Supplementary Information

Synthesis-enabled functional group deletions reveal key underpinnings of amphotericin B ion channel and antifungal activities

Daniel S. Palacios, Ian Dailey, David M. Siebert, Brandon C. Wilcock, and Martin D. Burke*

Howard Hughes Medical Institute, Roger Adams Laboratory, Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801

I. General Methods

Materials.

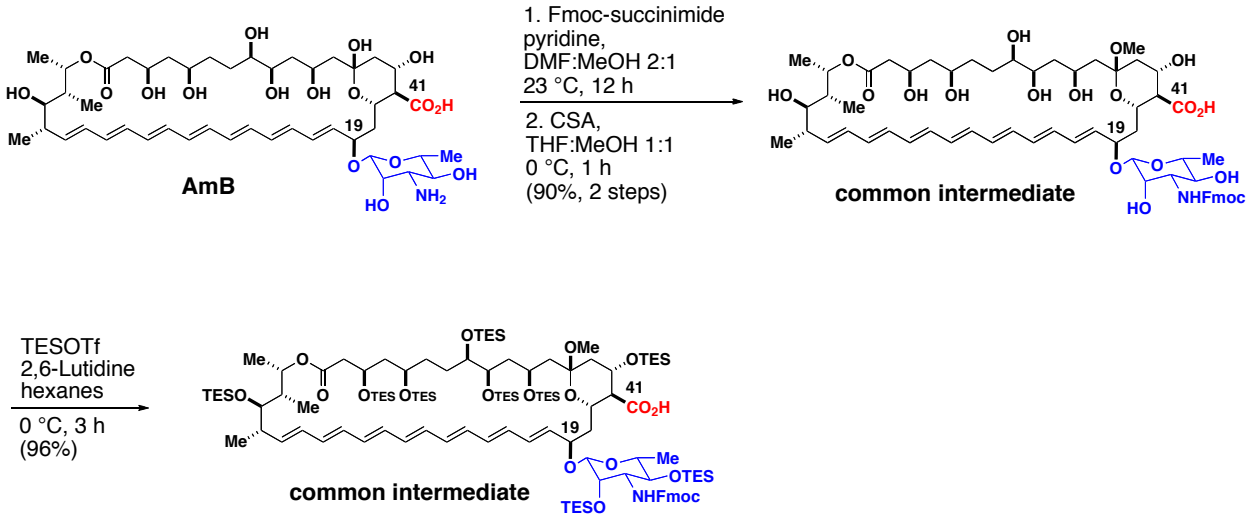
Commercially available materials were purchased from Aldrich Chemical Co. (Milwaukee, WI), Fisher Scientific (Hampton, NH), Avanti Polar Lipids (Alabaster, AL), and Small Parts Inc. (Miramar, FL) and were used without further purification unless noted otherwise. Amphotericin B was a generous gift from Bristol-Myers Squibb and was purified by preparative RP-HPLC using an Agilent (Santa Clara, CA) 1100 series HPLC system equipped

with a Waters (Milford, MA) Sunfire Prep C_{18} 30 x 150 mm column, as described previously.[1] Ergosterol and camphorsulfonic acid were recrystallized from ethyl acetate. Water was obtained from a Millipore (Billerica, MA) Gradient A10 water purification system. Amphotericin B derivatives were prepared and purified as described previously.[1]

II. Expanded Synthesis Schemes (for full experimental details, see reference 49).

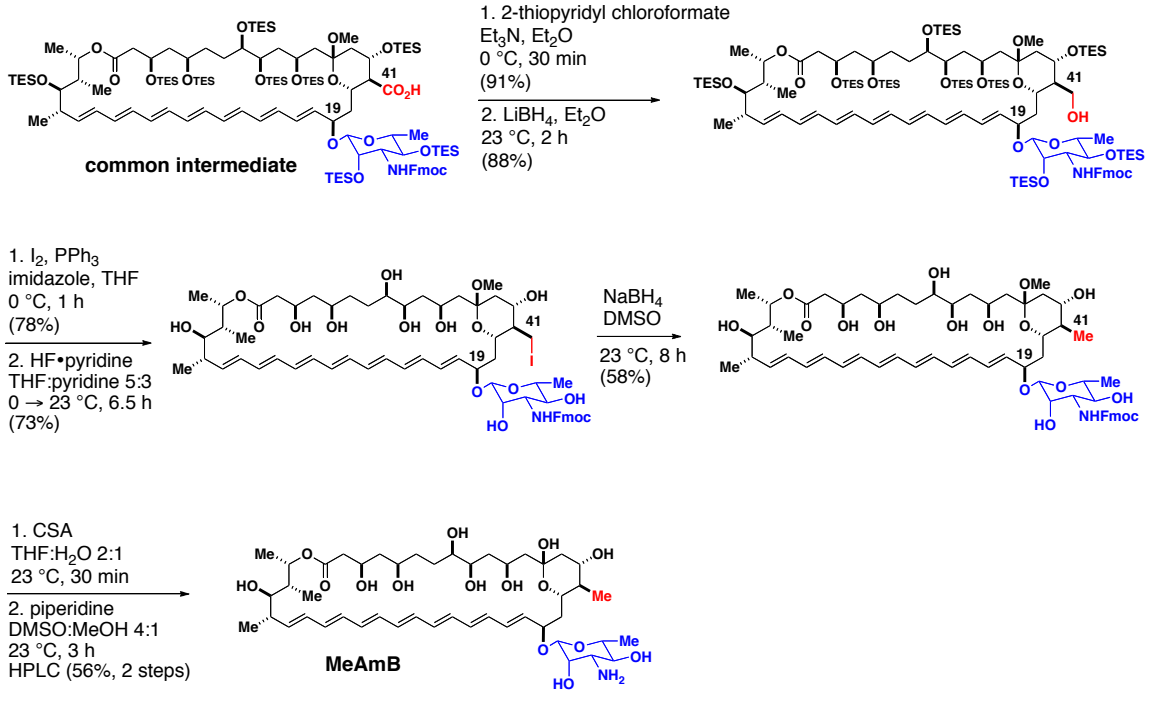
Glossary of abbreviations: AcOH = Acetic acid, AmdeB = Amphoteronolide B, CBS = Corey-Bakshi-Shibata, CSA = Camphorsulfonic acid, DDQ = 2,3-Dichloro-5,6-dicyanobenzoquinone, DMF = *N,N* Dimethylformamide, DMSO = Dimethyl sulfoxide, DMPU = 1,3-Dimethyl-3,4,5-tetrahydro-2(1*H*)-pyrimidinone, d.r. = Diastereomeric ratio, Et = Ethyl, Fmoc = 9-Fluorenylmethyl, HPLC = High performance liquid chromatography, *i*-Pr = isopropyl, Me = Methyl, MeAmB = C(41)-Methyl amphotericin B, MeAmdeB = C(41)-Methyl amphoteronolide B, MeOH = Methanol, TESOTf = Triethylsilyl trifluoromethanesulfonate, THF = Tetrahydorfuran.

