ELUCIDATION OF THE MYCOSAMINE DEPENDENT STEROL BINDING AND ANTIFUNGAL ACTIVITES OF AMPHOTERICIN B

BY

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DISSERTATION

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ABSTRACT

Amphotericin B (AmB) is a clinically vital, yet highly toxic antifungal agent that is dependent on the presence of membrane sterols to exert its biological activity. This natural product has been shown to form ion channels in model membrane systems and therefore represents a molecule with the capacity to perform protein-like function, i.e. the formation of a transmembrane ion channel. Efforts to improve the therapeutic index of AmB or harness its potential to act as a molecular prosthetic to treat diseases that arise from a lack of protein function would benefit from an atomistic understanding of the mechanism of action.

To elucidate this poorly understood mechanism, we developed a strategy focused upon the synthesis-enabled deletion of functional groups from the macrolide skeleton followed by determination of the biological and biophysical consequences. The first functional groups we targeted for deletion were the carboxylic acid and the mycosamine sugar appendages. Both of these functional groups had been predicted to be critical for the following roles: 1) stabilizing ion channel formation via an intermolecular salt-bridge interaction, 2) anchoring AmB to phospholipid bilayers and 3) binding to sterols. However, an alternative hypothesis states that AmB does not directly bind sterols but rather that sterol-induced global membrane properties are the source of the sterol dependency of AmB. This debate has been ongoing since the early 1970’s and had yet to be resolved at the time this work began.

The functional group deletion strategy proved to be remarkably effective. Ultimately, we found that polar interactions between the acid and mycosamine are not required for ion channel formation or antifungal activity. In addition, we found that electrostatic interactions between the acid and/or mycosamine and the zwitterionic phospholipid headgroup are not required for AmB to partition into phospholipid bilayers. Finally, it was discovered that AmB directly binds ergosterol and cholesterol and that the mycosamine appendage is strictly required for this binding interaction. Furthermore, this binding interaction was shown to be absolutely required for forming ion channels and killing yeast cells. Consequently, the long contentious theory that AmB and membrane sterols participate in a functionally vital small molecule-small molecule interaction was finally confirmed. Based upon these results, and related studies with another natural product, natamycin, we proposed a novel, potentially general, two mechanism model to account for AmB’s potent antifungal activity. These discoveries provide a foundation for the
more effective utilization of AmB and demonstrate the capacity of synthetic organic chemistry to illuminate even the most elusive aspects of small molecule function.
To C.E.C.
Acknowledgements

First, I would like to thank my research advisor Prof. Marty Burke who taught me how powerful it is to understand problems at the fundamental level and to not be afraid to think big. I will come back to the lessons throughout my scientific career. Also his support, confidence and guidance allowed me to go far outside my comfort zone to build the knowledge and infrastructure necessary to expand the biophysical capabilities of the group, for which I am very grateful. I would also like to thank my thesis committee, Prof. Scott Denmark, Prof. Paul Hergenrother and Prof. Wilfred van der Donk, for holding me to a high standard of scholarship during the course of my graduate studies. My undergraduate advisor Prof. Yitzhak Tor continued to be a valued source of advice and encouragement. Also, the organic secretaries, Becky Duffield, Susan Lighty and Stacy Olson, thanks for answering all the random questions I had over the years.

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I also need to thank my parents, uncle, grandparents and brother for shaping who I am as a person and for their endless and tireless encouragement (even when I didn’t want it). My successes so far are a testament to their unconditional love and support. Finally I want thank Jenn for being such a wonderful and incredible partner.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>Ac</td>
<td>acetate</td>
</tr>
<tr>
<td>ACME</td>
<td>amplitude constrained multiplet evaluation</td>
</tr>
<tr>
<td>AmB</td>
<td>amphotericin B</td>
</tr>
<tr>
<td>AmdeB</td>
<td>amphoteronolide B</td>
</tr>
<tr>
<td>AmE</td>
<td>amphotericin B methyl ester</td>
</tr>
<tr>
<td>CBS</td>
<td>Corey-Bakshi-Shibata oxazaborolidine catalyst</td>
</tr>
<tr>
<td>COSY-PS</td>
<td>phase sensitive correlation spectroscopy</td>
</tr>
<tr>
<td>CSA</td>
<td>(±)-10-camphorsulfonic acid</td>
</tr>
<tr>
<td>d.r.</td>
<td>diastereomeric ratio</td>
</tr>
<tr>
<td>DDQ</td>
<td>2,3-dichloro-5,6-dicyano-1,4-benzoquinone</td>
</tr>
<tr>
<td>diS-C$_3$(5)</td>
<td>3,3’-dipropylthiacarbocyanine iodide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-(dimethylamino)-pyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethyl formamide</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DMPU</td>
<td>1,3-dimethyl-3,4,5,6-tetrahydro2(1H)-pyrimidinone</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPPA</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphate</td>
</tr>
<tr>
<td>DPPC</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>EYPC</td>
<td>egg yolk phosphatidylcholine</td>
</tr>
</tbody>
</table>

(This is the dominant species, EYPC is a mixture of phosphocholines)
FMOC-OSu  9-Fluorenylmethyl N-succinimidyl carbonate

HPLC  high performance liquid chromatography

ITC  isothermal titration calorimetry

LUV  large unilamellar vesicle

MeAmB  C(41)-methyl amphotericin B

MeAmdeB  C(41)-methyl amphoteronolide B

MIC  minimum inhibitory concentration

MOE  molecular operating environment

NAcAmB  N-acetyl amphotericin B
NAcAmE  N-acetyl amphotericin B methyl ester

NBS   N-bromosuccinimide

NOE   nuclear Overhauser effect

NOESY  nuclear Overhauser effect spectroscopy

OPBC  1-oleoyl-2-(9,10-dibromosteryl)-sn-glycero-phosphocholine

POPC  1-palmitoyl-2-oleoyl-sn-3-glycero-phosphocholine

pyr  pyridine

REDOR  rotational double echo resonance spectroscopy

SEC  size exclusion chromatography

SSNMR  solid-state nuclear magnetic resonance spectroscopy

SUV  small unilamellar vesicle

TBDPS  t-butyldiphenyl silyl
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>TES</td>
<td>triethylsilyl</td>
</tr>
<tr>
<td>Tf</td>
<td>trifluoromethane sulfonate</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethyl silyl</td>
</tr>
</tbody>
</table>
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AMPHOTERICIN B IS AN INDISPENSABLE ANTIFUNGAL AGENT

Systemic fungal infections are a major and mounting source of morbidity and mortality. For example, an epidemiological study of blood-borne infections (sepsis) found that incidences of reported fungal septicemias in the United States increased by 207% over the 21-year period from 1979 to 2000.¹ Moreover, fungal pathogens account for approximately 10 percent of all reported hospital acquired infections, and Candida species are statistically tied for the third most common source of microbial bloodstream infections.² In addition, the rate of fungal infections may actually be considerably underestimated because fungal pathogens are particularly challenging to diagnose, and there is a high occurrence of false negative results for fungal blood cultures.³ The toll extracted by these invasive mycoses on the American health care system is severe. Crude mortality rates for blood borne Candidiasis are very high, with an average of 39 percent, although this rate can reach 60 percent for C. krusei infections.² Additionally, the associated cost for treating these systemic fungal infections has been estimated to cost between 2 to 4 billion dollars annually.⁴ Finally, the medical and demographic trends that are producing a population at risk for fungal septicemia, such as an ageing population, the overall increase in the length of hospital stays and the growing population of immunocompromised individuals, are expected to continue.⁵ Thus, there is an urgent and pressing need for safe and effective antifungal therapies.

Initially approved for clinical use in 1958 by the FDA, the natural product amphotericin B (AmB, Figure 1.1) to this day remains the last line of defense in treating for invasive mycoses. The paradigmatic and most extensively studied member of a large class of antifungal natural products known as the polyene macrolides,⁶ amphotericin B has enjoyed a remarkable six decades of continuous clinical use. This extraordinary medicinal success can be traced to two key pharmacological properties: broad-spectrum fungicidal activity⁷
and the lack of significant microbial resistance.\(^8\) The therapeutic effectiveness of this molecule is encumbered, however, by the substantial toxicity associated with treatment, including cardio and renal toxicity as well as hemolytic anemia.\(^9\) In fact, the harshness of these adverse reactions have become rather infamous over the lifetime of AmB and have earned the molecule the label “amphoterrible” amongst the clinical community. While the clinical efficacy of AmB can be partially improved through the use of lipid-based delivery vehicles,\(^10\) a limited understanding of its mechanism of action has obstructed the further improvement of the therapeutic index of this indispensible member of the modern pharmacopeia.

Currently, the leading hypothesis for the mechanism of action of AmB states that this molecule inserts into sterol-containing lipid bilayers and subsequently self-assembles into a transmembrane ion channel that disrupts the cellular electrochemical gradient, eventually leading to cell death.\(^11\) In this manner, amphotericin B also represents a small molecule with the capacity to perform a protein-like function \(i.e.\) the formation of a membrane-spanning ion channel. It has been envisioned\(^12\) that this functional mimicry could be harnessed to develop small molecules capable of replacing the role of missing or defective proteins in living systems, thereby operating as \textit{molecular prosthetics}. Importantly, the realization of this vision, in addition to the development of new AmB derivatives with better pharmacological profiles, requires an atomistic understanding of the AmB ion channel. However, despite fifty years of intensive investigation, the nature of the AmB ion channel and the underlying physical basis for its formation and stabilization remain unclear. The barrel-stave ion channel model is currently accepted as the leading hypothesis for the mechanism of action of AmB. The evolution of this now textbook classic theory\(^13\) and the key studies that led to its formulation will be overviewed in the following section.

1-2 AMPHOTERICIN B: FOUNDATIONAL STUDIES

In 1955 when AmB was initially isolated and found to be a potent antifungal agent,\(^14\) systemic fungal infections were a virtual death sentence because of the lack of any effective therapy. For example, a commonly advocated treatment for oral thrush infections at the time was the inhalation of ethyl iodide.\(^15\) AmB moved very quickly, therefore, into clinical investigation\(^15\) and use\(^16\) and has remained the standard of care for antifungal chemotherapy since the late 1950’s.
At the same time AmB was being taken forward into the clinic, mechanistic studies aimed at determining the source of AmB’s fungicidal action were also being initiated. A short three years after the initial isolation of AmB, Gottlieb and coworkers\textsuperscript{17} discovered that the addition of a crude hexanes extract of carrots to the culture medium of the mold \textit{P. oxalicum} could provide protection against the antifungal action of AmB. Chemical analysis of the hexanes extract suggested that the protective agent was a mixture of sterol molecules and this was confirmed when chemically isolated sterol mixtures were shown to antagonize the biological activity of AmB. The observed inhibitory effects of sterols led Gottlieb and coworkers to propose that polyene macrolides exert their antifungal activity by either interfering with the biosynthesis of sterols or by competitively inhibiting essential sterol dependent enzymes. However, Kinsky\textsuperscript{18} and Feingold\textsuperscript{19} alternatively suggested that the function of AmB was related to the presence of sterols in the membrane of the target organism.

For example, Kinsky found that both rat erythrocytes (containing cholesterol) and \textit{N. crassa} protoplasts (containing ergosterol) were susceptible to AmB. Conversely, the eubacterium \textit{B. megaterium}, which lacks membrane sterols, was completely resistant to AmB.\textsuperscript{18} However, the interpretation of this experiment was complicated by the presence of the bacterial cell wall because it was also possible that the cell wall was trapping and sequestering the AmB, thereby preventing cellular toxicity. Hence, the next major step in the mechanistic understanding of AmB came with comparative studies using the model mycobacterium \textit{A. laidlawii}. This microorganism does not have the protein machinery to biosynthesize sterols, but it will incorporate sterols into its plasma membrane if they are present in the environment. Importantly, however, sterols are not required for cellular viability, and \textit{A. laidlawii} cultured in rigorously sterol-depleted media will simply have sterol-free plasma membranes. Taking advantage of this unique physiological property, Feingold set up an experiment using two parallel batches of \textit{A. laidlawii} cultured under identical conditions except for one key difference. One batch was grown in the presence of cholesterol while the other was cultured under sterol-free conditions.\textsuperscript{19} Exposing each culture stock to AmB produced the striking result that \textit{A. laidlawii} grown with cholesterol were rapidly killed by AmB, while the sterol-free group was totally refractory to AmB. Moreover, transferring the “resistant” strain to cholesterol-rich media rendered it susceptible to AmB and, conversely, dilution of the vulnerable organisms in sterol-free media induced AmB resistance. Although
these studies showed that the antimicrobial action of AmB is dependent on the presence of membrane sterols, the role of sterols in the mechanism of AmB very much remained unclear.

Following these early studies, two different proposals were formulated to explain the absolute sterol dependence of AmB. These two models are termed the direct sterol hypothesis and the indirect sterol hypothesis. Originally proposed by Feingold and HsuChen in 1973, the indirect sterol hypothesis asserts that the action of AmB is dependent upon the sterol mediated global properties of the cell membrane. According to this hypothesis, the differences in the preorganization of the membrane induced by individual sterols are the source of the membrane selectivity of AmB. Thus, by the indirect sterol model, ergosterol best modulates the physiochemical characteristics of membrane bilayers in a manner that is conducive to the mechanism of action of AmB, leading to the overall selectivity of AmB for fungal membranes. The direct sterol hypothesis, advanced by Medoff and coworkers in 1974, alternatively states that AmB and membrane sterol engage in a direct binding interaction. Therefore, by the direct sterol hypothesis, the selective toxicity of AmB for fungal cells is derived from the higher binding affinity of AmB for ergosterol (the main fungal sterol) versus cholesterol (the main mammalian sterol). These two competing hypotheses have been hotly debated in the literature since their inception but so far the issue has remained unresolved. Importantly, distinguishing between these two hypotheses is critical to the rational development towards an improved therapeutic index. This subject will be discussed in more depth later in the chapter.

These early studies with AmB, in addition to investigating the important, though undefined role of membrane sterols, were also focused on elucidating the mechanism of AmB’s cellular toxicity. For example, in 1961 Kinsky found that AmB induced growth inhibition of the mold *N. crassa* was accompanied by a significant decrease in the dry weight of the mycelial mats. Kinsky therefore proposed that AmB compromised the permeability properties of cellular membranes and this permeability enhancement was the source of AmB’s antifungal activity. Kinsky’s supposition was further supported by the finding that *B. megaterium*, which had also been shown to be resistant to AmB, was not susceptible to AmB induced cellular lysis. Further studies by de Kruijff and coworkers, using the same *A. laidlawii* model system discussed above, were also useful in testing Kinsky’s permeability hypothesis. For example, exposing cholesterol-free and resistant *A. laidlawii* to AmB did not increase the membrane permeability of these organisms. In contrast, when cholesterol-laden *A. laidlawii* were treated with AmB, a
marked loss of intracellular potassium was observed. Additional permeability studies with the pathogenic yeast *C. albicans* were also consistent with Kinsky’s hypothesis.\(^{25}\) Considering the capacity of AmB to permeabilize membranes and the possible role of permeability enhancement in antifungal activity, three different mechanisms of membrane disruption were formulated: gross membrane destruction, ionophoric transport and discrete single ion channel formation.

Distinguishing between these three scenarios relied heavily on the then newly established planar lipid bilayer technology, which proved to be a very valuable tool.\(^{26}\) This *in vitro* technique allows for precise electrophysiological measurements on isolated patches of membrane, the composition of which can be controlled to suit the experiment. In 1968, Andreoli and coworkers employed this technique and found that AmB decreased the electrical resistance of cholesterol containing thin lipid membranes by over six orders of magnitude without disrupting the physical integrity of the phospholipid bilayer. Moreover, consistent with the biological data, treating sterol-free planar lipid bilayers with AmB did not alter the resistance properties of the membrane.\(^{27}\) The stability of the membrane in the presence of AmB strongly argued against the gross destruction mechanism, but this initial result was unable to resolve the ion channel and carrier models.

