillin. For strains RH6611 and RH6613 SD medium was used (1.7 g/liter yeast nitrogen base without amino acids, 20 g/liter glucose, 2 mg/liter trace components, 5 g/liter ammonium sulfate) supplemented with vitamins and the appropriate amino acids minus histidine and leucine (SD-His-Leu). Yeast strains used in this study are listed with their relevant genotypes in Table 1 and the plasmids in Table 2.

MIC Value Determinations—Minimum inhibitory concentrations (MICs) were determined by diluting the polyene antibiotics in YPUADT (with 0.1 mg/ml ampicillin) to concentrations of 400, 350, 300, and 250 μM of which 100 μl was added to the first row of a 96-well suspension culture plate (U-form, Greiner Bio One). This was followed by a 1:1 dilution series in medium. Overnight cultures were diluted back to an A_{600} 0.0001, of which 100 μl was added to the culture plate. The total volume per well was 200 μl. Strains RH6611 and RH6613 (in SD-His-Leu medium) were diluted to an A_{600} 0.01, because they had a very slow growth rate. The MIC value was determined to be the lowest concentration of antibiotic, which inhibits the growth of the yeast strain and could be determined by eye on the 96-well plate after an incubation of 24 h at 30 °C. The experiments were performed in triplicate.

Preparation of Large Unilamellar Vesicles (LUVs)—LUVs with a mean diameter of 200 nm were prepared using the following protocol. Aqueous phospholipid suspensions were prepared by premixing ergosterol or cholesterol with DOPC in the desired molar ratios as solutions in chloroform and evaporating the solvent in a stream of nitrogen, followed by drying the lipid film for 20 min under vacuum. Sterols were present in a range of 10 to 30 mol %. All following handlings were performed at 50 °C. The lipid film was hydrated and repeatedly vortexed until all lipid was removed from the walls of the test tube. Then a freeze-thaw cycle was repeated eight times using liquid nitro-

FIGURE 1. **Structures of several polyene antibiotics and ergosterol.** *A*, natamycin; *B*, nystatin; *C*, filipin; *D*, ergosterol.

TABLE 1
Strains used in this study
The source of these strains is described in Ref. 5.

Strain	Name	Genotype
Wild type	RH448	*MATa his4 leu2 ura3 lys2 bar1*
Erg2Δ	RH2897	*MATa erg2(end11)-1Δ::URA3 leu2 ura3 his4 lys2 bar1*
Erg2Δerg6Δ	RH3616	*MATa erg2(end11)-1Δ::URA3 erg6Δ leu2 ura3 bar1*
Erg6Δ	RH3622	*MATa erg6Δ::LEU2 leu2 ura3 his4 bar1*
Erg3Δ	RH4213	*MATa erg3Δ::LEU2 leu2 ura3 his4 lys2 bar1*
Erg3Δerg6Δ	RH5225	*MATa erg3Δ::LEU2 erg6Δ::LEU2 leu2 ura3 his4 lys2 bar1*
Erg2Δerg3Δ	RH5228	*MATa erg2Δ (end11)-1Δ::URA3 erg3Δ::LEU2 leu2 ura3 his4 lys2 bar1*
Erg4Δerg5Δ	RH5233	*MATa erg4Δ::URA3 erg5Δ::kanMX4 leu2 ura3 his4 lys2 bar1*
Wild type	RH6611	*MATa his3 ura3 leu2* (pRS423) (pRS425)
Cholesterol	RH6613	*MATa erg5Δ::TRP1 erg6::TRP1 his3 ura3 leu2 trp1* (pRS423-DHCR7) (pRS425-DHCR24)

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TABLE 2
Plasmids used in this study

Plasmid	Characteristics	Ref.
pRS423	Multicopy vector containing *GDP* promoter and *HIS3*	30
pRS423-DHCR7	pRS423 derivative vector containing *DHCR7* gene	—[a]
pRS425	Multicopy vector containing *GDP* promoter and *LEU2*	30
pRS425-DHCR24	pRS425 derivative vector containing *DHCR24* gene	—[a]

[a] C. M. Souza, H. Pichler, E. Leitner, X. Guan, M. R. Wenk, I. Tornare, and H. Riezman, submitted for publication.

gen and a water bath. Subsequently, the lipid suspension was extruded 8 times through a polycarbonate membrane filter with a pore size of 0.2 μm (Whatman International). The size of the vesicles was determined after extrusion by using the Zetasizer 3000 (Malvern Instruments). The average of the size of the vesicles was 168 ± 3.7 nm for vesicles without sterols, 165 ± 1.2 nm for vesicles with 10% cholesterol, and 173 ± 8 nm for vesicles with 10% ergosterol. Thus no significant differences in size were observed. The resulting vesicle suspension was stored at 4 °C. The final phospholipid concentration was determined by phosphate analysis according to Rouser *et al.* (6).

ITC Measurements—Titration experiments were carried out on a MCS titration calorimeter from Microcal Inc. LUVs were prepared as described above in 50 mM MES, 100 mM K_2SO_4, pH 6.0, or 10 mM HEPES, 100 mM NaCl, pH 7.0. Similar results were obtained with the different buffers. The vesicles were injected into a sample cell (volume = 1.345 ml) containing 50 μM antibiotic in the same buffer as used for the vesicle suspension. Because the polyene antibiotics are dissolved in Me_2SO, an equal amount was added to the LUV suspension to compensate for any heat generated by dilution of this solvent. No more than 1% Me_2SO was present. The solutions were degassed, before the start of the titration. The experiments consisted of 44 injections, 5 μl each, of a stock solution of vesicles at 25 °C (8 mM final phospholipid concentration). The results were analyzed using the ORIGIN software (version 2.9) provided by Microcal Inc. The interaction between the vesicles and the antibiotics was complex in that no clear saturation of this interaction was observed. Therefore the stoichiometry of the interaction could not be determined. An approximation of the binding constant was made using the ORIGIN software, where the value of integrated heat of the last injection was subtracted from all data and the model of one set of sites was fitted to the resulting data.

Binding Assay Using Centrifugation of Model Membranes—Vesicles were prepared as described above in 10 mM MES/Tris, 15 mM K_2SO_4 at pH 7. The reduced ion strength facilitated the pelleting of the vesicles. The concentrations of antibiotics and vesicles were varied from 0 to 0.1 and 0.5 to 5 mM, respectively, unless indicated otherwise. Vesicles were incubated with the polyene antibiotics for 1 h in an Eppendorf incubator (22 °C, 650 rpm), with a maximum of 1% Me_2SO present. To spin down the vesicles and the bound antibiotic, 1 ml of the mixture was centrifuged in a TLA 120.2 rotor in a Beckman Ultracentrifuge (TL-100) for 1.5 h at 100 krpm and 20 °C. The amount of antibiotic before centrifugation and in the supernatant and pellet was determined by UV absorption after 7 times dilution in methanol followed by centrifugation to remove any precipitated salts. The phospholipid concentrations were determined by phosphate analysis according to Rouser *et al.* (6). Under these conditions less than 10% of the phospholipids remained in the supernatant. The antibiotics were not pelleted in the absence of lipid below a concentration of 75, 34, and 30 μM, respectively, of natamycin, nystatin, and filipin. The binding isotherms of the interaction of natamycin with ergosterol could be described by the Langmuir adsorption model assuming that ergosterol was the only binding site for natamycin in the DOPC vesicles and that only the ergosterol in the outer leaflet of the bilayer could have an interaction with natamycin. The Langmuir adsorption model was applied to the data of the amount of natamycin bound to the vesicles *versus* the amount of free natamycin in the supernatant (7). From using this model in Sigmaplot (10.0), the binding constant and the binding saturation of natamycin with ergosterol could be determined.

Binding Assay Using Centrifugation of Intact Cells—Yeast were grown to the mid-logarithmic phase in 200 ml of YPUADT (with 0.1 mg/ml ampicillin) or SD medium. As a negative control the *Escherichia coli* strain DH5α was used that was grown to the logarithmic phase in 100 ml of Luria Broth (LB) medium at 37 °C. The cells were harvested by centrifugation at room temperature at 3600 × *g* for 10 min in a Sorvall RC 5B centrifuge (SLA 1500), washed two times in 100 ml of 10 mM MES/Tris, 15 mM K_2SO_4 at pH 7, and resuspended in a small volume of buffer. The A_{600} of the cell suspensions was determined and a series of 1-ml cell suspensions were prepared ranging from an A_{600} of 0 to 15. The cells were centrifuged at 3000 × *g* for 5 min at room temperature and resuspended in the same buffer containing 30 μM natamycin. As a control, cells were resuspended in buffer with no natamycin. The cells were incubated for 1 h in an Eppendorf incubator (900 rpm at room temperature) and spun down for 15 min at 3000 × *g*. The amount of natamycin in the supernatant was determined by UV absorption as described above (spectrum from 250 to 350 nm) and used to calculate the amount of natamycin bound to the yeast cells.

Carboxyfluorescein Permeability Assay in Large Unilamellar Vesicles—Carboxyfluorescein (CF)-loaded vesicles were prepared as described above in 50 mM MES/KOH buffer at pH 7 (8). To remove the untrapped CF, a Sephadex G-50 spin column equilibrated with 50 mM MES, 100 mM K_2SO_4 buffer at pH 7 was used. The CF-loaded vesicles were diluted in 1200 μl of 50 mM MES, 100 mM K_2SO_4 buffer at pH 7 followed by the addition of the antibiotic. The antibiotic-induced CF leakage from the vesicles was monitored by measuring the fluorescence intensity at 513 nm (excitation set at 430 nm) on a SLM AMINCO Spectrofluorometer (SPF-500). The detergent Triton X-100 was added at the end of the experiment to destroy the lipid vesicles and the resulting fluorescence was taken as the 100% leakage value.

Proton Permeability Assay in Large Unilamellar Vesicles—Proton permeability was determined in an assay with HPTS-loaded vesicles as performed by van Kan *et al.* (9). The assay is based on the strong pH dependence of the fluorescence of HPTS. Vesicles were prepared as described above in a 2 mM HPTS solution in 0.2 M NaH_2PO_4/Na_2HPO_4 buffer at pH 7. To create a lower pH at the outside and remove all the untrapped

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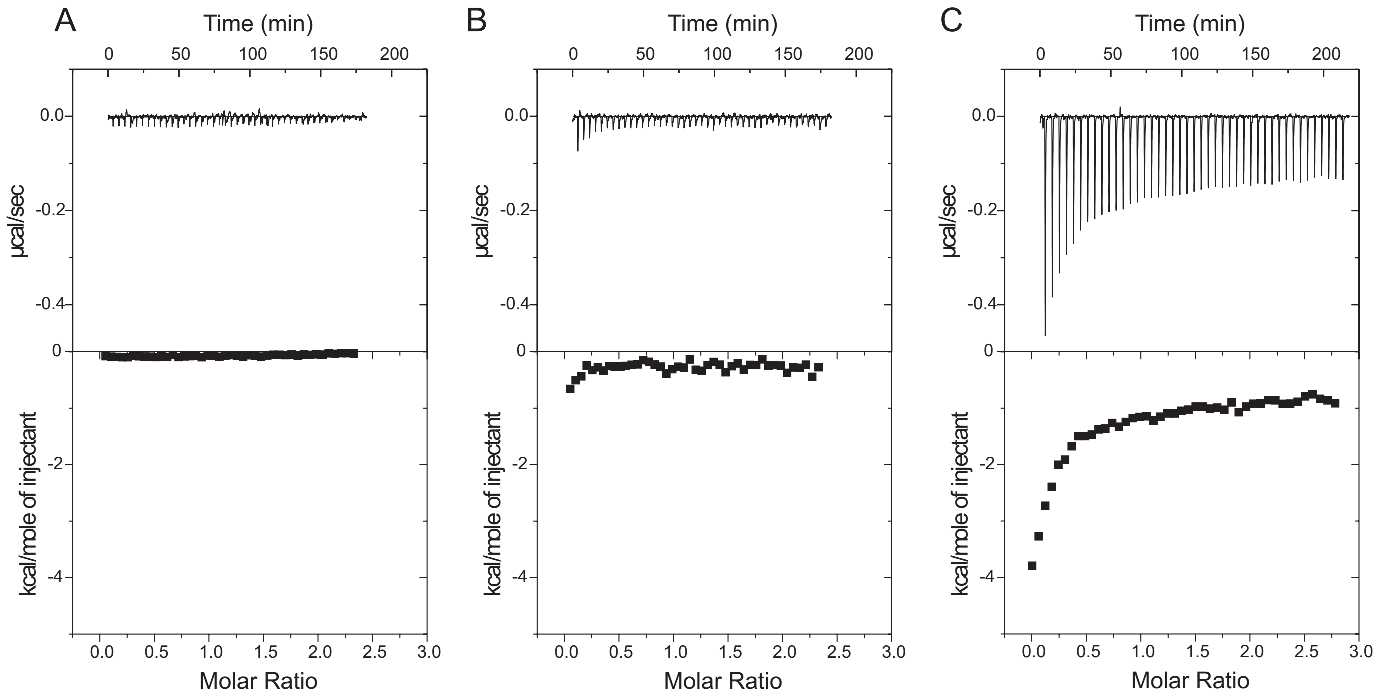

FIGURE 2. **Calorimetric titrations of natamycin with DOPC vesicles.** Vesicles contained no sterol (*A*), 10% cholesterol (*B*), and 10% ergosterol (*C*) and were dissolved in 50 mM MES, 100 mM K_2SO_4, pH 6.0. The *top graph* displays the heat peaks after consecutive injections of 5-μl vesicles with an 8 mM final phospholipid concentration into the sample cell containing 50 μM natamycin. The *bottom graph* shows the integrated heat per injection, which is normalized to the injected amount of moles of sterol and is displayed against the molar ratio of sterol *versus* natamycin. When no sterols are present, 10% of phospholipid is used to determine and display the integrated heat per injection.

HPTS, a Sephadex G-25 spin column was used equilibrated with 10 mM MES, 0.2 M Na_2SO_4 buffer at pH 5.5. To determine the phospholipid concentration of the resulting vesicles the lipids were first extracted according to Bligh-Dyer (10) to exclude the phosphate from the buffer in the following phosphate analysis according to Rouser *et al.* (6). The effects of the polyene antibiotics on the proton permeability of the lipid vesicles was monitored by adding aliquots of antibiotic to 1200 μl of 10 mM MES and 0.2 M Na_2SO_4 buffer, pH 5.5, containing HPTS-loaded vesicles (35 μM phospholipid phosphorous). The fluorescence emission was detected at 508 nm (excitation at 450 nm) on a SLM AMINCO Spectrofluorometer (SPF-500). Differing from van Kan *et al.* (9), the detergent DDAO was used instead of Triton X-100, because DDAO did not have any effect on the fluorescence of the probe where Triton X-100 did have an effect (not shown). DDAO was added at the end to destroy the lipid vesicles and the resulting fluorescence was taken as the 100% leakage value, whereas the blank without antibiotic was used as a reference for 0% leakage. Nigericin, a polyether ionophore known to collapse proton gradients, was used as a positive control (11).

Proton Permeability Assay in Yeast—The assay was based on the loading of yeast cells with the probe CFDA-SE as described by Bracey *et al.* (12, 13). CFDA-SE is a non-polar molecule that spontaneously penetrates cell membranes and is converted to the anionic pH-sensitive 5-(and-6)-carboxyfluorescein succinimidyl ester (CF-SE) by intracellular esterases (9). Once the probe is internalized, amine reactive coupling of succinimidyl groups of CF-SE to aliphatic amines of intracellular proteins results in the formation of membrane-impermeable pH-sensitive probe conjugates.

Wild type yeast cells from an overnight culture were diluted to an A_{600} of ~0.8 and then centrifuged at 3000 × *g* for 3 min. The cells were washed and resuspended in an equal volume of 100 mM citric/phosphate buffer at pH 4 (100 mM citric acid, 50 mM NaH_2PO_4, and 50 mM KOH). CFDA-SE (100 μM) was added and the cells were incubated overnight while shaking at 37 °C. The viability of the cells was not significantly compromised by the loading conditions. Loaded cells were harvested (3000 × *g* for 3 min), washed, and resuspended in YPUADT buffered with 50 mM citric/phosphate (pH 4) to an A_{600} of 0.4. To recover from the stress imposed by the probe loading conditions, the cultures were left for 1 h at 30 °C with shaking. The effects of the polyene antibiotics on the proton permeability of the yeast cells were monitored by adding aliquots of antibiotic to 5 ml of culture and measuring the A_{600} and fluorescence at regular intervals. The A_{600} was determined on a Helios Epsilon UNICAM spectrometer and the fluorescence emission was detected at 525 nm (excitation at 495 nm) on a SLM AMINCO Spectrofluorometer (SPF-500).

RESULTS

Sterol Specificity of Natamycin Binding to Membranes—To test whether sterols are required for membrane affinity of natamycin we used phosphatidylcholine model membranes containing ergosterol, the main fungal sterol or cholesterol, the main sterol in mammals.

The interaction between natamycin and sterols in the model membrane was first studied using ITC. ITC measurements were performed where LUVs containing either no sterols, cholesterol, or ergosterol were titrated into a solution of natamycin (Fig. 2). Natamycin displayed no interaction with vesicles containing no sterols as the resulting heats were no different from

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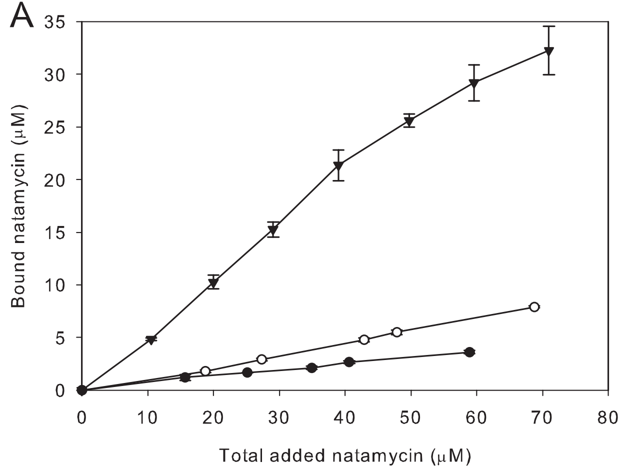

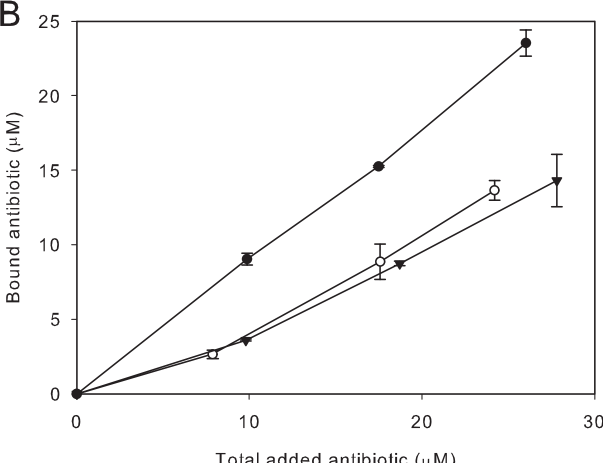

FIGURE 3. **Interaction of polyene antibiotics with model membranes.** *A*, binding of natamycin to vesicles containing 10% ergosterol (▼), 10% cholesterol (○), or no sterols (●). *B*, the interaction of filipin (●), nystatin (○), and natamycin (▼) on 10% ergosterol containing vesicles was examined. The assay was performed in duplicate in 10 mM MES/Tris, 15 mM K_2SO_4, pH 7.0, and the vesicles had a 2 mM final phospholipid concentration.

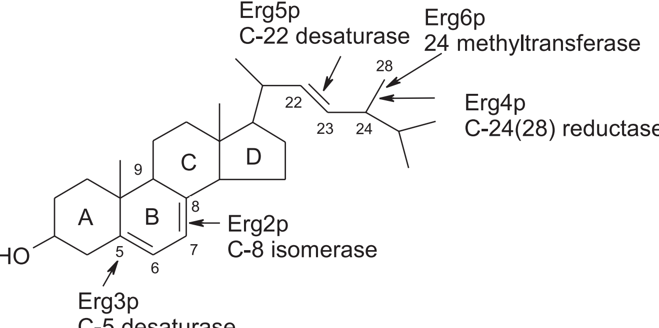

FIGURE 4. **Ergosterol molecule with the assignment of the ring structure.** Erg proteins and their functions are indicated. The corresponding genes are inactivated in ergΔ strains (5).

the control (Fig. 2*A*). LUVs containing 10 mol % cholesterol produced only minor heat effects during the first injections, which indicates that natamycin displayed only a very small interaction with cholesterol containing vesicles (Fig. 2*B*). Interestingly, 10 mol % ergosterol containing vesicles displayed a significant amount of interaction with natamycin as evidenced by the consecutive heat effects (Fig. 2*C*). This titration curve differs from a normal titration curve as no clear saturation of the interaction was observed. The binding constant between natamycin and ergosterol was estimated to be 5.7×10^4 M^{-1} (see "Experimental Procedures"). Comparable large differences in effects between cholesterol and ergosterol were observed for sterol concentrations of 20 mol % (data not shown).

Furthermore, the binding of natamycin to vesicles was studied by separating the bound from the free natamycin by centrifugation. Fig. 3*A* shows a representative graph of these results, from which can be concluded that ergosterol containing vesicles had a significant interaction with natamycin. In the absence of sterols or in the presence of cholesterol very little interaction with natamycin was observed consistent with the ITC experiments (Fig. 2). A similar sterol dependence of natamycin binding was observed when varying the concentrations of vesicles (data not shown). The binding constant was determined by the Langmuir adsorption model in SigmaPlot (10.0) to be $2.5 \pm 1.0 \times 10^4$ M^{-1}, which is in reasonable agreement with the binding constant determined in the ITC measurements. The binding saturation from the Langmuir adsorption model was determined at 72 ± 12 μM by extrapolating the data in SigmaPlot (10.0). By assuming that only the sterol in the external leaflet of the lipid vesicles could establish an interaction with the antibiotic, the sterol to antibiotic ratio was calculated to be ~1:1. If all sterols would be available for the interaction, because of sterol flip-flop, the ratio would be 2:1.

The affinity of natamycin for ergosterol containing vesicles was compared with that of filipin and nystatin to get insight into the relative strength of this interaction. Fig. 3*B* shows a representative graph of the results obtained with these antibiotics. Of the three polyene antibiotics filipin showed the highest affinity, followed by natamycin and nystatin.

Sterol Specificity in the Antibiotic Action—To test if ergosterol is needed for natamycin to exert its antifungal activity *in vivo*, yeast strains carrying specific mutations in the ergosterol biosynthesis pathway (ergΔ) were used. Because of these mutations, the strains cannot synthesize ergosterol. However, they each accumulate a distinct set of sterols that, compared with ergosterol, have structural differences in the side chain and double bonds in the B or C ring (Fig. 4). The availability of these strains allows us to address the sterol specificity for polyenes, in relation to their inhibitory activity.

The most prominent sterols present in the ergΔ mutants are tabulated in percentage of total sterol present, together with their MIC values for the polyene antibiotics natamycin, nystatin, and filipin in Table 3. The sterol composition of the erg strains given in Table 3 was taken from Heese-Peck *et al.* (5) and specifies the percentage of a listed sterol compared with the total sterol composition of a cell. The most sensitive erg strain is erg4Δerg5Δ, which has a MIC value of the wild type strain. The least sensitive toward natamycin was erg2Δerg6Δ, which contained mostly zymosterol. From the strain with the highest sensitivity toward the lowest, the most striking sterol structural feature that causes the loss of activity is the loss of double bonds in ring B. For example, the sterols in erg3Δ have one double bond at position C-7,8 and it is only 3 times less sensitive to natamycin compared with the wild type, whereas erg2Δerg6Δ has lost both double bonds at C-5,6 and C-7,8 and is 37 times

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TABLE 3

The minimum concentration of the polyene antibiotics needed to inhibit the growth of different ergΔ mutants

The MIC values for natamycin (MIC_{natam}), nystatin (MIC_{nyst}), and filipin (MIC_{filip}) are given for the different ergΔ strains, together with the structure and percentage of the most abundant sterols in an ergΔ strain, as stated in Ref. 5. The MIC values were determined in triplicate.