Synthesis of common intermediate



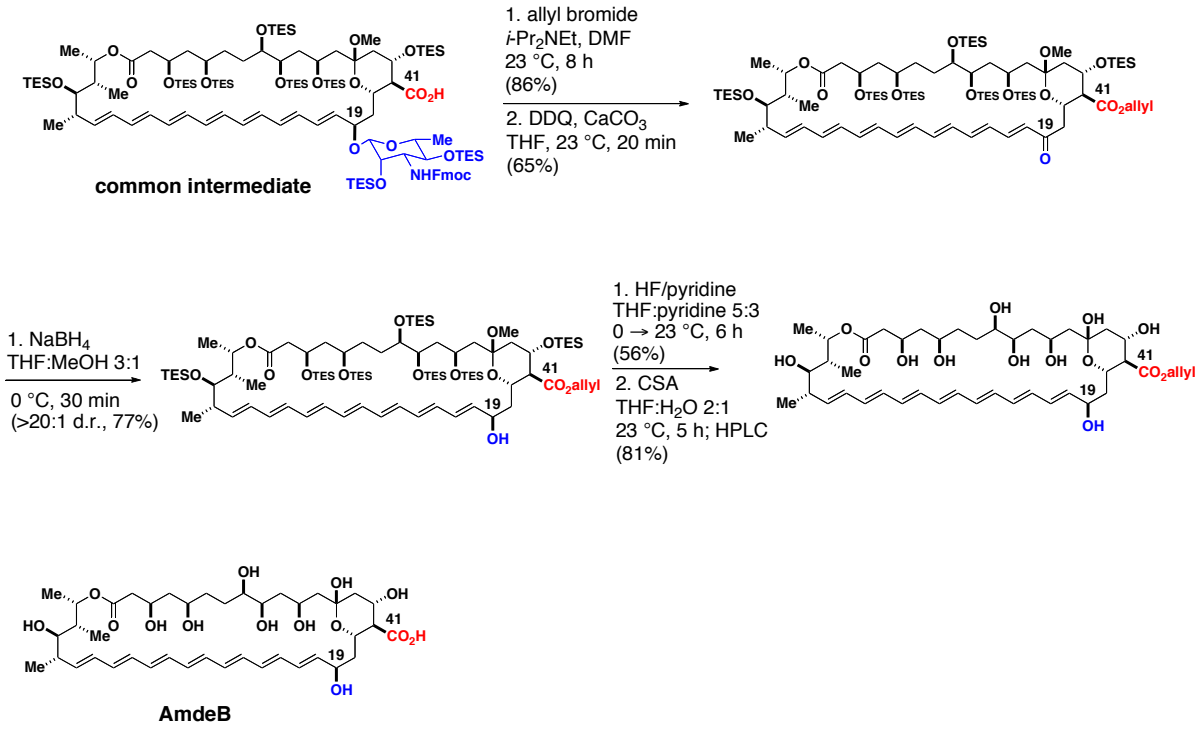
Scheme S1.

Synthesis of MeAmB



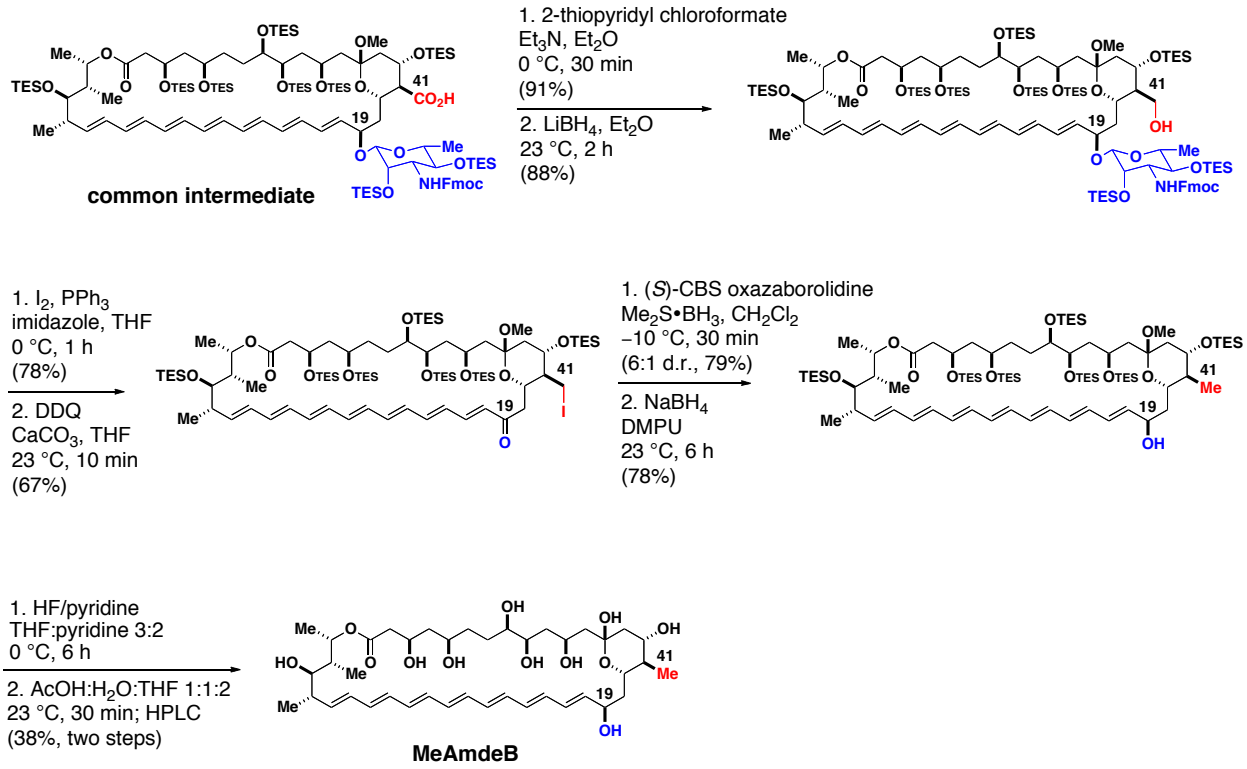
Scheme S2.

Synthesis of AmdeB



Scheme S3.

Synthesis of MeAmdeB



Scheme S4.

III. Antifungal Assays

Growth Conditions for *S. cerevisiae*.

S. cerevisiae was maintained with yeast peptone dextrose (YPD) growth media consisting of 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose, and 20 g/L agar for solid media. The media was sterilized by autoclaving at 250 °F for 30 min. Dextrose was subsequently added as a sterile 40% w/v solution in water (dextrose solutions were filter sterilized). Solid media was prepared by pouring sterile media containing agar (20 g/L) onto Corning (Corning, NY) 100 x 20 mm polystyrene plates. Liquid cultures were incubated at 30 °C on a rotary shaker and solid cultures were maintained at 30 °C in an incubator.

Growth Conditions for *C. albicans*.

C. albicans was cultured in a similar manner to *S. cerevisiae* except both liquid and solid cultures were incubated at 37 °C.

Broth Microdilution Minimum Inhibitory Concentration (MIC) Assay.