To differentiate these two mechanisms, Finkelstein and coworkers employed the model ionophore valinomycin for a series of comparative studies with AmB (Table 1.1).\(^{28}\) By measuring the membrane conductance as a function of the antibiotic concentration or of the temperature, strikingly different behaviors were seen for valinomycin and AmB. For example, in the presence of valinomycin, the conductance of the membrane increased linearly with increasing concentrations of valinomycin. Furthermore, increasing the temperature of the experiment produced an overall increase in the conduction of valinomycin treated membranes, behaviors consistent with the shuttling mechanism of valinomycin. AmB, however, produced very different results that we not consistent with the characteristics of ionophores. For instance, in the presence of increasing concentrations of AmB, the membrane conductance exhibited a power

<table>
<thead>
<tr>
<th>Relation between concentration ((c)) and conductance ((i))</th>
<th>Valinomycin</th>
<th>AmB</th>
</tr>
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<tbody>
<tr>
<td>(i) proportional to (c)</td>
<td>(i) proportional to (c^n)</td>
<td></td>
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<table>
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<tr>
<th>Effect of temperature ((T)) on conductance</th>
<th>Valinomycin</th>
<th>AmB</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) increases with (T)</td>
<td>(i) decreases with (T)</td>
<td></td>
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</table>

*Table 1.1* The differential effects of valinomycin and AmB on planar lipid bilayers supported the hypothesis that AmB forms ion channels.
dependence on the concentration of the antibiotic. Additionally, when the temperature of AmB exposed membranes was increased, a significant decrease in membrane conductance was observed. The characteristics of AmB, therefore, were much more consistent with the formation of a self-assembled aggregate. Upon increasing temperature, Finkelstein proposed that complex was dissociating and consequently reducing the membrane conductivity.

Further evidence consistent with the channel model was gained by studying the membrane permeability of small non-ionic solutes in the presence of AmB. These experiments showed a strong correlation between the hydrodynamic radius of the solutes and the ability of these molecules to pass through AmB treated membranes. All of the solutes used in this study had limited membrane permeability in the absence of AmB, but relatively small solutes such as urea and glycerol were able to permeate the bilayer to a much greater extent upon the addition of AmB. The AmB enabled permeability enhancement did not, however, translate to larger molecules such as glucose, which remained impermeable in the presence of AmB. Thus, this data indicated that AmB was forming a water filled pore of a defined size and, given the relative impermeability of glucose, Andreoli and coworkers predicted that this pore was between 7 and 10 angstroms in diameter.

Conclusive evidence for the putative AmB ion channel was finally obtained in 1976 with the direct experimental observation of single AmB ion channels using planar lipid bilayers. Remarkably, this small molecule ion channel displayed characteristics such as gating and ion selectivity that are typically associated with protein ion channels. Furthermore, the AmB ion channel could be reversibly blocked in a voltage-dependent fashion by the tetraethylammonium cation, similar to some protein-based ion channels. However, while the ability of AmB to form ion channels in model membranes had been established, the role of this ion channel in antifungal activity remained unclear and has been disputed. Most prominently, it has been proposed that the cellular toxicity of AmB is derived from the polyene-induced oxidative damage of phospholipid bilayers.
Collectively, these studies formed the basis for the AmB barrel-stave ion channel model. This hypothesis was independently and simultaneously advanced by de Kruijff, Andreoli, and Finkelstein in 1973 and is shown in Figure 1.3. This model proposes that eight AmB units self-assemble to form a transmembrane ion channel with the polyol portion of each molecule collectively pointing inward to create a water-filled pore that is conducive to ion conductance. Conversely, the non-polar polyene portions of the AmB monomers are oriented outward to interact with the hydrophobic lipid environment. In addition, later refinements of the barrel-stave model have made predictions regarding the role of specific chemical groups in forming and stabilizing this small molecule ion channel. Most prominently, three specific proposals have been made for the role(s) of the C(41) carboxylate and C(19) mycosamine, as shown in Figure 1.4. The first proposal (Figure 1.4A) is that the C(41) carboxylate and C(3') amine participate in an intermolecular salt bridge/hydrogen bond to create a peripheral ring of polar interactions predicted to be critical for ion channel formation. Additionally, polar interactions between zwitterionic phospholipid headgroups and the C(41) carboxylate and/or C(19) mycosamine been proposed to anchor AmB into the membrane bilayer (Figure 1.4B). Finally, the carboxylate and/or mycosamine have also been predicted to mediate the direct binding of membrane sterols via a hydrogen bond to the 3β-sterol hydroxyl group (Figure 1.4C). As was mentioned previously, however, a competing hypothesis states that AmB does not directly bind membrane.
sterols, but the sterol dependence of AmB instead arises from the indirect global effects that sterols impose on phospholipid bilayers.

The putative importance of the carboxylate and mycosamine is reinforced by the conspicuous biosynthetic origin of these two groups. When the biosynthesis of AmB was elucidated in 2001 it was found that the initial product of the polyketide synthase machinery is actually aglycone 1.2 (Figure 1.5), noticeably lacking both the C(41) carboxylic acid and C(19) mycosamine. These functionalities are installed post-polyketide synthase by dedicated enzymes that exhaustively oxidize the C(41) methyl group and glycosylate C(19) with mycosamine.
Similar tailoring enzyme modifications have also been found for the related natural products\textsuperscript{41} natamycin,\textsuperscript{42} nystatin,\textsuperscript{43} rimocidin,\textsuperscript{44} and candicidin.\textsuperscript{45} Additionally pointing to the potential critical role of the acid and mycosamine is the conservation of these two moieties in the same relative position in all but one of this collection of molecules, over 200 of which have been described.\textsuperscript{46}

Until recently, each one of these three hypotheses remained experimentally unconfirmed. The inability to furnish definitive conclusions to these questions of central mechanistic importance over five decades of research is symptomatic of the broader challenges associated with investigating molecular events occurring in the phospholipid bilayer environment. For example, the dynamic nature of membrane-localized phenomena, such as the self-assembled AmB ion channel aggregate, challenges current state of the art spectroscopic\textsuperscript{47} and computational\textsuperscript{48} techniques. In addition, two other commonly employed methods in membrane biophysical chemistry that have been used to interrogate the AmB ion channel, the covalent modification of lipids and/or the covalent modification of AmB, also have associated experimental complications. For instance, derivatization of a membrane component to investigate a particular chemical interaction can also substantially alter the physical characteristics of the parent bilayer. This is especially true for membrane sterols\textsuperscript{49} because small structural modifications, such as inverting the stereocenter of the 3β-hydroxyl group, can drastically change the molecular and macroscopic effects that membrane sterols impart on phospholipid bilayers.\textsuperscript{50} Changing these global membrane properties can also independently impact membrane-localized binding events, making the isolation and validation of binding events occurring within lipid bilayers extremely difficult. Furthermore, such lipid modification studies are challenging to translate to an \textit{in vivo} experiment because of the high degree of lipid structural specificity that is often required for the proper maintenance of cellular physiology.\textsuperscript{51}

Alternatively, covalent modification of AmB itself can introduce unfavorable steric interactions, and therefore it may be unclear if a positive, stabilizing interaction is being removed or if negative steric clashing is being introduced. This potential consequence is particularly critical when considering the formation of the AmB ion channel, because of the exquisite sensitivity of small molecule self-assembly to steric effects.\textsuperscript{52}
Given the inherent limitations of these general strategies, this thesis describes a different approach involving the synthesis enabled-deletion of protic functional groups from the macrolide skeleton and determination of the biological and biophysical consequences. This experimental algorithm is shown in Figure 1.6 and consists of three related modules: 1) synthesis of functional group deficient derivatives, 2) elucidation of the ground state three dimensional conformation of these derivatives and 3) determination of the biological and biophysical consequences of each functional group deletion. This strategy is akin to the alanine scanning methodology that has proven to be extremely successful for determining the function of specific amino acid residues in complex proteins. In addition, Lemeuix used a similar deletion based approach in his pioneering studies of the blood group lectins. An additional and very advantageous element of the functional group deletion strategy is that the same probe molecules can be employed in vitro and in vivo. Thus, the functional group deletion strategy can directly link structure-function relationships determined via biophysical assays to the consequences of perturbing such functions in living cells.

As will be discussed in detail in the following chapters, this experimental strategy proved to be remarkably effective. Ultimately, it was discovered that the putative polar interactions between functional groups at C(41) and C(19) are not necessary for pore formation (Figure 1.4A). We also found that polar interactions between the C(41) carboxylate and/or C(19) mycosamine and the phospholipid headgroups are not required to anchor AmB to the membrane (Figure 1.4B). Alternatively, it was discovered that AmB directly binds ergosterol and that the mycosamine appendage is strictly required for this interaction (Figure 1.4C). Furthermore, this binding interaction was shown to be strictly required for forming ion channels and killing yeast cells. Consequently, the long contentious theory that AmB and membrane sterols participate in a functionally vital small molecule-small molecule interaction was finally confirmed. To put these
results in context, the literature supporting each one of the hypotheses summarized in Figure 1.4 will be critically overviewed.

1-3 THE SALT-BRIDGE HYPOTHESIS

The initial evidence suggesting a polar interaction between the functional groups at C(41) and the mycosamine appendage at C(19) came from in vitro studies of covalently modified AmB derivatives. During electrophysiological investigations of AmB in 1979, Ermiskin and coworkers noted dramatic changes in the ion channel characteristics of AmB according to the state of the carboxylate and mycosamine. The chemistry of these two groups was varied by methylating the acid to produce amphotericin B methyl ester (AmE), and by acetylating the amine to give N-acetyl amphotericin B (NAcAmB). The results of these experiments are summarized in Table 1.2, which shows the dramatic decrease in channel lifetime that accompanied neutralization of one of the charged groups. In addition to reducing the channel lifetime, neutralization of one of the charged groups also had the effect of raising the concentration threshold required to observed single ion channels. However, once the derivative channels did form, both the AmE and NAcAmB ion channels appeared to have similar conductance properties to the native channel. Based upon these data, the authors proposed that blocking either of the charged groups disrupted electrostatic interactions between

![Image: The salt bridge hypothesis.](image)

### Table 1.2

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>conc. (M)</th>
<th>pH</th>
<th>conductance (pS)</th>
<th>channel lifetime (s)</th>
<th>charges</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmB</td>
<td>$2 \times 10^{-8}$</td>
<td>7.0</td>
<td>6.5</td>
<td>$260 \pm 40$</td>
<td>+–</td>
</tr>
<tr>
<td>AmE</td>
<td>$1 \times 10^{-6}$</td>
<td>7.0</td>
<td>6.5</td>
<td>$2.5 \pm 0.3$</td>
<td>+</td>
</tr>
<tr>
<td>NAcAmE</td>
<td>$3 \times 10^{-7}$</td>
<td>7.0</td>
<td>5.0</td>
<td>$3.0 \pm 0.3$</td>
<td>–</td>
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</tbody>
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Table 1.2. The channel lifetime of AmB derivatives is drastically reduced when either of the two charged groups is neutralized. Adapted from reference 37.

the C(41) acid and C(3′) amine. This hypothesis was later supported by molecular dynamics
studies of the barrel stave AmB ion channel by Khutorsky, McCammon, and Borowski. In each one of these molecular dynamics simulations a strong hydrogen bonding interaction between the C(41) carboxylate and C(3’) amine was noted, and thus was predicted to play a critical role in channel self-assembly.

In vivo studies were further interpreted as supporting the putative salt bridge hypothesis in a physiologically relevant context. Specifically, in addition to the reduced capacity to form ion channels in planar lipid bilayers, it was also known that acetylating the C(3’) amine leads to a significant reduction in the capacity to permeabilize yeast cells and to exert antifungal activity. This was interpreted as supporting the requirement for this putative polar interaction in the membrane of living yeast cells. Interestingly, a variety of C(41) esters have been found to retain yeast permeabilizing and antifungal activities. However, as pointed out by Bolard and Borowski, these covalently modified derivatives retain a polar carbonyl group and thereby possess the capacity to form charge-dipole interactions with the mycosamine appendage and perhaps thereby stabilize channel self-assembly (Figure 1.8).

In an attempt to probe the alleged salt bridge interaction, Murata and coworkers recently synthesized the covalently linked dimers 1.3-1.5 (Figure 1.9). Following their synthetic preparation, the biological activity of these molecules was investigated, and it was found that the dimers displayed little to no antifungal activity. Based on the limited activity of these molecules, the authors proposed that while the amine and acid may form a strong hydrogen bond in the AmB ion

![Figure 1.8](image1.png)

**Figure 1.8.** A. Steric clashing of the amine could alternatively underlie the reduced activity of AmB. B. The methyl ester functionality is still capable of forming a hydrogen bond with the C(3’) amine.

![Figure 1.9](image2.png)

**Figure 1.9.** Structures of the covalent dimers prepared to investigate the importance of the salt bridge. See reference 61.
channel assemblage, some amount of space and/or flexibility is required for this interaction to be fully effective. Murata and coworkers reported further support for this prediction with molecular mechanics force field calculations of 1.3-1.5. The structure of directly linked 1.3 suffered a large degree of structural distortion, which Murata and coworkers attribute to the strain induced by the short amide covalent linkage. Thus, the inherent steric interference caused by covalently modified derivatives made this approach unable to cleanly probe the proposed importance of the salt bridge interaction between the C(41) carboxylate and C(3’) ammonium.

1-4 THE PHOSPHOLIPID ANCHORING HYPOTHESIS

The hypothesis that phospholipids anchor AmB into the membrane via polar interactions between the phospholipid headgroup and the C(41) carboxylate and/or C(19) mycosamine was first prompted by a series of solution-phase NMR studies.\(^{37}\) Easwaran and coworkers studied the interaction of AmB with the saturated phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) in CDCl\(_3\) and observed significant AmB dependent line broadening in the \(^{31}\)P spectra of the phospholipid headgroup. This data was interpreted by Easwaran and coworkers to be consistent with an interaction between the polar functionality on the C(19) mycosamine and the phospholipid headgroup. But since these data were collected under non-physiological conditions in CDCl\(_3\), the results of this study must be interpreted with caution. Nonetheless, using this NMR data to constrain the calculations, a molecular dynamics simulation of the proposed AmB:DPPC complex was also performed.\(^{62}\) In the simulated complex, the C(2’) and C(4’) oxygens of mycosamine were observed to form hydrogen bonds with the glycerol oxygens of the DPPC headgroup but no electrostatic interactions between the C(41) carboxylate or the C(3’) ammonium and the charged groups on the phospholipid were observed. However, other molecular dynamics simulations performed in the presence of phospholipid bilayers did observe electrostatic interactions between the zwitterionic phospholipid headgroup and the C(41) carboxylate and C(3’) ammonium.\(^{63}\)
The nature of this putative AmB-phospholipid interaction has also been investigated using surface pressure measurements of thin films of sterol-free lipids and AmB. The first of these studies used the simple model lipid 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA), which does not contain a choline headgroup. Analysis of a series of surface pressure-area curves with differing molar ratios of phospholipid and AmB showed highly non-ideal behavior for AmB-DPPA thin films. Miñones and coworkers interpreted these deviations from ideality to be evidence of AmB-DPPA complex formation. In addition, the maximum deviation from ideality occurred at a ratio of 2:1 AmB to DPPA and the authors ascribed this phenomenon to the formation of a relatively stable 2:1 AmB:DPPA complex held together via electrostatic interactions between the charged groups on AmB and DPPA. Murata and coworkers also claimed evidence for the formation of an AmB-lipid complex held together by electrostatic interactions based on solid-state nuclear magnetic resonance (SSNMR) studies of AmB in DMPC membranes. However, it is currently not clear how well fully saturated lipids, such as DPPA and DMPC, can serve as good models for eukaryotic cell membranes. More importantly, neither of these studies employed membrane sterols, which are necessary for the antifungal activity of AmB, and, therefore, the results of these experiments are difficult to interpret.