Strain	MIC_{natam} (μM)	MIC_{nyst} (μM)	MIC_{filip} (μM)
Wild Type	**1.7 ± 0.5**	**1.1 ± 0.2**	**1.2 ± 0.1**
Ergosterol (77 %)			
Erg4Δerg5Δ	**2.1±0.6**	**2.1±0.2**	**2.5±0.9**
Ergosta-5,7,24-trienol (72 %)			
Erg3Δ	**5.7 ± 0.8**	**3.4 ± 0.5**	**2.5 ± 0.2**
Ergosta-7,22-dienol (46 %)	Episterol (13 %)	Ergosta-7-enol (11 %)	
Erg6Δ	**8.3 ± 0.9**	**7.8 ± 1.6**	**2.9 ± 0.2**
Zymosterol (39 %)	Cholesta-5,7,24-trienol (32 %)		
Erg3Δerg6Δ	**18 ± 3.6**	**14 ± 4.1**	**5.2 ± 0.9**
Zymosterol (41 %)	Cholesta-7,24-dienol (40 %)		
Erg2Δ	**22 ± 0.1**	**16 ± 1.6**	**3.4 ± 1.4**
Ergosta-5,8,22-trienol (13 %)	Fecosterol (33 %)	Ergosta-8-enol (35 %)	
Erg2Δerg3Δ	**46 ± 14**	**21 ± 14**	**6.8 ± 2.3**
Fecosterol (39 %)	Ergosta-8-enol (40 %)		
Erg2Δerg6Δ	**63 ± 0.1**	**52 ± 13**	**6.8 ± 0.9**
Zymosterol (86 %)			

less sensitive compared with the wild type. Variations in the C_{17} side chain of the sterols did not have very large effects on the sensitivity toward natamycin, which can be observed when comparing erg4Δerg5Δ with the wild type. The yeast strain sensitivities toward nystatin were similar compared with natamycin. Filipin sensitivity seemed not to be so dependent on the sterol structure. The results demonstrate that double bonds in the B ring of the sterols are very important for natamycin to inhibit the growth of yeast, whereas changes of the C_{17} side chain are of less importance.

TABLE 4

The minimum concentration of the polyene antibiotics needed to inhibit the growth of strains RH6611 and 6613

The MIC values for natamycin (MIC_{natam}), nystatin (MIC_{nyst}), and filipin (MIC_{filip}) are given for the different strains, together with the sterol structure and percentage of the most abundant sterols in the strain, as stated (C. M. Souza, H. Pichler, E. Leitner, X. Guan, M. R. Wenk, I. Tornare, and H. Riezman, submitted for publication). The MIC values were determined in triplicate.

	MIC_{natam} (μM)	MIC_{nyst} (μM)	MIC_{filip} (μM)
Wild Type	**2.0 ± 0.4**	**0.9 ± 0.1**	**2.7 ± 0.4**
Ergosterol (78 %)			
Cholesterol	**31 ± 6**	**13 ± 2.4**	**3.3 ± 0.6**
Cholesterol (68 %)	Cholesta-5,7-dienol (10 %)	Cholesta-5,24-dienol (10 %)	

Recently a yeast strain was constructed (RH6613) that is unable to synthesize ergosterol or its related precursors, but instead was programmed to synthesize cholesterol. This enabled us to test the strong preference of natamycin for ergosterol over cholesterol as noted in the model membrane experiments. The results of growth inhibition are shown in Table 4 and show that the cholesterol producing strain was 16-fold less sensitive toward natamycin compared with the corresponding wild type. This demonstrates that also *in vivo* natamycin has a strong specificity for ergosterol over cholesterol. Moreover, given the difference in chemical structures of ergosterol and cholesterol, the importance of the double bonds of the B-ring for interaction with natamycin is further emphasized consistent with the results of the erg strains. Nystatin had the same effect on the yeast strains as natamycin, whereas filipin is apparently less specific as it was almost as effective in killing the cholesterol producing strain as the wild type strain.

To determine whether the inhibition of growth was related to the amount of binding of natamycin to these yeast strains, a binding assay with the different strains was performed. All the strains were tested and in addition an *E. coli* wild type strain was taken as a negative control, because it contains no sterols in the plasma membrane. For clarity only 6 strains are depicted in Fig. 5*A*. The highest amount of binding of natamycin was observed for the wild type (both strain RH448 and RH6611), together with erg4Δerg5Δ. The least amount of binding was observed for the negative control, the *E. coli* wild type strain, whereas strain erg2Δerg6Δ showed the least amount of binding of the yeast strains. The relation of the amount of binding of natamycin to the MIC values is depicted in Fig. 5*B*, at a cell density corresponding to an A_{600} of 10. The figure shows an inverse relation between the amount of bound natamycin to the MIC value of a particular strain, strongly suggesting that the differences in MIC value toward natamycin are directly related to the difference in binding of natamycin to the yeast cells. In addition, binding studies with vesicles made from lipid extracts of plasma membrane-enriched yeast membrane fractions were per-

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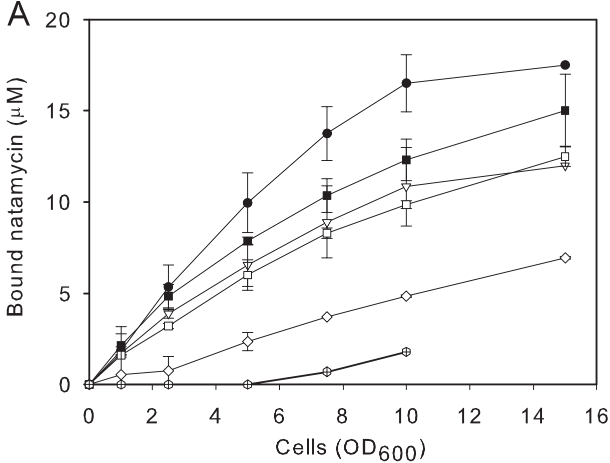

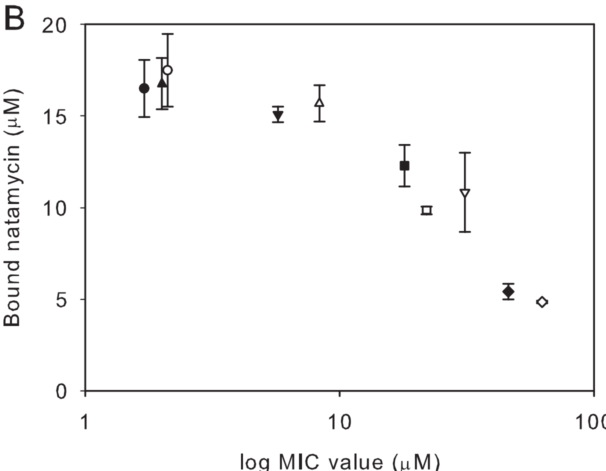

FIGURE 5. **Binding of natamycin to different yeast strains.** In *A*, the binding of natamycin with the yeast strains is depicted by addition of 30 μM natamycin to varying cell densities (A_{600}). In *B* the binding of natamycin to yeast at an A_{600} of 10 is plotted against the MIC values of the different strains. The binding was determined in duplicate in 10 mM MES/Tris, 15 mM K_2SO_4, pH 7.0, and the strains examined were the wild type RH448 (●), the wild type RH6611 (▲), erg4Δerg5Δ (○), erg3Δ (▼), erg6Δ (△), erg3Δerg6Δ (■), erg2Δ (□), cholesterol (▽), erg2Δerg3Δ (◆), erg2Δerg6Δ (◇) and the *E. coli* wild type strain (197).

formed and resulted in a similar binding pattern as compared with intact yeast cells (data not shown).

Effect of Polyene Antibiotics on Proton Permeability in Vitro—The binding assays as well as the MIC determinations show that there is a specific interaction of natamycin with ergosterol, which leads to an inhibition of cell growth. To test if the interaction of natamycin with ergosterol leads to changes in membrane permeability, different leakage assays were employed. Natamycin did not produce any carboxyfluorescein leakage from DOPC vesicles containing 10 mol % ergosterol in contrast to filipin, which did cause carboxyfluorescein leakage (results not shown). Because nystatin, which is known to form pores, also did not cause carboxyfluorescein release from the vesicles, the pores formed by this antibiotic are apparently too small to allow passage of this dye. A similar situation could be the case for natamycin. Therefore, we tried an assay based on leakage of protons that should be small enough to pass such pores. This assay makes use of a pH-dependent fluorescent probe (HPTS), which has a high fluorescent intensity at neutral pH and a low fluorescent intensity at low pH (9). An example of the effect of 5 μM polyene antibiotics on 10% ergosterol containing vesicles is given in Fig. 6*A*. Trace *1* was recorded by addition of the vesicles to the cuvette and following the fluorescence intensity in time (the blank). After ~300 s, the detergent DDAO was added to dissipate the vesicles and the fluorescent intensity reaches its lowest point. Nigericin (trace 2) was used as a positive control and resulted in an immediate dissipation of the proton gradient over the model membrane. Indeed, filipin (trace *3*) and nystatin (trace *4*) both resulted in leakage of the membrane vesicles. Strikingly, natamycin (trace *5*) did not result in proton leakage at this concentration. A more quantitative analysis of the effect of the antibiotics on H^+ leakage in model membranes is given in Fig. 6, *B–D*.

The results show that in strong contrast to filipin and nystatin, natamycin did not induce any significant proton leakage in ergosterol containing vesicles even at very high concentrations (Fig. 6*D*). This would indicate that natamycin does not act via a perturbation of the membrane barrier and thus has a completely different mode of action compared with filipin or nystatin. To test if similar effects could be observed *in vivo*, a proton leakage assay in yeast was performed.

Effect of Polyene Antibiotics on Proton Permeability and Growth in Vivo—To correlate the results from the in vitro leakage assay to an in vivo effect, yeast cells were loaded with the pH-sensitive probe CFDA-SE. The effect of the polyene antibiotics added at 2-fold the MIC value on the wild type yeast strain is displayed in Fig. 7. The fluorescence of the loaded yeast strain was monitored after different time intervals (Fig. 7*A*). In the absence of antibiotic, the yeast cells displayed a steady fluorescence intensity that decreased slightly in time. When natamycin was added, no further decrease in fluorescence intensity was observed. When nystatin was added to the yeast cells an immediate decrease in fluorescence intensity was observed, most likely due to the formation of pores in the plasma membrane. After the decrease of fluorescence, a gradual increase of the fluorescence intensity was observed, which indicates that the yeast cells try to restore the ion gradient over the plasma membrane. Fig. 7*B* shows that with the same conditions used to study the antibiotic-induced release of protons, growth was inhibited by both natamycin and nystatin further emphasizing the difference in mode of action between these polyene antibiotics. In conclusion, natamycin does not kill yeast cells by permeabilizing the plasma membrane.

DISCUSSION

In this study we have demonstrated that natamycin kills yeast by specifically binding to ergosterol but without permeabilizing the plasma membrane. This novel mechanism sets natamycin apart from other polyene antibiotics studied so far. We included two of these as a reference in this study.

The ITC and direct binding studies in both model and yeast membrane systems demonstrated that natamycin binds with an apparent affinity of ~100 μM specifically to ergosterol with a stoichiometry of ~1:1 or 1:2 depending upon whether the sterol is available for interaction only in the outer leaflet or in both leaflets of the membrane. This stoichiometry range is in good agreement with the stoichiometry reported before for other

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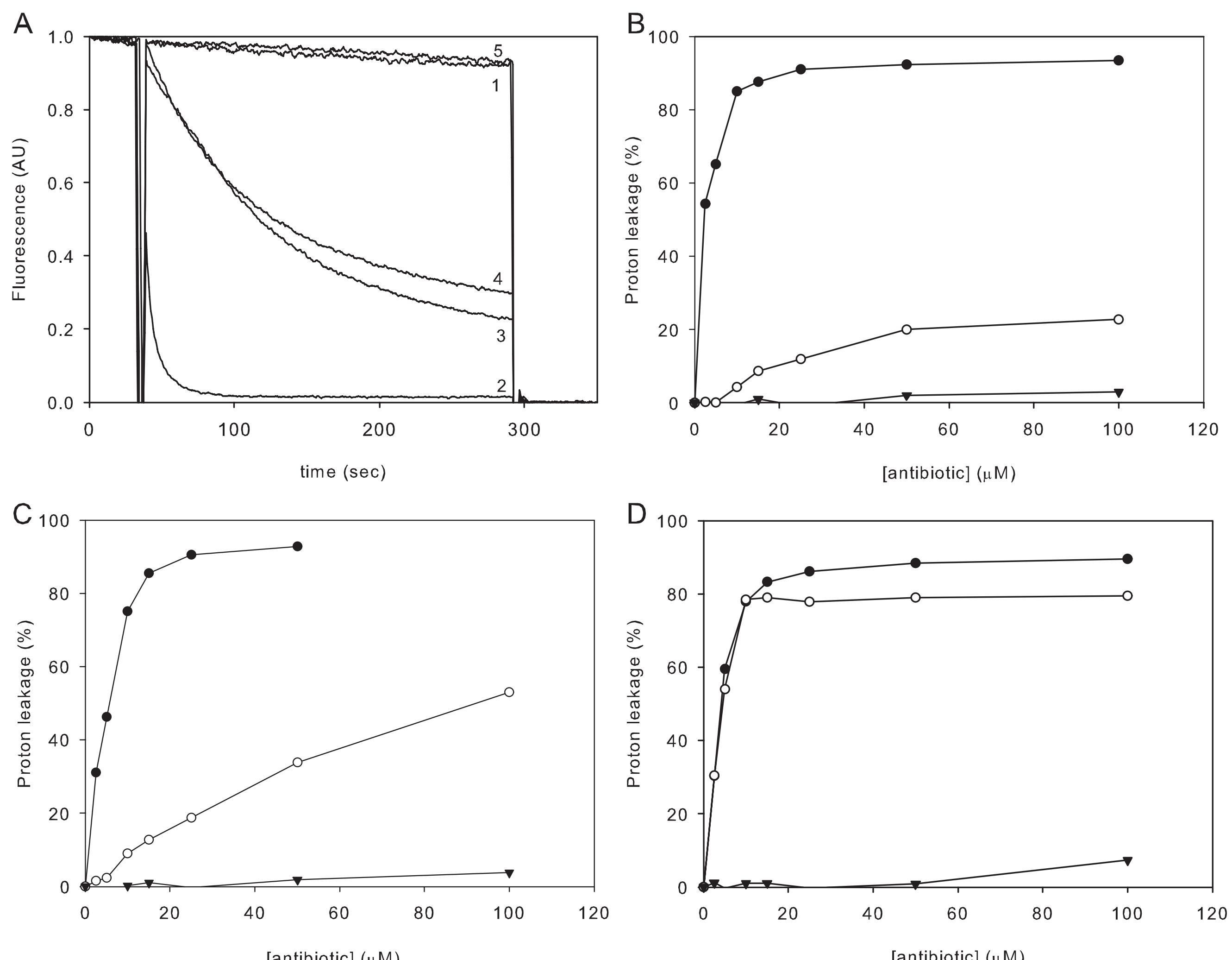

FIGURE 6. **Effect of the polyene antibiotics on the proton permeability of membrane vesicles.** *A*, time courses of HPTS fluorescence, which was influenced by: *1*) no addition, or the addition of: *2*) nigericin; *3*) filipin; *4*) nystatin; and *5*) natamycin (5 μM antibiotic) to 10% ergosterol containing vesicles. *B–D*, the percentage of proton leakage was determined by adding various concentrations of filipin (●), nystatin (○), and natamycin (▼) to vesicles containing (*B*) no sterols, (*C*) 10% cholesterol, or (*D*) 10% ergosterol. Measurements were performed in 10 mM MES, 0.2 M Na_2SO_4 buffer, pH 5.5, and the vesicles had a 35 μM final phospholipid concentration.

polyene antibiotic-sterol interactions (14). However, given the complexity of the binding data and the unknown nature of the natamycin-ergosterol complex a more quantitative discussion of the binding data is not possible.

Both results from the model system and the yeast mutants gave a clear picture of the requirements within the sterol structure for the binding to natamycin, where only variations in the double bonds of the B-ring resulted in large differences in interaction, especially the sp^2 hybridization of C-7. The packing of the sterol molecule together with natamycin is probably related to this structural requirement. The conformation of ring B in ergosterol differs from the conformation of this ring in cholesterol, which is clearly illustrated in Fig. 8. The sp^2 hybridization at C-7 in ergosterol (indicated with an *arrow*, Fig. 8*A*) results in a 1,3-diplanar chair conformation, which is lacking in cholesterol giving a half-chair conformation (Fig. 8*B*) (15). Natamycin has a tightly constrained molecular topology that gives a very high apparent structural order (16). Therefore it is very likely that the diplanar chair conformation of the B-ring in ergosterol will result in a more efficient interaction. For amphotericin B, similar results were observed, where the sp^2 hybridization at C-7 was of critical importance for the interaction of this antibiotic with sterols in model membranes, whereas the double bond at C-5,6 was not essential (17).

The sterol specificity of natamycin in model and biomembranes was more comparable with nystatin than to filipin. This can also be observed from the additional ITC experiments that are given as supplemental data. The observed order of binding for filipin in the ITC experiment was 10% ergosterol > 10% cholesterol > 0% sterol leading to the values of 41.3, 20.4, and 17.4 × 10^4 M^{-1}, respectively. Filipin did not seem to be as dependent on sterol structure nor the presence of sterols as the apparent *K* values to different membranes did not vary much (in agreement with literature) (18–20). The binding of nystatin seemed more similar to natamycin and the *K* value is slightly lower compared with natamycin, 2.72 to 5.7 × 10^4 M^{-1}.

We have shown that the interaction between natamycin and ergosterol leads to an inhibition of yeast growth and cell death, but, this is not via a permeabilization of the membrane as is exhibited by nystatin. The structure of the natamycin-ergos-

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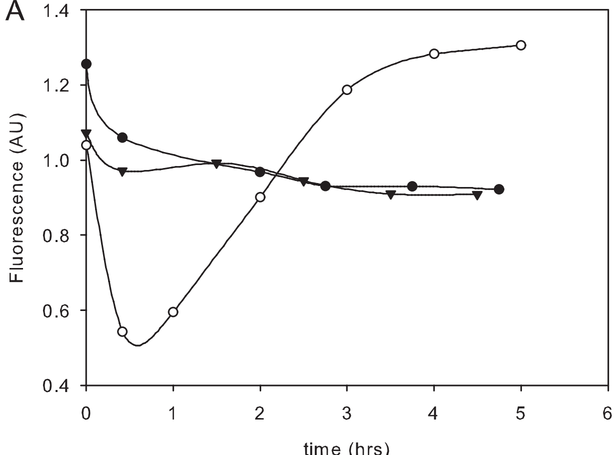

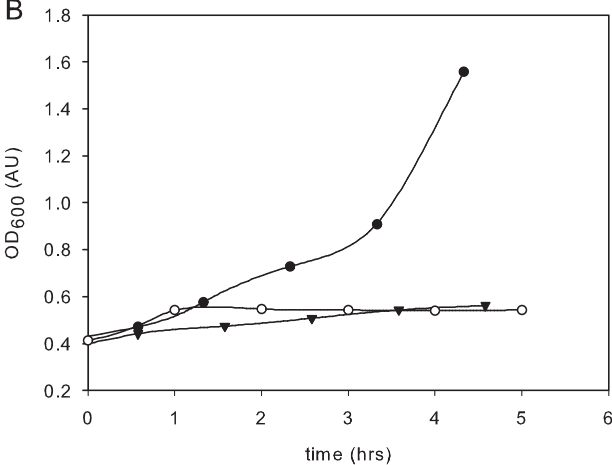

FIGURE 7. **Effect of the polyene antibiotics on CFDA-SE loaded wild type yeast cells.** Yeast cells in YPUADT medium buffered with 50 mM citric/phosphate (pH 4) were followed in time after no addition of antibiotic (●) or the addition of nystatin (○) or natamycin (▼) (2.5 μM). *A*, the fluorescent intensities; and *B*, the optical densities were monitored at regular time intervals.

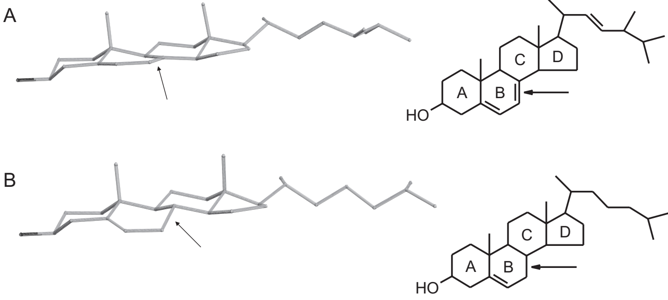

FIGURE 8. **The conformation of the ring structures in ergosterol (*A*) and cholesterol (*B*) viewed from the side at approximately the same angle, together with the flat structures.** The *arrow* indicates the C-7,8 bond, resulting in different B-ring conformations; a 1,3-diplanar chair conformation in ergosterol and a half-chair in cholesterol. Structures were taken from crystal structures given in Refs. 28 and 29.

terol complex is unknown, but assuming that it is similar to nystatin-ergosterol complexes, two possible explanations can account for the difference in mode of action. One is that the formed complex of natamycin and ergosterol might be too tight to pass even an ion as small as a proton. Second, the formed complex could be too small to span the complete bilayer. If the mode of action of natamycin does not involve permeabilization, then how does it act? In this light it is worth recalling that for the polyene antibiotics that are known to permeabilize the membrane, also other modes of actions have been proposed such as oxidative damage of membrane structures (21–23). The mode of action of natamycin must be related to an important function of ergosterol in the yeast cells. For example, sterols are known to have an ordering effect on the membrane, it is thought that they reside in specific sterol-rich domains in membranes and they are also known to be involved in endocytosis, exocytosis, and vacuolar fusion (24–27). Natamycin might inhibit these important processes by binding to ergosterol such that the sterol cannot perform its functional effects.

