Natamycin Blocks Fungal Growth by Binding Specifically to Ergosterol without Permeabilizing the Membrane

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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 consequent 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 fluorocytosines, and the azole derivatives. One important problem is the increase of drug resistance, particularly against azole antimitoticos 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 pentaeene in filipin, which is already considered as a small polynene 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) 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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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 Me2SO, whereas natamycin was dissolved in 85:15 Me2SO/H2O (v/v); all were obtained from Sigma. All antibiotics 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 \times 10^4 M^{-1} cm^{-1} (318 nm), 6.7 \times 10^4 M^{-1} cm^{-1} (318 nm), and 8.5 \times 10^4 M^{-1} cm^{-1} (356 nm) for nystatin, nystatin, and filipin, respectively. The molar extinction coefficient of ergosterol was measured in methanol to be 0.97 \times 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 Me2SO) and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) were both purchased from Invitrogen. N,N-Dimethyldecylamine-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 (YPUDPAC) 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 YPUDPAC (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 A600 0.0001, of which 100 μl was added to the culture plate. The total volume per well was 200 μl. Strains RH6611 and RH6613 (SD-His-Leu medium) were diluted to an A600 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.](image)

**FIGURE 1. Structures of several polyene antibiotics and ergosterol.** A, natamycin; B, nystatin; C, filipin; D, ergosterol.

**TABLE 1**

| Strain   | Name   | Genotype                        |
|----------|--------|---------------------------------|
| Wild type| RH448  | MATa his4 leu2 ura3 lys2 bar1   |
| ErgΔ     | RH2897 | MATa erg2(Δ1-Δ3)Ira3 leu2 ura3 his4 lys2 bar1 |
| Erg2Δerg6Δ| RH3616 | MATa erg2(Δ1-Δ3)Ira3 erg6Δ leu2 ura3 bar1 |
| ErgΔ     | RH6622 | MATa ergΔIra2 leu2 ura3 his4 bar1 |
| ErgΔ     | RH4213 | MATa ergΔIra2 leu2 ura3 his4 lys2 bar1 |
| Erg2Δerg6Δ| RH5225 | MATa ergΔIra2 erg6ΔIra2 leu2 ura3 his4 lys2 bar1 |
| Erg2ΔergΔ| RH5228 | MATa erg2(Δ1-Δ3)Ira3 erg3ΔIra2 leu2 ura3 his4 lys2 bar1 |
| ErgΔerg5Δ| RH5233 | MATa ergΔIra3 erg5ΔkanMX4 leu2 ura3 his4 lys2 bar1 |
| Wild type| RH6611 | MATa his3 ura3 leu2 (pRS423) (pRS425) |
| Cholesterol| RH6613 | MATa ergΔTRP1 erg6:TRP1 his3 ura3 leu2 trp1 (pRS423-DHCR7) (pRS425-DHCR24) |
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Protein 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 K2SO4 buffer at pH 7 was used. The CF-loaded vesicles were diluted in 1200 µl of 50 mM MES, 100 mM K2SO4 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 NaH2PO4/Na2HPO4 buffer at pH 7. To create a lower pH at the outside and remove all the untrapped

tated 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 K2SO4 at pH 7, and resuspended in a small volume of buffer. The A600 of the cell suspensions was determined and a series of 1-ml cell suspensions were prepared ranging from an A600 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.

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 | —   |
| pRS425          | Multicopy vector containing GDP promoter and LEU2 | 30   |
| pRS425-DHCR24   | pRS425 derivative vector containing DHCR24 gene | —   |

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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 Zetamizer 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 K2SO4, 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 Me2SO, an equal amount was added to the LUV suspension to compensate for any heat generated by dilution of this solvent. No more than 1% Me2SO 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 K2SO4 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% Me2SO 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 100000 rpm 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 precipi-
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Wild type yeast cells from an overnight culture were diluted to an $A_{600}$ of $\sim 0.8$ and then centrifuged at 3000 $\times$ 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$_2$PO$_4$, and 50 mM KOH). CFDA-SE (100 $\mu$m) 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 $\times$ 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.

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 (8), and 10% ergosterol (C) and were dissolved in 50 mM MES, 100 mM K$_2$SO$_4$, pH 6.0. The top graph displays the heat peaks after consecutive injections of 5-$\mu$l vesicles with an 8 mM final phospholipid concentration into the sample cell containing 50 $\mu$m 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.