The covalent modification approach has also been used to probe the phospholipid anchoring hypothesis. For example, utilizing a reductive amination to alkylate the C(3') amine with an aldehyde bearing phospholipid, Murata and coworkers prepared dimers 1.6-1.9 (Figure 1.11) to investigate the AmB-phospholipid interaction. The extremely poor aqueous solubility of these molecules made the experimental evaluation of their biophysical properties very difficult, however. For example, while all four dimers displayed membrane permeabilization
activity against sterol-free egg yolk phosphatidylcholine (EYPC) liposomes in a potassium efflux assay, the dimers had to be premixed with the lipids prior to liposome formation. Attempting to perform this experiment under more physiologically relevant conditions, that is adding a solution of the conjugates to a suspension of liposomes, was prevented by the insolubility of all four dimers. In addition, Murata and coworkers also cited the insolubility of the conjugates as a possible reason for the total lack of antifungal activity of these molecules. Thus, the potential importance of polar interactions between AmB and phospholipids to anchor AmB to the membrane remained ambiguous.

1-5 THE INDIRECT STEROL HYPOTHESIS

In 1973 Feingold and HsuChen reported that, although cholesterol promoted the action of AmB in many lipid bilayer systems, in certain model membranes AmB function was attenuated by the presence of membrane embedded cholesterol (Figure 1.12). This unexpected result was interpreted to be the first experimental evidence that the mechanism AmB is dependent on the indirect, physiochemical effects of membrane sterols. This study used a glucose release assay to investigate the effect of different lipids on the membrane permeabilizing activity of AmB. When the glucose release assay was performed with egg lecithin vesicles, the incorporation of cholesterol into the liposomes markedly enhanced the membrane permeabilizing activity of AmB. However, when this same experiment was repeated with vesicles alternatively composed of dipalmitoyl lecithin, the addition of cholesterol to these vesicles greatly suppressed the extent of AmB induced glucose leakage. In light of the then current molecular understanding of phospholipid membranes, Feingold proposed that AmB is active only against phospholipid membranes with a relatively high degree of overall molecular order. For inherently disordered unsaturated lipids, such as egg lecithin, cholesterol confers a degree of ordering to the lipid chains. In contrast, for saturated lipids such as dipalmitoyl lecithin, cholesterol disrupts the tight interactions of the saturated acyl tails and leads to an overall decrease in ordering.

<table>
<thead>
<tr>
<th>Vesicle system</th>
<th>Egg lecithin (+) Cholesterol</th>
<th>Dipalmitoyl lecithin (+) Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impact on AmB activity</td>
<td>Overall increase in membrane permeabilization</td>
<td>Overall decrease in membrane permeabilization</td>
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**Figure 1.12.** Cholesterol had opposing effects on the activity of AmB for the two different vesicles systems used in reference 20.
Later liposome-based studies were also interpreted to be consistent with this hypothesis. For example, Murata and coworkers also found that cholesterol could act as an AmB antagonist under certain conditions. In this experiment, the addition of cholesterol to egg yolk phosphatidylcholine (EYPC) liposomes, potentiated the membrane permeabilizing activity of AmB until the concentration of cholesterol in the membrane reached 20 percent (relative to lipid). When the amount of cholesterol was further increased to 30 percent, a reduction in the degree of ion permeability was recorded. Murata and coworkers proposed that the inhibitory effects of cholesterol were derived from the propensity of this sterol to thicken membrane bilayers.

Carreira and coworkers observed similar effects and concluded that the membrane selectivity and activity of AmB arises from the differential preorganization of the membrane imposed by different sterols. In their study, Carreira and coworkers used 100 nm 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposomes mixed with various amounts of ergosterol, cholesterol, 7-dehydrocholesterol or dihydrocholesterol (Figure 1.12) in a potassium efflux assay to investigate the impact of sterol structure on the permeabilizing activity of AmB. Ergosterol or cholesterol-loaded liposomes displayed the maximum degree of efflux at five percent sterol (relative to lipid). Increasing the sterol content beyond five percent led to an overall decrease in the extent of ionic flux. Alternatively, 7-dehydrocholesterol or dihydrocholesterol liposomes suffered the highest level of permeabilization at 13 percent sterol. Additional 7-dehydrocholesterol or dihydrocholesterol beyond 13 percent inhibited the efflux activity of AmB. Carreira and coworkers thus interpreted this data as being consistent with the indirect sterol hypothesis.
Even the notion that AmB requires membrane bound sterol for channel formation has been questioned. For example, Hartsel and coworkers\textsuperscript{71} demonstrated that AmB potently promoted the efflux of potassium from sterol-free EYPC small unilamellar vesicles (SUVs).\textsuperscript{72} Membrane permeabilization was observed at AmB to lipid ratios as low as 1:1000 for these sterol-free SUV’s. Other groups have also documented this type of sterol independent permeabilization of liposomes by AmB.\textsuperscript{73} Moreover, utilizing patch clamp technology, Ortega-Blake and coworkers reportedly observed the formation of single AmB ion channels in sterol-free liposomes composed of DMPC, egg lecithin or isolated bacterial membrane extracts.\textsuperscript{74} The ion channels that formed in these in vitro sterol-free systems displayed similar electrophysiological properties as the AmB ion channels that have been documented in sterol containing membranes. Ortega-Blake and coworkers interpreted this observation as evidence that that sterols are not involved in the supramolecular AmB channel architecture. However, all of these studies were performed in model membranes, and the authors noted that supraphysiological concentrations of AmB were required to observe ion channels in sterol-free membranes, making the results of this experiment difficult to interpret. In addition, small unilamellar vesicles are considered to be poor experimental models to use for investigating membrane active species. Their small size correlates to a high radius of curvature and consequently makes them much more susceptible to membrane permeabilization than the more physiologically representative large unilamellar vesicles (LUV’s).\textsuperscript{75}
Originally advanced in 1974, the alternative theory for the sterol dependency of AmB proposes that AmB directly binds membrane sterols. In this model, the selective fungal toxicity of AmB therefore arises from the greater binding affinity of AmB for ergosterol (the main fungal sterol) over cholesterol (the main mammalian sterol). More specifically, the carboxylate at C(41) and/or the mycosamine sugar are predicted to mediate this putative small molecule interaction.

Early data that was interpreted as supporting a direct AmB-sterol binding interaction came from sterol-dependent shifts in the UV/Vis and CD spectra of AmB in both solution phase and liposome based studies. These spectral shifts were mainly correlated to the permeability of liposomes, but not to any additional structural data. Thus, it has been conversely proposed that these sterol-dependent changes in the electronic absorption spectra of AmB are due to aggregation of the AmB monomers and not an AmB sterol interaction.

Attempting to elucidate the putative AmB-sterol interaction, Borowski and coworkers used the sterol modification strategy to investigate the role of the C(19) mycosamine and C(41) carboxylate in sterol binding. This study examined the ability of AmB, NAcAmB and AmE to permeabilize liposomes loaded with one of the sterols shown in Figure 1.15. Borowski and coworkers found that NAcAmB had a reduced activity relative to AmB against liposomes containing any one of the sterols shown in Figure 1.15. Based upon this result, they suggested that the C(3') amine hydrogen bonds with the 3β sterol hydroxyl group to promote the AmB-sterol binding interaction. This interaction has
also been proposed based upon molecular dynamics simulations of the AmB-sterol complex.\(^{58}\) However, as with the salt-bridge studies outlined in Section 1.3, it is difficult to dissociate the effect of removing a hydrogen bonding interaction from steric clashing when covalently modified derivatives are used as probes.

In contrast to NAcAmB, AmE selectively permeabilized liposomes that contained either ergosterol or brassicasterol. These two sterols share the same unsaturated isoprenoid tail that is not common to the remaining four sterols (Figure 1.14). Borowski and coworkers therefore proposed that AmB binds sterols through two complementary forces: a water mediated hydrogen bond to the 3β sterol hydroxyl group (Figure 1.16), and van der Waals contacts between the sterol nucleus and the AmB polyene subunit. For AmB derivatives bearing a free acid, Borowski and coworkers proposed that the hydrogen bond network is very strong and therefore hydrogen bonding dominates the sterol-AmB binding interaction (Figure 1.16A). For AmB derivatives with an alkyl ester at C(41), however, the authors hypothesize that this hydrogen-bonding network is disrupted (Figure 1.16B) and consequently, van der Waals contacts drive the binding event. A similar model was also advanced by Gary-Bobo.\(^{79}\) However, this model does not take into account the ability of the C(41) alkyl esters to maintain polar interactions through the ester moiety.\(^{60}\)

![Figure 1.16](image1.png)  
**Figure 1.16.** The proposed hydrogen bonding network that promotes the AmB-sterol binding interaction. Adapted from reference 39.

![Figure 1.17](image2.png)  
**Figure 1.17.** Structures of the intramolecularly tethered derivatives used to investigate the AmB sterol interaction in reference 80.

Alternatively, it has also been proposed that the C(2’) alcohol present on mycosamine may form a hydrogen bond to the 3β sterol hydroxyl group. Using the covalent modification approach, the intramolecularly tethered AmB derivatives shown in Figure 1.17 were synthesized.\(^{80}\) However, reflecting the inherent limitation of the covalent modification approach, conformationally restrained 1.10-1.12 displayed significantly reduced antifungal activity relative to AmB. Nevertheless, Murata
and coworkers used the results of a conformational analysis of the linked derivatives to try to predict which polar groups on mycosamine interact with the $3\beta$-sterol hydroxyl group. Based upon the conformation of the mycosamine appendage relative to the polyene subunit for the six-carbon linked 1.11, Murata and coworkers proposed that the C(2’) hydroxyl group was the functional group on mycosamine in the best spatial orientation to interact with the sterol $3\beta$ hydroxyl group. This interaction predicted by Murata and coworkers has also been proposed based upon molecular dynamics simulations of AmB-sterol complexes.\textsuperscript{81} However, the prediction that the C(2’) alcohol forms the critical hydrogen bond with membrane sterols has been questioned by the recent demonstration by Carreira and Croatt that epi-C(2’) AmB fully retains native antifungal activity.\textsuperscript{82}

Further attempts to investigate the AmB-sterol interaction used enantiomeric cholesterol\textsuperscript{83} as a mechanistic probe for the role of sterol in the AmB ion channel. In contrast to the sterol-free patch clamp experiment described in the previous section, Rychnovksy and coworkers found that planar lipid bilayers containing either cholesterol or ent-cholesterol produced two distinct AmB ion channels. The electrophysiological divergence for the enantiomeric sterol systems was interpreted by the authors as evidence that AmB was directly binding the membrane sterols and this was leading to the formation of two different diastereomeric AmB ion channels.\textsuperscript{84} Furthermore, the concentration of AmB required to observe the formation of ion channels was greater for the ent-cholesterol system, which the authors interpreted as consistent with a reduced binding affinity of AmB for this unnatural sterol. However, it is challenging to reproduce this experiment in a live yeast cell to determine if this putative binding interaction is functionally relevant \textit{in vivo}.\textsuperscript{85}

Finally, a recent SSNMR study utilizing deuterated versions of AmB, ergosterol and

![Figure 1.18](image-url)  
**Figure 1.18.** Structures of the deuterated isotopologues used in the SSNMR study claiming an AmB-ergosterol interaction. Adapted from reference 67.
cholesterol (Figure 1.17) produced conflicting results. Using the quadrupolar splitting of the deuterium peaks as a measure of molecular mobility in a POPC bilayer, Murata and coworkers observed that AmB and cholesterol have dissimilar mobilities under these conditions, suggesting little or no binding interaction between these two molecules. In contrast, when AmB was added to a POPC bilayer loaded with deuterated ergosterol analogue 1.14, the two molecules displayed similar mobilities, which the authors interpreted as being consistent with a direct binding interaction between AmB and ergosterol. Seeming to contradict this conclusion, when a rotational double echo resonance (REDOR) experiment was performed with untethered 13C-labelled AmB and 19F labeled ergosterol, Murata and coworkers did not observe a through space interaction between the two molecules. Such an interaction would be expected if the 19F ergosterol and AmB were participating in a direct binding interaction. Conversely, a dephasing effect was observed for covalently tethered AmB-ergosterol conjugate 1.16 but this molecule was not biologically active. Thus, the importance, or even the existence, of the putative AmB-sterol interaction has remained unclear.

Figure 1.19. Structures of the AmB-sterol covalent conjugates from reference 87.
1-7 SUMMARY

Collectively, the decades of research outlined above has shown that, despite being the standard of care for systemic mycoses for over fifty years, the molecular mechanism of action of AmB remains ambiguous. In particular, the role(s) of the C(41) carboxylate and C(19) mycosamine have eluded a precise understanding, though both are predicted to be vital for biological activity. Elucidating the function of these two groups is extremely challenging, however, because of the dynamic, multimolecular and membrane-based action of AmB. Investigations of this molecule have focused on four key experimental strategies: covalent modification of AmB, covalent and/or stereochemical sterol modifications, spectroscopic studies and computational methods. However, each one of these strategies has its limitations and, hence, the mechanism of AmB’s antifungal activity, and especially the specific roles of key functional groups, remains enigmatic. Therefore, there is a clear and unmet need for the definitive clarification of these ambiguities, particularly those surrounding the role(s) of the carboxylic acid and mycosamine. Our contributions to this goal utilizing the alternative, functional group deletion approach, are outlined in the following two chapters.
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Whyte, B.S.; Peterson, R.P.; Hartsel, S.C.

Zumbuehl, A.; Stano, P.; Heer, D.; Walde, P.; Carreira, E.M.


Small unilamellar vesicle is the term generally used for liposomes that are below 100 nm in diameter.