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REFERENCES

1. Ghannoum, M. A., and Rice, L. B. (1999) *Clin. Microbiol. Rev.* **12,** 501–517
2. de Kruijff, B., and Demel, R. A. (1974) *Biochim. Biophys. Acta* **339,** 57–70
3. Bolard, J. (1986) *Biochim. Biophys. Acta* **864,** 257–304
4. Aparicio, J. F., Colina, A. J., Ceballos, E., and Martin, J. F. (1999) *J. Biol. Chem.* **274,** 10133–10139
5. Heese-Peck, A., Pichler, H., Zanolari, B., Watanabe, R., Daum, G., and Riezman, H. (2002) *Mol. Biol. Cell* **13,** 2664–2680
6. Rouser, G., Fkeischer, S., and Yamamoto, A. (1970) *Lipids* **5,** 494–496
7. Langmuir, I. (1916) *J. Am. Chem. Soc.* **38,** 2221–2295
8. Breukink, E., van Kraaij, C., Demel, R. A., Siezen, R. J., Kuipers, O. P., and de Kruijff, B. (1997) *Biochemistry* **36,** 6968–6976
9. van Kan, E. J., Demel, R. A., Breukink, E., van der Bent, A., and de Kruijff, B. (2002) *Biochemistry* **41,** 7529–7539
10. Bligh, E. G., and Dyer, W. J. (1959) *Can J. Biochem. Physiol.* **37,** 911–917
11. Boyer, M. J., and Hedley, D. W. (1994) *Methods Cell Biol.* **41,** 135–148
12. Bracey, D., Holyoak, C. D., Nebe-von Caron, G., and Coote, P. J. (1998) *J. Microbiol. Methods* **31,** 113–125
13. Bracey, D., Holyoak, C. D., and Coote, P. J. (1998) *J. Appl. Microbiol.* **85,** 1056–1066
14. de Kruijff, B., Gerritsen, W. J., Oerlemans, A., Demel, R. A., and van Deenen, L. L. (1974) *Biochim. Biophys. Acta* **339,** 30–43
15. Baginski, M., Tempczyk, A., and Borowski, E. (1989) *Eur. Biophys. J.* **17,** 159–166
16. Volpon, L., and Lancelin, J. M. (2002) *Eur. J. Biochem.* **269,** 4533–4541
17. Clejan, S., and Bittman, R. (1985) *J. Biol. Chem.* **260,** 2884–2889
18. Milhaud, J., Mazerski, J., Bolard, J., and Dufourc, E. J. (1989) *Eur. Biophys. J.* **17,** 151–158
19. Milhaud, J. (1992) *Biochim. Biophys. Acta* **1105,** 307–318
20. Lopes, S. C., Goormaghtigh, E., Cabral, B. J., and Castanho, M. A. (2004) *J. Am. Chem. Soc.* **126,** 5396–5402
21. Brajtburg, J., Elberg, S., Schwartz, D. R., Vertut-Croquin, A., Schlessinger, D., Kobayashi, G. S., and Medoff, G. (1985) *Antimicrob. Agents Chemother.* **27,** 172–176
22. Sokol-Anderson, M. L., Brajtburg, J., and Medoff, G. (1986) *J. Infect. Dis.* **154,** 76–83
23. Mishra, P., Bolard, J., and Prasad, R. (1992) *Biochim. Biophys. Acta* **1127,** 1–14
24. Wachtler, V., and Balasubramanian, M. K. (2006) *Trends Cell Biol.* **16,** 1–4
25. Takeda, T., and Chang, F. (2005) *Curr. Biol.* **15,** 1331–1336
26. Munn, A. L. (2001) *Biochim. Biophys. Acta* **1535,** 236–257
27. Kato, M., and Wickner, W. (2001) *EMBO J.* **20,** 4035–4040
28. Rodrigues, M. L., Archer, M., Martel, P., Miranda, S., Thomaz, M., Enguita, F. J., Baptista, R. P., Pinho e Melo, E., Sousa, N., Cravador, A., and Carrondo, M. A. (2006) *Biochim. Biophys. Acta* **1764,** 110–121
29. Lascombe, M. B., Ponchet, M., Venard, P., Milat, M. L., Blein, J. P., and Prange, T. (2002) *Acta Crystallogr. D Biol. Crystallogr.* **58,** 1442–1447
30. Mumberg, D., Muller, R., and Funk, M. (1995) *Gene* (*Amst.*) **156,** 119–122

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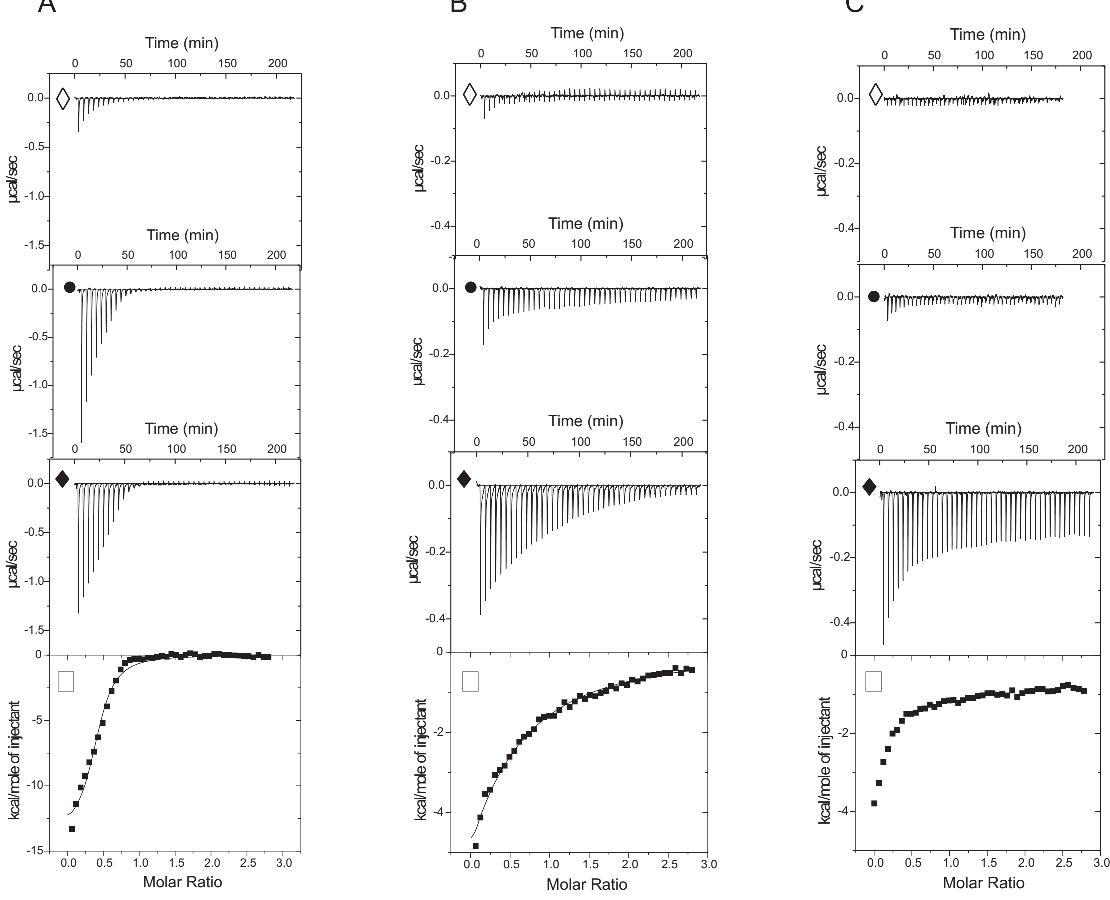
Supplemental Data

Supplementary Figure 1. Calorimetric titrations of the polyene antibiotics with vesicles. Filipin (A), nystatin (B) and natamycin (C) were dissolved in 50 mM MES, 100 mM K_2SO_4 pH 6.0. The top pannels display the heat peaks after consecutive injections of vesicles with no sterol (◊), 10 % cholesterol (•) or 10 % ergosterol (♦) (8 mM final phospholipid concentration) into the sample cell containing 50 mM antibiotic. The bottom graphs ( ) show the integrated heat per injection for 10% ergosterol containing vesicles, which is normalized to the injected amount of moles of sterol and is displayed against the molar ratio of sterol versus antibiotic.

Supplementary Figure 1.



Polyene antibiotic that inhibits membrane transport proteins

Yvonne Maria te Welscher[a], Martin Richard van Leeuwen[b], Ben de Kruijff[a], Jan Dijksterhuis[b], and Eefjan Breukink[a,1]

[a]Department of Biochemistry of Membranes, Institute of Biomembranes, Utrecht University, 3584 CH Utrecht, The Netherlands; and [b]Applied and Industrial Mycology, Fungal Biodiversity Centre-Royal Netherlands Academy of Arts and Sciences (CBS-KNAW), 3584 CT Utrecht, The Netherlands

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The limited therapeutic arsenal and the increase in reports of fungal resistance to multiple antifungal agents have made fungal infections a major therapeutic challenge. The polyene antibiotics are the only group of antifungal antibiotics that directly target the plasma membrane via a specific interaction with the main fungal sterol, ergosterol, often resulting in membrane permeabilization. In contrast to other polyene antibiotics that form pores in the membrane, the mode of action of natamycin has remained obscure but is not related to membrane permeabilization. Here, we demonstrate that natamycin inhibits growth of yeasts and fungi via the immediate inhibition of amino acid and glucose transport across the plasma membrane. This is attributable to ergosterol-specific and reversible inhibition of membrane transport proteins. It is proposed that ergosterol-dependent inhibition of membrane proteins is a general mode of action of all the polyene antibiotics, of which some have been shown additionally to permeabilize the plasma membrane. Our results imply that sterol-protein interactions are fundamentally important for protein function even for those proteins that are not known to reside in sterol-rich domains.

In recent years, advances in the treatment of transplant recipients and patients with cancer or AIDS have been accompanied by a dramatic increase in the incidence of life-threatening fungal infections (1). Sepsis, characterized by a whole-body inflammatory state, is the 10th most common cause of death overall, and the number of cases of sepsis caused by fungal organisms has doubled in 20 y (2, 3). Fungal infections are a major therapeutic challenge, because the therapeutic arsenal is limited and the use of drugs is restricted as a result of toxicity or unfavorable pharmacokinetic profiles. Fungal resistance has been turned into a global public health crisis, especially with fungi showing resistance to more than one antifungal agent (4, 5). In contrast to many other antifungal agents, resistance to polyene antibiotics is considered an exceptionally rare event (4, 5).The polyene antibiotics are the only group of antifungal antibiotics that directly target the plasma membrane via a specific interaction with the main fungal sterol, ergosterol (6). Natamycin, a member of the polyene antibiotic family, is widely used in the food industry and in pharmacotherapy for topical treatment. Unlike other polyene antibiotics, the mode of action of natamycin is not based on the ergosterol-dependent permeabilization of the plasma membrane (7). However, the immediate cessation of growth of yeasts by natamycin treatment indicates that there might be an instantaneous effect of natamycin at the level of the plasma membrane (7), which also contains the highest levels of ergosterol (8). Both the rapid action and lack of pore formation lead us to the hypothesis that natamycin may target plasma membrane proteins in an ergosterol-based manner.

Results

We performed transcriptome analysis on germinating conidia of the fungus *Aspergillus niger* to identify proteins that may be targeted by natamycin. Germination of these conidia has previously been shown to be blocked by natamycin (9). In the analysis, we focused on the effect of natamycin on transcription of plasma membrane proteins. The most prominent plasma membrane proteins in these germinating spores that were affected in their transcription by natamycin treatment were transport proteins. The majority of sugar and amino acid transporters especially showed a clear tendency of up-regulation in the presence of natamycin. (Table 1). The individual gene regulations of 20 and 31 genes for the sugar and amino acid transporters, respectively, are shown in Table S1. An examination of a subselection of the 29 most strongly up-regulated sugar and amino acid transporters after treatment with natamycin shows that the increase in expression was dose- and time-dependent (Fig. 1). Overall, these results show that conidia react to treatment with natamycin by up-regulating many sugar and amino acid transporters. The expression of other membrane proteins was also affected, resulting in either increased or decreased levels of expression (Table S2). For some membrane proteins, the expression was only slightly affected. These data demonstrate that the binding of natamycin to ergosterol has a strong effect on the expression of a subset of plasma membrane proteins.

Next, we determined whether the up-regulation of plasma membrane proteins is attributable to a direct effect of natamycin on the functioning of these proteins. For this, we used the yeast *Saccharomyces cerevisiae*, which is a well-described model system to study functioning of membrane transport proteins (10–12). First, we examined the effects of natamycin on the plasma membrane transporter Can1p. This protein specifically transports arginine into the cell via proton/arginine symport (10). The uptake of arginine was determined in yeast cells that were incubated with different concentrations of natamycin. Fig. 2*A* shows that natamycin causes a dose-dependent decrease in the uptake of arginine, which was complete at 20 μM natamycin. This can be attributable to either natamycin-dependent inhibition of the transport process or natamycin-induced leakage of arginine. This was examined by allowing the cells to take up arginine for 8 min, after which natamycin was added. Natamycin addition immediately blocked uptake without causing release of the arginine that had already been taken up (Fig. 2*B*). This indicates that natamycin directly inhibited the arginine import. Next, we tested the effect of natamycin on the growth and the viability of the yeast cells under identical conditions as used during the arginine uptake assay (Fig. S1). These results show that natamycin inhibits arginine uptake at concentrations that inhibit cell growth but without killing the cells. In addition, we could show that natamycin-induced blockade of arginine uptake in yeast cells can be reversed (Fig. S2*A*). However, relieving the blockade in arginine uptake does not result in immediate regrowth of the yeast culture (Fig. S2*B*). This suggests that natamycin also affects other plasma membrane proteins that possibly take longer to recuperate after removing the natamycin pressure on the outside of the cells, which is supported by the transcriptome analysis.

Author contributions: Y.M.t.W., M.R.v.L., B.d.K., J.D., and E.B. designed research; Y.M.t.W. and M.R.v.L. performed research; Y.M.t.W., M.R.v.L., and J.D. analyzed data; and Y.M.t.W., M.R.v.L., B.d.K., J.D., and E.B. wrote the paper.

The authors declare no conflict of interest.

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[1]To whom correspondence should be addressed. E-mail: e.j.breukink@uu.nl.

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BIOCHEMISTRY

Table 1. Effect of natamycin on the differential expression of sugar and amino acid transporters in *A. niger* conidia

		2h + natamycin			8h + natamycin	
	2h	2h, 3μM	2h, 10μM	8h	8h, 3μM	8h, 10μM
Cumulative values, arbitrary units						
Sugar transporters (*n* = 20)	3,840	5,976	7,843	3,421	14,983	20,678
Amino acid transporters (*n* = 31)	7,054	10,059	12,541	4,596	10,498	12,051
Ratio natamycin/control						
Sugar transporters	1	1.56	2.04	1	4.38	6.04
Amino acid transporters	1	1.43	1.78	1	2.28	2.62
All expressed proteins	1	1.23	1.34	1	1.21	1.25

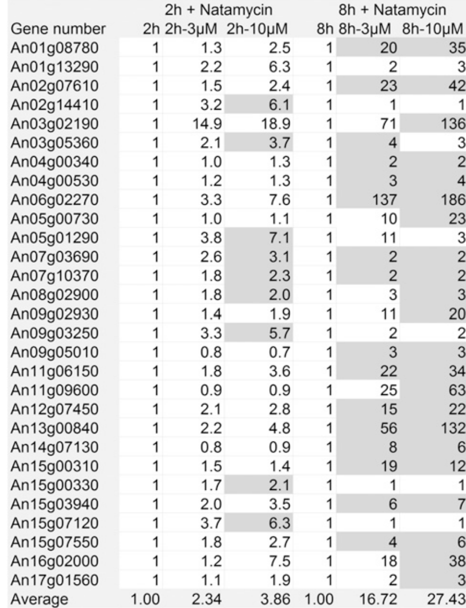
Next, the effects of natamycin on the transport of two other substrates (glucose and proline) were determined. Glucose is transported in yeast via hexose transporters, and of the 20 genes that encode these proteins, 7 are known to encode functional glucose transporters. All of them transport their substrates by passive, energy-independent, facilitated diffusion, with glucose moving down the concentration gradient (12). Proline can be taken up by a specific high-affinity permease Put4p or the general amino acid permease Gap1p, both of which are regulated by nitrogen (10, 13). Other low-affinity systems are also present, such as Agp1p and Gnp1p (13). The uptake of glucose and proline was completely blocked by the addition of natamycin (Fig. 3) at similar antibiotic-to-cell ratios that blocked arginine uptake. These results show that natamycin blocks the uptake of several important nutrients over the plasma membrane of yeast and indicate that natamycin can inhibit different membrane transport proteins.

Because the effect of natamycin on transcription of the sugar and amino acid transporters was shown in germinating conidia of *A. niger*, we adapted the arginine, proline, and glucose uptake assays to these conidia. Fig. 4 shows that natamycin is also able to inhibit the import of all tested substrates in germinating *A. niger* conidia after only a 5-min incubation. After 5 h of incubating the conidia with natamycin, the inhibition of the uptake of the amino acids was at its maximal level, whereas the conidia seemed to be able to recuperate partially from the blockade in glucose. The twofold general increase in the expression of sugar transporters over that of the amino acid transporters may be the reason for this (Table 1). These results show that natamycin is able to block the uptake of different substrates in baker's yeast as well as in fungal conidia. Taken together, these data demonstrate that natamycin has a general effect on transport proteins and that the binding of ergosterol by the antifungal has dramatic effects on the expression and functioning of membrane proteins.

Discussion

Natamycin is the only member of the polyene antibiotic family that does not exert its antifungal action by forming pores and permeabilizing the plasma membrane. It does, however, require binding to the same lipid receptor, ergosterol, as other polyene antibiotics. We have found that on binding ergosterol in the plasma membrane, natamycin is able to inhibit a broad class of essential membrane transport proteins without compromising the barrier function of the membrane.

The differences in substrate specificities, transport mechanisms, and lack of common domains/motifs of the amino acid and glucose transporters make it likely that natamycin inhibits these proteins



Gene number	2h	2h + Natamycin 2h-3μM	2h-10μM	8h	8h + Natamycin 8h-3μM	8h-10μM
An01g08780	1	1.3	2.5	1	20	35
An01g13290	1	2.2	6.3	1	2	3
An02g07610	1	1.5	2.4	1	23	42
An02g14410	1	3.2	6.1	1	1	1
An03g02190	1	14.9	18.9	1	71	136
An03g05360	1	2.1	3.7	1	4	3
An04g00340	1	1.0	1.3	1	2	2
An04g00530	1	1.2	1.3	1	3	4
An06g02270	1	3.3	7.6	1	137	186
An05g00730	1	1.0	1.1	1	10	23
An05g01290	1	3.8	7.1	1	11	3
An07g03690	1	2.6	3.1	1	2	2
An07g10370	1	1.8	2.3	1	2	2
An08g02900	1	1.8	2.0	1	3	3
An09g02930	1	1.4	1.9	1	11	20
An09g03250	1	3.3	5.7	1	2	2
An09g05010	1	0.8	0.7	1	3	3
An11g06150	1	1.8	3.6	1	22	34
An11g09600	1	0.9	0.9	1	25	63
An12g07450	1	2.1	2.8	1	15	22
An13g00840	1	2.2	4.8	1	56	132
An14g07130	1	0.8	0.9	1	8	6
An15g00310	1	1.5	1.4	1	19	12
An15g00330	1	1.7	2.1	1	1	1
An15g03940	1	2.0	3.5	1	6	7
An15g07120	1	3.7	6.3	1	1	1
An15g07550	1	1.8	2.7	1	4	6
An16g02000	1	1.2	7.5	1	18	38
An17g01560	1	1.1	1.9	1	2	3
Average	1.00	2.34	3.86	1.00	16.72	27.43

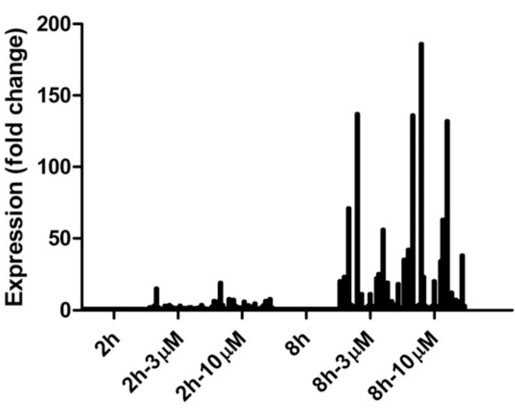


Fig. 1. Expression of a selection of sugar and amino acid transporters. The expression of 29 sugar and amino acid transporters that were most strongly upregulated on treatment with natamycin (>120 arbitrary units after 2 or 8 h) is shown. Together, these dominant transporters make up over 90% of the expression intensity of all sugar and amino acid transporters after natamycin treatment. A detailed description of the genes is provided in Table S1. Expression values are given of conidia during different stages of germination (controls) or in the presence of natamycin at different concentrations. The data are given as fluorescence data from three independent microarray experiments. The gray values are significant below the $P = 0.01$ level.

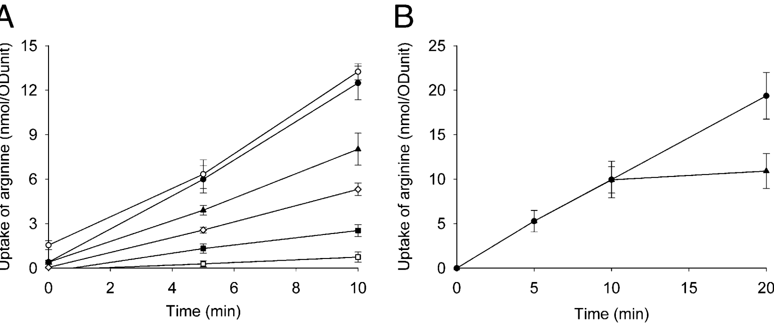


Fig. 2. Effect of natamycin on the uptake of arginine by yeast cells. At time 0, ^{14}C-arginine (30 μM) was added to the cells. The uptake of arginine was followed in time and corrected for the amount of cells (ODunit). (*A*) Yeast cells were incubated for 5 min before the addition of arginine with natamycin at 0 μM (●), 2 μM (▲), 5 μM (◇), 10 μM (■), and 20 μM (□), and DMSO (○) was added as a control. (*B*) Release of arginine from yeast cells was studied by adding 20 μM natamycin (▲) or no natamycin (●) at 8 min after the addition of arginine, and the uptake of arginine was followed in time. The results shown are the averages of three separately performed experiments with SD.

via a general mechanism (11, 12, 14). What these transporters do have in common is their similarity in length and hydrophobicity profiles (11, 15, 16). Therefore, it is most likely that natamycin affects these proteins through alterations in membrane properties instead of inhibiting each protein involved in a specific way. Moreover, considering the amount of transport proteins that were up-regulated by natamycin treatment in addition to those tested in our assays, we propose that natamycin is able to block virtually all transport processes in the plasma membrane of fungi.

Answering the question of how natamycin changes the membrane properties starts with the premise that the antibiotic action is attributable to binding to ergosterol (7), thereby affecting the functional properties the sterol has on membrane proteins either via ergosterol-rich domains (17) or more directly by interfering with a direct ergosterol-protein interaction. The different oxygen functions of natamycin may play a role in the latter mechanism, as is also suggested for other polyenes (6, 18). A disruption of the equilibrium of free sterols to sphingolipids by natamycin may also be responsible for the inhibition of ergosterol-dependent protein functions because sterols and sphingolipids work together to carry out a wide variety of functions (19).

The unique mechanism revealed in this study for natamycin may also be applicable to other polyene antibiotics that permeabilize the plasma membrane because these antibiotics all exert their action via binding to ergosterol. This is supported by the observation that nystatin affected properties of the arginine and glucose transporters in yeast plasma membrane vesicles (20). Further, the amphotericin B methyl ester inhibited the replication, assembly, and release of HIV-1 by interfering with the ion channel viral protein U (VPU) (18, 21). Microarray studies with *S. cerevisiae* clearly show an increase of expression of transport proteins on treatment with the polyenes amphotericin B and nystatin (22, 23). However, membrane permeabilization by these polyene antibiotics does mask these effects as a result of the fast collapse of vital ion or substrate gradients.

Recently, studies from the laboratory of Burke have shown that deleting one hydroxyl group (at position 35) on the ring structure of such a pore-forming polyene antibiotic, amphotericin B, left this derivative unable to form pores but still able to bind ergosterol and to retain its antifungal activity to a significant extent (24). Gray et al. (24) concluded that amphotericin B primarily kills yeast by simply binding ergosterol and that membrane permeabilization via pore formation represents a second complementary mechanism. Earlier studies from the laboratory of Carreira showed that substituting the hydroxyl group at position 35 of the methyl-ester version of amphotericin B led to a significant but not complete reduction in K^+ efflux from model membrane vesicles (25, 26). This finding pointed to the importance of this hydroxyl group for pore formation and led to the conclusion that channel formation is a necessary

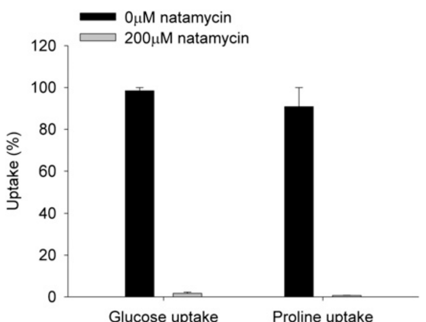


Fig. 3. Effect of natamycin on uptake of glucose and proline by yeast cells. Cells were incubated with 0 or 200 μM natamycin, after which the uptake of ^{14}C-glucose or ^{14}C-proline was assayed. The uptake of the different compounds by yeast cells is expressed as the percentage to the uptake of a compound by cells untreated with natamycin after 10 min. Additional details are provided in *Experimental Methods*. The results shown are the averages of two separately performed experiments with the spread of the data.

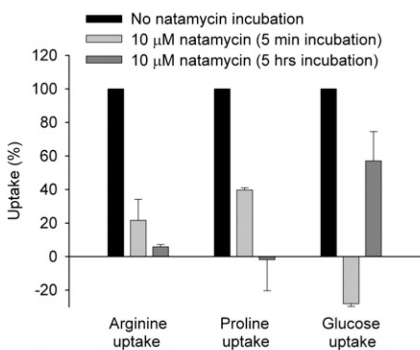


Fig. 4. Effect of natamycin on uptake of arginine, proline, and glucose by *A. niger* conidia. Conidia were incubated for either 5 min or 5 h with 0 or 10 μM natamycin, after which the uptake of ^{14}C-arginine, ^{14}C-proline, or ^{14}C-glucose was assayed. The uptake of the different compounds by conidia incubated for 0 or 10 min at 30 °C without antibiotic is normalized to 100% and compared with the effect of natamycin on substrate uptake. Negative uptake values occur because a smaller amount of compound was taken up by the spores after incubation with natamycin in comparison to the fast uptake in the absence of the inhibitor. Additional details are provided in *Experimental Methods*. The results shown are the averages of two separately performed experiments with the spread of the data.

condition for the antifungal activity of amphotericin B. However, no explanation was given for the residual activity of the compound, and the residual membrane permeabilization that was observed in this study was likely attributable to an aspecific effect caused by the positively charged nature of the methyl-ester version of the Carreira group, as previously suggested (24). Interestingly, the antifungal activity of natamycin is comparable to the activities of both amphotericin derivatives (24, 26). This implies that these amphotericin B derivatives are also able to inhibit a broad class of essential membrane transport proteins by binding to ergosterol, thus explaining their antifungal activities. This is strengthened by the observation that both natamycin and the variant of amphotericin B of the Burke laboratory require ergosterol for interacting with the membrane and executing their antifungal activity (7, 24). Amphotericin B may therefore be a good example of a polyene antibiotic that, via the binding of ergosterol, has a dual mode of action by inhibiting membrane proteins and permeabilizing the plasma membrane. A similar dual mode of action has been observed before in antibacterial lantibiotics that are able to block cell wall synthesis and form pores (27, 28). Likewise, the antibiotics chloramphenicol and tetracycline were shown to act both by inhibiting protein synthesis and by blocking protein translocation into the bacterial membrane (29–31). Because natamycin is the only natural family member known that does not permeabilize the membrane, it is the ideal candidate to study this previously undescribed basic mode of action of the polyene antibiotics further. This unique mode of action by completely blocking the transport function of many (if not all) membrane proteins via binding to a specific lipid receptor in the fungal membrane implies that sterol-protein interactions are fundamentally important for protein function even for those proteins that are not known to reside in sterol-rich membrane domains.