The protocol for the broth microdilution assay was adapted from the Clinical and Laboratory Standards Institute document M27-A2.[2] 50 mL of YPD media was inoculated and incubated overnight at either 30 °C (*S. cerevisiae*) or 37 °C (*C. albicans*) in a shaker incubator. The cell suspension was then diluted with YPD to an OD_{600} of 0.10 (~5 x 10^6 cfu/mL) as measured by a Shimadzu (Kyoto, Japan) PharmaSpec UV-1700 UV/Vis spectrophotometer. The solution was diluted 10-fold with YPD, and 195 μL aliquots of the dilute cell suspension were added to sterile Falcon (Franklin Lakes, NJ) Microtest 96 well plates in triplicate. Compounds were prepared either as 400 μM (AmB, MeAmB) or 2 mM (AmdeB, MeAmdeB) stock solutions in DMSO and serially diluted to the following concentrations with DMSO: 1600, 1200, 800, 400, 320, 240, 200, 160, 120, 80, 40, 20, 10 and 5 μM. 5 μL aliquots of each solution were added to the 96 well plate in triplicate, with each column representing a different concentration of the test compound. The concentration of DMSO in each well was 2.5% and a control well to confirm viability using only 2.5% DMSO was also performed in triplicate. This 40-fold dilution gave the following final concentrations: 50, 40, 30, 20, 10, 8, 6, 4, 1, 0.5, 0.25 and 0.125 μM. The plates were covered and incubated at 30 °C (*S. cerevisiae*) or 37 °C (*C. albicans*) for 24 hours prior to analysis. The MIC was determined to be the concentration of compound that resulted in no visible growth of the yeast. The experiments were performed in duplicate and the reported MIC represents an average of two experiments.

IV. Potassium Efflux Assays

General Information.

Ion selective measurements were obtained using a Denver Instruments (Denver, CO) Model 225 pH meter equipped with a Denver Instruments potassium selective electrode. The pH meter was connected to a desktop computer by an RS232 connection and the data were collected using Labtronics (Guleph, Ontario) Collect SL software. The electrode was conditioned in a 1000 ppm KCl standard solution overnight prior to ion selective measurements. Measurements were made on 15 mL solutions that were magnetically stirred in 40 mL I-Chem (Rockwood, TN) vials incubated in a 30 °C stirred water bath (*S. cerevisiae*) or at 23 °C (LUVs). The instrument was calibrated daily with KCl standard solutions to 10, 100, and 1000 ppm potassium. The potassium concentration was sampled every 30 seconds throughout the course of the efflux experiments.

Potassium Efflux from *S. cerevisiae.*

The protocol to determine potassium efflux from *S. cerevisiae* was adapted from a similar experiment utilizing *C. albicans*.[3] An overnight culture of *S. cerevisiae* in YPD was centrifuged at 1200 g for 5 minutes at 4 °C. The supernatant was decanted and the cells were washed twice with sterile water. After the second wash step, the cells were suspended in 150 mM NaCl, 5 mM HEPES pH 7.4 (Na buffer) to an OD_{600} of 1.5 (~$1x10^9$ CFU/mL). A 15 mL sample of the cell suspension was then incubated in a 30 °C water bath with stirring for approximately 10 minutes before data collection. The probe was then inserted and data was collected for 5 minutes before adding 150 μL of the compound in question as a 300 μM solution in DMSO. The cell suspension was stirred and data were collected for 30 minutes and then 150 μL of a 1% aqueous solution of digitonin was added to effect complete potassium release and data were collected for an additional 15 minutes. The experiment was performed independently three times for each small molecule.

Data Analysis.

The data from each run was normalized to the percent of total potassium release, from 0 to 100%. Thus for each experiment a scaling factor S was calculated using the following relationship:

$$\left[\frac{[K^+]_{final}}{[K^+]_{initial}} - 1\right] \cdot S = 100$$

Each concentration data point was then multiplied by S before plotting as a function of time.

Efflux from 10% ergosterol LUVs.

LUV Preparation.

Egg yolk phosphatidylcholine (EYPC) was obtained as a 10 mg/mL solution in $CHCl_3$ from Avanti Polar Lipids (Alabaster, AL) and was stored at -20 °C under an atmosphere of dry argon and used within 3 months. A 4 mg/mL solution of ergosterol in $CHCl_3$ was prepared monthly and stored at -20 °C under an atmosphere of dry argon. Prior to preparing a lipid film, the solutions were warmed to ambient temperature to prevent condensation from contaminating the solutions. A 13 x 100 mm test tube was charged with 1.6 mL EYPC and 230 μL of the ergosterol solution. The solvent was removed with a gentle stream of nitrogen and the resulting lipid film was stored under high vacuum for a minimum of eight hours prior to use. The film was then hydrated with 1 mL of 150 mM KCl, 5 mM HEPES pH 7.4 (K buffer) and vortexed vigorously for approximately 1 minute to form a suspension of multilamellar vesicles (MLVs). The resulting lipid suspension was pulled into a Hamilton (Reno, NV) 1 mL gastight syringe and the syringe was placed in an Avanti Polar Lipids Mini-Extruder. The lipid solution was then passed through a 0.20 μm Millipore (Billerica, MA) polycarbonate filter 21 times, the newly formed large unilamellar vesicle (LUV) suspension being collected in the syringe that did not

contain the original suspension of MLVs to prevent the carryover of MLVs into the LUV solution. To obtain a sufficient quantity of LUVs, three independent 1 mL preparations were pooled together for the dialysis and subsequent potassium efflux experiments. The newly formed LUVs were dialyzed using Pierce (Rockford, IL) Slide-A-Lyzer MWCO 3,500 dialysis cassettes. The samples were dialyzed three times against 600 mL of Na buffer. The first two dialyses were two hours long, while the final dialysis was performed overnight.

Determination of Phosphorus Content.

Determination of total phosphorus was adapted from the report of Chen and coworkers.[4] The LUV solution was diluted tenfold with Na buffer and three 10 μL samples of the diluted LUV suspension were added to three separate 7 mL vials. Subsequently, the solvent was removed with a stream of N_2. To each dried LUV film, and a fourth vial containing no lipids that was used as a blank, was added 450 μL of 8.9 M H_2SO_4. The four samples were incubated open to ambient atmosphere in a 225 °C aluminum heating block for 25 min and then removed to 23 °C and cooled for 5 minutes. After cooling, 150 μL of 30% w/v aqueous hydrogen peroxide was added to each sample, and the vials were returned to the 225 °C heating block for 30 minutes. The samples were then removed to 23 °C and cooled for 5 minutes before the addition of 3.9 mL water. Then 500 μL of 2.5% w/v ammonium molybdate was added to each vial and the resulting mixtures were then vortexed briefly and vigorously five times. Subsequently, 500 μL of 10% w/v ascorbic acid was added to each vial and the resulting mixtures were then vortexed briefly and vigorously five times. The vials were enclosed with a PTFE lined cap and then placed in a 100 °C aluminum heating block for 7 minutes. The samples were removed to 23 °C and cooled for approximately 15 minutes prior to analysis by UV/Vis spectroscopy. Total phosphorus was determined by observing the absorbance at 820 nm and comparing this value to a standard curve

obtained through this method and a standard phosphorus solution of known concentration.

Determination of Ergosterol Content.

Ergosterol content was determined spectrophotometrically. The LUV solution was diluted tenfold with Na buffer, and 50 μL of the dilute LUV suspension was added to 450 μL 2:18:9 hexane:isopropanol:water (v/v/v). Three independent samples were prepared and then vortexed vigorously for approximately one minute. The solutions were then analyzed by UV/Vis spectroscopy and the concentration of ergosterol in solution was determined by the extinction coefficient of 10400 L mol^{-1} cm^{-1} at the UV_{max} of 282 nm and was compared to the concentration of phosphorus to determine the percent sterol content. The extinction coefficient was determined independently in the above ternary solvent system. LUVs prepared by this method contained between 7 and 14% ergosterol.

Efflux from LUVs.