Chapter 2

“Top-Down” Degradative Syntheses of C(41)-Methyl amphotericin B, amphoteronolide B and C(41)-Methyl amphoteronolide B

Investigating the role(s) played by mycosamine and the C(41) carboxylate in the mechanism(s) of action of amphotericin B required the development of a synthetic route to three functional group deficient derivatives: C(41)-Methyl amphotericin B, amphoteronolide B and C(41)-Methyl amphoteronolide B. This chapter details the synthetic strategy used to achieve all three derivatives from a common intermediate derived through synthetic manipulation of the natural product. In the course of these syntheses, significant hurdles had to be overcome. For example, the natural product itself presents a challenge because it is insoluble in common organic solvents and water, and is light, oxygen and acid sensitive, making the molecule difficult to handle. In addition, the mycosamine and the carboxylic acid had to be selectively deleted in this polyfunctional molecule without harming other sensitive chemical moieties, such as the lactone or the polyene. Building on important literature precedent from chemists at Smith Kline-Beecham, we found that activating the C(41) carboxylic acid as a 2-pyridyl thioester was an effective method for selective reduction. Similarly building on studies by Nicolaou and Masamune, we found that mycosamine could be removed oxidatively using 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in the presence of either an allyl ester or a primary iodide at the C(41)-position. Moreover, we found that the functional group at the C(41) position heavily influenced the diastereoselectivity of the carbonyl reduction at C(19) that was performed directly after mycosamine removal. The configuration of the newly formed stereocenter at C(19) was established using coupling constant and NOE data in comparison to the natural product. In addition, the 3-dimensional conformation of the three functional group deficient derivatives and AmB were calculated using a Monte-Carlo simulation constrained by extensive two-dimensional NMR data. Thomas M. Anderson contributed to the development of the oxidative removal of mycosamine using DDQ and performed the NMR experiments used to determine the configuration at C(19) and the 3D ground state conformation of the four molecules with assistance from the author. Thomas Anderson also contributed the calculated structures shown in Figure 2.3. Portions of this chapter were taken with permission from Palacios, D.S.; Anderson, T.M.; Burke, M.D. *J. Am. Chem. Soc.* **2007**, *129*, 13804-13805.
2-1 BACKGROUND

When I first joined Prof. Marty Burke’s research group in October of 2005, he suggested that I pursue a degradative synthesis of AmB to prepare the three functional group deficient derivatives shown in Scheme 2.1: amphoteronolide B (AmdeB, 2.2), C(41)-Methyl amphoteronolide B (MeAmdeB, 2.3), and C(41)-Methyl amphotericin B (MeAmB, 2.4). The object of preparing these molecules was to enable a suite of biological and biophysical assays to illuminate the poorly understood molecular mechanism of action of AmB. As of 2005, the only complete total synthesis of AmB was the report from the Nicolaou group in 1987. These authors also prepared the aglycone of AmB, amphoteronolide B (AmdeB). Although it was a landmark achievement that has since become a classic in the field, the length and complexity of the Nicolaou synthesis made its use challenging for the preparation of mechanistic probes. Alternatively, degradative syntheses of AmdeB and MeAmB were known in the literature, but, prior to our investigations, MeAmdeB had not been prepared. Finally, MeAmB and the doubly modified 8-deoxy amphoteronolide B have been prepared via genetic engineering of the AmB producing organism S. nodosus. However, despite extensive attempts, it was not possible to obtain pure samples of MeAmB or the aglycones for biological testing.

The MeAmB synthesized by chemists at Smith-Kline Beecham through degradative chemical synthesis was reported to have a four-fold diminished minimum inhibitory concentration (MIC) relative to the natural product against C. albicans. The biological activity of the AmdeB synthesized by Nicolaou and coworkers was not determined. Therefore, to provide much needed insight into the mechanistic role(s) played by the carboxylic acid and the mycosamine sugar (See Chapter 1), we pursued modular degradative syntheses of MeAmB, AmdeB and MeAmdeB that could provide these compounds in chemically pure form and in

Scheme 2.1. Three functional group deficient derivatives were targeted for synthesis using the natural product amphotericin B as the starting material.
quantities suitable for biological and biophysical investigation.

2-2 SYNTHETIC STRATEGY

Recognizing that greater synthetic throughput and efficiency could be achieved by maximizing the number of common intermediates in the route, we designed a synthesis that consisted of four synthetic modules (Scheme 2.2). The first step would be a global protection to generate common intermediate 2.5, in part to provide a compound that was more amendable to manipulation. It had been shown previously that protected AmB derivatives were soluble in common organic solvents such as acetone and diethyl ether and could be purified by simple flash chromatography. From 2.5, we had to choose whether to first target the mycosamine or the acid for removal in order to access aglycone 2.9, en route to MeAmdeB. We decided to first implement the carboxylic acid reduction module to generate common intermediate 2.6, because we hypothesized that retention of mycosamine at C(19) would impart greater chemical stability than a secondary alcohol at this position. Continuing the path towards MeAmdeB, the carboxylic acid reduction would be followed by a deglycosylation sequence to generate aglycone 2.9, which would be globally deprotected to yield MeAmdeB 2.3. Similarly, AmdeB 2.2 could be obtained from a deglycosylation/global deprotection sequence from common intermediate 2.5. Finally,
global deprotection of common intermediate 2.6 could provide access to MeAmB 2.4. Initial efforts towards realizing this overall synthetic plan were focused on developing conditions to selectively reduce the carboxylic acid.

**2-3 DEVELOPMENT OF THE REDUCTION MODULE**

Our objective was to find mild, chemoselective conditions to reduce the carboxylic acid without simultaneously reducing the macrolactone moiety. Our first attempt to achieve this goal is shown in Scheme 2.3. First, the amine was protected as an Fmoc-carbamate, and subsequent exposure to trimethylsilyl diazomethane generated methyl ester 2.10 in 71% yield over two steps. Dehydration of hemiketal 2.10 with trimethylsilyl trifluoromethanesulfonate (TMSOTf) effectively protected the hemiketal as a C(13)-C(14) dihydropyran. However, the TMS protecting groups were not expected to be stable to the downstream chemistry, so the TMS ethers were deprotected using HF/pyridine and the resulting free alcohols were reprotected as more robust triethylsilyl (TES) ethers with triethylsilyl trifluoromethane sulfonate (TESOTf). This provided methyl ester 2.11 in 65% yield over the deprotection/reprotection sequence.

Unluckily, this methyl ester proved to be completely resistant to reduction, presumably because of steric congestion about the C(41) position. The use of more forcing conditions for this
reduction led to extensive decomposition of the starting material with loss of the Fmoc-carbamate only being observed by mass spectrometry of crude reaction mixtures.

Nicolaou reported that a methyl ester at the C(41) position could be selectively reduced in the presence of an unprotected alcohol at C(15). We therefore used this report to develop the second generation route as shown in Scheme 2.4. In stark contrast to the previous route, addition of sodium borohydride to a methanolic solution of free alcohol 2.11 rapidly reduced the methyl ester, providing the desired C(41) primary alcohol 2.15 in only 15 minutes at room temperature. Assisted delivery of the hydride to the methyl ester such as depicted by 2.14 may be responsible for the greatly enhanced reactivity of unprotected 2.11 versus silyl protected 2.12. The newly formed C(41) primary alcohol 2.15 could not, however, be site-specifically converted into a bromide, iodide or tosylate, despite extensive efforts. Therefore, to isolate the C(41) position, the sterically hindered t-butyldiphenylsilyl chloride (TBDPSCl) was employed to selectively protect the primary alcohol, giving the silyl ether in 79% yield. After this silation, the remaining secondary alcohols were orthogonally protected as acetates, giving the fully protected dihydropyan 2.16. However, all attempts to deprotect the silyl ether yielded only Ferrier rearrangement product 2.17 and not the desired silyl ether removal, despite efforts at optimization. Because the bulky TBDPS group requires relatively harsh conditions to be cleaved,

\[ \text{Scheme 2.4. (a) NaBH}_4, \text{THF:MeOH}} \text{1:3, 23 °C, 15 min, 65\%; (b) t-BuPh}_2\text{SiCl, imidazole, DMF, 0 °C, 2 h, 79\% (c) Ac}_2\text{O, DMAP, pyridine, 0 → 23 °C, 12 h, 94\%; (d) KF, 18-crown-6, THF, 5 h, yield n.d.} \]
the silyl group could not be selectively removed without the Ferrier rearrangement also occurring. Consequently, the problems associated with silicon deprotection led us to abandon this second generation route.

The second generation route showed that the C(41) carboxylate could be selectively reduced, but the presence of nine free secondary hydroxyl groups made the site-selective manipulation of the newly formed primary alcohol at C(41) extremely challenging. With this in mind, we drew from the degradative synthetic work performed by chemists at Smith-Kline Beecham, to generate a third generation route. Our strategy with this synthesis was to establish the chemical isolation of the C(41) position in the beginning of the route, thereby simplifying the chemistry downstream of the carboxylic acid reduction. As shown in Scheme 2.5, the synthesis began by converting AmB into the silyl protected intermediate 2.18 in 47% yield. This three-step sequence involved Fmoc protection of the amine, conversion of the hemiketal to a methyl ketal, and masking of the secondary alcohols as TES ethers using TESOTf. For the TESOTf step, it was critical that the reaction be run heterogeneously in hexanes because use of more polar solvents such as dichloromethane gave mixtures of the methyl ketal and the dihydropyran at

\[ \text{Scheme 2.5.} \ (a) \text{Fmoc-OSu, DMF:MeOH 2:1, 23 °C, 12 h; (b) CSA, THF:MeOH 1:1, 0 °C, 1 h, 90% over 2 steps; (c) TESOTf, 2,6 lutidine, hexanes, 0 °C, 3 h, 96%; (d) 2-pyrindyl thiochloroformate, Et,N, Et,O, 30 min, 0 °C, 91%; (e) LiBH}_4, \text{ Et,O, 23 °C, 2 h, 88%; (f) Tf}_2\text{O, pyridine, CH}_2\text{Cl}_2, -10 °C, 5 min, then Bu}_3\text{NI, 0 °C, 1 h, 26%; (g) LiEt}_3\text{BH, THF, -10 °C, 20 min, 34%.} \]
C(13)-C(14). With the reactivity of the secondary alcohols sequestered, the free acid was then selectively activated using 2-pyridyl thiochloroformate. The resulting thioester could then be reduced under mild conditions with lithium borohydride to furnish primary alcohol \(2.20\) in 88% yield. In an attempt to obviate the use of 2-pyridyl thiochloroformate, which necessarily had to be freshly prepared from phosgene and 2-mercapto pyridine prior to use, we explored isobutyl chloroformate as a more convenient activator for the acid. However, the resulting mixed anhydride simply reverted back to free acid \(2.18\) upon reaction with lithium borohydride, and so ultimately 2-pyridyl thiochloroformate was used to prime the acid for reduction.

At this point in the route, the primary alcohol at C(41) was now the only free alcohol present on intermediate \(2.20\), and so the only challenge left in the route was to simply activate the alcohol and displace the activated intermediate with an additional equivalent of hydride. Accordingly, the alcohol was primed for removal by the \textit{in situ} generation of the primary triflate using triflic anhydride, which was immediately displaced with iodide to produce primary iodide \(2.21\) in 26% yield over the two steps. Exposure of iodomethyl \(2.21\) to the nucleophilic hydride source lithium triethylborohydride (Super-Hydride\textsuperscript{®}),\textsuperscript{10} generated protected C(41)-Methyl AmB \(2.22\), thereby successfully concluding the reduction sequence.

Surveying the route post-completion, it was apparent that the final activation/reduction sequence would require significant optimization efforts to provide assay quantities of MeAmB. The yield for each reaction was poor (26% for the two-step iodide installation and 34% for the reduction) and the product of the reduction, \(2.22\), could not be obtained in pure form because of chromatographically inseparable byproducts. Therefore, a final fourth generation route was developed to improve upon these significant yield deficiencies.

\[\text{Scheme 2.6. (a) } \text{I}_2, \text{PPh}_3, \text{imidazole, THF, } 0 \text{ °C, 1 h, 78%; (b) HF/pyr, THF:pyr 5:3, 0 \rightarrow 25 \text{ °C, 6.5 h, 73%; (c) NaBH}_4, \text{DMSO, 23 °C, 8 h, 58%}.\]
The ultimate solution to the reduction module (Scheme 2.6) began with primary alcohol 2.20 (produced as shown in Scheme 2.5), which was converted into primary iodide 2.21 under the action of triphenylphosphine and iodine\textsuperscript{11} in a 78% isolated yield. This new method of iodide installation was remarkably successful and tripled the yield of the analogous two-step iodide installation in the third generation route. Next, given the poor results obtained with very aggressive reductant lithium triethylborohydride, we instead chose to use the much milder sodium borohydride, which necessitated the use of highly polar aprotic solvents to effectively reduce the carbon-iodide bond.\textsuperscript{12} When primary iodide 2.21 was reacted with sodium borohydride in 1,3 dimethyl-3,4,5,6-tetrahydro-2-(1H)-pyrimidinone (DMPU) the primary iodide was indeed reduced to a methyl group. Unfortunately, under these conditions we also observed concomitant cleavage of the Fmoc carbamate. The yield for this double reduction, despite efforts to improve the reaction, was poor and irreproducible. The alternative use of dimethyl sulfoxide (DMSO) as the solvent for the reaction was also problematic, in this case because the very non-polar TES protected 2.21 was insoluble in DMSO. To resolve this solubility issue, the TES ethers were cleaved with HF/pyridine to give primary iodide 2.23. The deprotected 2.23 was then subjected to sodium borohydride in DMSO to successfully reduce the iodide and give C(41)-Methyl AmB 2.24 in 58% yield and only two deprotection steps away from MeAmB. Thus, the goals of this fourth generation route were satisfactorily met. By chaining the method of iodine installation and using a less aggressive hydride source we were able to improve the overall yield of the carboxylic acid reduction relative to the third generation route. With the route for reduction module complete, our attention then focused on removal of mycosamine from the macrolactone.

2-4 DEVELOPMENT OF THE DEGLYCOSYLATION MODULE

Achieving the modular synthetic plan outlined in Scheme 2.2 required that the mycosamine appendage be removable in the presence of either a protected acid or an activated methylene at the C(41) position. Pioneering work done by the Nicolaou\textsuperscript{3} and Masamune\textsuperscript{13} groups demonstrated that mycosamine could be removed oxidatively to yield a heptaenone with a methyl ester at C(41) under the action of either N-bromosuccinimde (NBS)\textsuperscript{2} or DDQ.\textsuperscript{14} Oxidative conditions were used because the acid sensitivity of AmB, and even more so the resulting aglycone, precluded the use of traditional deglycosylation conditions (\textit{i.e.} strong aqueous acid).\textsuperscript{3}
The reported yield for the deglycosylation was higher with DDQ than with NBS (50% vs. 18-30%), so we began our deglycosylation studies on primary iodide 2.21 using DDQ as the oxidant.

![Scheme 2.7](image)

Scheme 2.7. (a) DDQ, CaCO₃, THF, 23 ºC, 10 min, 62%

The oxidative deglycosylation proved to be a robust reaction- 2.21 reacted cleanly with DDQ (Scheme 2.7), to provide the desired heptaenone 2.25 in 62% yield as a beautiful, rich red solid. The next step was the reduction of the newly formed carbonyl at C(19). During the degradative synthesis of AmdeB, (Scheme 2.8 and Section 2-6) we observed that the reduction of heptaenone 2.27 with sodium borohydride proceeded with excellent diastereoselectivity; only the desired diastereomer was observed. In addition, Nicolaou and coworkers had also observed a highly stereoselective reduction for a similar substrate during their degradative synthesis of AmdeB.² We were therefore confident that we would achieve a similar level of selectivity for the reduction of heptaenone 2.25. Instead we found that the reaction of 2.25 with sodium borohydride gave only a 2:1 diastereomeric ratio (d.r.) in favor of the natural 19-(R) configuration (Table 2.1). Use of the (S)-Corey-Bakshi-Shibata (CBS) oxazaborolidine catalyst,¹⁴ however, provided the desired diastereomer in a synthetically useful 6:1 d.r. Interestingly, the (R)-CBS catalyst gave an equimolar ratio of the two diastereomers, slightly overturning the inherent selectivity of this large, complex substrate (Table 2.1). The mechanism behind the large difference in stereoselectivity based upon the substituent at C(41) is not currently understood. With the unexpected stereochemical outcome in the

![Table 2.1](image)

Table 2.1 List of reagents and the corresponding diastereoselectives for the conversion of 2.25 to 2.26

The mechanism behind the large difference in stereoselectivity based upon the substituent at C(41) is not currently understood.
reduction of heptaenone 2.25, we set out to firmly establish the stereochemistry at the C(19) position using nuclear magnetic resonance (NMR) spectroscopy.