Experimental Methods

A unique RNA extraction method for conidia of *A. niger* was developed resulting in high-quality intact RNA. Full experimental details are included in *SI Experimental Methods*. RNA samples from three independent biological replicates were used for hybridization of Affymetrix microarray chips representing 14,509 ORFs of *A. niger*. Each experiment had conidia pooled from three cDNA labeling experiments. Microarray hybridization and scanning were performed at ServiceXS (Leiden, The Netherlands) according to Affymetrix protocols. The Functional Catalogue (Munich Information Center for Protein Sequences) (32) was used for systematic classification of genes according to their cellular and molecular functions (33).

Transport assays for arginine, glucose, and proline in *S. cerevisiae* were based on the methods of Robl et al. (10) and Malínská et al. (34). The uptake of arginine was determined in yeast cells that were incubated with different concentrations of natamycin. The substrate uptake assays in fungal conidia were performed in a similar way to the assays with baker's yeast to allow a comparison of the results. Full experimental details are included in *SI Experimental Methods*.

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1. Cuenca-Estrella M, et al. (2008) Update on the epidemiology and diagnosis of invasive fungal infection. *Int J Antimicrob Agents* 32(Suppl 2):S143–S147.
2. Martin GS, Mannino DM, Eaton S, Moss M (2003) The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 348:1546–1554.
3. Llewelyn MJ, Cohen J (2007) Tracking the microbes in sepsis: Advancements in treatment bring challenges for microbial epidemiology. *Clin Infect Dis* 44:1343–1348.
4. Monk BC, Goffeau A (2008) Outwitting multidrug resistance to antifungals. *Science* 321:367–369.
5. Kanafani ZA, Perfect JR (2008) Antimicrobial resistance: Resistance to antifungal agents: Mechanisms and clinical impact. *Clin Infect Dis* 46:120–128.
6. Bolard J (1986) How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochim Biophys Acta* 864:257–304.
7. te Welscher YM, et al. (2008) Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *J Biol Chem* 283:6393–6401.
8. Zinser E, Paltauf F, Daum G (1993) Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. *J Bacteriol* 175: 2853–2858.
9. Van Leeuwen MR, Van Doorn TM, Golovina EA, Stark J, Dijksterhuis J (2010) Water- and air-distributed conidia differ in sterol content and cytoplasmic microviscosity. *Appl Environ Microbiol* 76:366–369.
10. Robl I, Grassl R, Tanner W, Opekarová M (2001) Construction of phosphatidylethanolamine-less strain of Saccharomyces cerevisiae. Effect on amino acid transport. *Yeast* 18:251–260.
11. Regenberg B, Düring-Olsen L, Kielland-Brandt MC, Holmberg S (1999) Substrate specificity and gene expression of the amino-acid permeases in Saccharomyces cerevisiae. *Curr Genet* 36:317–328.
12. Ozcan S, Johnston M (1999) Function and regulation of yeast hexose transporters. *Microbiol Mol Biol Rev* 63:554–569.
13. Andréasson C, Neve EP, Ljungdahl PO (2004) Four permeases import proline and the toxic proline analogue azetidine-2-carboxylate into yeast. *Yeast* 21:193–199.
14. Chervitz SA, et al. (1999) Using the Saccharomyces Genome Database (SGD) for analysis of protein similarities and structure. *Nucleic Acids Res* 27:74–78.
15. Sophianopoulou V, Diallinas G (1995) Amino acid transporters of lower eukaryotes: Regulation, structure and topogenesis. *FEMS Microbiol Rev* 16:53–75.
16. Horák J (1997) Yeast nutrient transporters. *Biochim Biophys Acta* 1331:41–79.
17. Zumbuehl A, Stano P, Heer D, Walde P, Carreira EM (2004) Amphotericin B as a potential probe of the physical state of vesicle membranes. *Org Lett* 6:3683–3686.
18. Waheed AA, et al. (2006) Inhibition of HIV-1 replication by amphotericin B methyl ester: Selection for resistant variants. *J Biol Chem* 281:28699–28711.
19. Guan XL, et al. (2009) Functional interactions between sphingolipids and sterols in biological membranes regulating cell physiology. *Mol Biol Cell* 20:2083–2095.
20. Opekarová M, Tanner W (1994) Nystatin changes the properties of transporters for arginine and sugars. An in vitro study. *FEBS Lett* 350:46–50.
21. Waheed AA, et al. (2008) Inhibition of human immunodeficiency virus type 1 assembly and release by the cholesterol-binding compound amphotericin B methyl ester: Evidence for Vpu dependence. *J Virol* 82:9776–9781.
22. Zhang L, et al. (2002) Response of gene expression in Saccharomyces cerevisiae to amphotericin B and nystatin measured by microarrays. *J Antimicrob Chemother* 49: 905–915.
23. Agarwal AK, et al. (2003) Genome-wide expression profiling of the response to polyene, pyrimidine, azole, and echinocandin antifungal agents in Saccharomyces cerevisiae. *J Biol Chem* 278:34998–35015.
24. Gray KC, et al. (2012) Amphotericin primarily kills yeast by simply binding ergosterol. *Proc Natl Acad Sci USA* 109:2234–2239.
25. Szpilman AM, Cereghetti DM, Wurtz NR, Manthorpe JM, Carreira EM (2008) Synthesis of 35-deoxy amphotericin B methyl ester: A strategy for molecular editing. *Angew Chem Int Ed Engl* 47:4335–4338.
26. Szpilman AM, Manthorpe JM, Carreira EM (2008) Synthesis and biological studies of 35-deoxy amphotericin B methyl ester. *Angew Chem Int Ed Engl* 47:4339–4342.
27. Breukink E, et al. (1999) Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* 286:2361–2364.
28. Hasper HE, et al. (2006) An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science* 313:1636–1637.
29. van Stelten J, Silva F, Belin D, Silhavy TJ (2009) Effects of antibiotics and a proto-oncogene homolog on destruction of protein translocator SecY. *Science* 325:753–756.
30. Pioletti M, et al. (2001) Crystal structures of complexes of the small ribosomal subunit with tetracycline, edeine and IF3. *EMBO J* 20:1829–1839.
31. Schlünzen F, et al. (2001) Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. *Nature* 413:814–821.
32. Ruepp A, et al. (2004) The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Res* 32:5539–5545.
33. Pel HJ, et al. (2007) Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. *Nat Biotechnol* 25:221–231.
34. Malínská K, Malínský J, Opekarová M, Tanner W (2003) Visualization of protein compartmentation within the plasma membrane of living yeast cells. *Mol Biol Cell* 14: 4427–4436.

Earth, Environmental and Life Sciences
Utrechtseweg 48
3704 HE Zeist
P.O. Box 360
3700 AJ Zeist
The Netherlands

www.tno.nl

T +31 88 866 60 00
F +31 88 866 87 28
infodesk@tno.nl

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Literature study on antifungal resistance in general and natamycin resistance in particular

Date	October, 2012
Author(s)	Tineke van den Hoorn, Annelotte Vonk, Jos van der Vossen
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1 Introduction

Natamycin or pimaricin is a polyene macrolide antimycotic substance active against a broad variety of yeasts and moulds present in nature. The compound has no antibacterial properties. As such it is commercially exploited as an antifungal product as a food preservative in cheeses and sausages, and to a lesser extend as antimycotic drug in topical medication.

DSM Food Specialties B.V. (DFS) has natamycin on the market as food preservative. Since antibiotic drug resistance is a serious concern that is also acknowledged by DFS, they requested TNO to perform a literature study on the potential of fungi to become resistant to natamycin. Such exploration is particularly relevant in view of supporting submissions for their natamycin product in different applications with an independent literature study on recently (2000 – present) published antifungal resistance data. The study includes the identified papers and the qualifying criteria to determine their relevance. This should lead to a good insight in published information specifically related to possible natamycin resistance development and resistance transfer via horizontal gene transfer. Furthermore, information on cross resistance to other polyene antimycotics and other antimycotics was searched for and processed as well as the relation between mode of action of natamycin and development of cross-resistance in fungi. After collection of published data, arguments are given 'for' and 'against' findings of natamycin resistance risk.

DFS requested TNO to perform this literature study in which TNO looked specifically at published material on natamycin resistance. In agreement with DFS, literature on the subject was obtained through an independent library search. The library search was focused on literature published from the year 2000 until present. The search was performed on April 23, 2012 by using the STN databases covering the clusters: BioScience, Food, Chemistry, Medicine, Pharmacology. The different databases are listed in Appendix I. The search terms used for the literature search were provided by DFS included:

- natamycin: natamycin(e); pimaricin(e); natamax; delvocid; pimafucin;
- resistance : resistance; resistant; resistentie; tolerant; sensitive; sensitivity; gevoelig(heid); cross(-)resistentie; cross(-)resistance.

After analysis of relevant literature material, additional search terms were used to obtain supplementary information mainly using search databases Pubmed and Scopus on June 3, 2012. These search terms included:

- fungal/fungi (drug) resistance; fungal (drug) cross-resistance; mould (drug) resistance;
- natamycin (drug) resistance; polyene antibiotics; polyene (drug) resistance; antifungal (drug) resistance;

For drafting the report, a selection was made as to which literature was relevant for this review and this selection is listed in separate folders in Refworks including given arguments (Appendix II provided in supplement). The literature considered relevant is subdivided into subjects.

The basis for including literature for reviewing was:

- its reference to antimycotics in combination with resistance or failing inhibition.
- Its reference to cross resistance of polyene antimycotics and other antimycotics.
- nature of antibiotic drug resistance
- nature of acquisition of antibiotic drug resistance
- recent review papers on natamycin.

Furthermore, as a criterion for relevant literature, only sources considered reliable were selected. These sources mainly included recent reviews (2000- present) and scientific magazines generally accepted as publishing sound, substantiated research studies. Some studies published prior to 2000 were also included, when considered relevant. These were included because observations of natamycin resistance were not described in recent literature and only referred to older publications. Individual cases of fungal or natamycin resistance were summarized as not every described case was considered relevant or trustworthy.

Literature was excluded when considered not reliable or describing studies that were not sufficiently substantiated. Also literature that was written in foreign language without an appropriate English translation was excluded, unless abstracts provided sufficient and relevant information. Publications of described studies with individual cases of natamycin or polyene resistance were not selected when medical circumstances of treatment were not described in detail

2 Natamycin: the compound and applications

2.1 The compound

Natamycin ($C_{33}H_{47}NO_{13}$) is a polyene macrolide antimycotic compound with a molecular weight of 665.7 Daltons. It is an amphoteric molecule containing one basic and one acidic group. It is produced by fermentation of sugar-based substrates by *Streptomyces natalensis*. Natamycin is recovered by extraction, filtration and drying. As a dry powder it can be stored for several years with minimal loss of activity. Gao et al. (2010) described the stability of natamycin covering the effects of temperature, light, pH and inorganic salts on the biological stability of water dissolved natamycin. Natamycin in aqueous solutions at temperatures below 100 °C exhibits strong biological stability. However, the exposure to 121 °C results in the reduction of biological stability of natamycin solution. Natamycin activity in an aqueous solution was completely abolished by heat treatment at 121 °C during 30 minutes. Natamycin was also shown sensitive to UV radiation and inactivated after UV treatment for 90 minutes. Moreover, fluorescent light radiation could also result in inactivation of natamycin. The complete inactivation of natamycin was observed by fluorescent light illumination for 10 days. Natamycin appeared to be relatively stable in aqueous solutions in the range of pH 4-8 (Gao et al., 2010). Natamycin has a limited solubility in water, around 40 ppm. The minimum inhibitory concentration (MIC) for natamycin against almost all foodborne fungi is less than 20 ppm. It is fungicidal and has a no inhibitory effect on bacteria. The activity towards fungi and yeasts is summarized in tables published by Stark, 2003 and Delves-Broughton et al. 2006.

Natamycin is considered a safe food additive and is an approved application on cheese by the Food and Drug Administration (FDA) according to its last revision in 2011 (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/ CFRSearch.cfm?fr=172.155). It is also approved by the European Food Safety Authority (EFSA) for application on cheese and sausages in 2009. The safety of natamycin uptake was reviewed and confirmed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA,) (Stark 2003; EFSA, 2009).

2.2 Applications

Below acknowledged food and medical application areas of natamycin are described.

In the food area, natamycin is mainly used in protection of product surfaces of cheese and sausages for fungal growth. For application of natamycin on cheese, three different methods are applied:

- spraying on the cheese surface
- dipping the cheese in natamycin suspension
- applying natamycin in a polyvinyl acetate suspension to coat the cheese surface.

Shredded cheese, blue cheese and block cheese are all treated with natamycin by spraying. Natamycin can also be added to block cheese by dipping or coating.

Other dairy applications of natamycin have been described (Dos Santos Pires et al. 2008; Vinderola et al. 2002; Basilico et al. 2001) but are currently not applied in Europe.

For application of natamycin on fermented sausages prone to spoilage by growth of moulds and yeasts, spraying or dipping are the preferred treatments.
Natamycin does not migrate into the interior and is tasteless. Thereby it will not affect the bacterial starter cultures essential for the fermentation process (Stark 2003, Delves-Broughton et al. 2006).

In the medical area, Natamycin finds its main application in the treatment of fungal keratitis. This is a ophthalmic condition that requires medical treatment with antimycotics and/or antibiotics, depending on the cause of the keratitis which can be caused by bacteria, viruses, fungi or parasites. Treatment of this disease often includes natamycin eye drops or natamycin in combination with antifungal agents belonging to the azole group. This is due to natamycin showing poor penetration of the skin which renders it only effective against topical or superficial infections. It is especially effective against keratitis caused by filamentous fungi like *Fusarium* sp. and *Aspergillus* sp (Pouyeh et al. 2011).

Natamycin has also efficiently been used in other medical treatments against fungal infections like vulvovaginal candidosis (Vrablik et al. 2007), but is less efficient against systemic infections like systemic mycoses as its absorption trough gastrointestinal mucosa is almost nonexistent (for review, see Vandeputte et al., 2012).

To conclude, natamycin is an effective antifungal that can be used as a food preservative and in specific topical medical applications. The use of natamycin in these applications has wide regulatory status throughout the world.

3 Mechanism of action of polyenes

Polyene antibiotics can act as fungistatic and fungicidal compounds. After their first discovery they were classified into two groups; I and II.

- Group I polyenes, including filipin, etruscomycin and natamycin, were thought to destroy the fungal membrane in an all-or-none effect showing mainly fungicidal effects.
- Group II polyenes, including candicidin, nystatin and amphotericin B, were thought to induce ion leakage at much lower concentrations having a more fungistatic effect (Kotler-Brajtburg et al, 1979). These group II polyenes are larger molecules that interact in a hydrophobic manner with sterols to form a half pore. The combination of two half-pores builds up a membrane spanning aqueous pore that alters the selective permeability of the lipid bilayer (Baginski et al, 1997).

Filipin, a much smaller compound than natamycin that lacks a charged carboxyl group and a bulky mycosamine, is thought to form a planar complex with sterols. Two of these planar aggregates associate with their hydrophilic sides to form a double sandwich like structure. The entire complex is thought to be embedded within the hydrophobic core of the lipid bilayers, which results in membrane fragmentation and cellular leakage (De Kruijf and Demel, 1974, De Kruijf et al., 1974) and as reviewed by Van Leeuwen et al. (Van Leeuwen et al. 2009). For amphotericin B, it was shown that formation of ion channels causing cellular leakage is not the only mechanism of its antifungal action. This polyene was shown to kill the fungal cells also by simply binding ergosterol and without ion-channel formation (Gray et al., 2012).

For natamycin, like for more polyene antibiotics, the exact mode of action was unknown until recently. In 2008, Te Welscher et al. showed through isothermal titration calorimetric measurements and direct binding studies that natamycin binds specifically to ergosterol present in model membranes. Yeast sterol biosynthetic mutants revealed the importance of the double bonds in the B-ring of ergosterol for the natamycin-ergosterol interaction and the consecutive block of fungal growth. However, in contrast to nystatin and filipin, natamycin did not change the permeability of the yeast plasma membrane under conditions where growth was blocked. In ergosterol containing model membranes, natamycin was not able to change bilayer permeability. These results show that natamycin acts via a novel mode of action and blocks fungal growth by binding specifically to ergosterol (te Welscher et al., 2008, Jones et al. 2010).

Van Leeuwen et al. (2009) studied the role of natamycin, nystatin and filipin in conidial germination of the food spoilage mold *Penicillium discolor*. Germination is characterized by an initial stage of isotropic growth followed by polarization and outgrowth of the germ tube. Membrane permeabilization was observed when germinating conidia from *Penicillium discolor* were treated with nystatin or filipin. However, treatment with natamycin did not result in membrane permeabilization. Instead, natamycin was found to inhibit endocytosis, a cellular process that enables active uptake of membrane vesicles into the cell (Van Leeuwen et al. 2009).
Using isolated yeast vacuoles Te Welscher et al. (2010) showed that natamycin inhibited the vacuole fusion without affecting the barrier function of vacuolar

membranes. Sublethal doses of this antimycotic perturbed vacuolar morphology by formation of many more small vacuolar structures in yeast cells. Deletions in the *ERG genes,* which lead to changes in the tail of ergosterol, did not affect vacuolar fusion rates. However vacuoles from *ERG3* or *ERG*2 deletions, causing a loss of double bonds in the B-ring of ergosterol, did result in vacuoles with reduced fusion capabilities. This suggests that the inhibitory activity of natamycin is dependent on the presence of specific chemical features in the structure of ergosterol that allows binding of natamycin (te Welscher et al. 2010).

Recently published results by Te Welscher et al. (2012) demonstrated fungal growth inhibition by natamycin via inhibition of ergosterol-specific membrane transport proteins. Transport inhibition affected the uptake of glucose and the amino acids proline and arginine. They speculated about the fact that this mechanisms is also applicable to other polyene antibiotics via their binding to ergosterol. This recent information on ergosterol binding of polyenes combined with the observation of Gray et al. (2012) made Te Welscher et al. (2012) propose that ergosterol-dependent inhibition of membrane proteins is a general mode of action of all polyene antibiotics and that some of them have a dual activity, displaying also a plasma membrane permeabilizing action in fungi.

4 Resistance in fungi

4.1 Definitions

Many terms are used in literature to describe antibiotic drug resistance and related phenomena. In order to interpret these definitions in this literature research correctly we tried to clarify the meaning of these widely used terms. Formal definitions were determined by descriptions obtained from the Merriam-Webster dictionary. In combination with how definitions are explained and described in literature we made a choice as to how these definitions should be used to describe phenomena related to resistance.

In relation to antibiotic drug **resistance** of micro-organisms the Merriam-Webster dictionary describes the definition of this word as follows:
"the power or capacity to resist: as
a: the inherent ability of an organism to resist harmful influences (as disease, toxic agents, or infection)
b: the capacity of a species or strain of microorganism to survive exposure to a toxic agent (as a drug) formerly effective against it." (http://www.merriam-webster.com/dictionary/ resistance?show=0&t=1344858508).

When interpreting the meaning of the word resistance in relation to micro-organisms able to resist antibiotics, a distinction has to be made between multiple terms that are frequently used in relation to resistance. Terms as **microbiological resistance** and **clinical resistance** are both used in literature although these refer to different events. **Microbiological resistance** often refers to non-susceptibility of a micro-organism to an antimicrobial agent by *in vitro* susceptibility testing, in which the minimal inhibitory concentration (MIC) of the drug exceeds the susceptibility breakpoint for that organism (Kanafani et al. 2008). Microbiological resistance can be natural (primary) or acquired (secondary). Here, **natural resistance** refers to the micro-organisms natural ability to be insensitive to a drug or compound without prior exposure to that drug. **Acquired resistance** on the other hand refers to a lack of sensitivity to a drug only after it has been exposed prior to this event (Loeffler et al. 2003, Kanafani et al. 2008). **Clinical resistance** is defined as the failure to eradicate an infection despite the administration of a toxic agent at a level with *in vitro* activity against the organism or lack of response even if the maximum dosage is used (Loeffler et al. 2003, Kanafani et al. 2008, Sanglard et al, 2009).

Next to the various terms used in combination with antibiotic drug resistance, another widely used term is (in)**sensitivity**. The Merriam-Webster dictionary describes the definition of this word as follows:
"a: not responsive or susceptible <insensitive to the demands of the public"
"b : not physically or chemically sensitive."
(http://www.merriam- webster.com/dictionary/insensitivity?show=0&t= 1344852153)

More related to the microbiological interpretation of antibiotic drug (in)sensitivity the next description was found in literature:
'The "susceptible" category implies that isolates (i.e. microorganisms) *are inhibited by the usually achievable concentrations of antimicrobial agent when the recommended dosage is used for the site of infection.'*

(http://www.clsi.org/Content/NavigationMenu/Committees/Microbiology/AST/ArchiveofPreviousEvents/Summary_MinutesJune2005_2.pdf)

Also **tolerance** is a word that is often used in relation to decreased drug efficacy on microorganisms. Tolerance according to the Merriam-Webster dictionary is described as :
"(1): the capacity of the body to endure or become less responsive to a substance (as a drug) or a physiological insult especially with repeated use or exposure <developed a tolerance to painkillers>; also: the immunological state marked by unresponsiveness to a specific antigen
(2): relative capacity of an organism to grow or thrive when subjected to an unfavorable environmental factor."
(http://www.merriam-webster.com/dictionary/tolerance)

(In) susceptibility according to the Merriam- Webster dictionary can be described as follows:

1: the quality or state of being; especially: lack of ability to resist some extraneous agent (as a pathogen or drug): sensitivity.
(http://www.merriam-webster.com/dictionary/susceptibility)

Susceptibility refers to the general state of an organism being able to resist a toxic agent regardless the cause of this fact. **Cross resistance** refers to non-susceptibility to several/multiple drug agents as a consequence of one general cause. The interpretation of cross-resistance according to the Merriam-Webster dictionary is:
Tolerance (as of a virus) to a usually toxic substance (as an antibiotic) that is acquired not as a result of direct exposure but by exposure to a related substance (http://www.merriam-webster.com/dictionary/cross-resistance).

In relation to antibiotic resistance, many terms are used in literature with different and sometimes unframed interpretations. To correctly interpret the literature covering this subject we here summarize the following distinctions between the definitions and its related context:

- **Acquired resistance** is used in relation to micro-organisms showing secondary antibiotic resistance that is not of natural origin;
- **(In)sensitivity** or **natural resistance** is used in relation to micro-organisms showing primary resistance against a drug;
- **(In) susceptibility** is referred to decreased or no efficacy of a compound or drug on a micro-organisms without clear information whether this is due to natural or acquired resistance.
- **Microbiological resistance** is related to non-susceptibility of a micro-organism to an antimicrobial agent by *in vitro* susceptibility testing, in which the minimal inhibitory concentration (MIC) of the drug exceeds the susceptibility breakpoint for that organism.
- **Clinical resistance** is related to the failure to eradicate an infection despite the administration of a toxic agent at a level with *in vitro* activity against the organism or lack of response even if the maximum dosage is used.
- **Cross-resistance** is related to antibiotic resistance of a micro-organism against a drug that is not a consequence of direct exposure to this drug but of exposure to a related substance.

4.2 Fungal resistance

4.2.1 Mechanisms of resistance

The discovery of antimicrobial drugs has led to the ability to effectively treat and fight infectious diseases. However, since the discovery of antimicrobials, and in particular antibiotics directed against bacteria, resistance has become an emerging and ever increasing problem mainly in medical treatment. Acquired resistance in bacteria occurs through a variety of mechanisms that all imply genetic changes in several ways. On an individual genetic level, bacteria can undergo mutations that can lead to a changed sensitivity or resistance to antibiotics (Tenover 2006; Livermore 2012). Another significant cause of bacterial resistance is the occurrence of horizontal gene transfer of antibiotic resistance genes that occurs within or even between species, which will be explained later in paragraph 4.2.2.

Besides antibiotic resistance in bacteria, also antibiotic resistance in fungi has become an increasing problem in the past 40 years (Ghannoum et al. 1999, Anderson 2005, Kanafani et al. 2008). Especially due to the increase of immunosuppressed patients as a consequence of HIV and clinical treatments related to organ transplantations and chemotherapy, opportunistic fungal infections have become an increasing problem. With the rise of treatments against fungal infections, more cases of antifungal insusceptibility have been detected (Sanglard et al, 2009). Some fungal strains can show natural insensitivity against antifungals, but in some cases also acquired resistance is observed (Kanafani et al. 2008, Sanglard et al, 2009).