The LUV solutions were adjusted to 1 mM in phosphorus using Na buffer. 15 mL of the 1 mM LUV suspension was added to a 40 mL I-Chem vial and the solution was gently stirred. The potassium ISE probe was inserted and data were collected for one minute prior to the addition of the compound. Then, 150 μL of a 100 μM DMSO solution of the compound in question was added and data were collected for five minutes. Then to effect complete potassium release, 150 μL of a 10% v/v solution of triton X-100 was added and data were collected for an additional five minutes. The experiment was duplicated with similar results.

Data Analysis.

The data from each run were analyzed in the same manner as the efflux data from *S. cerevisiae.*

V. Planar Lipid Bilayer Experiments

General Information.

All data were acquired using a Warner Instruments (Hamden, CT) BC-535 amplifier and the data were filtered using a built in 4 pole Bessel filter with a cutoff frequency of 5 kHz. The headstage and delrin cell were housed within a Warner Instruments model FC-1 Faraday cage. The solutions were stirred using a Warner Instruments SUNstir-3 stirplate. The signal was passed through a Warner Instruments low pass 8 pole Bessel filter with a frequency cutoff of 1 kHz. The filtered data were sampled at a rate of 10 kHz using a Molecular Devices (Sunnyvale, CA) Digidata 1440 data acquisition system and the data were analyzed using Molecular Devices pClamp 10 software. Following acquisition, the data were digitally filtered to 20 Hz. Salt bridges were prepared monthly and were fabricated from 1.5 mm OD, 0.86 mm ID borosilicate capillary tubing and were filled with 1 M aqueous KCl with 2.5% agar. Prior to a day's experiments, silver electrodes were plated by submerging in commercial bleach for 15 to 30 minutes. The electrodes were plated periodically throughout the day.

Preparation of Teflon® Sheets.

Prior to use, Teflon® sheets of 125 μM thickness (Small Parts Inc, Miramar, FL) were washed sequentially with 10 mM tribasic sodium phosphate, 1% HCl and then MilliQ water. Pores of approximately 100-150 μm in diameter were formed with the spark method[5] using a home built sparking apparatus. The Teflon® sheet was scored with a sewing needle and then the sheet was placed on a grounded sheet of copper and the Teflon® was sparked 10 times. The sheet was then flipped over and sparked an additional 10 times. Pore sizes were analyzed via light microscopy.

Preparation of Lipid Solution.

Lipids were obtained from Avanti Polar Lipids as 10 mg/ml solutions in $CHCl_3$. The

solutions were stored at -20 °C under dry argon and used within 3 months. A 4 mg/mL solution of ergosterol in $CHCl_3$ was prepared monthly and stored at -20 °C under dry argon. Lipid films were prepared by charging a 12 x 75 mm test tube with 40 μL porcine brain phosphatidylcholine, 20 μL porcine brain phosphatidylethanolamine and 3.8 μL ergosterol. The solvent was removed with a gentle stream of nitrogen. The lipid film was then dissolved in 30 μL *n*-decane to give the 20 mg/ml solution of lipids used for the electrophysiology experiments. The decane solutions were used within 3 hours of preparation.

Formation of planar lipid bilayers.

Teflon® sheets were cut to approximately 1 cm^2 and adhered to a home fabricated delrin cell[6] using Dow Corning (Midland, MI) high vacuum grease. The area around the hole was then primed with the decane lipid solution. The primed sheet was left to stand for approximately 10 minutes such that some of the decane evaporated. Then 3.5 mL of 2 M KCl, 10 mM potassium phosphate pH 7.0 buffer was added to each chamber. The membrane was formed by sequential vertical swabs across the hole using a flame polished glass applicator that had been previously dipped into the lipid solution. The formation of a membrane was detected by a reduction in the current to 0 pA. The integrity of this membrane was confirmed by applying a potential of 150 mV for approximately one minute. If the current increased by >1 pA upon voltage introduction, the membrane was rejected. Membranes were between 20 and 45 pF in size.

Interrogating Channel Formation.

If the membrane was acceptable, 3.5 μL of a compound in DMSO was added to both chambers and the solutions were stirred with zero applied potential for 10 minutes. After 10 minutes the stirring was stopped, and 150 mV of potential was applied across the membrane. The formation of single AmB channels under similar conditions has been well

documented.[7,8,9,10,11,12,13,14] The concentration of AmB and MeAmB required to observe channel activity varied based upon the lot number and age of the lipids used to make the membrane. For AmB, single channel formation was observed at concentrations between 0.5 and 5 nM while MeAmB displayed single channel activity between 30 and 80 nM. The concentration of MeAmdeB was raised to 1000 nM without observing any channel activity. At concentrations greater than 100 nM MeAmdeB and AmdeB tended to grossly destroy the membrane, as evidenced by an abrupt change from zero current to an offscale reading. To verify the inability of MeAmdeB and AmdeB to form channels, 5 independent experiments were performed at concentrations ranging from 50 to 100 nM, each with 15 minutes of applied potential. In every case, AmdeB and MeAmdeB failed to produce channel activity. These same conditions consistently led to channel formation with AmB and MeAmB.

VI. Yeast Binding Assay

Determination of Binding.

The yeast binding assay was adapted from the report by Kobayashi and coworkers that demonstrated binding of AmB to intact *S. cerevisiae* cells.[15] 10 mL of an overnight culture of *S. cerevisiae* in YPD was centrifuged at 1200 g for 5 min at 4 °C. The supernatant was decanted, and the cells were washed twice with sterile water using the same centrifuge conditions. The washed cells were then suspended in sterile water to an OD_{600} of 0.10 (~$5x10^6$ CFU/mL), and 990 µl of this suspension was added to a 1.5 mL microcentrifuge tube. 10 µL of a 200 µM solution of compound in DMSO was added to the suspension, which was vortexed for approximately 10 seconds and then incubated at 30 °C for 15 minutes. The samples were subsequently centrifuged at 5000 RPM with a Savant HSC10K Speedfuge for 5 min to pellet the cells. The concentration of AmB in such aqueous solutions cannot be accurately determined

because of aggregate formation.[16,17] Thus, 950 μL of the supernatant was removed and incubated at -20 °C for approximately 20 minutes before being lyophilized overnight. The lyophilized sample was dissolved in 400 μL of MeOH and the concentration of compound in solution was determined by UV/Vis analysis using the known extinction coefficent of each compound.[1] This analysis gives the percent recovery, the percent incorporation being equal to 1-(percent recovery). The samples were prepared in triplicate and the entire experiment was duplicated. The values represent the average of 5 or 6 trials plus or minus the standard deviation.

Recovery Control.

To ensure that the compounds were not binding to the walls of the microcentrifuge tube or decomposing during the course of the experiment, a control was run using the experimental protocol outlined above but substituting pure water for the *S. cerevisiae* suspension. As shown in Figure S1, greater than 90% recovery was achieved with all four compounds.