2.5 DETERMINATION OF CONFIGURATION AT C-19

Based upon the known stereochemical preference of the (S)-CBS catalyst\(^{14}\) we were confident that the major diastereomer resulting from the (S)-CBS reduction of heptaenone 2.25 was the desired 19-(R) stereoisomer, but we wanted additional verification of this assignment. For our stereochemical analysis, we compared the \(^3J\) H-H coupling constant data of the major diastereomer resulting from the reduction of heptaenone 2.25 (Scheme 2.7) to the analogous coupling constants for two reference compounds, N-Fmoc-C(41) methyl AmB 2.4 and N-acetyl AmB methyl ester (NAcAmE 2.27). The known rigidity of the AmB macrolactone, found by our own (See Section 2-7)\(^{15}\) and previous\(^{16,17}\) studies, allowing coupling constants to be accurately predicted using the Altona-Karplus equation\(^{18}\), as shown in Figure 2.1. The Altona-Karplus predicted that both the \(J_{18a-19}\) and \(J_{18e-19}\) coupling constants should be less than 8 Hz for the 19-(R) diastereomer. In contrast, for the 19-(S) stereoisomer, the \(J_{18a-19}\) coupling constant was expected to be greater than 8 Hz because of the antiperiplanar relationship of these two protons (Figure 2.1). The \(J_{18a-19}\) and \(J_{18e-19}\) coupling constants for 2.3 were therefore determined unambiguously using phase-sensitive correlation spectroscopy (COSY-PS)\(^{19}\) experiments and the Amplitude Constrained Multiplet Evaluation (ACME) program\(^{20}\) and compared to the calculated

\[
\begin{array}{c|cc}
\text{A} & \text{Calculated Coupling Constants (Hz)} & \\
& 19-(R) & 19-(S) \\
\hline
J_{18a-19} & < 7 \text{ Hz} & > 8 \text{ Hz} \\
J_{18e-19} & < 8 \text{ Hz} & < 8 \text{ Hz} \\
\end{array}
\]

\[
\begin{array}{c|ccc}
\text{B} & \text{Observed coupling constant} & \text{Hz} & \\
& 5.5 & 4.5 & 4.6 \\
\hline
J_{18a-19} & 5.5 & 4.5 & 4.6 \\
J_{18e-19} & 3.9 & 2.0 & 2.5 \\
\end{array}
\]

Table 2.2. Observed coupling constant data derived from ACME processed COSYPS data for 2.3, 2.27, and 2.29.

![Figure 2.1. A) The predicted coupling constants for the 19-(R) and 19-(S) diastereomers given by the Altona-Karplus equation. B) Perspective and Newman projections (looking down the C_{18}-C_{19} bond) of the two diastereomers.](image)

values (See Section 2-7 for more details of the NMR experiments. The final compound 2.3
was used for the stereochemical determination because the C(19) diastereomers were inseparable until the final HPLC purification, see Section 2-6 for details). Coupling constants for reference compounds C(41) methyl AmB 2.4 and NAcAmE 2.27 were also determined. As shown in Table 2.1, both of the $J_{18-19}$ coupling constants for MeAmdeB are significantly less then 8 Hz, consistent with the theoretical prediction for the 19-(R) configuration. Furthermore, the coupling constants for the reference compounds (for which the stereochemistry at C(19) was not altered) are very similar to 2.3, further confirming the assigned 19-(R) configuration.

2-6 THE SYNTHESIS OF MEAMB, AMD EB AND MEAMDEB

With the development and optimization of the deglycosylation and reduction modules complete, the synthetic plan shown in Scheme 2.9 was implemented. The synthesis began with a global protection step to give carboxylic acid 2.18, an intermediate common to all three targeted derivatives. Next, as discussed in Section 2-3, the primary alcohol was generated via the intermediacy of the activated 2-pyridyl thioester. This primary alcohol was then converted to primary iodide 2.21, a valuable intermediate that could be funneled to either MeAmdeB or MeAmB. In addition, this molecule was persistent to storage, so this material was accumulated in significant quantities. To access MeAmdeB, the mycosamine appendage was removed with DDQ as discussed previously and the resulting heptaenone was reduced with the (S)-CBS catalyst to give secondary alcohol 2.30. Unfortunately, alcohol 2.30 proved to be impersistent to even short term (2-4 weeks) storage or acidic conditions. Because of the fragility of this molecule, once the path towards MeAmdeB was initiated, the intermediates had to be taken through the entire sequence without any delay. In addition, the two stereoisomers could not be separated at this point and the undesired diastereomer was carried through the route and separated from the final product by high performance liquid chromatography (HPLC). Reduction of the primary iodide proceeded smoothly using sodium borohydride in DMPU in 78% yield, setting the stage for a final global deprotection sequence. This highly optimized protocol began with global cleavage of the TES ethers using HF/pyridine. The crude product of this reaction was then immediately taken forward to generate the hemiketal using 2:1:1 THF:H$_2$O:acetic acid. Subsequent purification by preparative HPLC completed the first synthesis of MeAmdeB, delivering the molecule in 38% yield over the final deprotection steps and >90% purity as determined by HPLC.
The synthesis of MeAmB began from the common intermediate, primary iodide 2.21. The silyl protecting groups were removed using HF/pyridine in 73% yield and the resulting
deprotected primary iodide was treated with sodium borohydride in DMSO to give C(41) methyl intermediate 2.24. Following cleavage of the iodide, the methyl ketal was deprotected with aqueous camphorsulfonic acid (CSA) the crude the hemiketal was carried forward into the final deprotection step without further purification. A portion of this intermediate was purified by reverse phase HPLC for the purpose of determining the ground state conformation of this molecule (See Section 2-7). Deprotection of the Fmoc carbamate proceeded readily using the mild amine base piperidine and the resultant material was purified by preparative HPLC to provide MeAmB in 58% yield and >90% purity over the final two steps.

The synthesis of AmdeB began with free acid 2.18 the compound common to all three syntheses. The path towards AmdeB began with the protection of the carboxylic acid as an allyl ester using allyl bromide and diisopropyl ethylamine in 86% yield. The allyl ester was subsequently treated with DDQ to cleanly remove mycosamine in 65% yield. The secondary alcohol 2.28 was formed without incident in 77% yield and greater than >20:1 d.r. using methanolic sodium borohydride as the reducing agent. Next, the TES groups were removed using HF/pyridine but initial attempts were met poor yields. Given the acid sensitivity of the conjugated allylic secondary alcohol, we suspected that the material was decomposing during silica gel chromatography and this conceit was confirmed by 2D-thin layer chromatography (TLC) analysis. Buffering the silica gel with triethylamine did not attenuate this decomposition and so the crude product necessarily had to be purified by C18 reverse phase flash chromatography. With this reverse phase purification protocol, we were able to obtain the deprotected allyl ester in an acceptable 56% yield. Next, the methyl ketal was deprotected using aqueous CSA and, remarkably, the time required for this hydrolysis to be complete was ten times longer than the equivalent transformation for MeAmB, presumably due to the electronically deactivating nature of the allyl ester. Following preparative reverse phase HPLC purification of the hydrolysis reaction, the allyl ester was removed using tetrakis(triphenyl phosphine)palladium and thiosalicylic acid.22 The crude deallylation product was also purified by preparative reverse phase HPLC to provide AmdeB in 48% yield and >90% purity as judged by HPLC.

In summary, the modular synthetic route originally proposed in Scheme 2.2 proved to be very successful in providing the desired functional group deficient derivatives. In all, 24 grams of the common intermediate 2.18 was synthesized and this material provided at least 10 mg of each of the three final products, after HPLC purification. In addition, the completion of these
syntheses accomplished the first stated goal in the research algorithm outlined in Figure 1.6, namely the synthesis of key functional group deficient AmB derivatives in quantities suitable for biophysical investigations. Before moving on to study the biological and biophysical impacts of these deletions, we had to determine of the ground state three-dimensional conformation of these derivatives. This endeavor will be described in the succeeding section.

2-7 DETERMINATION OF THE GROUND STATE CONFORMATION

Interpretation of the biological and biophysical activity of AmdeB, MeAmB and MeAmdeB could be complicated if removal of the mycosamine and/or carboxylic acid engendered changes in the three-dimensional conformation of the macrolactone. If the functional group deletions alter the conformation of the molecule then differences in activity could be ascribed to shape change, rather than a loss of an intermolecular interaction. To control for this possibility, the second objective of the functional group deletion research program is to evaluate the ground state conformation of all synthesized derivatives. Unprotected AmB, AmdeB and MeAmB were not amenable to these studies because of their poor solubility properties, so we instead used the suitably protected analogues of these molecules shown in Scheme 2.10. Importantly, it has been shown\textsuperscript{22} that covalent modifications of the acid or the amine do not affect the ground state conformation of the macrolactone. A combination of solution phase COSY-PS and nuclear Overhauser effect spectroscopy (NOESY) experiments were used to investigate the ground state conformation of the three functional group deficient derivatives and AmB. Unlike conventional COSY experiments, the COSY-PS pulse sequence can distinguish between active and passive coupling constants in the two dimensional crosspeak.\textsuperscript{24} Individual two dimensional crosspeaks are then processed using ACME, which fits these data to yield very
accurate coupling constant information. We used this process to generate a detailed map of the coupling constants for the macrolides in Scheme 2.10. These data were then converted into dihedral angles using the Altona-Karplus equation. Additional conformational constraints were obtained through two-dimensional NOESY measurements of inter-proton distances.

The coupling constant derived dihedral angles and through-space proton correlations were then used as parameters for a Monte-Carlo based energy minimization using Molecular Operating Environment (MOE)\textsuperscript{23} to perform the calculations. The results of these minimizations, as shown in Figure 2.2, clearly demonstrate that the conformation of the macrolactone is unaffected by the functional group deletions. The conformations of the four macrolides are virtually identical, with a computed RMSD of only 0.08 Å. This conservation of shape greatly facilitates interpretation of biological and biophysical data because any observed differences in activity would be the direct result of a lack of a chemical interaction, rather than deformation of molecular shape.

\textbf{Figure 2.2.} Overlay of the ground state of 2.3, 2.27, 2.29, and 2.30 as determined by solution-phase NMR studies. Only the macrolactone is shown for clarity.


2-8 SUMMARY

The initial implementation of the functional group deletion strategy required facile access to the targeted derivatives AmdeB, MeAmB and MeAmdeB. A flexible synthetic route that took advantage of common synthetic intermediates and reaction conditions was designed and implemented. Essential to the success of this synthetic plan was the discovery of conditions for the selective reduction of the C(41) carboxylic acid, and the removal of mycosamine under non-acidic conditions. It was found that the carboxylic acid could be reduced after activation as a 2-
pyridyl thioester and the mycosamine subunit could be removed by the action of DDQ. The overall synthesis of all three derivatives proved successful, and at least 10 mg of each derivative was readily synthesized. Additionally, extensive NMR data was collected and used to calculate ground state three-dimensional conformation of the three functional group deficient derivatives and the natural product. The calculations showed that the macrolide conformation is not distorted as a result of the chemical deletions, a discovery that greatly simplifies interpretation of the planned biological and biophysical experimentation that will be discussed in the succeeding chapter.
2-9 EXPERIMENTAL SECTION

Materials. Commercially available materials were purchased from Aldrich Chemical Co. (Milwaukee, WI), Fisher Scientific (Hampton, NH), and Silicycle (Quebec, Canada) and used without further purification unless noted otherwise. Amphotericin B was a generous gift from Bristol-Myers Squibb Company. All solvents were dispensed from a solvent purification system that passes solvents through packed columns according to the method of Pangborn and coworkers\textsuperscript{24} (THF, Et\textsubscript{2}O, CH\textsubscript{2}Cl\textsubscript{2} : dry neutral alumina; DMSO, DMF, CH\textsubscript{3}OH : activated molecular sieves). Hexanes, 2,6-lutidine, triethylamine, and pyridine were freshly distilled under nitrogen from CaH\textsubscript{2}. Camphorsulfonic acid was recrystallized from ethyl acetate. Water was doubly distilled or obtained from a Millipore (Billerica, MA) MilliQ water purification system.

Reactions. Due to the light and air sensitivity of amphotericin B, all manipulations were carried out under low light conditions and compounds were stored under an anaerobic atmosphere. All reactions were performed in oven- or flame-dried glassware under an atmosphere of argon unless otherwise indicated. Reactions were monitored by analytical thin layer chromatography performed using the indicated solvent on E. Merck silica gel 60 F\textsubscript{254} plates (0.25mm). Compounds were visualized using a UV (\textlambda\textsubscript{254}) lamp or stained by an acidic solution of p-anisaldehyde. Alternatively, reactions were monitored by RP-HPLC using an Agilent 1100 Series HPLC system equipped with a Sunfire\textsuperscript{TM} C\textsubscript{18} 5 micron 10 x 250 mm column (Waters Corp. Milford, MA) with UV detection at 406 nm and the indicated eluent and flow rate.

Purification and Analysis. Flash chromatography was performed as described by Still and coworkers\textsuperscript{25} using the indicated solvent on E. Merck silica gel 60 230-400 mesh or on Silicycle 17% carbon C\textsubscript{18} 230-400 mesh reverse phase silica gel. \textsuperscript{1}H NMR spectra were recorded at 23 °C on a Varian Unity Inova Narrow Bore spectrometer operating at a \textsuperscript{1}H frequency of 500 MHz with a Varian 5 mm \textsuperscript{1}H\{\textsuperscript{13}C/\textsuperscript{15}N\} pulsed-field gradient Z probe or a Varian Unity Inova spectrometer operating at a \textsuperscript{1}H frequency of 600 MHz with a Varian 5 mm \textsuperscript{1}H\{\textsuperscript{13}C/\textsuperscript{15}N\} pulsed-field gradient X,Y,Z probe. Chemical shifts (\textdelta) are reported in parts per million (ppm) downfield from tetramethylsilane and referenced internally to the residual protium in the NMR solvent (CHD\textsubscript{2}OD, \textdelta = 3.31, center line, CD\textsubscript{3}C(O)CHD\textsubscript{2}, \textdelta = 2.05, center line) or to added tetramethylsilane. Data are reported as follows: chemical shift, multiplicity (s = singlet, d =
doublet, t = triplet, dd = doublet of doublets, m = multiplet, b = broad, app = apparent), coupling constant \( J \) in Hertz (Hz) and integration. For compounds S5 (a more soluble derivative of 1), 2, 20 (a more soluble derivative of 3), and 22 (a more soluble derivative of 4), proton and coupling constant assignments were made using a variety of two-dimensional NMR techniques including phase-sensitive COSY experiments combined with amplitude constrained multiplet evaluation (ACME)\(^{20}\) (see Section III for a detailed discussion). \(^{13}\)C spectra were recorded at 23 °C with a Varian Unity Inova spectrometer operating at a \(^{13}\)C frequency of 125 MHz with a 5 mm Nalorac gradient \({^{13}\text{C}}/^{15}\text{N}\) \(^1\)H quad probe or a Varian Unity Inova spectrometer operating at a \(^{13}\)C frequency of 150 MHz and equipped with a Varian 5 mm 600 DB Auto X probe. Chemical shifts (\(\delta\)) are reported downfield of tetramethylsilane and are referenced to the carbon resonances in the NMR solvent (CD\(_3\)OD, \(\delta = 49.0\), center line, CD\(_3\)C(O)CD\(_3\), \(\delta = 29.8\), center line) or to added tetramethylsilane (\(\delta = 0.00\)). MS analysis was performed with an Applied Biosystems Micromass Ultima system with ESI ionization. High resolution mass spectra (HRMS) were obtained at the University of Illinois mass spectrometry facility. All synthesized compounds (2-4 and 7-22) gave HRMS within 5 ppm of the calculated values. The purity of amphotericin B and its derivatives was determined by HPLC analysis using a Waters SunFire Prep C\(_{18}\) OBD 5 micron 30 x 150 mm Lot # 168I161701 column with detection at 406 nm and an eluent of acetonitrile and aqueous ammonium acetate unless otherwise indicated.