Out of the four main classes of widely used antimycotic drugs, being azoles, polyenes, allyamines and echinocandines, recent literature has mainly described acquired resistance in fungi for the group of azoles (Anderson 2005, Kanafani et al. 2008, Chandrasekar 2011). In general, acquired resistance in fungi is caused by several mechanisms of which four are described in literature:

- *Over expression of naturally present genes* results in an increase of active efflux pumps leading to decreased drug concentrations at the site of action. This mechanism has been shown for azole resistant *Candida* strains by the presence and overexpression of two transporter genes *CDR1* and *CDR2*. Presence and overexpression of other transporter genes like *MDR1* and *FLU1* also lead to azole resistant *Candida* strains (Ghannoum 1999, Anderson 2005, Kanafani et al.2008, Sanglard et al 2009);
- *Alteration of target enzyme(s)* results in the prevention of binding an antifungal drug by alteration of the target protein as a consequence of mutations. This has been shown for *Candida* strains in which mutations in the *ERG11* gene leads to resistance to azoles. Total absence of a target can also lead to resistance. This was described for a defective *ERG6* gene leading to absence of ergosterol in the cell membrane. This leads to insusceptibility of *Candida glabrata* to amphotericin B. (Anderson 2005, Kanafani et al. 2008, Sanglard et al. 2009);
- *Up-regulation of the target enzyme* increased production of the target protein leading to incomplete functional breakdown by the drug as has been shown for some *Candida* species in azole insusceptibility (Kanafani et al. 2008);

- *Utilization of compensatory and catabolic pathways:* some antifungals have an effect on the ergosterol biosynthesis pathway resulting in the depletion of ergosterol from the fungal membrane. During this incomplete synthesis a toxic product is formed. Some fungi are able to modify the biosynthesis of these toxic products to their advantage. Azole resistance by mutations in the ERG3 gene illustrates this mechanism. During exposure the ergosterol synthesis is disturbed and a toxic product like 14alpha-methyl-3,6-dihydroxy is formed. Mutations in ERG3 can result in the alteration of this product leading to functional membranes with no or less ergosterol. This mechanism has been described for azole- resistant *Candida* strains. As a consequence of the lack of ergosterol in the cell wall this also leads to polyene insusceptibility (Kanafani et al. 2008, Sanglard et al. 2009).

Mechanisms of resistance to antifungals are mainly shown for azoles and are to a lesser extend described for polyenes or other classes of antifungals. Occurrence of decreased susceptibility to antifungals has also been shown mainly in a variety of *Candida* species and not as frequent in other fungal species (Sanglard et al. 2009). However, the last mentioned mechanism, utilization of compensatory and catabolic pathways, has been described to lead to polyene insusceptibility through cross-resistance as will be described in paragraph 4.2.3 (Kontoyiannis et al. 2002, Anderson 2005, Kanafani et al. 2008).

In the review by Hof in 2008, the widespread acquisition of fungal resistance against azoles is considered unlikely to occur. Apart from the mentioned reported resistance mechanisms, fungi do not have any comparable way of acquiring resistance compared to the various efficient ways bacteria can acquire antibiotic resistance. It has not been described for fungi to actively produce enzymes that break down azoles or other antifungals. Furthermore transmission of specific resistance genes via horizontal gene transfer in fungi is unknown. Resistance to newer azoles is reported being rare (Hof 2008).

Insusceptibility against antifungal drugs can also arise in fungal biofilm formation. Some fungal species, especially *Candida* species but also some filamentous fungi like *Aspergillus fumigatus*, can be involved in highly structured biofilm communities. Biofilm formation leads to advantages for the organisms involved since the biofilm structure environment influences and protects them against physical and chemical stress. Biofilms can cause problems in human infection since some species can efficiently adhere and colonize onto organic and anorganic surfaces. Protection against antifungals by an extracellular matrix occurs because the penetration of antifungal drugs is decreased, although the composition and regulation of these matrices might also play a central role in insusceptibility at least for some *Candida* species (Ramage et al. 2011). Persister cells in biofilms are dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics. Especially for several *Candida* species it has been shown by several studies that subpopulations of these types of cells occur within biofilms with high insusceptibility against amphotericin B. The underlying cause of insusceptibility by persister cells to antifungals has not been elucidated yet but it is potentially linked to expression of Hsp90 and calcineurin upon environmental stress (Lafleur et al. 2006, Cowen 2008).

4.2.2 *Transmission of fungal resistance.*

In bacteria, horizontal gene transfer (HGT) is a very common phenomenon by which genetic material can be exchanged within or between species. This also leads to the efficient distribution and acquisition of widespread resistance against many antibiotics. Mechanisms of HGT in bacteria are possible through conjugation, transduction or transformation. Not much is known or described in detail about HGT in fungi (Ghannoum et al. 1999). It is suggested however, that local recombination within species or between closely related species might occur through a mating process, or in the case of filamentous fungi, through vegetative cell fusion and subsequent para-sexual shuffling of genes (Anderson 2005). Furthermore, there is evidence that HGT between fungal species must have had a role in the evolution of fungi based on phylogenetic analysis (Richards et al. 2011, Rosewich et al 2000). However, current prevalence of gene transfer between fungal species and transmission of genetic material is low. Fungi, like other eukaryotes, have many characteristics that plead against the frequent occurrence of HGT (Richards et al. 2011). Fungi have a rigid, less permeable cell wall, recognize foreign DNA and have lost the ability to perform phagocytosis. However, there are indications for additional routes for HGT in fungi. Laboratory experiments have shown HGT of plasmids between fungal species. Transfer of plasmids has been shown between *Agrobacterium tumefaciens* and *Aspergillus niger*, *Agaricus bisporus*, *Colletotrichum gloeosporioides*, *Fusarium venenatum* and *Neurospora crassa* (de Groot et al, 1998) in laboratory experiments (reviewed by Richards et al. 2011). However, all these observations have only been found in artificial laboratory circumstances, induced under very selective conditions and at low frequency. Interspecies HGT as a natural phenomenon in fungi is considered not existing or happening at a very low frequency. Its occurrence cannot be fully disputed, but it is considered highly unlikely that HGT or other DNA transmission mechanisms currently play a role in fungi to acquire resistance (Rosewich et al. 2000, Anderson 2005, Hof 2008, Richards et al. 2011). We also could not find literature that describes examples of acquired resistance in fungi as a consequence of HGT.

4.2.3 *Cross resistance*

Cross resistance as a consequence of acquired resistance has mainly been described between different types of azoles (Ghannoum et al. 1999, Kontoyiannis et al. 2002). Cross-resistance from azoles to other antifungal groups has not been described frequently although strains have been found to exhibit resistance to terbinafine (an allylamine) after showing resistance to fluconazole (Ghannoum et al. 1999, Kontoyiannis et al. 2002). Also cross-resistance has been shown between azoles and amphotericin B (see next chapter, Kelly et al. 1997).

As the bacterial cell wall does not contain ergosterol bacteria are not sensitive to natamycin (MIC for bacteria are ≥ 250 µg/ml) or other polyenes since the bacterial cell wall does not contain sterol (EFSA. 2009). Therefore cross-resistance between antifungal and antibacterial agents is considered highly unlikely (Ghannoum et al. 1999). We also could not find examples in literature describing cross-resistance between antifungal drugs and antibacterial agents.

4.3 Polyene drug resistance

While considering literature describing polyene resistance or insusceptibility it appeared that recent publications hardly provide new information. Therefore it is necessary to include relevant literature published before the year 2000 to get a better understanding on general polyene resistance.

Published information from before the year 2000 demonstrates insusceptibility of fungi to polyenes as being associated with a low(er) abundance or modification of ergosterol in the fungal membrane at least in yeast strains. This was first suggested by Hamilton-Miller in 1973. *Candida* strains that are resistant to nystatin, for example, contained less ergosterol than sensitive strains which has been shown by Athar and Winner (1971) and Safe et al. (1977). Dick et al. (1980) confirmed this by finding that defects or mutations in the *ERG3* gene involved in ergosterol biosynthesis lead to accumulation of other sterols in the fungal membrane. Consequently, polyene-resistant *Candida* and *Cryptococcus* isolates had relatively low ergosterol content (74-85%) compared to the polyene-susceptible isolates. Other similar observations regarding correlation between polyene insusceptibility and ergosterol pattern for several fungal species have been published, mainly for yeast strains like *Candida* spp. and *Saccharomyces cerevisiae*.

Interestingly, Kelly et al. (1997) described the acquisition of azole resistance for two clinical isolates from AIDS patients due to mutations in sterol $\Delta^{5,6}$- desaturase (*ERG3p* gene). This occurrence shows an example of the mechanism of utilization of compensatory and catabolic pathways to attain resistance as described before. The alteration of ergosterol into 14α-methyl-3,6-diol presence in the cell wall also led to amphotericin B resistant *Candida* strains (Kelly et al. 1997). Since the polyene acts on the end product of the pathway affected by the azole antifungal, insusceptibility to polyenes was a consequence of acquired resistance against azoles. The fungal isolates described in this study showed cross resistance against amphotericin B.

Detailed research on the mechanisms as to how polyene resistance can be acquired is uncommon. This is mainly due to the fact that specific polyene resistance is rarely reported before the year 2000 and is also well acknowledged being rare in recent literature (Ghannoum et al. 1999, Kanafani et al. 2008, Hof 2008, Vandeputte et al. 2011). There is limited knowledge on the specific mechanism of polyene resistance and that is probably a consequence of the limited number of studies with relevant or publishable data. Besides the mechanism of utilization of compensatory and catabolic pathways causing cross-resistance between azoles to amphotericin B, none of the previously described mechanisms of fungal resistance could be found in literature published before the year 2000 specifically for polyenes. The mechanism of increased efflux leading to resistance of fungi against polyenes, as described for azoles, is according to literature not considered likely since polyenes do not require entrance to the cell (Ghannoum et al, 1999).

In the recent literature from 2000 to present most cases of insusceptibility against polyenes are described being related to natural insensitivity or clinical resistance of fungal strains, which occurs mainly in filamentous fungi and certain *Candida* species (Anderson. 2005, Junie et al. 2005, Kanafani et al. 2008, Kontoyiannis et al. 2002).

The site of infection, however, is also a very important factor in the occurrence of insusceptibility of antifungals. Furthermore, there are many more reasons for antifungals not being effective in clinical therapies like an incorrect diagnosis, patients with severe immunosuppression or a fungal burden that is too high or too virulent for antifungals to overcome. Also the toxicity of antifungals or the non-efficient polypharmacy approach and the length of treatment can influence the clinical outcome (Kanafani et al, 2008). For the polyene amphotericin B, clinicians generally use a MIC of ≥ 1µg/ml to indicate microbiological resistance (insusceptibility) (Kanafani et al. 2008). Official breakpoints for determination of fungal insusceptibility to polyenes like amphotericin B have not been standardized (Kontoyiannis et al. 2002, Kanafani et al. 2008). Thereby, it can also be debated whether the MIC value is an appropriate standard for defining insusceptibility of fungal strains to polyenes since interpretation of these tests are difficult and are not always a good prediction of the clinical outcome (Anderson 2005, Kanafani et al. 2008).

Although acquisition of polyene resistance is not described frequently after the year 2000, there have been reports about increasing MICs to amphotericin B in *Candida* species (Anderson 2005). Rare cases of polyene resistance are also reported by Kanafani et al. (2008) and Vandeputte et al. (2011). Filamentous fungi are naturally less sensitive to polyenes. Messer et al. (2006) described the increased prevalence of polyene resistance among *Aspergillus* spp. with only 11.5% of *Aspergillus fumigatus* isolates inhibited at ≤ 1 µg/ml which would imply acquisition of polyene resistance. Large longitudinal studies, however, do not support this published observation (Kontoyiannis et al. 2002, Kanafani et al. 2008). Broad spectrum acquired polyene-resistance has been described for some isolated strains (*Aspergillus flavus, Candida sp; Saccharomyces cerevisiae*) due to alterations in the membrane ergosterol content (Kontoyiannis et al. 2002). The formerly described mutations in the *ERG3* gene has not often been described as a cause for high-level resistance, but does lead to cross-resistance between azoles and amphotericin B as was already described before 2000 (Kelly et al. 1997, Kontoyiannis et al. 2002). Cross resistance in these cases is a consequence of altered or decreased ergosterol abundance in the cell wall due to mutations in the ERG3 gene that mainly seems a side effect of acquired resistance against azoles (Kontoyiannis et al. 2002, Kanafani et al. 2008,).

To summarize, publications before the year 2000 about acquired resistance to polyenes in general have mainly been described for strains with a decreased or modified presence of ergosterol in the cell wall, which is often due to either natural insensitivity (Ghannoum et al. 1999) or acquired resistance against azoles (Kelly et al. 1996). After the year 2000, similar observations have been made (Kanafani et al, 2008) but rarely described in literature. We conclude that the occurrence of polyene resistance is a rare event. More importantly, polyene resistance most often seems due to cross resistance where mutations in the *ERG3* gene actively leading to acquired azole resistance also leads to polyene resistance. In the recent publication

of Gray et al. (2012) the authors suggest that binding a physiological important lipid as ergosterol is a powerful and clinically validated antimicrobial strategy that may be inherently refractory to resistance. They advocate the dual mode of action (i.e., lipid binding and membrane permeabilization) of amphotericin B, contributing to the resistance-refractory nature of the polyene antibiotic.

4.4 Natamycin resistance in fungi

Publications on resistance to natamycin are hard to find in the literature published since the year 2000 until present. The development of resistance against antimicrobials due to frequent specific application in the food industry is rarely described and investigated (Davidson et al. 2002). Therefore older publications from before the year 2000 were used to obtain useful information and to get a better view concerning resistance or insensitivity of fungi against natamycin.

In older publications it can be found that induction of natamycin-resistance in fungi is reported being difficult to achieve. Athar and Winner (1971) investigated whether polyene resistance could be induced in several *Candida* species. The resulting mutants invariably show a reduced metabolic growth rate *in vitro*, and in the absence of polyenes they readily revert to normal metabolism, growth and sensitivity towards natamycin. Resistance of *Candida albicans* has been induced after 25 passages in media with increasing concentrations of natamycin. This resistance gradually developed and the MIC increased from 2.5-12 to 12-50 mg/L (EFSA, 2009). JECFA reported in 1976 that the selection of natamycin-resistant strains *in vitro* has not induced cross-resistance to other polyenes. This was based on the studies of Hejzlar et al. in 1970 where fungal strains with indicated resistance to fungicidine (nystatin) were still sensitive to natamycin (Hejzlar et al. 1970, EFSA 2009).

De Boer and Stolk-Horsthuis (1977) attempted to induce tolerance in strains of fungi by transferring each culture 25-31 times in media containing concentrations of natamycin equal to and greater than the MIC. Following multiple transfers, the MIC increased in only 8 of 26 strains by a maximum of 4 mg/L instead of <1 mg/L. The overall lack of increased resistance was due to the lethal (fungicidal) mode of action, and the compound's instability.
Surveys in cheese warehouses and in dry sausage factories where the natamycin containing product Delvocid was used for up to 10 years showed no change in the composition or the sensitivity of the contaminating fungal flora (de Boer and Stolk-Horsthuis, 1977; de Boer et al., 1979; Hoekstra and Van der Horst, 1998). All previously described studies are summarized by the EFSA (2009).

Recent cases of specific acquired natamycin resistance have not been reported in literature between 2000 until present. Resistance of fungi against polyenes in general is not often described and this also goes for natamycin. This is well acknowledged in literature where natamycin is considered a broad range and highly effective antifungal with little reported cases of resistance or insensitivity (Ghannoum et al. 1999, Ganegoda et al. 2004, Delves-Broughton et al. 2006, Kanafani et al. 2008, EFSA 2009). Occurring cases of natamycin insusceptibility have been reported almost exclusively for clinically isolated fungal strains (Ghannoum et al. 1999, Shiraishi et al. 2011, Gajjar et al. 2011, Edelstein et al.

2012, Al-Badriyeh et al. 2009, Ford et al. 2008, Patel et al. 2006). In these publications mainly clinical resistance was described for individual cases of natamycin not being effective in topical treatment of ocular infections like keratitis. It is described, however, that natamycin (as some other polyenes like amphotericin B) shows poor penetration of the intact epithelium which might also explain the lack of effectiveness in the clinical treatment (Iqbal et al. 2008, Lalitha et al. 2007, Ford 2004). As previously described in chapter 4.3, also other factors can interfere with efficient clinical treatment. However, often these isolated clinically insusceptible fungal strains also showed a very high minimal inhibitory concentration (MIC) ranging from 2 µg/ml (Shiraishi et al. 2011a) to 64 µg/ml (Gajjar et al. 2011) for natamycin, but in most cases it is not clearly described whether this is due to natural insensitivity or acquired resistance. Good interpretation of the publications reporting clinical observations of the insusceptibility of fungi for natamycin, made us conclude that such is caused by clinical resistance and not by acquired resistance.

Bacteria are not sensitive for natamycin or other polyenes (MIC for bacteria are ≥ 250 µg/ml) since the bacterial cell wall does not contain sterols. Therefore antifungals like natamycin are specifically directed against fungi and are not effective against bacteria (Ghannoum 1999, Anonymous 2009). For this reason the effect of natamycin on the gastrointestinal flora can also be considered not being an issue. The human gastrointestinal flora may be exposed to trace quantities of ingested natamycin residues, but in healthy humans the intestinal microflora is predominated by bacterial species, whereas yeast and fungal species are only ca. 0.001% of the total flora. Several studies in experimental animals indicate that natamycin and any potential degradation products do not display antibiotic activity in the colon. As bacteria are generally not affected by polyenes, and fungi are found in low quantities in the intestinal tract, the consequences of exposure to ingested traces of natamycin could be considered as minimal (EFSA, 2009).
Looking at literature between 2000 to present there is no recent and convincing experimental evidence of fungi acquiring specific resistance to natamycin. Also no genes have been described specifically leading to high prevalence of natamycin resistance. Transmission of acquired resistance between fungi is unlikely and since there is no specific gene for acquired resistance against natamycin the chance of this occurrence are extremely low. No examples have been found in literature of acquired resistance specifically against natamycin due to cross-resistance. However, it must be noted that the previously described mechanism of utilization of compensatory and catabolic pathways can play a role in general polyene resistance. Since acquired resistance of fungi against azoles causes either a lack or modification of ergosterol present in the fungal cell wall, this will generally lead to cross-resistance against polyenes and therefore also in natamycin. We do not consider this as an acquired resistance specifically against polyenes or natamycin but rather as a natural or subsequential consequence of acquired resistance against antifungals belonging to the azole group.

5 Conclusions

Natamycin is an effective and safe food preservative with fungicidal activity and has wide regulatory status throughout the world. In addition, it is a well-recognized antifungal compound effective in curing topical fungal infections but not against systemic infections like mycoses.. Because of its chemical structure, the compound has low solubility in aqueous solutions. It interacts selectively with fungal membranes resulting in changed membrane function and ultimately causing cell death. Natamycin interacts with ergosterol in the fungal membranes resulting in obstruction of the membrane functionality causing cell death.

Information on resistance against natamycin is limited available. Where mentioned, resistance is intrinsically caused by low presence of ergosterol in the fungal membranes which may be caused by mutation in the ERG3 gene that mainly seems a side effect of acquired resistance against azoles.. Recent literature reporting natamycin resistance as an issue is exceptional. Resistance is generally described as a natural trait in some strains. The mechanism of increased drug efflux in fungi in the development of resistance against polyenes is considered not likely since polyenes do not require entrance to the cell. It appears that mechanisms by which resistance can spread to other fungal strains are rather limited compared to the situation encountered in bacterial strains where horizontal gene transfer is a far more common phenomenon. Described natamycin resistance is mainly due to natural resistance and no reports have been published claiming acquired natamycin resistance in fungi due to horizontal gene transfer. Experimental evidence of fungi acquiring resistance to natamycin is not encountered so far.

6 References

AL-BADRIYEH, D., LEUNG, L., DAVIES G. E., STEWART, K. and KONG, D., 2009.Successful salvage treatment of Scedosporium apiospermum keratitis with topical voriconazole after failure of natamycin. *The Annals of pharmacotherapy*, **43**(6), pp. 1139-42.

ANDERSON, J.B., 2005. Evolution of antifungal-drug resistance: Mechanisms and pathogen fitness. *Nature Reviews Microbiology,* **3**(7), pp. 547-556.

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**(4) pp. 505-517.

BAGINSKI, M., RESAT, H. and MCCAMMON, J. A. 1997. Molecular properties of amphotericin B membrane channel: a molecular dynamics simulation. *Molecular Pharmacology,* **52**, pp. 560-570.

BASILICO, J.C., DEBASILICO, M.Z., CHIERICATTI, C. and VINDEROLA, C.G., 2001. Characterization and control of thread mould in cheese. *Letters in Applied Microbiology,* **32**(6), pp. 419-423.

CHANDRASEKAR, P., 2011. Management of invasive fungal infections: A role for polyenes. *Journal of Antimicrobial Chemotherapy,* **66**(3), pp. 457-465.

COWEN, L. 2008. The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype. *Nature*. **6** (3), pp. 187-798.

DAVIDSON, P.M. and HARRISON, M.A., 2002. Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. *Food Technology,* **56**(11), pp. 69-78.

DE BOER, E. AND STOLK- HORSTHUIS, M., 1977. Sensitivity to natamycin of fungi isolated in cheese warehouses. *Journal of food protection*, **40**, pp. 533-536.

DE BOER, E., LABOTS, H., STOLK-HORSTHUIS, M., and VISSER, J.N., 1979. Sensitivity to natamycin of fungi in factories producing dry sausage. *Fleischwirtschaft*, **59**, pp. 1868-1869.

DE GROOT, MJ, HOOYKAAS, P. and BEIJERSBERGEN, A.G.1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nature Biotechnology, **16**, 839-842.

DE KRUIJF, B. and DEMEL, R.A. 1974. Polyene antibiotic-sterol interactions in memebranes of Acholeplasma laidlawii cells and lecithin liposomes -III. Molecular structure of the polyene antibiotic-cholesterol complexes. *Biochimica et Biophysica Acta*, **339**, pp. 57-70.

DE KRUIJF B., GERRITSEN W.J., OERLEMANS, A., DEMEL R.A. and VAN DEENEN, L.L.M.1974. Polyene antibiotic-sterol interactions in membranes of Acholeplasma laidlawii cells and lecithin liposomes. -I. Specificity of the membrane permeability changes induced by the polyene antibiotics. *Biochimica et Biophysica Acta,* **339**, 30-43.

DELVES-BROUGHTON, J., THOMAS, L.V. and WILLIAMS, G., 2006. Natamycin as an antimycotic preservative on cheese and fermented sausages. *Food Australia,* **58**(1/2), pp. 19-21.

DICK, J.D., MERZ, W.G. AND SARAL, R., 1980. Incidence of polyene-resistant yeasts recovered from clinical specimens. *Antimicrobiological Agents and Chemotherapy,* **18**, pp. 158-163.

DOS SANTOS PIRES, A.C., DE FERREIRA SOARES, N.F.., DE ANDRADE, N.J., MENDES DA SILVA, L.H., PERUCH CAMILLOTO, G. and CAMPOS BERNARDES, P., 2008. Development and evaluation of active packaging for sliced mozzarella preservation. *Packaging Technology and Science,* **21**(7), pp. 375-383).

EDELSTEIN, S.L., AKDUMAN, L., DURHAM, B.H., FOTHERGILL, A.W. and HSU, H.Y., 2012. Resistant fusarium keratitis progressing to endophtalmitis. *Eye Contact Lens*, **38**(5), pp. 331-335.

EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS); Scientific Opinion on the use of natamycin (E 235) as a food additive. *EFSA Journal* 2009, **7**(12), pp.1412.

FORD M. M., 2004. Antifungals and their use in veterinary ophthalmology. *Veterinary Clinics of North America - Small Animal Practice*, **34**(3), pp. 669-691.

FORD, J.G., AGEE, S. and GREENHAW, S.T., 2008. Successful medical treatment of a case of Paecilomyces lilacinus keratitis. *Cornea,* **27**(9), pp. 1077-1079.

GAJJAR, D.U., PAL, A.K., SANTOS, J.M., GHODADRA, B.K. and VASAVADA, A.R., 2011.Severe pigmented keratitis caused by Cladorrhinum bulbillosum. *Indian Journal of Medical Microbiology*, **29**(4), pp. 434-437.

GANEGODA, N., RAO SRINIVAS K., GANEGODA, N., 2004. Antifungal therapy for keratomycoses. *Expert Opinion on Pharmacotherapy*, **5**(4),pp.865-874.

GAO, Y., WANG, X. and LIU, Y., 2010. Biological stability of natamycin solution. *Shipin Kexue (Beijing, China),* **31**(9), pp. 41-44.