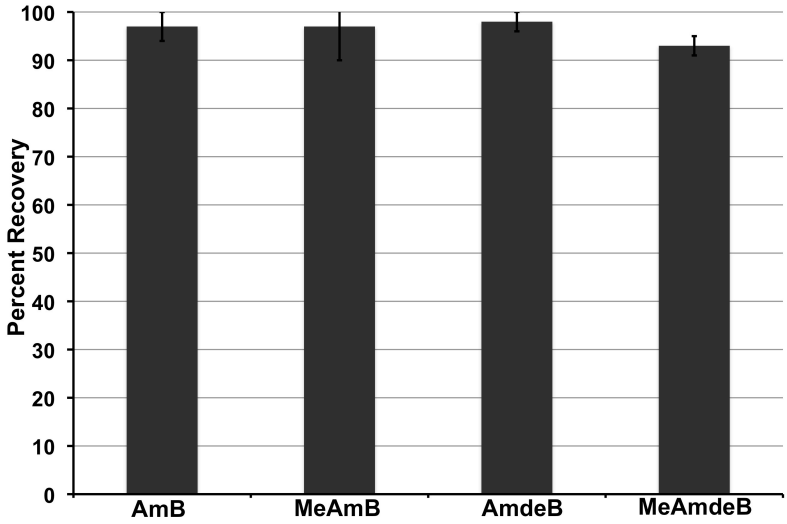


Figure S1. At least 90% of each compound was recovered in the *S. cerevisiae* incorporation assay control. Error bars indicate standard deviation.

VII. LUV Binding Assay

Preparation of LUVs.

LUVs were prepared as described in Section III except dialysis was not performed and the newly extruded vesicles were purified via size exclusion chromatography using Sephadex G50-150 resin as the stationary phase and K buffer as the mobile phase. The concentration of phosphorus and the sterol content of the LUVs were determined as described in Section III.

LUV Binding.

The partitioning of AmB into both sterol-containing[18,19,20] and sterol-free[21,22,23] LUVs has been previously demonstrated. While many prior methods relied upon a measureable change of a physical property (such as electronic absorption spectra) upon the interaction of AmB with a phospholipid bilayer, the SEC based method is advantageous because it physically separates bound from unbound compound, and thereby avoids assumptions regarding the underpinnings of the observed spectral changes.[24] A LUV solution of known phosphorus concentration was diluted to a concentration of 2.05 mM using K buffer, and the solution was gently vortexed. Then, a 975 μL sample of the LUV suspension was added to a 7 mL screw cap vial. Subsequently, 25 μL of a 0.8 mM DMSO solution of the compound under investigation was added and the sample was gently vortexed. The sample was then incubated at 30 °C for one hour. The sample was then purified via size exclusion chromatography using a 1.5 x 30 cm Sephadex G50-150 column, with K buffer as the mobile phase (LUVs typically eluted from the column between 9 and 11 ml of eluent). After the LUVs eluted from the column, the column was flushed with 100 mL of K buffer to remove any small molecules left on the resin.

The concentration of the purified LUVs was then determined through analysis of phosphorus content, as described above. The concentration of compound within the lipid bilayer was determined by dissolving triplicate 50 μL samples of the LUV solution in 450 μL of 2:18:9

hexane:isopropanol:water (v/v/v) and analyzing the sample by UV/Vis spectroscopy. The amount of compound incorporation was determined by comparing the final ratio of lipid to compound to the theoretical max of 100:1. The experiments were performed in quadruplicate for each compound; thus, the reported values represent the average of four runs plus or minus the standard deviation. The binding to sterol-free vesicles was determined in similar fashion except no ergosterol was added to the initial lipid film. As shown in Figure S2, AmB, MeAmB, AmdeB and MeAmdeB readily partition into sterol-free vesicles.

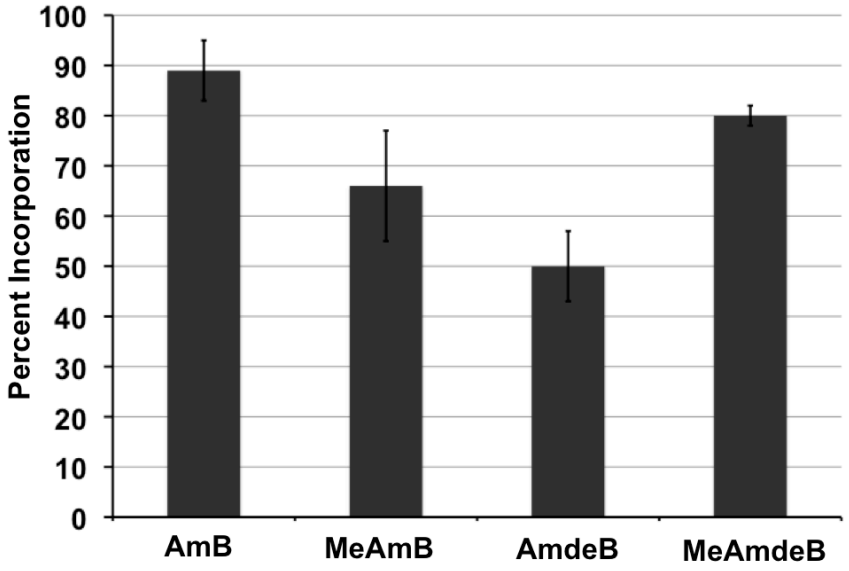


Figure S2. AmB, MeAmB, AmdeB and MeAmdeB all readily partition into sterol-free vesicles.

LUV-free Control Studies.

As a control, the same procedure described above was repeated without LUVs to determine the amount of compound that elutes from the column at the approximate elution volume of the LUVs (the LUVs typically eluted between 9 and 11 mL). Five fractions of 5 mL elution volume were collected, frozen and lyophilized overnight. Then, the resulting solid was suspended in 1 mL of MeOH and vortexed vigorously for approximately two minutes. The samples were then centrifuged at 4000 rpm with a Savant HSC10K Speedfuge for approximately

30 minutes to pellet the inorganic salts. The supernatant was removed and analyzed by UV/vis spectroscopy to determine the amount of compound in solution. As shown in Figure S3, for all cases, less than 10% of the compound loaded onto the column eluted at the retention time of the LUVs. The measurement was performed in duplicate.

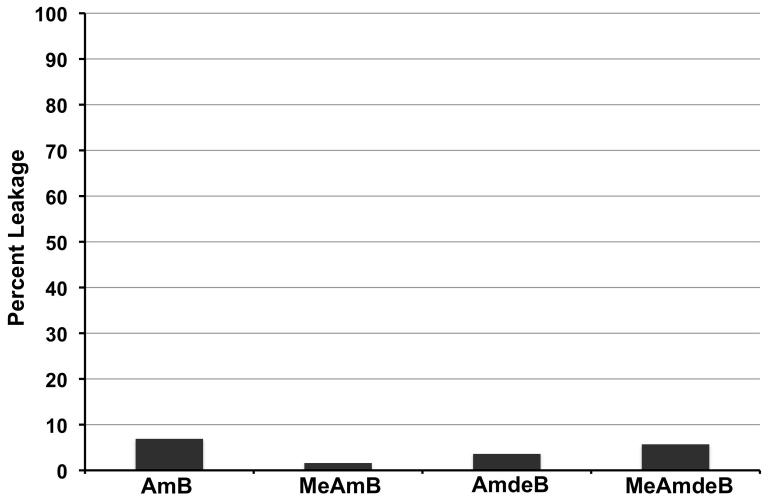


Figure S3. When solutions of compound without LUVs are added to the size exclusion column, less than 10% of each compound elutes at the LUV retention volume.

VIII. Isothermal Titration Calorimetry

General Information.