Part 1. Synthesis of AmB Derivatives

Methyl ketal 2.31
A round bottom flask was charged with amphotericin B (1.5 g, \(\sim 55\%\) pure, \(ca. 0.891 \text{ mmol, 1 eq}\)) and Fmoc-succinimide (0.840 g, 2.48 mmol, 2.8 eq) which were dissolved in a mixture of DMF:MeOH 2:1 (105 mL) at 23 °C. Pyridine (0.84 mL, 10.22 mmol, 11.5 eq) was subsequently added and the reaction was stirred for 12 hours. The reaction mixture was then poured into
diethyl ether (1.8 L) stirring at 0 °C. After stirring for 30 minutes at 0 °C the resulting yellow precipitate was isolated via Büchner filtration using Whatman 50 filter paper to afford a yellow solid. The residual solvent was removed by coevaporating with acetonitrile (3 x 20 mL) and storing under vacuum for one hour. The resulting powder (1.69 g, ca. 0.8 mmol) was dissolved in THF:MeOH 1:1 (50 mL) and cooled to 0 °C. To this solution was added camphorsulfonic acid (0.100 g, 0.438 mmol, 0.55 eq) and the resulting mixture was stirred for 1 hour at 0 °C. The reaction was then quenched at 0 °C with triethylamine (0.06 mL, 0.438 mmol, 0.55 eq) and gravity filtered. The filtrate was concentrated in vacuo until a yellow solid began to precipitate. The resulting supersaturated solution was poured into hexanes:diethyl ether 1:1 (800 mL) and the yellow precipitate was collected via Büchner filtration and washed with ethyl acetate:diethyl ether 1:1 (200 mL) to yield 2.31 as a yellow solid (1.33 g, ~70% purity, ca. 0.80 mmol, ca. 90% over two steps). This material was carried forward without further purification.

![Chemical structure](image)

TLC (CH₂Cl₂:MeOH 5:1)

Rᵓ = 0.15, stained by anisaldehyde

HPLC

tR = 13.2 min; flow rate = 4 mL/min, gradient of 5 → 95% MeCN in 5 mM ammonium acetate over 15 min.

¹H NMR (500 MHz, pyridine d-5:CD₃OD 10:1)

δ 7.86 (d, J = 7.5 Hz, 2H), 7.73 (t, J = 8 Hz, 2H), 7.43 (t, J = 7.5 Hz, 2H), 7.28 (dd, J = 2, 8 Hz, 2H), 6.59-6.34 (m, 12H), 6.23 (dd, J = 6.5, 14 Hz, 1H), 5.66 (dd, J = 10, 15 Hz, 1H), 4.97 (bs, 1H), 4.92 (bs, 1H), 4.69 (bs, 1H), 4.54 (app t, J = 9.5 Hz, 1H), 4.52-4.31 (m, 4H) 4.31 (bs, 1H), 4.26-4.20 (m, 3H), 4.07-4.01 (m, 2H), 3.87 (app dd, J = 7.5, 16.5 Hz, 2H), 3.67-3.63 (m, 2H), 3.58 (app d, 10.5 Hz, 1H) 3.48 (app d, J = 8.5 Hz, 1H), 3.28
(s, 3H), 2.77-2.54 (m, 5H), 2.45 (dd, J = 3.5, 16.5 Hz, 1H), 2.08-2.00 (m, 4H), 1.88-1.72 (m, 7H), 1.68-1.64 (app d, J = 14 Hz, 1H), 1.52 (d, J = 6 Hz, 3H), 1.39 (d, J = 6.5 Hz, 3H), 1.29 (d, J = 6.5 Hz, 3H), 1.20 (d, J = 7 Hz, 3H).

13C NMR (125 MHz, pyridine d-5:CD3OD 10:1)
δ 171.7, 158.0, 144.8, 144.7, 141.8, 137.3, 134.6, 134.5, 134.3, 134.0, 133.4, 133.3, 133.2, 133.1, 132.8, 132.5, 132.2, 128.2, 127.6, 125.9, 125.8, 120.4, 101.9, 99.1, 78.1, 75.4, 74.6, 74.5, 71.8, 71.6, 71.1, 70.7, 68.2, 67.7, 67.2, 66.8, 58.1, 46.3, 44.6, 43.5, 43.1, 42.7, 42.5, 41.5, 41.3, 39.3, 36.1, 34.1, 30.8, 30.5, 30.1, 29.7, 29.3, 24.9, 24.2, 23.3, 18.7, 18.4, 17.4, 14.1, 14.0, 12.2, 11.0.

HRMS (ESI)
calculated for C63H85NO19 (M + Na)⁺: 1182.5614
found: 1182.5608

Nonatriethylsilyl ether 2.18
Prior to the reaction 2.31, was azeotropically dried via coevaporation with acetonitrile (3 x 20 mL) and was left under vacuum for a minimum of eight hours. The resulting yellow powder (3.9 g, ~ 70% purity, ca. 2.35 mmol, 1 eq) was suspended in hexanes (110 mL). 2,6-lutidine (7.0 mL, 60.5 mmol, 26 eq) was added and the resulting suspension was then cooled to 0 °C. Triethylsilyl triflate (10.6 mL, 47.0 mmol, 20 eq) was added dropwise over 10 minutes and the resulting yellow suspension was stirred for 2 hours at 0 °C. Additional 2,6-lutidine (1.8 mL, 18.3 mmol, 6.5 eq) was then added followed by additional triethylsilyl triflate (2.5 mL, 11.1 mmol, 5 eq) dropwise over 5 minutes. After 15 minutes of stirring additional 2,6-lutidine (1.8 mL, 18.3 mmol, 6.5 eq) was added followed by additional triethylsilyl triflate (2.5 mL, 11.1 mmol, 5 eq)
dropwise over 5 minutes. The mixture was stirred at 0 °C for 1 hour following completion of the final addition and was then quenched at 0 °C with saturated aqueous sodium bicarbonate (250 mL). The resulting emulsion was diluted with diethyl ether (500 mL) and the layers were separated. The organic phase was washed with saturated aqueous sodium bicarbonate (1 x 100 mL) and water (3 x 50 mL), and the combined aqueous washings were back-extracted with diethyl ether (3 x 100 mL). The combined organic phases were then washed with 1M aqueous copper sulfate (10 x 25 mL). The combined copper sulfate layers were then back-extracted with diethyl ether (5 x 50 mL). The combined organic layers were washed with water (3 x 50 mL) and brine (1 x 50 mL). This second set of aqueous washings was back extracted with diethyl ether (3 x 50 mL) and the combined organic layers were dried over sodium sulfate and concentrated in vacuo. Purification of the crude yellow oil by flash chromatography (SiO₂; hexanes:diethyl ether 20:1 → 7:1) furnished 2.18 as an orange solid (4.94 g, 2.26 mmol, 96%).

![Structural formula](image)

2.18

P = Triethylsilyl

TLC (hexanes:diethyl ether 2:1)

R_f = 0.38, visualized by UV

\(^1\)H NMR (500 MHz, acetone d-6)

\[\delta = 7.90\ (d, J = 7.5\ Hz, 2H), 7.72\ (d, J = 7\ Hz, 2H), 7.45\ (t, J = 7.5\ Hz, 2H), 7.37\ (t, J = 7.5\ Hz, 2H)\]

\[6.63-6.56\ (m, 3H), 6.49-6.14\ (m, 9H)\]

\[5.98\ (dd, J = 5.5, 15.5\ Hz, 1H), 5.52\ (dd, J = 10, 15\ Hz, 1H), 5.42\ (d, J = 10\ Hz, 1H), 4.74\ (bs, 1H), 4.67\ (bs, 1H), 4.57\ (s, 1H), 4.54-4.45\ (m, 2H), 4.37\ (dd, J = 6.5, 10\ Hz, 1H), 4.28\ (app t, J = 6\ Hz, 2H), 4.17\ (t, J = 10\ Hz, 1H), 4.02\ (app t, J = 9\ Hz, 2H), 3.92\ (d, J = 2.5\ Hz, 1H), 3.89\ (app d, J = 8.5\ Hz, 1H), 3.73\ (bs, 1H), 3.65-3.60\ (m, 2H), 3.48\ (app t, J = 9\ Hz, 2H), 3.33\ (dd, J = 6.5, 8.5\ Hz, 1H), 3.16\ (s, 4H), 2.62\ (d, J = 7\ Hz, 1H)\]

\[2.51-2.46\ (m, 2H), 2.35\ (t, J = 10.5\ Hz, 1H)\]

\[2.17\ (dd, J = 7, 15\ Hz, 1H), 2.04\ (dd, 7.5, 15.5\ Hz, 1H), 1.97-1.62\ (m, 10H), 1.56-1.52\]
(m, 1H) 1.27 (d, J = 6.5 Hz, 3H), 1.20 (d, J = 6 Hz, 3H), 1.11-0.92 (m, 87H), 0.82-0.59 (m, 54H).

\(^{13}\)C NMR (125 MHz, acetone \(d\-6\))

\[\delta 174.3, 170.5, 158.2, 156.4, 145.0, 142.2, 139.5, 137.2, 135.8, 135.6, 135.2, 134.5, 134.2, 134.0, 132.4, 132.3, 132.1, 132.0, 131.2, 130.6, 128.5, 127.9, 127.8, 125.8, 125.7, 120.8, 120.6, 101.5, 98.0, 76.8, 75.0, 74.5, 74.1, 73.5, 73.4, 71.1, 68.9, 67.5, 67.3, 67.2, 58.1, 57.5, 48.1, 48.0, 44.3, 43.4, 42.2, 41.5, 40.7, 35.6, 27.6, 24.4, 20.0, 19.3, 18.9, 11.1, 7.65, 7.61, 7.51, 7.49, 7.34, 7.30, 7.27, 7.15, 6.37, 6.18, 5.88, 5.87, 5.83, 5.78, 5.76, 5.63.\]

HRMS (ESI)
calculated for \(C_{117}H_{211}O_{19}N_{19}Si_9\) (M + Na): 2209.3397
found: 2209.3303

2-pyridylthioester 2.32

To a stirred solution of 2.18 (4.9 g, 2.24 mmol, 1 eq) in diethyl ether (90 mL) at 0 °C was added triethylamine (0.40 mL, 2.91 mmol, 1.3 eq). 2-thiopyridyl chloroformate (4 mL, 4 mmol, 1.8 eq, 1M in \(CH_2Cl_2\)) was added and the solution was stirred for 30 minutes at 0 °C. The formation of a precipitate was observed as the reaction progressed. The mixture was then diluted with diethyl ether (200 mL) and the solids were removed via Büchner filtration using Whatman 50 filter paper. The filtrate was concentrated \textit{in vacuo} to give crude 2.32 as a yellow solid. Purification by flash chromatography (SiO\(_2\); hexanes:diethyl ether 10:1 \(\rightarrow\) 3:1) afforded 2.32 as an orange solid (4.65 g, 2.04 mmol, 91%).
TLC (hexanes:diethyl ether 2:1)

R_f = 0.76, visualized by UV

^1^H NMR (500 MHz, acetone d-6)

\[ \delta \ 8.71 \ (d, \ J = 3.5 \ Hz, \ 1H), \ 7.94 \ (dt, \ J = 1.5, 7.5 \ Hz, \ 1H), \ 7.86 \ (d, \ J = 7.5 \ Hz, \ 2H), \ 7.73-7.68 \ (m, \ 3H), \ 7.43-7.31 \ (m, \ 5H), \ 6.59-6.52 \ (m, \ 3H), \ 6.46-6.11 \ (m, \ 9H) \ 5.97 \ (dd, \ J = 5.5, 16 \ Hz, \ 1H), \ 5.49 \ (dd, \ J = 9.5, 15 \ Hz, \ 1H), \ 5.28 \ (d, \ J = 10 \ Hz, \ 1H), \ 4.78 \ (bs, \ 1H), \ 4.65 \ (bs, \ 1H), \ 4.62 \ (s, \ 1H), \ 4.52-4.43 \ (m, \ 3H), \ 4.33 \ (dd, \ J = 7, 10.5 \ Hz, \ 1H), \ 4.23 \ (app \ t, \ J = 6.5 \ Hz, \ 2H), \ 4.14 \ (t, \ J = 10 \ Hz, \ 1H), \ 4.06 \ (t, \ J = 9.0 \ Hz, \ 1H), \ 4.00 \ (bs, \ 1H), \ 3.91 \ (d, \ J = 2.5 \ Hz, \ 1H), \ 3.85 \ (dd, \ J = 2, 8.5 \ Hz, \ 1H), \ 3.70-3.66 \ (m, \ 3H), \ 3.61 \ (dd, \ J = 4, 10.5 \ Hz, \ 1H), \ 3.44-3.37 \ (m, \ 2H), \ 3.13 \ (s, \ 3H), \ 2.71 \ (t, \ J = 10 \ Hz, \ 1H), \ 2.58 \ (d, \ J = 6.5 \ Hz, \ 1H), \ 2.43 \ (app \ dd, \ J = 7, 9 \ Hz, \ 1H), \ 2.32 \ (dd, \ J = 6.5, 15 \ Hz, \ 1H) \ 2.12-2.08 \ (m, \ 2H), \ 1.93-1.83 \ (m, \ 3H), \ 1.79-1.62 \ (m, \ 7H), \ 1.53-1.50 \ (m, \ 1H), \ 1.22 \ (d, \ J = 5.5 \ Hz, \ 3H), \ 1.17 \ (d, \ J = 6 \ Hz, \ 3H), \ 1.10-0.90 \ (m, \ 87H), \ 0.81-0.56 \ (m, \ 54H). \]

^13^C NMR (125 MHz, acetone d-6)

\[ \delta \ 197.5, \ 169.9, \ 163.5, \ 155.6, \ 151.5, \ 151.0, \ 144.5, \ 141.6, \ 138.3, \ 137.8, \ 135.1, \ 135.0, \ 134.5, \ 133.7, \ 133.3, \ 131.9, \ 131.8, \ 131.6, \ 131.3, \ 130.7, \ 130.0, \ 129.5, \ 127.8, \ 127.2, \ 127.1, \ 125.2, \ 125.1, \ 124.1, \ 120.1, \ 100.7, \ 97.2, \ 76.0, \ 74.0, \ 73.7, \ 73.6, \ 73.4, \ 72.8, \ 72.7, \ 70.5, \ 68.5, \ 67.2, \ 66.8, \ 66.7, \ 66.6, \ 64.8, \ 57.2, \ 47.6, \ 47.4, \ 43.5, \ 42.7, \ 42.0, \ 40.9, \ 40.1, \ 35.0, \ 34.7, \ 26.9, \ 19.3, \ 18.7, \ 18.3, \ 10.5, \ 7.01, \ 6.89, \ 6.88, \ 6.72, \ 6.68, \ 6.65, \ 6.59, \ 5.80, \ 5.56, \ 5.29, \ 5.28, \ 5.24, \ 5.21, \ 5.16, \ 5.02. \]
Primary alcohol 2.20
To a stirred solution of 2.32 (4.6 g, 2.02 mmol, 1 eq) in diethyl ether (100 mL) at 23 °C was added dropwise a solution of lithium borohydride in THF (2M, 10 mL, 20 mmol, 10 eq). The solution was stirred for 2 hours and then cooled to 0 °C and quenched by addition over 5 minutes of saturated aqueous ammonium chloride (100 mL). The two layers were separated and the organic phase was diluted with diethyl ether (100 mL). The organic phase was washed with saturated ammonium chloride (1 x 20 mL), water (3 x 20 mL) and brine (1 x 20 mL). The combined aqueous washings were back-extracted with diethyl ether (1 x 50 mL) and the combined organic layers were dried over sodium sulfate and concentrated *in vacuo*. Purification of the resulting residue by flash chromatography (SiO$_2$; hexanes:diethyl ether 20:1 → 5:1) furnished 2.20 as a yellow solid (3.87 g, 1.78 mmol, 88%).