GHANNOUM, M.A. and RICE, L.B., 1999. Antifungal agents: Mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clinical Microbiology Reviews,* **12**(4), pp. 501-517.

GRAY, K.C., PALACIOS, D.S., DAILEY, I., ENDO, M.M., UNO, B.E., WILCOCK, B.C., and BURKE, M.D. 2012. Amphotericin primarily kills yeast by simply binding ergosterol. *Proceedings of the National Academy of Sciences of the United States of America,* **109**(7):2234-2239.

HAMILTON-MILLER, J.M.T.,1973. Chemistry and biology of the polyene macrolide antibiotics. *Bacteriology Reviews*, **37**, pp.166-196.

HEJZLAR, M. and VYMOLA, F. 1970. Comparative study of pimaricin and fungicidin activity *in vitro*. *Journal of Hygiene, Epidemiology, Microbiology & Immunology,* **14**, pp. 211–213.

HOEKSTRA, E.S. AND VAN DER HORST, M.I., 1998. Survey of the fungal flora in Dutch cheese factories and warehouses. *Journal of Food Mycology*, **1**, pp. 13-22.

HOF, H., 2008. Is there a serious risk of resistance development to azoles among fungi due to the widespread use and long-term application of azole antifungals in medicine? *Drug resistance updates*, **11**, pp. 25-31.

IQBAL, N.J., BOEY, A., PARK, B.J. and BRANDT, M.E., 2008. Determination of in vitro susceptibility of ocular Fusarium spp. isolates from keratitis cases and comparison of Clinical and Laboratory Standards Institute M38-A2 and E test methods. *Diagnostic Microbiology and Infectious Disease,* **62**(3), pp. 348-350.

JUNIE, M. and COSTACHE, C., 2005. Compared sensitivity of C. albicans isolated from different pathological produces to antimycotic drugs. *Revista Scientia Parasitologica,* **6**(1/2), pp. 141-146.

KANAFANI, Z.A. and PERFECT, J.R., 2008. Resistance to antifungal agents: Mechanisms and clinical impact. *Clinical Infectious Diseases,* **46**(1), pp. 120-128.

KELLY, S.L., LAMB, D.C., KELLY, D.E., MANNING, N.J., LOEFFLER, J. HEBART, H. SCHUMACHER, U. AND EINSELE, H. 1997. Resistance to fluconazole and cross-resistance to amphotericin B in Candida albicans from AIDS patients caused by defective sterol Δ 5,6- desaturation. *FEBS Letters*. Vol. **400** (1), pp. 80-82.

KONTOYIANNIS, D.P. and LEWIS, R.E., 2002. Antifungal drug resistance of pathogenic fungi. *Lancet,* **359**(9312), pp. 1135-1144.

KOTLER-BRAJTBURG, J., MEDOFF, G., KOBAYASHI, G. S., BOGGS, S., SCHLESSINGER, D., PANDEY, R. C.and RINEHART JR., K. L. 1979. Classification of polyene antibiotics according to chemical structure and biological effects. *Antimicrobial Agents and Chemothery* **15** , pp. 716-722.

LAFLEUR, M., KUMAMOTO, C.A. and LEWIS, K. 2006. Candida albicans biofilms produce antifungal-tolerant persister cells. *Antimicrobial Agents and Chemotherapy.* Vol. **50**(11), pp. 3839-3846.

LALITHA, P., SHAPIRO, B.L., SRINIVASAN, M., PRAJNA, N.V., ACHARYA, N.R., FOTHERGILL, A.W., RUIZ, J., CHIDAMBARAM, J.D., MAXEY, K.J., HONG, K.C., MCLEOD, S.D. and LIETMAN, T.M., 2007. Antimicrobial susceptibility of Fusarium, Aspergillus, and other filamentous fungi isolated from keratitis. *Archives of Ophthalmology,* **125**(6), pp. 789-793.

LIVERMORE, D.M., 2012. Fourteen years in resistance. *International Journal of Antimicrobial Agents,* **39**(4), pp. 283-294.

LOEFLER, J. and STEVENS, D.A. 2003. Antifungal Drug Resistance. *Clinical Infectious Diseases*, **36**(Suppl. 1), pp. S31-S41.LU, Y., HE, J. and LI, T., 2010. HPLC determination of content and related substances of natamycin. *Yaowu Fenxi Zazhi,* **30**(5), pp. 929-932.

MESSER, S.A., JONES, R.N. and FRITSCHE, T.R. 2006. International surveillance of Candida spp. and Aspergillus spp.: report from the SENTRY Antimicrobial Surveillance program (2003). *Journal of Clinical Microbiology*, **44**, pp. 1782-1787.

PATEL, S.R., HAMMERSMITH, K,M., RAPUANO, C.J. and COHEN, E.J., 2006. Exophiala dermatitidis keratitis after laser in situ keratomileusis. *Journal of Cataract and Refractive Surgery,* **32**(4), pp. 681-684.

POUYEH, B., GALOR A., MILLER, D., and ALFONSO E.C., 2011. New horizons in one of ophthalmology's challenges: Fungal keratitis. *Expert Review of Ophthalmology,* **6**(5), pp. 529-540.

RAMAGE, G., RAJENDAN, R., SHERRY, L. AND WILLIAMS, C. 2011. Fungal biofilm resistance. *International Journal of Microbiology*. Vol 2012, pp. 1-14.

RICHARDS, T.A., LEONARD, G., SOANES, D.M. and TALBOT, N.J., 2011. Gene transfer into the fungi. *Fungal Biology Reviews,* **25**(2), pp. 98-110.

ROSEWICH, U.L. and KISTLER, H.C., 2000. Role of horizontal gene transfer in the evolution of fungi. *Annual Review of Phytopathology,* **38**, pp.325-363.

SAFE, L.M., SAFE, S.H., SUBDEN, R.E., and MORRIS, D.C., 1977. Sterol content and polyene antibiotic resistance in isolates of Candida krusei, Candida parakrusei, and Candida tropicalis. *Canadian Journal of Microbiology* 23(4), pp. 398-401.

SANGLARD, D., COSTE, A. and FERRARI, S., 2009. Antifungal drug resistance mechanisms in fungal pathogens from the perspective of transcriptional gene regulation. *FEMS Yeast Research,* **9**(7), pp. 1029-1050.

SHIRAISHI, A., ARAKI-SASAKI, K., MITANI, A., MIYAMOTO, H., SUNADA, A., UEDA, A., ASARI, S., ZHENG XIAODONG, YAMAMOTO, Y., HARA, Y., OHASHI, Y. and ZHENG, X.D., 2011. Clinical characteristics of keratitis due to Colletotrichum gloeosporioides. *Journal of Ocular Pharmacology and Therapeutics,* **27**(5), pp. 487-491.

STARK, J., 2003. Natamycin: an effective fungicide for food and beverages. *Natural Antimicrobials for the Minimal Processing of Foods,* pp. 82-97

TE WELSCHER, Y.M., JONES, L., VAN LEEUWEN, M.R., DIJKSTERHUIS, J., DE KRUIJFF, B., EITZEN, G. and BREUKINK, E., 2010. Natamycin inhibits vacuole fusion at the priming phase via a specific interaction with ergosterol. *Antimicrobial Agents and Chemotherapy,* **54**(6), pp. 2618-2625.

TE WELSCHER, Y.M., TEN NAPEL, H.H., BALAGUE, M.M., SOUZA, C.M., RIEZMAN, H., DE KRUIJFF, B. and BREUKINK, E., 2008. Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *The Journal of biological chemistry,* **283**(10), pp. 6393-6401.

TE WELSCHER, Y.M., VAN LEEUWEN, M.R., DE KRUIJFF, B., DIJKSTERHUIS, J., and BREUKINK, E. 2012, Polyene antibiotic that inhibits membrane transport proteins. *Proceedings of the National Academy of Sciences of the United States of America,* **109**(28):11156-11159.

TENOVER, F.C., 2006. Mechanisms of Antimicrobial Resistance in Bacteria. *American Journal of Medicine,* **119**(6 SUPPL. 1), pp. S3-S10.

TUOMANEN, E., DURACK, D.T.,TOMASZ, A. 1986. Antibiotic Tolerance among Clinical Isolates of Bacteria. *Antimicrobial Agents and Chemotherapy*, **30** (4).p. 521-527.

VANDEPUTTE, P. FERRARI, S. AND COSTE, A.T. 2012. Antifungal resistance and new strategies to control fungal infections. *International Journal of Microbiology*. Vol. 2012, pp. 1-25.

VAN LEEUWEN, M.R., GOLOVINA, E.A., DIJKSTERHUIS, J., 2009. The polyene antimycotics nystatin and filipin disrupt the plasma membrane, whereas natamycin inhibits endocytosis in germinating conidia of Penicillium discolor. *Journal of Applied Microbiology,* (2009 Jun) **106**(6), pp. 1908-18.

VINDEROLA, C.G., COSTA, G.A., REGENHARDT, S. and REINHEIMER, J.A., 2002. Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. *International Dairy Journal 12*(7), pp. 579-589.

VRABLIK, J., MASATA, J., JEDLICKOVA. A, and HAJICKOVA, M. 2007. Prospective study the prevalence of different candida strains and their sensitivity to different antimycotic treatment in women with vulvovaginal candidiasis. *Ceska Gynekologicko,* **72**(1), pp. 27-32 .

Thesis:

Leeuwen, M.R. 2009. Thesis: Natamycin and the germinating spore. Universiteit Utrecht. (ISBN: 978-90-393-50614)

Te Welscher, Y.M. 2010. Thesis: The antifungal activity of Natamycin.A novel mode of action of the polyene antibiotics. Universiteit Utrecht. (ISBN 978 - 90 - 393 - 5349–3)

Internet resources:
Thesis MR. van Leeuwen :
http://www.google.nl/url?sa=t&rct=j&q=&esrc=s&frm=1&source=web&cd=4&ved=0CGAQFjAD&url=http%3A%2F%2Figitur-archive.library.uu.nl%2Fdissertations%2F2009-0630-200908%2Fleeuwen.pdf&ei=tpXZT8bjBsKg0QW-t-H1Aw&usg=AFQjCNH6B4ZxPZjfG0MJs5k1Q1Z8iAUgqA&sig2=rATsvp5NBuY3cr-eMcszwA, 01 Aug 2012.

Thesis YM te Welscher :
http://www.google.nl/url?sa=t&rct=j&q=&esrc=s&frm=1&source=web&cd=3&ved=0CF4QFjAC&url=http%3A%2F%2Figitur-archive.library.uu.nl%2Fdissertations%2F2010-0604-200205%2Fwelscher.pdf&ei=NpfZT9rhF-Wm0QW5qKz5Aw&usg=AFQjCNHX50njBZQTkpkKl82kT7pvVkBa7Q&sig2=WovvN2YbkUpDHgeNdKHBjA, 01 Aug 2012.

(http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=172.155), 01 Aug 2012.

(http://www.medterms.com/script/main/art.asp?articlekey=2276), 02 Aug 2012.

(http://groups.molbiosci.northwestern.edu/holmgren/Glossary/Definitions/Def-A/antibiotic_resistance.html), 02 Aug 2012.

(http://www.edinformatics.com/biotechnology/antibiotic_resistance.htm), 02 Aug 2012.

(http://www.merriam-webster.com/dictionary/cross-resistance,) 01 Aug 2012.

http://www.merriam-webster.com/dictionary/insensitivity?show=0&t=1344852153, 02 Aug 2012.

http://www.merriam-webster.com/dictionary/tolerance, 02 Aug 2012

http://www.merriam-webster.com/dictionary/resistance?show=0&t=1344858508, 02 Aug 2012.

http://www.merriam-webster.com/dictionary/susceptibility, 02 Aug 2012.

http://www.clsi.org/Content/NavigationMenu/Committees/Microbiology/AST/ArchiveofPreviousEvents/Summary_MinutesJune2005_2.pdf, 8 Oct 2012.

7 Signature

Zeist, August 31, 2012

Placeholder

Dr. Roy Montijn
Head of department

Dr. Jos van der Vossen
Author

Dr. Nynke van Berkum
Project Manager

8 Appendix I: STN-sub-databases

Defined STN-databases used for literature search:

ADISCTI
ADISINSIGHT
ADISNEWS
AGRICOLA
ALUMINIUM
ANABSTR
ANTE
APOLLIT
AQUALINE
AQUASCI
BIOENG
BIOSIS
BIOTECHABS
BIOTECHNO
CABA
CAPLUS
CBNB
CEABA-VTB
CERAB
CIN
COMPENDEX
CONFSCI
COPPERLIT
CORROSION
CROPB
CROPU
DDFB
DDFU
DGENE
DISSABS
DRUGB
EMBAL
EMBASE
ENCOMPLIT2
ESBIOBASE
FOMAD
FROSTI
FSTA
GENBANK
HEALSAFE
IFIPAT
IMSPATENTS
IMSRESEARCH
INSPEC
INSPHYS
IPA
KOSMET
LIFESCI
MEDLINE
METADEX
NAPRALERT
NTIS
OCEAN
PASCAL
PCTGEN
PS
RAPRA
SCISEARCH
TOXCENTER
TULSA2
USAN
VETU
WATER
WELDASEARCH
WPINDEX
WSCA

Federal Food, Drug, and Cosmetic Act (FFDCA) Considerations for Natamycin

Docket ID Number: EPA-HQ-OPP-2015-0811 (NOF)
Date: June 16, 2016

Section 408(c)(2)(A)(i) of FFDCA allows the U.S. Environmental Protection Agency (EPA or the Agency) to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if the EPA determines that the exemption is "safe." Section 408(c)(2)(A)(ii) of FFDCA defines "safe" to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." This includes exposure through drinking water and in residential settings but does not include occupational exposure. Pursuant to FFDCA section 408(c)(2)(B), in establishing or maintaining in effect an exemption from the requirement of a tolerance, the EPA must take into account the factors set forth in FFDCA section 408(b)(2)(C), which require the EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance exemption, and to "ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue...." Additionally, FFDCA section 408(b)(2)(D) requires that the EPA consider "available information concerning the cumulative effects of [a particular pesticide's] . . . residues and other substances that have a common mechanism of toxicity."

The EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, the EPA determines the toxicity of pesticides. Second, the EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings.

I. Summary of Petitioned-for Tolerance Exemption

In the Federal Register of April 25, 2016 (81 FR 24044) (FRL-9944-86), EPA issued a notice pursuant to FFDCA section 408(d)(3), 21 U.S.C. 346a(d)(3), announcing the filing of a pesticide tolerance petition (PP 5F8407) by Keller and Heckman, LLP, 1001 G Street, NW, Washington, DC, 20001 on behalf of DSM Food Specialties (the Petitioner) B.V., P.O. Box 1, 2600 MA Delft, The Netherlands. The petition requested that 40 CFR part 180 be amended by establishing an exemption from the requirement of a tolerance for residues of natamycin in or on citrus, pome and stone

fruit crop groups, avocado, kiwi, mango and pomegranates when used as a fungistat and used as a dip drench, flood or spray in enclosed packing house facilities. The notice referenced a summary of the petition prepared by the Petitioner, which is available in Docket ID Number EPA-HQ-OPP-2015-0811 via http://www.regulations.gov.

II. Toxicological Profile

Consistent with section 408(b)(2)(D) of FFDCA, the EPA reviewed the available scientific data and other relevant information on natamycin, and considered its validity, completeness, and reliability, as well as the relationship of this information to human risk. The EPA also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children.

A. Overview of Natamycin

Natamycin is a naturally occurring antifungal agent produced during fermentation by the common soil microorganisms *Streptomyces natalensis, Streptomyces lydicus,* and *Streptomyces chattanoogensis.* Natamycin has a non-toxic mode of action. As an antifungal it binds to ergosterol, a sterol found in plants, fish liver oil, egg yolk, dairy products and in the cell membranes of many strains of fungi. In fungi, ergosterol helps regulate fluidity, permeability, stability, and resistance to physical stress of cell membranes similar to the function of cholesterol in animal cells. By binding to ergosterol, natamycin alters the cell membrane of the targeted fungus to the degree that the cell cannot grow, thus preventing germination of fungal spores. Natamycin is active against a broad variety of yeast and molds. However, natamycin has no antibacterial properties and therefore is not expected to cause resistance by human pathogenic bacteria.

Natamycin has been used as a food preservative worldwide for over 40 years and is approved as a food additive/preservative by the European Union, the World Health Organization, and by 70 individual countries including New Zealand and Australia for use as a fungistat to suppress mold on cheese, meats and sausage. In the United States, natamycin is approved by the Food and Drug Administration (FDA) as a direct food additive/preservative for the inhibition of mold and yeast on the surface of cheeses (21CFR 172.155) and as an additive to the feed and drinking water of broiler chickens to retard the growth of specific molds (21CFR 573.685). Natamycin is also FDA approved for use as a treatment to suppress fungal eye infections such as blepharitis, conjunctivitis, and keratitis (21CFR 449.40). In 2012, the EPA and Health Canada’s Pesticide Management Regulatory Agency (PMRA) jointly established a tolerance exemption for natamycin when used as a fungistat to prevent

the germination of fungal spores on mushrooms produced in enclosed production facilities (FR vol. 77, no. 97, p. 29543). PMRA has also registered natamycin under the active ingredient name "pimaricin" for use in laboratories for preserving milk samples (PMRA Reg. Nos. 22612 and 28530). On December 17, 2014, the EPA amended the tolerance by extending use to post-harvest pineapples when used as a fungistat in enclosed packinghouse facilities to prevent certain fungal diseases. (FR 79, no 242, p. 75065). Three products are currently registered containing natamycin for use as a biopesticide fungistat (87485-1, Natamycin TGAI; 87485-2 Natamycin L, (mushrooms), and 87485-3 Zivion P (pineapples)).

B. Biochemical Pesticide Toxicology Data Requirements

All applicable mammalian toxicology data requirements supporting the petition to amend the existing tolerance exemption for natamycin to include use on citrus, pome, stone fruit crop groups, avocado, kiwi, mango and pomegranate as a post harvest use have been fulfilled by bridging from existing data on file that supported the tolerance exemption for natamycin on pineapples. No significant toxicological effects were observed in the acute toxicity studies, the Tier I subchronic toxicity studies, or other information that was used to address the toxicity data requirements. Natamycin is not a contact dermal sensitizer and does not cause chromosomal aberration and is not a mutagen. Relative to Natamycin use, non-occupational exposure will be primarily dietary (consumption of treated citrus, pome, and stone fruit crop groups, avocado, kiwi, mango, and pomegranates). The following is a summary of EPA's review of the toxicity profile of this biochemical.

Acute Toxicity: Natamycin has a non-toxic mode of action as is categorized as follows: Acute Oral $LD_{50} > 3,000$ mg/kg (Toxicology Category III); Acute Dermal $LD_{50} > 5,050$ mg/kg (Toxicology Category IV); Acute Inhalation $LC_{50} > 2.39$ mg/L (Toxicology Category IV); Primary Eye Irritation was severely irritating but no positive effects after 24 hours (Toxicology Category III); Primary Dermal Irritation was slightly irritating (Toxicology Category IV); and natamycin is not a contact dermal sensitizer.

The acute toxicity studies on natamycin (98.17% and 98.27% pure), confirm a low toxicity profile. The acute toxicity data show virtual nontoxicity for all routes of exposure. Therefore, it can be concluded that any dietary risks associated with this biochemical would be negligible.

1. **Acute Oral Toxicity OSCPP 870.1100)** Natamycin was evaluated for acute oral toxicity to albino female rats with the administration via gavage dose at a level of 2,000 mg/kg. There were no clinical signs of toxicity in survivors (one

rat died, polyuria was observed). There were no effects on body weight gain in animals surviving to termination. The acute oral LD_{50} was estimated at greater than 2,000 mg/kg. (**MRID 48105505).**

2. **Acute Dermal Toxicity (OCSPP 870.1200)** An acute dermal toxicity test was conducted with rats to determine the potential for natamycin to produce toxicity from a single topical application. Under the conditions of this study, the single dose acute dermal LD_{50} of the test substance is **greater than 5,050 mg/kg** of body weight in male and female rats. There were no clinical signs of toxicity or signs of dermal irritation at any time throughout the study. The animals were observed for mortality, signs of gross toxicity, and behavioral changes at least once daily for 14 days. Body weights were recorded prior to application and again on Days 7 and 14 (termination). Necropsies were performed on all animals at terminal sacrifice **(MRID 48105506).**

3. **Acute Inhalation Toxicity (OCSPP 870.1300)** Natamycin, was evaluated for its acute inhalation toxicity potential in albino rats where five males and five females were exposed for four hours to an aerosol generated from the test substance (dry powder) at a level of 2.39 mg/L. There was no mortality during the study. Clinical signs included activity decrease and piloerection, which were no longer evident by Day 3. Animal body weight was unaffected. The gross necropsy revealed no observable abnormalities. As indicated by the data, the acute inhalation LC_{50} is greater than 2.39 mg/L **(MRID 48105507).**

4. **Primary Eye Irritation (OCSPP 870.2400)** An acute eye irritation study was conducted on three albino rabbits using test substance natamycin. The undiluted test substance (O.1 mL) was placed into the conjunctival sac of the right eye of each test animal. All treated eyes were washed with room temperature deionized water for one minute immediately after recording the 24-hour observation. The number of animals testing "positive" for each parameter (Table 1.0) over the number of animals tested is presented below.

Table 1.0. Results of Primary Eye Irritation Test (870.2400) for Natamycin

	Time After Treatment			
	1hr.	24hr.	48hr.	72hr.
Cornea Opacity	3/3	0/3	0/3	0/3
Iritis	3/3	0/3	0/3	0/3
Conjunctivae				
Redness	3/3	0/3	0/3	0/3
Chemosis	3/3	0/3	0/3	0/3

There were no positive effects exhibited in any eyes at 24 hours after

treatment. The test substance is rated severely irritating and assigned to Toxicity Category III (**MRID 48105508).**

5. **Primary Skin Irritation (OCSPP 870.2500)** A primary dermal irritation study was conducted on three albino rabbits using test substance natamycin. There was one intact test site per animal. Each test site was treated with 500 mg of test substance and covered with a semi-permeable dressing. The test substance was maintained in contact with the skin for 4 hours. Observations for dermal irritation and defects were made at 1, 24, 48, and 72 hours after removal of the dressings. Irritation scores derived from the respective erythema and edema scores through the 72-hour observations for each animal are presented in Table 2.0.

Table 2.0. Results of Primary Skin Irritation Test for Natamycin

	Erythema				Edema				Irritation Scores
	Hours after unwrap				Hours after unwrap				
	1	24	48	72	1	24	48	72	
5082-M	0	0	0	0	0	0	0	0	0.00
5084-M	1	0	0	0	0	0	0	0	0.25
5071-F	0	0	0	0	0	0	0	0	0.00

Based on the PII of 0.1, the test substance is rated slightly irritating. Based on the scores at the 72-hour observation, the test substance is assigned to Toxicity Category IV (**MRID 48105509**).

6. **Dermal Sensitization: (OCSPP 870.2600)** A skin sensitization study was conducted on 3 groups of 5 female mice to determine if test substance Natamycin produced a sensitizing reaction. Five females were assigned to each of three groups, I-III. Naive control group animals remained untreated during the induction phase of the study. Test group animals were treated with 25 ul on the dorsum of the ear. Test animals were treated once daily for three days. The test substance produced a stimulation index of <3 in all groups of test animals and is not considered a sensitizing agent in mice **(MRID 48105510).**

Subchronic Toxicity: : In a subchronic oral toxicity study using natamycin (98.17% and 98.27% pure) as the test substance, doses of 125 and 500 mg/kg/day showed no treatment related findings. The highest concentration level, 2,000 mg/kg/day, showed reduced weight for both male and female rats (MRID 48105511). The Agency does not consider the temporary decrease in body weight or food intake observed in the 2,000 mg/kg bw/day test group to be an adverse effect, as this is likely due to the palatability of the food containing this high dose of test substance. Therefore, the Agency establishes the NOAEL (No Observed Adverse Effect Level) for this study as 2,000 mg/kg bw/day. A LOAEL (Lowest

Observed Adverse Effect Level) was not identified, suggesting that the test animals could have tolerated a higher dose.

90-day Oral Toxicity (OCSPP 870.3100) Dose levels were 0, 125, 500, and 2000 ppm. Test substance was administered daily via diet for at least 90 days. One control group and three treatment groups were tested, each consisting of 10 males and 10 females Wistar rats. The following parameters were evaluated: clinical signs daily; body weight weekly and on the day preceding the first necropsy date, food consumption weekly, food scattered daily in weeks 4,7,8,10 and 12, functional observations during week 12-13, ophthalmoscopic examination at pretest and in week 13, clinical pathology, macroscopic examination and organ weight at termination. At concentrations 125 and 500 mg/kg/day there were no treatment related findings. The highest concentration level, 2000 mg/kg/day showed reduced weight for both male and female rats. Alanine aminotransferase activity increased in individual cases (males), urea and inorganic phosphate values increased for both male and female rats, potassium values increased for males, cholesterol and total protein values reduced (females) (**MRID 48105511).**

Developmental Toxicity: A developmental toxicity study using natamycin (98.17% and 98.27% pure) as the test substance (MRID 48105512) showed no discernable effects on growth, reproduction, teratological or mutagenic responses, or on gross and microscopic pathology, at concentration levels 0, 5, 15 and 50 mg/kg bw/day.