Experiments were performed using a VP-ITC isothermal titration calorimeter (MicroCal Inc., Piscataway, NJ). Solutions of the compounds to be tested were prepared by diluting a 5.0 mM stock solution of the compound in DMSO to 50 μM with K buffer. The final DMSO concentration in the solution was 1% v/v. LUVs were prepared and phosphorus and ergosterol content was quantified as described in Section VI. Ergosterol and lanosterol were also incorporated into POPC LUVs. The LUV solutions were diluted with buffer and DMSO to give a final phospholipid concentration of 8.0 mM in a 1% DMSO/K buffer solution. Immediately prior

to use, all solutions were degassed under vacuum at 17 °C for 10 minutes. The reference cell of the instrument was filled with a solution of 1% v/v DMSO/K buffer.

Titration Experiment.

Titrations were performed by injecting the LUV suspension at ambient temperature into the sample cell (volume = 1.4399 mL or 1.4495 mL) which contained the 50 μM solution of the compound in question at 25 °C. The volume of the first injection was 1 μL. Consistent with standard procedure,[25] due to the large error commonly associated with the first injection of ITC experiments, the heat of this injection was not included in the analysis of the data. Next, forty 5 μL injections of the LUV suspension were performed. The injection duration was 2.1 seconds and 10.3 seconds for the 1 μL and 5 μL injections, respectively. The spacing between each injection varied between 240 seconds and 480 seconds and was adjusted to allow the instrument to return to baseline before the next injection was made. The rate of stirring for each experiment was 300 or 310 rpm.

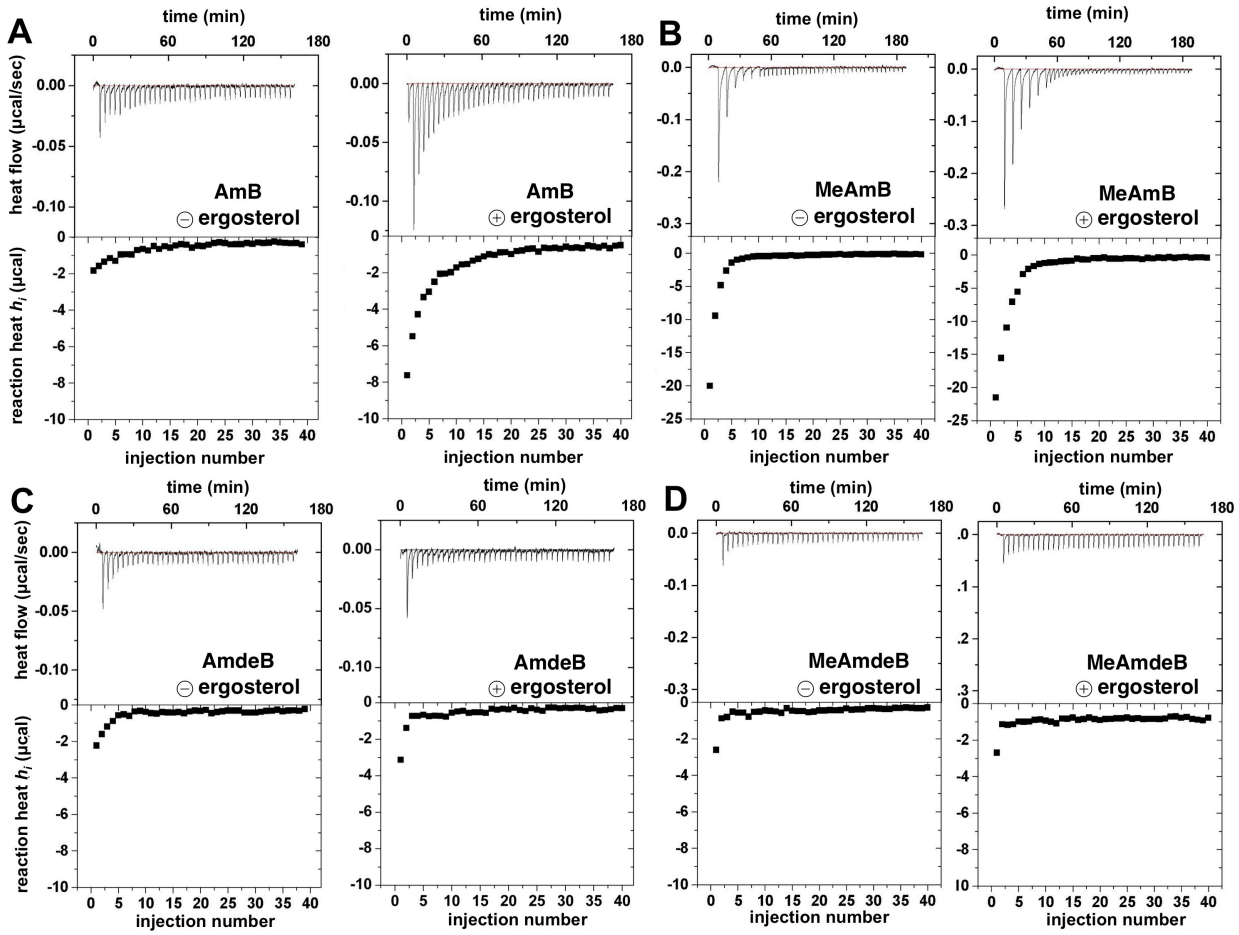
Data Analysis.

ORIGIN software (MicroCal, Inc.) was used for baseline determination and integration of the injection heats, and Microsoft Excel was used for subtraction of dilution heats and the calculation of overall heat evolved. To approximate the dilution heats, the final integrated heat from each run was subtracted from all the data for that particular experiment.[26] The overall heat evolved during the experiment was calculated using the following formula:

$$\mu\text{cal}_{overall} = \sum_{i=1}^{n} (\Delta h^{i}_{injection} - \Delta h^{n}_{injection})$$

Where i = injection number, n = total number of injections, $\Delta h^{i}_{injection}$ = heat of the i^{th} injection, $\Delta h^{n}_{injection}$ = the heat of the final injection of the experiment. Representative thermograms for each

molecule are shown in Figure S4 A-D. The average and standard deviation from at least three independent experiments using either sterol-free or 10% ergosterol containing LUVs for all four compounds is shown in Figure S4 E.



E

	(-) ergosterol $\sum_{i=1}^{40} h_i$(µcal)	(+) ergosterol $\sum_{i=1}^{40} h_i$(µcal)	$\Delta\sum_{i=1}^{40} h_i$(µcal)
AmB	**-8.8 ± 4.9**	**-38.0 ± 6.2**	**-29.2 ± 7.9* *P* = 0.003**
MeAmB	**-43.1 ± 7.3**	**-68.1 ± 6.9**	**-25.0 ± 10* *P* = 0.0003**
AmdeB	**-9.3 ± 3.8**	**-9.5 ± 2.0**	**-0.2 ± 4.3**
MeAmdeB	**-10.9 ± 5.0**	**-6.2 ± 2.1**	**4.7 ± 5.4**

Fig. S4. Representative thermograms and ITC data for all AmB, MeAmB, AmdeB, and MeAmdeB titrated with sterol-free or 10% ergosterol containing egg PC LUVs.

Cholesterol Binding Experiments.