$\mu$cal/sec

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$_2$SO$_4$, pH 6.0. The top graph displays the heat peaks after consecutive injections of 5-$\mu$l vesicles with an 8 mM final phospholipid concentration into the sample cell containing 50 $\mu$m 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$_2$SO$_4$ 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 yeast cells was monitored by adding aliquots of antibiotic to 1200 $\mu$l of 10 mM MES and 0.2 M Na$_2$SO$_4$ buffer, pH 5.5, containing HPTS-loaded vesicles (35 $\mu$m 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.

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 membrane-impermeable pH-sensitive probe conjugates.

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.

FIGURE 2. Natamycin-Ergosterol Interactions

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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. 3B 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 natamycin was erg2Δ, which is in reasonable agreement with the Langmuir adsorption model in SigmaPlot (10.0) to be $2.5 \pm 1.0 \times 10^4 \text{ M}^{-1}$, 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 $72 \pm 12 \mu M$ 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 $\sim 1:1$. If all sterols would be available for the interaction, because of sterol flip-flop, the ratio would be 2:1.

The binding of natamycin to vesicles was studied by separating the bound from the free natamycin by centrifugation. Fig. 3A shows a representative graph of these results, from which we can conclude 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 $2.5 \pm 1.0 \times 10^4 \text{ M}^{-1}$, 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 $72 \pm 12 \mu M$ 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 $\sim 1:1$. If all sterols would be available for the interaction, because of sterol flip-flop, the ratio would be 2:1.

The sterol specificity for polyenes, in relation to their inhibitory activity.

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The sterol specificity for polyenes, in relation to their inhibitory activity.
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TABLE 3
The minimum concentration of the polyene antibiotics needed to inhibit the growth of different ergΔ mutants

| Strain          | MICnatam (µM) | MICnyst (µM) | MICfilp (µM) |
|-----------------|---------------|--------------|--------------|
| Wild Type       | 1.7 ± 0.5     | 1.1 ± 0.2    | 1.2 ± 0.1    |
| Erg4Δerg5Δ      | 2.1 ± 0.6     | 2.1 ± 0.2    | 2.5 ± 0.9    |
| Erg3Δ           | 5.7 ± 0.8     | 3.4 ± 0.5    | 2.5 ± 0.2    |
| Erg6Δ           | 8.3 ± 0.9     | 7.8 ± 1.6    | 2.9 ± 0.2    |
| Erg3Δerg6Δ      | 18 ± 3.6      | 14 ± 4.1     | 5.2 ± 0.9    |
| Erg2Δ           | 22 ± 0.1      | 16 ± 1.6     | 3.4 ± 1.4    |
| Erg2Δerg3Δ      | 46 ± 14       | 21 ± 14      | 6.8 ± 2.3    |
| Erg2Δerg6Δ      | 63 ± 0.1      | 52 ± 13      | 6.8 ± 0.9    |

less sensitive compared with the wild type. Variations in the C17 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 C17 side chain are of less importance.

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. 5A. 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. 5B, at a cell density corresponding to an A600 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-

TABLE 4
The minimum concentration of the polyene antibiotics needed to inhibit the growth of strains RH6611 and 6613

| Strain          | MICnatam (µM) | MICnyst (µM) | MICfilp (µM) |
|-----------------|---------------|--------------|--------------|
| Wild Type       | 2.0 ± 0.4     | 0.9 ± 0.1    | 2.7 ± 0.4    |
| Ergosterol (78 %) |             |              |              |
| Cholesterol (68 %) | 31 ± 6       | 13 ± 2.4     | 3.3 ± 0.6    |

Cholesterol-5,7-dienol (10 %) | Cholesterol-5,7-dienol (10 %)
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**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 \( \sim 100 \mu M \) specifically to ergosterol with a stoichiometry of \( \sim 1:1 \) 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 polyene antibiotics.
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. 8A) results in a 1,3-diplanar chair conformation, which is lacking in cholesterol giving a half-chair conformation (Fig. 8B) (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 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 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-ergosterol interactions (14).
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 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.

Acknowledgments—We thank M. R. van Leeuwen and J. Dijksterhuis (Fungal Biodiversity Center (CBS), The Netherlands) for helpful comments and valuable research discussions.

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JOURNAL OF BIOLOGICAL CHEMISTRY 6401

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