Prenatal Developmental Toxicity (OCSPP 870.3700; 870.3800; 870.5450; 870.5915) Natamycin dosages of 5, 15, and 50 mg/kg per day administered by diet had no discernable effect on growth, reproduction, teratological or mutagenic response, or on gross and microscopic pathology (**MRID 48105512).**

Mutagenicity: Two mutagenicity studies, using natamycin (98.17% and 98.27% pure) as the test substance, were performed. These studies are sufficient to confirm that there are no expected dietary or non-occupational risks of mutagenicity with regard to food use of natamycin.

Bacterial Reverse Mutation Test (OCSPP 870.5100) Natamycin was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-required strains (TA1535, TA1537, TA100, and TA96) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain (WP_2uvrA). The test was performed in two independent experiments in the presence and absence of S9-mix (Aroclor-1254 induced rat liver S9-mix). All bacterial strains showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants in two independently repeated experiments. Based on the results of this study,

Natamycin is not a mutagen in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay (**MRID 48105513)**

***In Vitro* Mammalian Cell Gene Mutation (OCSPP 870.5300)** This test reported the effects of Natamycin on the number of chromosome aberrations in cultured peripheral human lymphocytes in the presence and absence of a metabolic activation system (Aroclor-1254 induced rat liver S9-mix). Positive control chemicals, mitomycin C and cyclophosphamide, both produced a statistically significant increase in the incidence of cell with chromosome aberrations, indicating that the test conditions were adequate and that the metabolic activation system (S9-mix) functioned properly. Natamycin did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations in the absence and in the presence of S9-mix, in two independently repeated experiments (**MRID 48105514).**

Table 3.0. Toxicological Data for Natamycin

Guideline # Test	Results/Toxicity Category	MRIDs	Study Conclusio
Acute Oral Toxicity OCSPP 870.1100	LD_{50} >3,000 mg/kg III	48105505 Kuhn, (2008)	Acceptable
Acute Dermal Toxicity OCSPP 870.1200	LD_{50} > 5,050 mg/kg IV	48105506 Kuhn, (2008)	Acceptable
Acute Inhalation Toxicity OCSPP 870.1300	LC_{50} > 2.39 mg/L IV	48105507 Crutchfield, (2008)	Acceptable
Primary Eye Irritation OCSPP 870.2400	Severely irritating n positive effects after 24 hours. III	48105508 Kuhn, (2008)	Acceptable
Primary Dermal Irritation OCSPP 870.2500	Slightly irritating IV	48105509 Kuhn, (2008)	Acceptable
Dermal Sensitizatior OCSPP 870.2600	Not a dermal sensitizer	48105510 Kuhn, (2008)	Acceptable
90 day Oral Toxicity-Rat OCSPP 870.3100	At 125 and 500 mg/kg/day no treatment related findings. At 2000 mg/kg/day showed reduced weight.	48105511 Otterdijk, (2003)	Acceptable

Guideline # Test	Results/Toxicity Category	MRIDs	Study Conclusio
870.3700, 3800, 5450, 5915. Prenatal Developmental Toxicity Study - Rat	Dosages of 5, 15 and 50 mg/kg/day administered by diet had no effect on growth, reproduction, teratology or mutagenic response.	48105512 Knickerbocker and Re, (1979)	Acceptable
870.5100 Bacterial Reverse Mutation Test	Not a mutagen	48105513 Verspeek-Rip, (2002)	Acceptable
870.5375 *In Vitro* Mammalian Chromosome Aberration Tests	No chromosomal aberrations	48105514 Meerts, (2002)	Acceptable

III. *Aggregate Exposure*

In examining aggregate exposure, FFDCA section 408 directs the EPA to consider available information concerning exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

Dietary Exposure: Dietary exposure to residues of natamycin are expected to be insignificant, even in the event of exposure. Furthermore, the active ingredient is of low acute toxicity and is not a developmental toxicant, a mutagen, or toxic via repeat oral exposure.

Drinking Water Exposure

Exposure of humans to natamycin in drinking water is not expected because natamycin is approved for application indoors only.

Other Non-occupational Exposure: Non-occupational exposure is not expected because natamycin is not approved for residential uses. The active ingredient is applied directly to commodities and degrades rapidly.

IV. *Cumulative Effects from Substances with a Common Mechanism of Toxicity*

Section 408(b)(2)(D)(v) of FFDCA requires that, when considering whether to

establish, modify, or revoke a tolerance, the EPA consider "available information concerning the cumulative effects of [a particular pesticide's] . . . residues and other substances that have a common mechanism of toxicity."

The EPA has not found natamycin to share a common mechanism of toxicity with any other substances, and natamycin does not appear to produce a toxic metabolite produced by other substances. For the purposes of this tolerance action, therefore, the EPA has assumed that natamycin does not have a common mechanism of toxicity with other substances. Following from this, the EPA concludes that there are no cumulative effects associated with natamycin that need to be considered. For information regarding the EPA's efforts to determine chemicals that have a common mechanism of toxicity and to evaluate the cumulative effects of su ch chemicals, see the EPA's website at *http://www.epa.gov/pesticides/cumulative.*

V. Determination of Safety for the United States Population, Infants and Children

FFDCA section 408(b)(2)(C) provides that, in considering the establishment of a tolerance or tolerance exemption for a pesticide chemical residue, the EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues, and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408(b)(2)(C) provides that the EPA shall apply an additional tenfold (10X) margin of safety for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the database on toxicity and exposure, unless the EPA determines that a different margin of safety will be safe for infants and children. This additional margin of safety is commonly referred to as the Food Quality Protection Act Safety Factor. In applying this provision, the EPA either retains the default value of 10X, or uses a different additional or no safety factor when reliable data are available to support a different additional or no safety factor.

Because there are no threshold effects associated with this biochemical, an additional margin of safety for infants and children is not necessary.

EPA has determined that there are no foreseeable dietary risks to the U.S. population, including infants and children, from the use of natamycin as a pesticide (fungstat) on mushrooms in enclosed mushroom facilities, pineapples, citrus, pome, stone fruit crop groups, avocado, kiwi, mango and pomegranates when label instructions and good agricultural practices are followed. The available data and information indicate that the chemical is of low toxicity and not a developmental

toxicant. Therefore, EPA concludes that there is a reasonable certainty that no harm will result to the U.S. population, including infants and children, from aggregate exposure to the residues of natamycin when it is used as labeled and in accordance with good agricultural practices. Such exposure includes all anticipated dietary exposures and all other exposures for which there is reliable information. EPA has arrived at this conclusion because the data and information available on natamycin do not demonstrate significant toxic potential to mammals, including infants and children.

VI. Conclusions

EPA concludes that there is a reasonable certainty that no harm will result to the U.S. population, including infants and children, from aggregate exposure to residues of natamycin. Therefore, the tolerance exemption is amended for residues of the biochemical pesticide natamycin when used on citrus, pome, stone fruit crop groups, avocado, kiwi, mango and pomegranates when used in accordance with label directions and good agricultural practices.

References

1. Crutchfield, V. (2008) Natamycin TGAI: Acute Inhalation Toxicity Study in Rats: Final Report. Project Number: 11405/07. Unpublished study prepared by Stillmeadow, Inc. 19 p. (MRID 48105507)

2. Devine, J.; Maryann C. (2013) Magnitude of Residues of Natamycin in Post-Harvest Treated Pineapples (Decline, Processing). Project Number: 197SRUS12R/1, 029653/1, 029653. Unpublished study prepared by SynTech Research. 204p. (MRID 49292403)

3. Knickerbocker, M. (1979) Natamycin TGAI: Teratological Evaluation of Pimaricin in Dutch-Belted Rabbits. Project Number: 5906. Unpublished study prepared by Food & Drug Research Laboratories, Inc. 290 p. (MRID 48105512)

4. Kuhn, J. (2008) Natamycin TGAI: Acute Oral Toxicity Study (UDP) in Rats: Final Report. Project Number: 11403/07. Unpublished study prepared by Stillmeadow, Inc. 12 p (MRID 48105505)

5. Kuhn, J. (2008) Natamycin TGAI: Acute Dermal Toxicity Study in Rat: Final Report. Project Number: 11404/07. Unpublished study prepared by Stillmeadow, Inc. 13 p. (MRID 448105506)

6. Kuhn, J. (2008) Natamycin TGAI: Acute Eye Irritation Study in Rabbits: Final Report. Project Number: 11406/07. Unpublished study prepared by Stillmeadow, Inc. 18 p. (MRID 48105508)

7. Kuhn, J. (2008) Natamycin TGAI: Acute Dermal Irritation Study in Rabbits: Final Report. Project Number: 11407/07. Unpublished study prepared by Stillmeadow, Inc. 13 p. (MRID 48105509)

8. Kuhn, J. (2008) Natamycin TGAI: Skin Sensitization: Local Lymph Node Assay in Mice: Final Report. Project Number: 11408/07. Unpublished study prepared by Stillmeadow, Inc. 13 p. (MRID 48105510)

9. Meerts, I. (2002) Natamycin TGAI: Evaluation of the Ability of Natamycin to Induce Chromosome Aberrations in Cultured Peripheral Human Lymphocytes. Project Number: NOTOX/PROJECT/339356. Unpublished study prepared by Notox B.V. 28 p. (MRID 48105514).
10. Otterdijk, F. (2003) Natamycin TGAI: 90 Day Oral Toxicity Study with Natamycin in the Rat. Project Number: NOTOX/PROJECT/339323, 002019. Unpublished study prepared by Notox B.V. 220 p. (MRID 48105511)

11. Van der Lee, J, and Jovanovich, A. 2011. 18-month Final Report for Storage Stability and Corrosion Characteristics; Natamycin L. Project No. STAB/SLD/09/008. (MRID 48544501).

12. Verspeek, C. (2002) Natamycin TGAI: Evaluation of the Mutagenic Activity of Natamycin in the Salmonella typhimurium Reverse Mutation Assay and the Escherichia coli Reverse Mutation Assay (with Independent Rat). Project Number: NOTOX/PROJECT/339345. Unpublished study prepared by Notox B.V. 29 p. (MRID 48105513).

WHO Technical Report Series
909

EVALUATION OF CERTAIN FOOD ADDITIVES AND CONTAMINANTS

Fifty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives

World Health Organization
Geneva

this compound. If use only in bread is assumed, it can be calculated that up to 720 g of bread containing hydrogenated poly-1-decene at a concentration of 500 mg/kg could be consumed by a 60-kg person before the ADI of 0–6 mg/kg of body weight was exceeded. However, it was considered highly unlikely that a person would consume this amount of bread containing hydrogenated poly-1-decene at the maximum level of use each day.

An addendum to the toxicological monograph was prepared.

3.1.6 *Preservative*

3.1.6.1 Natamycin (pimaricin)

Natamycin (pimaricin) is a polyene macrolide antibiotic produced by submerged aerobic fermentation of *Streptomyces natalensis* and related species. The fermentation process takes several days, after which the antibiotic is isolated by extraction from broth or by extraction of the mycelium.

Natamycin 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 of body weight. The present evaluation was conducted in response to a request by the Codex Committee on Food Additives and Contaminants at its Thirty-second Session (*4*).

The Committee considered information on the current uses of natamycin, data on its intake and biological data that had not been evaluated previously.

Uses. Because natamycin is active against yeasts and moulds, but not bacteria, it is used in foods that undergo a ripening period after processing. Its low solubility in water and most organic solvents makes it appropriate 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 fungal infections of the cornea and the prevention of such infections in contact lens wearers.

Assessment of intake. The Committee noted that as the draft Codex General Standard for Food Additives proposes restricted use of natamycin only in cheese and in dried, non-heat-treated meats, intake would not be expected to exceed the ADI.

Data submitted by Australia, Germany, New Zealand, the United Kingdom and the USA indicated that the intakes at mean and high percentiles 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 by consumers ranged from 0.01 to 0.03 mg/kg of body weight per day (representing 3% and 9% of the ADI in Germany and the United Kingdom, respectively), and those by consumers at high percentiles were 0.03–0.08 mg/kg of body weight 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 Codex General Standard for Food Additives. The estimated intakes of natamycin were lower when national levels of use were assumed.

Toxicological studies. The Committee considered eight studies that had not been evaluated previously and had been conducted before the 1980s. 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 given the highest dose (equivalent to 25 mg/kg of body weight 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 natamycin at 500 mg/l was given to groups of 20–26 rabbits at a dose of 0, 5, 15 or 50 mg/kg of body weight 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. The mean maternal body weight, pregnancy rate, number of implantation sites, number of resorption sites, numbers of live and dead fetuses, proportion of viable fetuses and incidence of soft-tissue anomalies were comparable in the treated groups and a control group given the vehicle only. The fetal body weight in the group dosed at 15 mg/kg of body weight by gavage was lower than that of fetuses in the control group given the vehicle only. The incidence of extra sternebrae was increased at the two highest doses in comparison with the control

group, but not in a dose-related manner. However, in view of the unusual sensitivity of the gastrointestinal tract of rabbits to poorly absorbed substances and to compounds with antimicrobial activity, this study was considered unsuitable for deriving the ADI.

Microbiological studies. The antifungal activities of natamycin and other polyenes depend on their binding to cell membrane sterols, primarily ergosterol, the principal sterol in fungal membranes. Oomycetes 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 indigenous microflora to trace quantities of antimicrobial residues. The human intestinal microflora is a complex mixture of more than 400 bacterial species, consisting primarily of bacterial cells at a concentration of approximately 10^{11}–10^{12} colony-forming units per gram (CFU/g). Fungi are much less abundant than bacteria in the human gastrointestinal tract, the concentration of yeast in stool samples from healthy subjects being up to 10^5 CFU/g. As bacteria are not affected by polyenes, natamycin residues should not harm them; 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 antimicrobial activity in the colon, suggesting that natamycin was degraded into microbiologically inactive compounds by bacterial flora. However, no data were 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 it is incompletely absorbed or degraded.

As emergence of resistance to antimicrobials is a concern, the Committee evaluated the possible development of resistance among microflora as a consequence of exposure to natamycin. A preparation containing 50% natamycin has been used since the 1980s 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 by similarly low concentrations (0.5–8 µg/ml). In another study, 26 strains of fungi were isolated in eight warehouses where natamycin was used and two warehouses where it had never been used, and were tested for sensitivity to the compound; no insensitive yeasts or moulds were found. The results of laboratory experiments to induce resistance to natamycin in strains of fungi 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 used, no significant differences were demonstrated.

It has been found difficult to induce resistance to polyenes, especially natamycin, in fungi in vitro. Resistant isolates 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 isolates resistant to natamycin is successive subculturing 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 concentration that inhibited *Candida albicans* was minimally increased, from 2.5–12 μg/ml to 12–50 μg/ml.

Evaluation. Natamycin is a polyene macrolide antibiotic that is effective against yeasts and moulds but not against bacteria or oomycetes fungi. The antifungal activities of natamycin depend on its binding to cell membrane sterols, primarily ergosterol, the principal sterol in fungal membranes, which is absent in bacteria. The use of natamycin as an antifungal agent in food may result in exposure of the indigenous flora to trace quantities of antimicrobial residues. As bacteria in the human gastrointestinal tract are not affected by polyenes, the Committee concluded that natamycin would not have an effect and that disruption of the barrier to colonization of the intestinal tract was therefore not a concern. Fungi are much less prevalent than bacteria in the human gastrointestinal tract, and, in light of the negative results of the studies of acquired resistance, selection of natamycin-resistant fungi was not considered 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 also reported. It considered, however, that administration of an antimicrobial agent to rabbits by gavage was an inappropriate way of testing for developmental toxicity. In addition, extra sternebrae have been described as a skeletal variation rather than a frank sign of teratogenicity. Thus, the Committee did not consider the finding of extra sternebrae to be evidence that natamycin is teratogenic.

The Committee confirmed the previously established ADI of 0–0.3 mg/kg of body weight for natamycin, which was based on observations of gastrointestinal effects in humans. The Committee noted that the estimated intakes of natamycin, based on maximum levels of use

in cheese and processed meats proposed in the draft Codex General Standard for Food Additives, do not exceed this ADI.

A toxicological monograph was prepared and the existing specifications were revised. The title of the specifications was changed from pimaricin to natamycin, the commonly used designation. The specifications were made tentative, pending the receipt of information on the level and determination of water content, limit for lead, specific rotation, assay value and method of assay for the commercial product. This information was required for evaluation in 2003.

3.1.7 *Sweetening agent*

3.1.7.1 D-Tagatose

D-Tagatose is a keto-hexose, an epimer of D-fructose inverted at C-4, with a sweet taste. It is obtained from D-galactose by isomerization under alkaline conditions in the presence of calcium.

D-Tagatose was evaluated by the Committee at its fifty-fifth meeting (Annex 1, reference *149*), when it concluded that the available data indicated that D-tagatose is not genotoxic, embryotoxic or teratogenic. It noted that the increased liver weights and hepatocellular hypertrophy seen in Sprague-Dawley rats occurred concurrently with increased glycogen deposition; however, the reversal of increased glycogen storage after removal of D-tagatose from the feed was more rapid than regression of the liver hypertrophy. Although the gastrointestinal symptoms seen in adult humans with the expected daily intake of D-tagatose were minor, the Committee was concerned about the increased serum uric acid concentrations observed in a number of studies in humans after administration of either single or repeated doses of D-tagatose. Similar increases were observed with other sugars, such as fructose, but D-tagatose appeared to be a more potent inducer of this effect. The Committee also noted that this effect of D-tagatose had not been studied in persons prone to high serum uric acid concentrations. The Committee concluded that an ADI could not be allocated for D-tagatose because of concern about its potential to induce glycogen deposition in the liver and liver hypertrophy and to increase the serum concentration of uric acid.

Two studies of up to 7 days' duration in Wistar and Sprague-Dawley rats given repeated doses of D-tagatose were submitted to the Committee at its fifty-fifth meeting, but the reports were received only in draft form and were not suitable for consideration at that time. The Committee therefore asked for the final reports and for further data to clarify the extent, mechanism and toxicological consequences of the increased serum uric acid concentrations observed in humans exposed to D-tagatose. At its present meeting, the Committee reviewed the

reports of the two studies in rats, the results of a study in volunteers (on the relevance of the glycogen deposition and liver hypertrophy) and some published studies on the increased uric acid concentrations in serum after intake of D-tagatose, other sugars and other food components.

Biological data. Review of the results of the studies considered by the Committee at its fifty-fifth meeting and comparisons with the data reviewed at the present meeting revealed a difference in sensitivity between Wistar and Sprague-Dawley rats. Sprague-Dawley rats given D-tagatose at a concentration of 50 g/kg of diet for 28 days showed increased hepatic glycogen only when they had not been fasted the night before necropsy, and this effect was not associated with any microscopic changes in the liver. In a 90-day study in which Sprague-Dawley rats were killed after fasting overnight, administration of D-tagatose at a concentration of 50 g/kg of diet had no adverse effect on the liver. In a 6-month study in Wistar rats in which the animals were killed after fasting 3, 7, 14 and 28 days and 3 and 5 months after treatment, administration of D-tagatose at concentrations of up to 100 g/kg of diet had no adverse effects. Wistar rats are therefore less susceptible to the hepatic effects of D-tagatose than Sprague-Dawley rats. As D-tagatose stimulated glycogen deposition to a similar degree in the two rat strains in short-term studies, the difference is likely to occur at a later stage, during glycogen-induced or other stimulation of liver growth.

The authors suggested that the increase in normal liver mass seen in fasted rats fed diets containing 100 or 200 g/kg D-tagatose is triggered by increased postprandial storage of liver glycogen resulting from simultaneous feeding of D-tagatose and glucose equivalents. In order to test this hypothesis, the effects of separate and simultaneous administration of D-tagatose and glycogen precursors on liver weight and glycogen level were investigated in Wistar and Sprague-Dawley rats. The results neither supported nor invalidated the hypothesis.

As several studies have been performed in healthy volunteers and in patients with diabetes, the number of persons varying from 4 to 73, the Committee based its toxicological evaluation on the data from these studies. The length of these studies varied from several days to several weeks; one study of 12 months' duration included only a limited number of patients with type 2 diabetes. The toxicological aspects investigated included gastrointestinal effects, increased serum uric acid concentrations and hepatic effects.

Mild gastrointestinal symptoms were reported in only one study, in 3 of 10 patients with type 2 diabetes receiving D-tagatose at 10 g/day for several days, whereas in other studies diarrhoea was observed only in patients receiving 25 g three times daily for 8 weeks. In healthy individuals, administration of a single dose of 30 g induced diarrhoea in

some persons only, whereas other studies showed no laxative effect of single doses of D-tagatose as high as 75 g.

The serum or plasma concentration of uric acid was increased transiently in some studies, but the increased uric acid concentration was above the normal range for a number of days in only one study of persons receiving 75 g/day. The other studies showed either no increase or a transient increase in serum uric acid concentrations within the normal range.

In a 28-day study in which 15 g of D-tagatose or 15 g of sucrose were given three times daily to volunteers, magnetic resonance imaging was used to determine liver volume, and glycogen concentrations and several clinical chemical parameters were measured. The results did not reveal any relevant effect on the liver. In addition, no diarrhoea and no increase in serum uric acid concentration were observed. Therefore, the NOEL was 45 g/person per day, equivalent to 0.75 g/kg of body weight per day (for a person weighing 60 kg).

Evaluation. The Committee considered the 28-day study in which humans received a daily dose of 45 g of D-tagatose or sucrose in three divided doses as most representative of human dietary intake and therefore most relevant for assessing the acceptable intake of D-tagatose accurately. While effects were observed after administration of a single dose of 75 g, no effects were seen following administration of three daily doses of 15 g of D-tagatose, equivalent to 0.75 g/kg of body weight per day. The Committee established an ADI of 0–80 mg/kg of body weight on the basis of this NOEL and a safety factor of 10.

Assessment of intake. D-Tagatose is proposed for use as a bulk sweetener in low-energy foods, such as edible ices (at a concentration of 3 g/kg), chewing-gum and confectionery (at 15 g/kg), breakfast cereals (at 15 g/kg) and soft drinks (at 1 g/kg). At its present meeting, the Committee considered that the predicted intakes of D-tagatose determined at the fifty-fifth meeting, which were based on the manufacturers' proposed levels of use and individual dietary records in several countries, were conservative. This was because use had been assumed in the entire food category rather than only in the low-energy food component. The mean consumer intakes of D-tagatose from all proposed uses (except chewing-gum, dietary supplements and meal replacements) predicted for Australia, the Member States of the European Union and the USA ranged from 3 to 9 g/day (63–190% of the ADI), and the predicted intakes by persons at high percentiles of consumption were up to 18 g/day (375% of the ADI). On the basis of the information on possible uses, the Committee concluded that the ADI for D-tagatose may be exceeded by some groups of the population.

A toxicological monograph was prepared. The specifications prepared by the Committee at its fifty-fifth meeting were maintained.

3.1.8 *Thickening agents*

3.1.8.1 Carrageenan and processed Eucheuma seaweed

Carrageenan, a substance with hydrocolloid properties owing to the presence of sulfated polyglycans with average relative molecular masses well above 100 000, is derived from a number of seaweeds of the family Rhodophyceae. It has no nutritional value and is used in food preparation for its gelling, thickening and emulsifying properties. Three main types of carrageenan, known as ι-, κ- and λ-carrageenan, are used commercially in the food industry. These names do not reflect definitive chemical structures but only general differences in the composition and degree of sulfation at specific locations in the polymer. Processed *Eucheuma* seaweed is derived from either *E. cottonii* (κ-carrageenan) or *E. spinosum* (λ-carrageenan), which are also Rhodophyceae.

Carrageenan is obtained by extraction of the seaweed into water or aqueous dilute alkali and may be recovered by precipitation with alcohol, by drying in a rotary drum or by precipitation with aqueous potassium chloride and subsequent freezing. In contrast, processed *Eucheuma* seaweed is prepared by soaking the cleaned seaweed in alkaline solution for a short time at elevated temperatures. The treated material is then thoroughly washed with water to remove residual salts and further washed with alcohol, dried and milled to a powder. For both carrageenan and processed *Eucheuma* seaweed, the alcohols that may be used during purification are restricted to methanol, ethanol and isopropanol. The articles of commerce may contain sugars added for standardization purposes, salts to obtain specific gelling or thickening characteristics, or emulsifiers carried over from the drum-drying process.