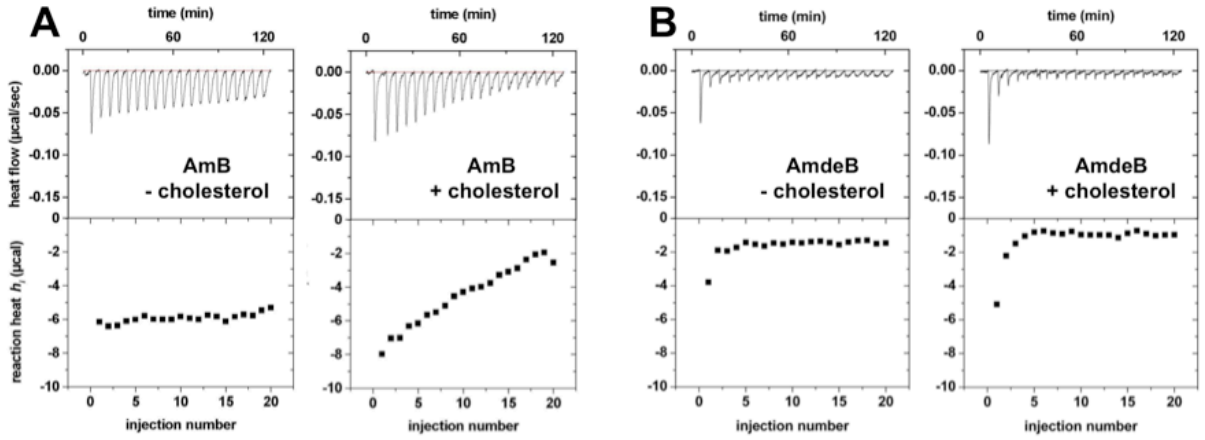
In order to detect the relatively weaker binding between AmB and cholesterol the concentrations of small molecule and liposomes were increased for these experiments. Solutions of AmB and AmdeB were prepared by diluting a 15.0 mM stock solution of the compound in DMSO to 150 μM with 5 mM HEPES (pH = 7.4) buffer. The final DMSO concentration in the solution was 1% v/v. LUVs were prepared and phosphorus content was quantified as described in Section VI. Cholesterol content was assumed based upon the molar ratio of sterol to lipid in the preparation of the lipid films. The LUV solutions were diluted with 1% v/v DMSO/5 mM HEPES to give a final phospholipid concentration of 12.0 mM. Immediately prior to use, all solutions were degassed under vacuum at 17 °C for 10 minutes. The reference cell of the instrument was filled with a solution of 1% v/v DMSO/5 mM HEPES.

Titration Experiment.

Titrations were performed by injecting the LUV suspension at ambient temperature into the sample cell (volume = 1.4399 mL or 1.4495 mL) which contained the 150 μM solution of AmB or AmdeB at 25 °C. The volume of the first injection was 1 μL. Consistent with standard procedure, due to the large error commonly associated with the first injection of ITC experiments, the heat of this injection was not included in the analysis of the data. Next, twenty 10 μL injections of the LUV suspension were performed. The injection duration was 2.1 seconds and 20.6 seconds for the 1 μL and 10 μL injections, respectively. The spacing between each injection varied between 240 seconds and 360 seconds and was adjusted to allow the instrument to return to baseline before the next injection was made. The rate of stirring for each experiment was 300 or 310 rpm.

Data Analysis.

The data analysis for the cholesterol binding experiments was performed as described above for the ergosterol binding experiments. Representative thermograms for AmB and AmdeB are shown in Figure S5 A and B. The average and standard deviation from at least three independent experiments using either sterol-free or 10% cholesterol containing LUVs for AmB and AmdeB compounds is shown in Figure S5 C.



C

	(-) cholesterol $\sum_{i=1}^{40} h_i$(µcal)	(+) cholesterol $\sum_{i=1}^{40} h_i$(µcal)	$\Delta\sum_{i=1}^{40} h_i$(µcal)
AmB	-14.3 ± 4.1	-38.8 ± 7.1	-24.5 ± 8.2* *P* = 0.0066
AmdeB	-9.7 ±6.5	-7.3 ± 2.8	2.4 ± 7.1

Fig. S5. Representative thermograms and ITC data for AmB and AmdeB titrated with sterol-free or 10% cholesterol containing egg PC LUVs using the more sensitive ITC experimental protocol.

IX. Natamycin Experiments

Antifungal Assay.

The antifungal assay for natamycin was run the in the same manner as described in Section II except the natamycin was dissolved in 85:15 DMSO:H_2O rather than pure DMSO as for AmB and its derivatives. The MIC values for natamycin represent the average of two independent runs.

MIC (μM)	AmB	MeAmB	Natamycin	AmdeB	MeAmdeB
S. cerevisiae	0.5	0.5	2	>50	>50
C. albicans	0.25	0.25	4	>50	>50

Figure S6. Antifungal activity of amphotericin B, its derivatives and natamycin against *S. cerevisiae* and *C. albicans*.

Potassium Efflux Assay.

The potassium efflux assay was run in the same manner as described in Section III except the natamycin was dissolved in 85:15 DMSO:H_2O rather than pure DMSO as for AmB and its derivatives.

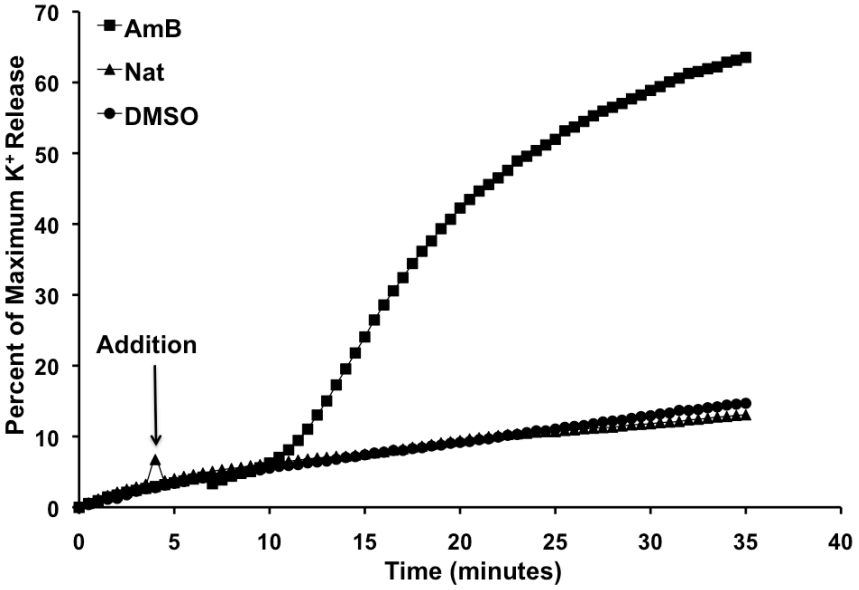
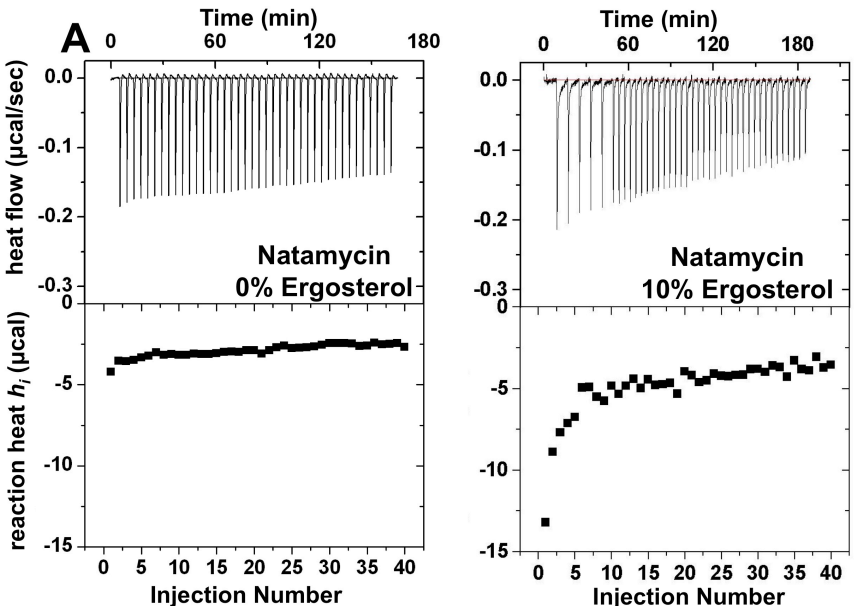


Figure S7. Natamycin fails to elicit significant postassium efflux from live *S. cerevisiae* cells.