TLC (hexanes:diethyl ether 1:1)

R$_f$ = 0.67, visualized by UV
\(^{1}\)H NMR (500 MHz, acetone \(d-6\))
\[\delta 7.86 (d, J = 8 \text{ Hz}, 2\text{H}), \ 7.69 (d, J = 7.5 \text{ Hz}, 2\text{H}), \ 7.42 (t, J = 7.5 \text{ Hz}, 2\text{H}), \ 7.33 (t, J = 7.5 \text{ Hz}, 2\text{H}), \ 6.53-6.10 (m, 12\text{H}), \ 6.05 (dd, J = 6.5, 15.5 \text{ Hz}, 1\text{H}), \ 5.50 (dd, J = 9.5, 15 \text{ Hz}, 1\text{H}), \ 5.32 (d, J = 10 \text{ Hz}, 1\text{H}) \ 4.77 (s, 1\text{H}), \ 4.68 (app t, J = 6 \text{ Hz}, 2\text{H}), \ 4.46 (dd, J = 6.5, 10.5 \text{ Hz}, 1\text{H}), \ 4.32 (dd, J = 6.5, 10.5 \text{ Hz}, 1\text{H}), \ 4.23 (app t, J = 6.5 \text{ Hz}, 2\text{H}), \ 4.18 (dt, J = 5, 10.5 \text{ Hz}, 1\text{H}), \ 4.11 (t, J = 10.5 \text{ Hz}, 1\text{H}), \ 3.99 (m, 1\text{H}), \ 3.91 (d, J = 3 \text{ Hz}, 2\text{H}), \ 3.86-380 (m, 4\text{H}), \ 3.70-3.67 (m, 2\text{H}), \ 3.65-3.59 (m, 3\text{H}), \ 3.46 (t, J = 9 \text{ Hz}, 1\text{H}), \ 3.34 (app dd, J = 6.5, 8.5 \text{ Hz}, 1\text{H}), \ 3.11 (s, 3\text{H}), \ 2.56-2.54 (m, 2\text{H}), \ 2.45-2.38 (m, 2\text{H}), \ 2.10 (dd, J = 4.5, 12.5 \text{ Hz}, 1\text{H}), \ 2.02-2.00 (m, 1\text{H}), \ 1.94-1.61 (m, 10\text{H}), \ 1.52-1.49 (m, 1\text{H}), \ 1.24 (d, J = 6.5 \text{ Hz}, 3\text{H}), \ 1.17 (d, J = 6 \text{ Hz}, 3\text{H}), \ 1.06-0.890 (m, 87\text{H}), \ 0.77-0.57 (m, 54\text{H}).\]

\(^{13}\)C NMR (125 MHz, acetone \(d-6\))
\[\delta 170.0, \ 155.8, \ 144.4, \ 141.6, \ 138.6, \ 135.2, \ 134.7, \ 134.1, \ 133.3, \ 132.5, \ 132.4, \ 132.2, \ 131.9, \ 130.8, \ 130.4, \ 130.1, \ 127.9, \ 127.3, \ 127.2, \ 125.2, \ 125.1, \ 120.2, \ 100.4, \ 97.3, \ 92.4, \ 76.1, \ 75.0, \ 73.8, \ 73.4, \ 72.8, \ 70.4, \ 67.4, \ 66.9, \ 66.7, \ 66.6, \ 66.1, \ 58.4, \ 57.5, \ 49.6, \ 47.3, \ 47.1, \ 44.0, \ 43.4, \ 42.7, \ 40.8, \ 35.1, \ 26.8, \ 19.3, \ 18.3, \ 7.01, \ 6.88, \ 6.72, \ 6.68, \ 5.72, \ 5.51, \ 5.23, \ 5.16, \ 5.12, \ 4.99.\]

HRMS (ESI)

Calculated for \(C_{117}H_{213}NO_{18}Si_{9}\) (M + Na): 2195.3604

Found: 2195.3503
Iodomethyl 2.21

Prior to the reaction, 2.20 was azeotropically dried via coevaporation with benzene (3 x 25 mL) and left under vacuum for a minimum of eight hours. The resulting yellow solid (3.8 g, 1.74 mmol, 1 eq) was dissolved in THF (60 mL) and cooled 0 °C. To this solution was added imidazole (0.355 g, 5.22 mmol, 3 eq), triphenyl phosphine (0.912 g, 3.48 mmol, 2 eq), and iodine (0.880 g, 3.48 mmol, 2 eq). The resulting brown solution was stirred for 1 hour at 0 °C and then quenched with the addition of saturated aqueous sodium bisulfite (50 mL). The two phases were separated and the organic layer was diluted with diethyl ether (50 mL). The organic layer was washed with saturated aqueous sodium bisulfite (1 x 20 mL), water (3 x 20 mL), and brine (1 x 20 mL). The combined aqueous washings were back-extracted with diethyl ether (1 x 20 mL). The combined organic layers were dried over sodium sulfate and concentrated in vacuo.

Purification of the resulting residue by flash chromatography (SiO$_2$; hexanes:diethyl ether 20:1 → 7:1) furnished 2.21 as an orange solid (3.10 g, 1.36 mmol, 78%) and recovered 2.20 as a yellow solid (0.545 g, 0.251 mmol, 14%).

\[
\text{TLC (hexanes:diethyl ether 2:1)}
\]
\[
R_f = 0.79, \text{ visualized by UV}
\]
\(^1\)H NMR (500 MHz, acetone \(d-6\))
\[\delta\]
- 7.86 (d, \(J = 7.5\) Hz, 2H), 7.69 (d, \(J = 6.5\) Hz, 2H), 7.41 (t, \(J = 7\) Hz, 2H), 7.32 (t, \(J = 7.5\) Hz, 2H), 6.54-6.10 (m, 12H), 6.04 (dd, \(J = 6\), 15.5 Hz, 1H), 5.50 (dd, \(J = 9.5\), 14.5 Hz, 1H), 5.30 (d, \(J = 10\) Hz, 1H), 4.87 (s, 1H) 4.72 (bs, 1H), 4.67 (bs, 1H), 4.46 (dd, \(J = 6.5\), 10.5 Hz, 1H), 4.34 (dd, \(J = 6.5\), 10.5 Hz, 1H), 4.22 (app t, \(J = 6\) Hz, 2H), 4.14-4.04 (m, 3H), 4.00 (bs, 1H) 3.84 (app d, \(J = 7\) Hz, 1H), 3.76 (t, \(J = 9\) Hz, 1H), 3.76-3.69 (m, 3H) 3.63-3.61 (m, 2H), 3.50 (app d, \(J = 8\) Hz, 1H), 3.46 (t, 9.5 Hz, 1H), 3.41-3.37 (m, 2H), 3.14 (s, 3H), 2.57 (bs, 2H), 2.43 (app dd, \(J = 9\), 15 Hz, 1H), 2.27 (dd, \(J = 7.5\), 14.5 Hz, 1H), 2.16 (dd, \(J = 5\), 12.5 Hz, 1H), 1.93-1.86 (m, 3H), 1.81-1.74 (m, 6H) 1.65-1.61 (m, 2H), 1.49 (bs, 1H), 1.25 (d, \(J = 6\) Hz, 3H), 1.16 (d, \(J = 6\) Hz, 3H), 1.06-0.86 (m, 87H), 0.77-0.55 (m, 54H).

\(^{13}\)C NMR (125 MHz, acetone \(d-6\))
\[\delta\]
- 170.0, 155.7, 144.7, 144.4, 141.6, 138.5, 135.1, 134.6, 134.5, 134.0, 133.2, 132.4, 132.3, 132.0, 130.9, 130.2, 127.9, 127.2, 125.2, 125.1, 120.2, 100.4, 97.3, 76.1, 74.0, 73.4, 72.8, 70.5, 69.9, 68.3, 66.9, 66.7, 66.5, 57.5, 47.4, 47.2, 45.9, 43.8, 42.8, 42.0, 40.6, 40.3, 35.1, 33.5, 26.8, 19.3, 18.6, 18.3, 10.7, 9.23, 7.04, 7.03, 6.92, 6.91, 6.79, 6.77, 6.73, 6.69, 5.80, 5.55, 5.48, 5.31, 5.28, 5.24, 5.20, 5.17.

HRMS (ESI)
- calculated for \(C_{117}H_{212}INO_{17}Si_9\) (M + Na): 2305.2621
- found: 2305.2617
Heptaenone 2.25

Prior to the reaction, calcium carbonate was dried via storage under vacuum for a minimum of eight hours in the presence phosphorus pentoxide desiccant. Also, 2.21 was azeotropically dried via coevaporation with benzene (3 x 25 mL) and left under vacuum for a minimum of eight hours. The resulting orange solid (2.45 g, 1.07 mmol, 1 eq) was dissolved in THF (50 mL) at 23 °C and calcium carbonate (1.07 g, 10.7 mmol, 10 eq) was added. DDQ (0.364 g, 1.61 mmol, 1.5 eq) was added and the reaction mixture immediately transitioned to a dark red color. The mixture was stirred for 10 minutes and then quenched with saturated aqueous sodium bicarbonate (250 mL). The resulting red emulsion was extracted with dichloromethane (10 x 100 mL). The combined organic extracts were washed with brine (1 x 100 mL), dried over sodium sulfate, and concentrated in vacuo. Purification of the resulting residue by flash chromatography (SiO$_2$; hexanes:diethyl ether 20:1 → 9:1) afforded 2.25 as a deep red solid (1.21 g, 0.717 mmol, 67%).

TLC (hexanes:diethyl ether 2:1)

$R_f = 0.48$, visualized by eye

$^1$H NMR (500 MHz, acetone $d$-6)

$\delta$ 7.84 (dd, $J = 11.5$, 16 Hz, 1H), 7.11 (dd, $J = 11.5$, 14.5 Hz, 1H), 6.80 (dd, $J = 11$, 15 Hz, 1H), 6.61-6.54 (m, 2H), 6.49-6.14 (m, 7H), 6.08 (d, $J = 15.5$ Hz, 1H), 5.52 (dd, $J =$
9.5, 15 Hz, 1H), 4.47-4.44 (m, 1H), 4.27 (app t, J = 10.5 Hz, 1H), 4.16-4.07 (m, 3H), 3.96 (dd, J = 3, 9 Hz, 1H), 3.75-3.63 (m, 4H), 3.46 (dd, J = 3, 10.5 Hz, 1H), 3.01 (dd, J = 10.5, 12 Hz, 1H), 2.90 (s, 3H), 2.66 (dd, J = 4, 18 Hz, 1H), 2.60 (dd, J = 9.5, 18 Hz, 1H) 2.47-2.42 (m, 2H), 2.26 (app t, J = 10.5 Hz, 1H), 2.10 (dd, J = 5, 12 Hz, 1H), 2.00 (app t, J = 10.5 Hz, 1H), 1.93-1.72 (m, 8H), 1.65 (app d, J =12.5 Hz, 1H), 1.47 (app t, J = 10.5 Hz, 1H), 1.15 (d, J = 6 Hz, 3H), 1.11 (t, J = 8 Hz, 10H), 1.05-0.96 (m, 51H), 0.89 (t, J = 7.5 Hz, 9H), 0.84 (q, J = 8 Hz, 6H), 0.76-0.51 (m, 36H).

^{13}C NMR (125 MHz, acetone d-6)
δ 199.4, 169.4, 148.1, 142.8, 140.2, 139.4, 138.6, 137.6, 136.5, 131.5, 131.1, 130.9, 130.4, 130.3, 130.2, 129.6, 100.4, 76.1, 73.5, 73.0, 72.9, 70.9, 68.2, 67.2, 66.4, 47.8, 46.5, 46.4, 43.2, 42.7, 42.6, 40.7, 40.3, 36.5, 34.6, 27.2, 24.6, 23.2, 19.5, 19.1, 7.11, 6.90, 6.84, 6.67, 6.62, 5.94, 5.50, 5.38, 5.23, 5.21, 5.08, 4.97.

HRMS (ESI)
calculated for C_{84}H_{161}O_{12}Si_{7}(M + Na)^{+}: 1707.9316
found: 1707.9270

Allylic alcohol 2.26
Prior to the reaction, 2.25 was azeotropically dried via coevaporation with benzene (3 x 10 mL) and was left under vacuum for a minimum of eight hours. To a stirred solution of borane dimethyl sulfide complex (27 µL, 0.270 mmol, 1.2 eq) and (S)-2-methyl-CBS-oxazaborolidine (0.225 mL, 0.225 mmol, 1 eq, 1M in toluene) in dichloromethane (1 mL) at -10 °C was added 2.25 (380 mg, 0.225 mmol, 1 eq) dropwise as a solution in dichloromethane (6.5 mL). The resulting solution was stirred for 30 minutes at -10 °C and during this time a color change from
deep red to pale orange was observed. The reaction was quenched at -10 °C with saturated aqueous ammonium chloride (10 mL) and diluted with dichloromethane (50 mL). The two layers were separated and the organic layer was washed with saturated aqueous ammonium chloride (1 x 20 mL), water (3 x 20 mL), and brine (1 x 20 mL). The combined aqueous washings were back-extracted with dichloromethane (1 x 50 mL). The combined organic extracts were dried over sodium sulfate and concentrated in vacuo. Purification of the resulting residue by flash chromatography (SiO₂; hexanes:diethyl ether 19:1 → 6:1) furnished 2.26 (300 mg, 0.177 mmol, 6:1 d.r., 79%).

\[ \text{P = Triethylsilyl} \]

TLC (hexanes:diethyl ether 2:1)

\[ R_f = 0.35, \text{stained by anisaldehyde} \]

\(^1\)H NMR (500 MHz, acetone \(d\)-6)

\[ \delta 6.48-6.10 \text{ (m, 13H), 5.53 (dd, } J = 9.5, 15 \text{ Hz, 1H), 4.71 (bs, 1H), 4.52 (bs, 1H), 4.25 (bs, 1H), 4.10 (t, } J = 10 \text{ Hz, 1H), 4.06-3.98 (m, 2H), 3.88 (app d, } J = 1.5, 1H) 3.80 (app t, } J = 9 \text{ Hz, 2H), 3.68-3.61 (m, 4H), 3.37 (app d, } J = 10 \text{ Hz, 1H), 3.13 (s, 3H), 2.55 (t, } J = 6 \text{ Hz, 2H), 2.42 (app dd, } J = 7, 14 \text{ Hz, 1H), 2.17 (dd, } J = 5, 10.5 \text{ Hz, 1H), 1.99-1.84 (m, 5H), 1.78-1.69 (m, 5H), 1.62-1.61 (m, 3H), 1.53-1.47 (m, 1H), 1.17 (d, } J = 6 \text{ Hz, 3H), 1.06-0.95 (m, 69H), 0.75-0.62 (m, 42H).} \]

\(^13\)C NMR (125 MHz, acetone \(d\)-6)

\[ \delta 173.6, 142.7, 141.7, 137.9, 137.7, 136.9, 136.3, 136.0, 135.9, 135.6, 135.5, 134.5, 134.4, 133.8, 130.7, 104.0, 79.6, 77.0, 76.1, 73.9, 73.4, 72.0, 71.9, 70.4, 70.3, 50.9, 50.8, 50.6, 49.1, 47.5, 46.4, 45.9, 44.2, 43.9, 42.4, 40.1, 38.6, 30.3, 28.2, 22.7, 22.0, 14.2, 12.5, 10.6, 10.4, 10.2, 9.32, 9.09, 9.03, 8.82, 8.75, 8.73.} \]
HRMS (ESI)
calculated for C_{84}H_{163}O_{12}ISi_{7} (M + Na)^+: 1709.9472
found: 1709.9456

C(41)-methyl 2.33
Prior to the reaction, 2.26 was azeotropically dried via coevaporation with benzene (3 x 25 mL) and left under vacuum for a minimum of eight hours. The resulting orange solid (775 mg, 0.459 mmol, 1 eq) was dissolved in DMPU (15 mL) at 23 °C and sodium borohydride (87 mg, 2.3 mmol, 5 eq) was added. The solution was stirred for 6 hours and was then quenched with saturated aqueous ammonium chloride (5 mL). The resulting yellow emulsion was diluted with hexanes (50 mL) and the mixture washed with water (5 x 10 mL) and brine (1 x 10 mL). The combined aqueous washings were back-extracted with hexanes (25 mL). The combined organic extracts were dried over sodium sulfate and concentrated in vacuo. Purification of the resulting residue by flash chromatography (SiO_{2}; hexanes:diethyl ether 20:1→10:1) yielded 2.33 as an orange solid (557 mg, 0.356 mmol, 78%).