Carrageenan was reviewed by the Committee at its thirteenth, seventeenth, twenty-eighth and fifty-first meetings (Annex 1, references *19*, *32*, *66* and *137*). At its twenty-eighth meeting, the Committee established an ADI "not specified"[1] on the basis of the results of a number

[1] ADI "not specified" is used to refer to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effect and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for reasons stated in the individual evaluation, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice, i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.

of toxicological studies on carrageenans obtained from various sources.

Processed *Eucheuma* seaweed was considered by the Committee at its thirtieth, thirty-ninth, forty-first, forty-fourth and fifty-first meetings (Annex 1, references *73*, *101*, *107*, *116* and *137*). At its forty-fourth meeting, the Committee concluded that, because of the chemical relationship between processed *Eucheuma* seaweed and traditionally refined carrageenan, the toxicological data on carrageenan were relevant to the safety assessment of the carrageenan polysaccharide constituents of processed *Eucheuma* seaweed, but could not replace adequate toxicological studies on processed *Eucheuma* seaweed itself. At its fifty-first meeting, the Committee reviewed the results of a 90-day study on toxicity in rats fed processed *Eucheuma* seaweed from *E. cottonii* and *E. spinosum*. The Committee concluded that the toxicity of this material was sufficiently similar to that of carrageenan to allow extension of the previous ADI "not specified" for carrageenan to a group ADI that covered processed *Eucheuma* seaweed. The Committee also considered all studies on carrageenan that had been published since its twenty-eighth meeting and, for the earlier studies, noted the identity of the source material and the type of carrageenan, when these could be identified. It expressed concern about the potential promotion of colon carcinogenesis by carrageenans and processed *Eucheuma* seaweed and therefore made the group ADI "not specified" temporary, pending clarification of the significance of the promotion of colon cancer observed in studies in rats. At its present meeting, the Committee reviewed the available evidence for the tumour-promoting and related effects of these compounds in rat colon.

Assessment of intake. Carrageenan and processed *Eucheuma* seaweed are used as thickeners, gelling agents, stabilizers or emulsifiers in a wide range of foods at concentrations of up to 1500 mg/kg. Per capita intakes in 1995 derived from "poundage" (disappearance) data in Europe and the USA ranged from 28 to 51 mg/day. These estimates corresponded to those reported for 1993 by the Seaweed Industry Association of the Philippines on the basis of sales of 44 mg/person per day for the populations of Canada and the USA and 33 mg/person per day for European populations.

The estimates derived from poundage data were also consistent with those derived for the population of the USA from model diets, with reported mean intakes of carrageenan of 20 mg/day for all consumers and 40 mg/day for persons at the 90th percentile of consumption (derived by multiplying the mean by a factor of 2). The intakes were derived from data on the food consumption of individuals aged

2 years and over that were available in 1976 from nutrition surveys in the USA, combined with the results of a 2-week study by the Marketing Research Corporation of America on the frequency of food consumption.

Biological data. Two studies showed that carrageenan administered before, during and after administration of known carcinogens (dimethylhydrazine, azoxymethane, *N*-methyl-*N*-nitrosourea) enhanced the tumorigenicity of these carcinogens. One of the studies involved administration of carrageenan at 150 g/kg of diet, which resulted in decreased body-weight gain. In the second study, involving administration of carrageenan at 60 g/kg of diet, the body-weight gain of treated animals was comparable to that of controls. The increased incidence of tumours seen under these circumstances may have resulted from promotion but may also have resulted from altered toxicokinetics or biotransformation of the carcinogen. In addition, there were indications that the bacterial flora had been altered as a result of administration of carrageenan. In a separate study conducted according to a classical tumour initiation–promotion protocol, in which rats were given dimethylhydrazine, subsequent administration of carrageenan at dietary concentrations of up to 50 g/kg did not result in a statistically significant increase in the incidence of colon tumours over that seen with dimethylhydrazine alone.

Two further studies in rats involved use of a conventional tumour initiation–promotion protocol but in which formation of aberrant crypt foci was the end-point, instead of tumour formation. Rats were given azoxymethane with or without subsequent administration of carrageenan in their drinking-water. The higher concentration of carrageenan, 25 g/kg, was given in the form of a solid gel, which may have altered the food and water consumption patterns of the animals. The first study demonstrated that dietary administration of carrageenan after the carcinogen decreased the number of aberrant crypt foci seen relative to the number observed with the carcinogen alone, but significantly increased their size. A subsequent study in rats injected with human faecal microflora showed no effect of carrageenan on either the number or size of aberrant crypt foci. As the relationship between aberrant crypt foci and tumorigenesis is still unclear, it is difficult to interpret the biological significance of these results.

Increased cell proliferation has frequently been postulated as a mechanism of non-genotoxic carcinogenicity or tumour promotion. The preferred methods of assessing cell proliferation are based on histological techniques, which allow identification of the nature and location of proliferating cells. There was no consistent pattern of colon damage in rats treated with carrageenan for prolonged periods.

Some studies showed caecal enlargement, but most did not show histological damage. In one study in which rats underwent autoradiographic examination, no significant difference from controls in the number of cells per crypt or in the proportion of labelled cells was seen in rats fed a diet containing carrageenan at 74 g/kg for 28 days.

Methods for measuring cell proliferation that are based on measurement of cell cycle-dependent enzyme activities, such as thymidine kinase activity, are cruder means of measuring overall cell proliferation in an entire tissue specimen. A significant increase in thymidine kinase activity, expressed relative to protein content, was found in homogenized mucosal scrapings from the colon of rats fed diets containing carrageenan at 26 or 50 g/kg for 4 weeks; no significant effects were observed in the animals fed 0, 6.5 or 13 g/kg carrageenan in the diet for 4 weeks. Histological examination revealed no evidence of infiltration by inflammatory cells in any of the treated groups. In another study, the increased thymidine kinase activity observed in rats fed diets containing carrageenan at 50 g/kg returned to the basal level within 28 days when the animals were returned to a diet with no carrageenan. No increase in thymidine kinase activity was seen in animals receiving diets containing 2 or 15 g/kg carrageenan for 28 days. Staining for proliferating cell nuclear antigen (PCNA) revealed a significant increase in PCNA-positive cells in the upper third of the crypts of rats receiving a diet containing carrageenan at 50 g/kg for 91 days, but not after 28 or 64 days followed by a 28-day recovery period on a normal diet. No PCNA-positive cells were observed at the luminal surface. The pattern of staining for PCNA seen with carrageenan was considered indicative of an adaptive response, which would not contribute to an increased risk for colonic neoplasia.

In one study, carrageenan inhibited gap-junctional intercellular communication in vitro. However, the mechanism of action was different from that of a known tumour-promoting agent, phorbol ester, and the relevance of this observation is unclear for a substance that is not absorbed in vivo.

Evaluation. In a recent study with a classical tumour initiation–promotion protocol, administration of carrageenan at concentrations of up to 50 g/kg of diet did not promote colon carcinogenesis in rats given dimethylhydrazine. The Committee noted, however, that, in two studies that showed enhancement of colon carcinogenesis in rats, higher dietary concentrations of carrageenan were used and carrageenan was administered before, during and after the carcinogens. The enhanced carcinogenicity seen under these circumstances may have resulted from promotion or from altered toxicokinetics or biotransformation of the carcinogen. Therefore, the mechanism of the

enhancement of colon carcinogenesis in these studies remains unresolved. Continuous feeding of high doses of carrageenan caused a generalized proliferative response, measured as increased thymidine kinase activity, in the mucosal tissue of the colon of male rats. This effect might play a role in the observed enhancement of the tumorigenicity of known colon carcinogens by high dietary concentrations of carrageenan. However, a proliferative effect of carrageenan on the mucosa of the colon was seen only at a dietary concentration of 26 g/kg or more. No effect was seen at a concentration of 15 g/kg in the diet, corresponding to 750 mg/kg of body weight per day, which greatly exceeded the estimated human intake of carrageenan and processed *Eucheuma* seaweed of 30–50 g/person per day from their use as food additives. Bearing in mind that the enhancement of colon carcinogenesis in rats was seen at much higher concentrations and that carrageenan at 50 g/kg of diet did not promote tumours in rat colon in a classical initiation–promotion study, the Committee considered that the intake of carrageenan and processed *Eucheuma* seaweed from their use as food additives was of no concern. It therefore allocated a group ADI "not specified"[1] to the sum of carrageenan and processed *Eucheuma* seaweed.

An addendum to the toxicological monograph was prepared. The existing specifications for both carrageenan and processed *Eucheuma* seaweed were revised by incorporating more complete descriptions of the analytical procedures for the determination of lead, cadmium and mercury and by raising the acceptable limit for lead from 2 mg/kg to 5 mg/kg and the acceptable limit for cadmium from 1 mg/kg to 2 mg/kg. These limits were raised to take into account new information on inadequacies of the analytical methods for determination of these elements, which are due to the high salt content of the polysaccharides of both processed *Eucheuma* seaweed and carrageenan. The changes were not made because of information about higher concentrations of lead and cadmium than those previously considered by the Committee. The Committee also observed that the new limits are consistent with the limits established for these heavy metals in specifications for other hydrocolloids, such as alginic acid.

3.1.8.2 Curdlan

Curdlan (synonym, β-1,3-glucan) is a linear polymer of high relative molecular mass, consisting of β-1,3-linked glucose units. Curdlan is produced by fermentation of pure cultures from a non-pathogenic, non-toxinogenic strain of *Agrobacterium* Biovar1 (identified as *Alcaligens faecalis* var. *myxogenes* at the time of its isolation) or

[1] See footnote on page 32.

Alcaligens radiobacter. Curdlan is recovered from the fermentation medium by addition of acid and alkali to disrupt the cells, which releases the curdlan into the medium, followed by separation by centrifugation. It is then washed with copious amounts of water to eliminate mineral salts and other water-soluble substances that may have been carried over from the fermentation broth. The commercial product is an odourless or nearly odourless, tasteless, white to nearly-white spray-dried powder.

The use of curdlan in a wide variety of foods is based on its ability to form an elastic gel upon heating in an aqueous suspension. Thus, it can be used in processed meat, fish and poultry products and in gelatins, puddings and fillings as a firming or gelling agent or as a stabilizer or thickener.

The Committee reviewed curdlan at its fifty-third meeting (Annex 1, reference *143*), when it allocated a temporary ADI "not specified",[1] pending information on the use of curdlan, including the maximum and typical expected levels in the food categories in which it is proposed for use in the draft Codex General Standard for Food Additives, and on the consumption in various regions of the world of foods that might contain curdlan.

Use of curdlan is based on its physical properties, which imply a self-limiting level of use in solid foods. A submission from the USA described a model constructed to predict the intakes of curdlan by a long-term consumer on the basis of a study of the frequency of consumption of foods in 1982–1988 from the Market Research Corporation of America, and average portion sizes from a 3-day national food consumption survey conducted in 1987–1988 by the United States Department of Agriculture. Intake was assessed on the basis of the self-limiting levels of use (20 mg/kg of processed meat, 15 mg/kg of processed poultry and fish, 10 mg/kg of dairy products, 35 mg/kg of egg products, 15 mg/kg of grain products and pasta, 30 mg/kg of cereals and starch desserts, 20 mg/kg of gravies and sauces and 40 mg/kg of gelatins). The resulting mean intake by consumers was estimated to be 3.6 g/person per day, corresponding to 60 mg/kg of body weight per day.

The sponsor submitted an estimate based on daily food intake per capita and typical levels of use in Japan (15 mg/kg of processed meat, 10 mg/kg of processed poultry and fish, 5 mg/kg of dairy products, 30 mg/kg of egg products, 10 mg/kg of grain products and pasta, 10 mg/kg of cereals and starch desserts, 10 mg/kg of gravies and sauces and 30 mg/kg of gelatins). The mean intakes were estimated to be

[1] See footnote on page 32.

0.77 g/person per day, corresponding to 13 mg/kg of body weight per day, for typical levels of use and 1.7 g/person per day, corresponding to 28 mg/kg of body weight per day, for maximum levels of use.

Estimates of the intake of curdlan based on individual dietary records were submitted by the USA on the basis of a survey by the Department of Agriculture and the Continuing Survey of Food Intakes by Individuals (1989–1992). When intake was estimated on the basis of the upper limit of the range of recommended use, the intake of curdlan by consumers was 20 mg/kg of body weight per day for consumption at the mean and 47 mg/kg of body weight per day for consumption at the 90th percentile. When intake was estimated on the basis of self-limiting levels of use, the intake of consumers was 30 mg/kg of body weight per day for consumption at the mean and 68 mg/kg of body weight per day for consumption at the 90th percentile.

The data on uses and intake requested by the Committee at its fifty-third meeting were provided and raised no safety concern. The Committee therefore established an ADI "not specified"[1] for use of curdlan as a food additive.

The existing specifications were revised, with minor changes.

3.1.9 *Miscellaneous substances*

3.1.9.1 *Acetylated oxidized starch*

Acetylated oxidized starch is a chemically modified root or grain starch. It is produced by oxidation of a slurry of starch granules in alkaline hypochlorite at low temperatures (21–38 °C). The alkaline medium is neutralized with sodium bisulfite, and the resulting organic salts are removed by washing with water. The oxidized starch is then esterified with acetic anhydride under mildly alkaline conditions. The product is neutralized with hydrochloric acid, washed and dried.

Acetylated oxidized starch had not been evaluated previously by the Committee. At the present meeting, it was proposed for use as a binding agent in soft confectionery at a concentration of about 300 mg/kg — it is mixed with water, sugars and flavours in a batch process until a clear solution with a dry-solid content of 70% is obtained. The characteristics of the end-product important for confectionery use are gel strength and clarity. Acid hydrolysis results in starch products that are relatively unclear, and oxidized starch products result in overly soft confectionery. Acetylation of oxidized starch enhances the desired properties, resulting in a gummy, clear jelly. It

[1] See footnote on page 32.

can be used as a substitute for gelatin or gum arabic and would replace a large amount of sugar.

Acetylated oxidized starch has a stable configuration under normal conditions in food. It is hydrolysed slowly in the presence of strong acids, yielding glucose, gluconic acid and acetic acid. No degradation products are expected or known to result from storage or use of this substance in the preparation of foods at neutral pH. The substance is not known to sequester minerals, nor does it interact with proteins or vitamins. It has no known effect on other nutrients.

In a 14-day range-finding study in rats, administration of a diet containing acetylated oxidized starch at a concentration of 300 or 500 mg/kg increased the weights of full and empty caeca, and dilated caeca were found at autopsy. At the higher concentration, soft faeces also occurred. The NOEL was 100 mg/kg of diet.

In a 90-day study in rats given a diet containing acetylated oxidized starch, increased full and empty caecal weights were seen at the highest concentration of 300 mg/kg of diet. Macroscopic examination showed a dilated caecum in one male rat. Histological examination did not reveal changes in the caecal wall or other parts of the digestive tract. Increased caecal weights are a known response to high dietary concentrations of poorly digested carbohydrates in rats, due perhaps to an increased osmotic load of short-chain fatty acids produced by microbial degradation and the associated water retention. Focal hyperplasia of the urinary bladder epithelium was seen in 4 out of 10 male rats that received the highest dietary concentration but not in males given lower concentrations, in controls or in females. The change was probably treatment-related and a consequence of irritation of the urinary bladder by calculi. The NOEL was 100 mg/kg of diet, equivalent to 5900 mg/kg of body weight per day.

If acetylated oxidized starch was to be used only in jelly confectionery at a concentration of 300 g/kg and if the maximum consumption by consumers was 200 g of jelly confectionery per day, the maximum intake of acetylated oxidized starch would be 60 g/day.

The effects seen in the 14-day and 90-day studies in rats were similar to those observed with high dietary concentrations of other slowly digested carbohydrates and are commonly seen in rats given other modified starches in the diet. Because of the nature of acetylated oxidized starch and its similarity to other modified starches with non-systemic effects, the Committee established an ADI "not specified",[1] on the basis of the known uses of acetylated oxidized starch as an ingredient in confectionery products.

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[1] See footnote on page 32.

A toxicological monograph was prepared. New specifications for acetylated oxidized starch were prepared and incorporated into the specifications for modified starches.

3.1.9.2 α-Cyclodextrin

α-Cyclodextrin is a non-reducing cyclic saccharide composed of six glucose units linked by α-1,4 bonds. It is produced by the action of cyclodextrin glucosyltransferase (CGTase, EC 2.4.1.19) on hydrolysed starch syrups at neutral pH (6.0–7.0) and moderate temperatures (35–40 °C). The annular structure of α-cyclodextrin provides a hydrophobic cavity that allows formation of inclusion complexes with a variety of non-polar organic molecules of appropriate size. The hydrophilic nature of the outer surface of the cyclic structure makes α-cyclodextrin water-soluble.

The principal method for the isolation and purification of α-cyclodextrin takes advantage of its complex-forming ability. At the end of the reaction, 1-decanol is added to the reaction mixture to form an insoluble 1:1 inclusion complex of α-cyclodextrin:1-decanol. The complex is continuously mixed with water and separated from the reaction mixture by centrifugation. The recovered complex is resuspended in water and dissolved by heating. Subsequent cooling leads to precipitation of the complex. The precipitate is recovered by centrifugation, and 1-decanol is removed by steam distillation. Upon cooling, α-cyclodextrin crystallizes from the solution. The crystals are removed by filtration and dried, yielding a white crystalline powder with a water content of less than 11%. The purity on a dried basis is at least 98%.

The hydrophobic cavity and the hydrophilic outer surface of α-cyclodextrin form the basis for its use in the food industry. α-Cyclodextrin, like its homologues β- and γ-cyclodextrin, can function as a carrier and stabilizer for flavours, colours and sweeteners; as an absorbent for suppression of undesirable flavours and odours in foods; as an absorbent for suppression of halitosis (in breath-freshening preparations); and as a water-solubilizer for fatty acids and vitamins.

α-Cyclodextrin had not been evaluated previously by the Committee, but the structurally related compound β-cyclodextrin was evaluated at the forty-first and forty-fourth meetings (Annex 1, references *107* and *116*), and γ-cyclodextrin was evaluated at the fifty-first and fifty-third meetings (Annex 1, references *137* and *143*). At its present meeting, the Committee noted the close structural similarity between α- and β-cyclodextrin (seven glucose units) and γ-cyclodextrin (eight glucose units), which permitted comparisons of the metabolism and toxicity of these compounds.

Biological data. α-Cyclodextrin, like β-cyclodextrin, is not digested in the gastrointestinal tract but is fermented by the intestinal microflora. In germ-free rats, α-cyclodextrin is almost completely excreted in the faeces, whereas γ-cyclodextrin is readily digested to glucose by the luminal and/or epithelial enzymes of the gastrointestinal tract. At low concentrations in the diet (about 20 g/kg), α-cyclodextrin is absorbed intact from the small intestine and is then excreted rapidly in the urine. The majority of the absorption takes place after metabolism of the substance by the microflora in the caecum. Although no studies of metabolism in humans in vivo were available, in vitro studies indicated that α- and β-cyclodextrin, unlike γ-cyclodextrin, cannot be hydrolysed by human salivary and pancreatic amylases.

The acute toxicity of α-cyclodextrin was studied in mice and rats that received the substance by intraperitoneal or intravenous injection. It caused osmotic nephrosis, probably because it was not degraded by lysosomal amylases. At high doses, this led to renal failure.

The results of short-term (28-day and 90-day) studies of the toxicity of α-cyclodextrin indicated that it had little effect when given orally to rats or dogs. After administration of a very high dietary concentration (200 g/kg), caecal enlargement and associated changes were seen in both species. This effect was probably the consequence of the presence of a high concentration of an osmotically active substance in the large intestine. No studies of intravenous administration were available to permit a comparison of the systemic toxicity of this compound with that of β- and γ-cyclodextrin.

Studies conducted in mice, rats and rabbits given α-cyclodextrin in the diet at concentrations of up to 200 g/kg did not indicate any teratogenic effects. Similarly, the results of assays for genotoxicity were negative. No long-term studies of toxicity, carcinogenicity or reproductive toxicity have been conducted with α-cyclodextrin, but the Committee concluded that, given the known fate of this compound in the gut, such studies were not required for an evaluation.

In vitro, α-cyclodextrin, like β-cyclodextrin, sequestered components of the membranes of erythrocytes, causing haemolysis. The threshold concentration for this effect was, however, higher than that observed with β-cyclodextrin.

While the potential interaction of α-cyclodextrin with lipophilic vitamins, which might impair their bioavailability, has not been studied directly, such an effect was considered unlikely, by analogy with the results of studies with β-cyclodextrin. Complexes between fat-soluble vitamins and β-cyclodextrin have been shown to have greater bioavailability than uncomplexed forms.

The enzyme cyclodextrin-glycosyltransferase, which is used in the production of α-cyclodextrin, is derived from a non-genotoxic, non-toxinogenic source and is completely removed from α-cyclodextrin during purification.

Assessment of intake. The predicted mean intake of α-cyclodextrin by consumers, based on individual dietary records for 1994–1998 in the USA and the proposed maximum levels of use in a variety of foods, would be 1.7 g/day (28 mg/kg of body weight per day) for the whole population and 1.6 g/day (87 mg/kg of body weight per day) for children aged 2–6 years. The main contributors to the total intake of α-cyclodextrin are likely to be soya milk and sweets. For persons at the 90th percentile of consumption, the predicted intake of α-cyclodextrin would be 3 g/day (50 mg/kg of body weight per day) for the whole population and 2.6 g/day (140 mg/kg of body weight per day) for children aged 2–6 years.

Evaluation. No studies of human tolerance to α-cyclodextrin were submitted to the Committee, despite the potentially high dietary intake. Nevertheless, the Committee was reassured by the relatively low toxicity of this compound in animals and the fact that it was less toxic than β-cyclodextrin, for which studies of human tolerance were available. Furthermore, the fact that it is fermented in the gastrointestinal tract in an analogous manner to β-cyclodextrin supported the conclusion that, as in laboratory animals, it would be fermented to innocuous metabolites before its absorption in the human gastrointestinal tract.

The Committee concluded that, on the basis of the available studies on α-cyclodextrin and studies on the related compounds β-cyclodextrin and γ-cyclodextrin, for which ADIs have been allocated, there was sufficient information to allocate an ADI "not specified".[1] This ADI was based on the known current uses of α-cyclodextrin within good manufacturing practice as a carrier and stabilizer for flavours, colours and sweeteners; as a water-solubilizer for fatty acids and certain vitamins; as a flavour modifier in soya milk; and as an absorbent in confectionery.

A toxicological monograph and new specifications for α-cyclodextrin were prepared.

3.1.9.3 Sodium sulfate

Sodium sulfate was evaluated by the Committee at its fifty-third meeting (Annex 1, reference *143*), when a temporary ADI "not specified"[1] was established. The ADI was made temporary because information was required on the functional effect and actual uses of

[1] See footnote on page 32.

sodium sulfate in food. This information was provided to the Committee at its fifty-fifth meeting (Annex 1, reference *149*), and the "tentative" designation was removed from the specifications. At that time, the temporary ADI was not reconsidered.

Sodium sulfate is used as a colour adjuvant. Worldwide consumption from its use in food is approximately 100 tonnes per year.

At its present meeting, the Committee noted that the results of the few published studies conducted in experimental animals do not raise concern about the toxicity of sodium sulfate. Little is absorbed from the gut, and it is therefore used clinically as a laxative. The small amount absorbed remains in the extracellular fluid space and is rapidly excreted via the kidneys. Minor adverse effects have been reported in a small number of clinical trials and in case reports. All of the effects were seen with preparations containing sodium sulfate and may have resulted from other components of the preparations.

In the absence of evidence of toxicity and given the current uses of this substance, the Committee allocated an ADI "not specified"[1] for sodium sulfate.

A toxicological monograph was not prepared. The specifications prepared by the Committee at its fifty-fifth meeting were maintained.

3.2 Revision of specifications

3.2.1 *Acesulfame K*

Acesulfame K is prepared in a three-step process in which sulfamic acid and diketene are reacted to produce an adduct, which undergoes cyclization to the acid form of acesulfame. This product is neutralized with potassium hydroxide to form the potassium salt.

The specifications for acesulfame K were revised. In addition to editorial revisions, a new criterion for purity with regard to the pH value of the aqueous solution was introduced, and the limit for lead was lowered from 10 mg/kg to 1 mg/kg.

3.2.2 *Blackcurrant extract*

Blackcurrant extract is obtained from blackcurrant pomace by aqueous extraction. The main colouring principles are four anthocyanins (cyanidin 3-rutinoside, delphinidin 3-rutinoside, cyanidin 3-glucoside and delphinidin 3-glucoside). Most of the extracted sugars are fermented to alcohol, and virtually all the alcohol is removed during concentration of the extract by vacuum evaporation. Sulfur dioxide is used during the extraction process, and residual sulfur dioxide may

[1] See footnote on page 32.