Isothermal Titration Calorimetry

Isothermal titration calorimetry experiments were run in as described in Section VII except for the preparation of the aqueous natamycin solution. Instead of diluting a 5 mM stock 100 fold to achieve the 50 μM solution, 1.1 x 10^{-4} mmol of natamycin was added to a 4 mL vial as a solution in 85:15 DMSO:H_2O. This solution was removed by leaving under high vacuum for

several hours. The resulting natamycin film was dissolved in 22 μL 85:15 DMSO:H_2O and then 2.178 mL of K buffer was added to give the 50 μM solution used in the titration experiment.



B

	(-) ergosterol $\sum_{i=1}^{40} h_i$(μcal)	(+) ergosterol $\sum_{i=1}^{40} h_i$(μcal)	$\Delta\sum_{i=1}^{40} h_i$(μcal)
Natamycin	-8.8 ± 2.3	-60.9 ± 6.0	-52.1 ± 6.4* P = 0.001

Figure S8. A. Thermograms for natamycin titrated with either sterol-free or ergosterol loaded EYPC liposomes. Ergosterol content ranged between 9.5 and 13.8%. **B.** Sum of the reaction heats for sterol-free and ergosterol loaded liposomes. The numbers represent the average of three independent experiments.

[1] Palacios DS, Anderson TM, Burke MD (2007) A post-PKS oxidation of the amphotericin B skeleton predicted to be critical for channel formation is not required for potent antifungal activity *J Am Chem Soc* 129:13804-13805.

[2] Clinical and Laboratory Standards Institute. Reference Method for Broth Dilution Antifungal Susceptibility Testing, M27-A2, Approved Standard 2nd Ed. Vol. 22, Number 15, 2002.
[3] Hammond SM, Lambert PA, Klinger BN (1974) Mode of action of polyene antibiotics-induced potassium leakage in *Candida albicans J Gen Microbiol* 81:325-330.
[4] Chen PS, Toribara TY, Warner H (1956) Microdetermination of phosphorus *Anal Chem* 28:1756-1758.
[5] Hanke W. Schule WR (1993) *Planar Lipid Bilayers* (Academic Press, London)
[6] The design of the cell was adapted from: Mayer M, Kriebel JK, Tosteson MT, Whitesides GM (2003) Microfabricated teflon membranes for low-noise recordings of ion channels in planar lipid bilayers *Biophys J* 85:2684-2695.
[7] Ermiskin LN, Kasumov KN, Potzeluyev VM (1976) Single ionic channels induced in lipid bilayers by polyene antibiotics amphotericin B and nystatine *Nature* 262:698-699.
[8]Ermishkin LN, Kasumov KN, Potzeluyev VM (1977) Properties of amphotericin B in a lipid bilayer *Biochem Biophys Acta* 470:357-367.
[9] Kasumov KhM, et al (1979) How do ionic channel properties depend on the structure of polyene antibiotic molecules *Biochem Biophys Acta* 551:229-237.
[10] Borosiva MP, Ermishkin LN, Silbertein AY (1979) Mechanism of blockage of amphotericin B channels in a lipid bilayer *Biochem Biophys Acta* 553:450-459.
[11] Borosova MP, Brutyan RA, Ermishkin LN (1986) Mechanism of anion-cation selectivity of amphotericin B channels *J. Membrane Biol* 90:13-20.
[12] Mickus DE, Levitt DG, Rychnovsky SR (1992) Enantiomeric cholesterol as a probe of ion channel structure *J Am Chem Soc* 114:359-360.
[13] Brutyan RA, McPhie P (1996) One sided action of amphotericin B on lipid bilayer membranes *J Gen Physiol* 107:69-78.
[14] Ibragimova V, Alieva I, Kasumov K, Khutorsky V (2006) Transient permeability induced by alkyl derivatives of amphotericin B in lipid membranes *Biochem Biophys Acta* 1758:29-37.
[15] Kotler-Brajtburg J, Medoff G, Schlessinger D, Kobayashi GS (1974) Characterization of the binding of amphotericin B to *Saccharomyces cerevisiae* and the relationship to antifungal effects *Antimicrob Agents Ch* 6:770-776.
[16] Millié P, Langlet J, Bergés J, Caillet J, Demaret J-P (1999) Self-association of amphotericin B in water. Theoretical energy and spectroscopy studies *J Phys Chem B* 103:10883-10891.
[17] Shervani Z, Etori H, Taga K, Yoshida T, Okabayashi H (1996) Aggregation of polyene antibiotics as studied by electronic absorption and circular dichroism spectroscopies *Colloid Surface B* 7:31-38.
[18] Mouri R, Konoki K, Matsumori N, Oishi T, Murata M (2008) Complex formation of amphotericin B in sterol-containing membranes as evidenced by surface plasmon resonance *Biochemistry* 47:7807-7815.
[19] Fujii G, Chang J-E, Coley T, Steere B (1997) The formation of amphotericin B ion channels in lipid bilayers *Biochemistry* 36:4959-4968.
[20] Vertut-Croquin A, Bolard J, Chabbert M, Gary-Bobo C (1983) Differences in the interaction of the polyene antibiotic amphotericin B with cholesterol-containing or ergosterol-containing phospholipid-vesicles- a circular dichroism and permeability study *Biochemistry* 22:2939-2944.

[21] Paquet M-J, Fournier I, Barwicz J, Tancréde P, Auger M (2002) The effects of amphotericin B on pure and ergosterol- or cholesterol-containing dipalmitoylphosphatidylcholine bilayers as viewed by H-2 NMR *Chem Phys Lipids* 119:1-11.
[22] Milhaud J, Michels B (1999) Binding of nystatin and amphotericin B with sterol-free L-dilauroylphosphatidylcholine bilayers resulting in the formation of dichroic lipid superstructures *Chem Phys Lipids* 101:223-235.
[23] Milhaud J, Hartmann M-A, Bolard J (1989) Interaction of the polyene antibiotic amphotericin B with model membranes- differences between small and large unilamellar vesicles *Biochimie* 71:49-56.
[24] White SH, Wimley WC, Ladokhin AS, Hristova K (1998) Protein folding in membranes: Determining energetics of peptide-bilayer interactions *Method Enzymol* 295:62-87.
[25] Heerklotz H, Seelig J (2000) Titration calorimetry of surfactant-membrane partitioning and membrane solubization *Biochem Biophys Acta* 1508:69-85.
[26] This is a standard protocol for ITC experiments, for example see; 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.