TLC (hexanes:diethyl ether, 2:1)
R_f = 0.28, stained by anisaldehyde
\(^1\)H NMR (500 MHz, acetone d-6)
\[ \delta 6.49-6.10 \text{ (m, 13H), 5.52 (dd, } J = 9.5, 15 \text{ Hz, 1H), 4.71 (app t, } J = 6.5 \text{ Hz, 1H), 4.47 (bs, 1H), 4.28-4.24 \text{ (m, 1H), 4.10 (t, } J = 10 \text{ Hz, 1H), 4.00-3.97 \text{ (m, 1H), 3.85 (d, } J = 4 \text{ Hz, 1H), 3.82 (dd, } J = 2.5, 9 \text{ Hz, 1H) 3.76-3.67 \text{ (m, 3H), 3.62 (dd, } J = 4.5, 10.5 \text{ Hz, 1H), 3.56 (app t, } J = 8.5 \text{ Hz, 1H), 3.10 (s, 3H), 2.55 \text{ (t, } J = 6.5 \text{ Hz, 2H), 2.42 (app dd, } J = 9, 16 \text{ Hz, 1H), 2.01-1.97 \text{ (m, 2H), 1.93-1.84 \text{ (m, 3H), 1.80-1.74 \text{ (m, 3H), 1.73-1.70 \text{ (m, 2H) 1.68-1.62 \text{ (m, 3H), 1.53-1.50 \text{ (m, 1H), 1.36-1.27 \text{ (m, 2H) 1.17 (d, } J = 6 \text{ Hz, 3H), 1.06-0.94 \text{ (m, 72H), 0.75-0.61 \text{ (m, 42H).}}}}}}
\]}

\(^{13}\)C NMR (125 MHz, acetone d-6)
\[ \delta 170.1, 139.1, 138.2, 134.3, 134.2, 133.4, 132.7, 132.5, 132.4, 132.1, 132.0, 131.0, 130.9, 130.2, 128.5, 127.2, 100.5, 76.1, 73.5, 72.6, 71.6, 70.7, 70.4, 68.7, 68.5, 67.0, 66.8, 47.2, 47.1, 44.1, 42.9, 42.8, 40.8, 40.4, 39.5, 35.1, 26.8, 19.2, 18.5, 13.3, 10.7, 10.6, 7.04, 7.01, 6.90, 6.71, 6.69, 5.78, 5.57, 5.31, 5.27, 5.24, 5.22.\]

HRMS (ESI)
- calculated for C\(^{84}\)H\(^{164}\)O\(^{12}\)Si\(^{7}\) (M+Na): 1584.0506
- found: 1584.0481

\[2.26\]

\[P = \text{Triethylsilyl}\]

\[2.4\]

C41-Methyl amphoteronolide B 2.4

To a stirred solution of 2.26 (50 mg, 0.032 mmol) in THF:pyridine 3:2 (5 mL) at 0 °C was added dropwise 70% HF/pyridine complex (320 μL). The solution was stirred for 6 hours at 0 °C and was then quenched with the addition of trimethylsilyl ethoxide (2 mL). The solution was concentrated in vacuo and the resulting residue was dissolved in THF (1 mL) and AcOH:H\(_2\)O 1:1 (1 mL). The solution was stirred for 30 minutes and was then concentrated in vacuo. The
resulting orange solid was immediately dissolved in DMSO (1.5 mL) and purified by preparative RP-HPLC (Waters SunFire Prep C\textsubscript{18} OBD 5 micron 30 x 150 mm Lot # 168I161701 300 \textmu L injection volume, 25 mL/min flow rate, MeCN:H\textsubscript{2}O 1:19 \rightarrow 19:1, over 25 minutes) to yield C(41)-methyl amphoteronolide B 2.4 as a yellow solid (9 mg, 0.012 mmol, 38% over two steps).

\[
\begin{align*}
38\text{Me} & \quad O \quad 37 & \quad 1 & \quad OH & \quad OH & \quad OH & \quad OH \\
34 & \quad 39\text{Me} & \quad OH & \quad OH & \quad OH & \quad OH & \quad 15
\end{align*}
\]

\[2.30\]

HPLC

t\text{R} = 24.9 min, flow rate = 25 mL/min, gradient of 5 \rightarrow 95% MeCN in H\textsubscript{2}O over 25 min.

\textsuperscript{1}H NMR (600 MHz, pyridine d-5, CD\textsubscript{3}OD 10:1)

\[\delta 6.83 (dd, J = 10.8, 14.7 \text{ Hz}, 1\text{H}, \text{H-22}), 6.77 (dd, J = 10.8, 15 \text{ Hz}, 1\text{H}, \text{H-24}), 6.70 (dd, J_{19,20} = 8.9 \text{ Hz}, J_{20,21} = 15 \text{ Hz}, 1\text{H}, \text{H-20}), 6.60 (dd, J = 11.4, 14.4 \text{ Hz}, 1\text{H}, \text{H-23}), 6.55-6.35 (m, 9\text{H}), 5.79 (app d, J_{36,37} = 3.1 \text{ Hz}, 1\text{H}, \text{H-37}), 5.55 (dd, J_{32,33} = 15.6, J_{33,34} = 9.9 \text{ Hz}, 1\text{H}, \text{H-33}), 4.87 (app t, J_{10a,11} = 1.3 \text{ Hz}, J_{10c,11} = 10.9 \text{ Hz}, J_{11,12a} = 1.2 \text{ Hz}, J_{11,12e} = 10.7 \text{ Hz}, 1\text{H}, \text{H-11}), 4.77 (m, J_{18a,19} = 3.9, J_{18e,19} = 5.5 \text{ Hz}, J_{19,20} = 8.9 \text{ Hz}, 1\text{H}, \text{H-19}), 4.65 (m, J_{2e,3} = 9.2 \text{ Hz}, J_{3,4a} = 4.3, J_{3,4e} = 10.9 \text{ Hz}, J_{16,17} = 10.8 \text{ Hz}, J_{17,18a} = 8.1 \text{ Hz}, 2\text{H}, \text{H-3}, \text{H-17}), 4.20 (app dt, J_{14e,15} = 2.1, J_{14a,15} = 11.5 \text{ Hz}, J_{15,16} = 10.8 \text{ Hz}, 1\text{H}, \text{H-15}), 4.12 (app t, J_{4a,5} = 1.8, J_{4e,5} = 10.3 \text{ Hz}, J_{5,6a} = 1.1, J_{5,6e} = 11.5 \text{ Hz}, 1\text{H}, \text{H-5}), 4.03 (app dd, J_{8,9} = 3.2 \text{ Hz}, J_{9,10a} = 2.3, J_{9,10e} = 11.7 \text{ Hz}, 1\text{H}, \text{H-9}), 3.60 (app dd, J_{7c,8} = 2.4, J_{7a,8} = 11.3 \text{ Hz}, J_{8,9} = 3.2 \text{ Hz}, 1\text{H}, \text{H-8}), 3.44 (app d, J_{34,35} = 10.0 \text{ Hz}, J_{35,36} = 2.6 \text{ Hz}, 1\text{H}, \text{H-35}), 2.68 (m, J_{33,34} = 9.9 \text{ Hz}, J_{34,35} = 10.0 \text{ Hz}, 1\text{H}, \text{H-34}), 2.62 (dd, J_{2a,2e} = 16.2 \text{ Hz}, J_{2e,3} = 9.2 \text{ Hz}, 1\text{H}, \text{H-2e}), 2.49-2.44 (m, J_{14e,15} = 2.1 \text{ Hz}, J_{18c,19} = 5.5 \text{ Hz}, 3\text{H}, \text{H-2a}, \text{H-14e}, \text{H-18e}), 2.37-2.34 (m, J_{6a,7a} = 10.6 \text{ Hz}, J_{6c,7a} = 5.7 \text{ Hz}, J_{7a,8} = 11.3, 1\text{H}, \text{H-7a}), 2.19-2.15 (m, J_{9,10e} = 11.7 \text{ Hz}, J_{10c,11} = 10.9 \text{ Hz}, 1\text{H}, \text{H-10e}) 2.13-2.10 (m, J_{35,36} = 2.6 \text{ Hz}, J_{36,37} = 3.1 \text{ Hz}, 1\text{H}, \text{H-36}), 2.02 (ddd, J_{17,18a} = 8.1 \text{ Hz}, J_{18a,18e} = 14.4 \text{ Hz}, J_{18a,19} = 3.9 \text{ Hz}, 1\text{H}, \text{H-18a}), 1.99-1.95 (m, J_{5,6e} = 11.5 \text{ Hz}, J_{6c,7a} = 5.7 \text{ Hz}, J_{6e,7c} = 13.5 \text{ Hz}, J_{11,12c} = 10.7 \text{ Hz}, 2\text{H}, \text{H-6e}, \text{H-12e}), 1.85-1.74 (m, J_{3,4e} = 10.9 \text{ Hz}, J_{4e,5} = 10.3 \text{ Hz}, J_{6c,7c} = 13.5 \text{ Hz}, J_{7c,8} = 2.4 \text{ Hz}, J_{11,12a} = 1.2 \text{ Hz}, J_{14a,15} = 11.5 \text{ Hz}, 4\text{H}, \text{H-4e}),
H-7e, H-12a, H-14a), 1.72-1.66 (m, $J_{5,6a} = 1.1$ Hz, $J_{6a,7a} = 10.6$ Hz, $J_{15,16} = 10.8$ Hz, 2H, H-6a, H-16), 1.63 (app dt, $J_{3,4a} = 4.3$ Hz, $J_{4a,4e} = 14.1$ Hz, $J_{4a,5} = 1.8$ Hz, 1H, H-4a), 1.56 (app dd, $J_{9,10a} = 2.3$Hz, $J_{10a,10e} = 14.4$ Hz, $J_{10a,11} = 1.3$ Hz, 1H, H-10a), 1.45 (d, $J_{37,38} = 6.6$ Hz, 3H, H-38), 1.34 (d, $J_{16,41} = 6$ Hz, 3H, H-41), 1.31 (d, $J_{34,40} = 6$ Hz, 3H, H-40), 1.24 (d, $J_{36,39} = 7.2$ Hz, 3H, H-39).

$^{13}$C NMR (150 MHz, pyridine d-5 : CD$_3$OD, 10:1)

$\delta$ 172.1, 141.4, 137.5, 135.1, 134.8, 134.2, 133.6, 133.4, 133.1, 132.8, 132.7, 129.1, 98.3, 78.7, 76.3, 75.3, 75.2, 72.2, 71.4, 70.4, 69.7, 68.5, 48.2, 46.7, 45.1, 44.4, 43.8, 43.0, 41.5, 41.2, 41.0, 36.6, 31.9, 30.2, 29.8, 19.1, 17.4, 14.1, 12.8.

HRMS (ESI)

calculated for C$_{41}$H$_{64}$O$_{12}$ (M + Na)$^+$: 771.4295

found: 771.4268

Figure 2.3. Representative chromatogram of HPLC purified MeAmdeB.
Allyl ester 2.34

To a stirred solution of 2.18 (4.95 g, 2.26 mmol, 1 eq) in DMF:MeOH 10:1 (82.5 mL) at 23 °C was added allyl bromide (7.5 mL, 85.9 mmol, 38 eq) and diisopropyl ethyl amine (1.75 mL, 9.9 mmol, 4.4 eq). The solution was stirred for 8 hours and then diluted with water:saturated aqueous sodium bicarbonate 1:1 (250 mL). The aqueous phase was extracted with diethyl ether (4 x 100 mL) and the combined organic extracts were washed with brine (1 x 50 mL), dried over sodium sulfate and concentrated in vacuo. Purification of the resulting residue by flash chromatography (SiO$_2$; hexanes:diethyl ether 20:1 → 10:1) yielded 2.34 as a yellow solid (4.33 grams, 1.94 mmol, 86%).

TLC (hexanes:diethyl ether 2:1)

R$_f$ = 0.85, visualized by UV

$^1$H NMR (500 MHz, acetone $d$-6)

$\delta$ 7.87 (d, $J$ = 7.5 Hz, 2H), 7.69 (d, $J$ = 7.5 Hz, 2H), 7.42 (t, $J$ = 7.5 Hz, 2H), 7.34 (t, $J$ = 7.5 Hz, 2H), 7.34 (t, $J$ = 7.5 Hz, 2H), 6.57-6.50 (m, 2H), 6.48-6.13 (m, 10H), 6.06-5.99 (m, 1H), 5.98 (dd, $J$ = 5.5, 16 Hz, 1H), 5.49 (dd, $J$ = 9.5, 14.5 Hz, 1H), 5.43 (dd, $J$ = 1.5, 17 Hz, 1H), 5.34 (d, $J$ = 10 Hz, 1H), 5.29 (d, $J$ = 10.5 Hz, 1H), 4.74 (dd, $J$ = 6, 13.5 Hz, 1H), 4.63-4.51 (m, 3H), 4.49-4.41 (m, 3H), 4.33 (dd, $J$ = 6.5, 10.5 Hz, 1H), 4.26-4.23 (m, 2H), 4.12 (t, $J$ = 10 Hz,
1H), 4.02-3.97 (m, 2H), 3.90 (d, J = 3 Hz, 1H), 3.85 (dd, J = 2.5, 6.5 Hz, 1H), 3.71-3.67 (m, 2H), 3.66-3.62 (m, 3H), 3.45 (t, J = 8.5 Hz, 1H), 3.43-3.30 (m, 1H), 3.13 (s, 3H), 2.58-2.56 (m, 2H) 2.43 (app dd, J = 9, 16 Hz, 1H), 2.36 (t, J = 10.5 Hz, 1H), 2.00-1.96 (m, 2H), 1.93-1.90 (m, 2H), 1.84-1.58 (m, 8H), 1.50 (bs, 1H), 1.23 (d, J = 6 Hz, 3H), 1.17 (d, J = 6 Hz, 3H), 1.07-0.89 (m, 87H), 0.78-0.56 (m, 54H).

$^{13}$C NMR (125 MHz, acetone $d$-6)

$\delta$ 172.9, 170.5, 156.3, 145.0, 144.9, 142.2, 142.1, 139.3, 135.6, 135.5, 135.0, 134.5, 134.2, 133.6, 133.4, 132.4, 132.3, 132.1, 131.6, 131.2, 130.6, 128.4, 127.8, 127.7, 125.8, 125.7, 120.7, 119.0, 101.4, 98.6, 76.6, 75.7, 74.5, 74.0, 73.9, 73.4, 73.2, 71.0, 68.9, 67.6, 67.4, 67.3, 67.2, 65.9, 58.1, 57.5, 48.1, 48.0, 47.8, 44.2, 43.3, 42.2, 41.4, 40.7, 36.2, 35.6, 27.4, 22.9, 19.9, 19.2, 18.8, 15.5, 11.1, 7.58, 7.53, 7.43, 7.42, 7.29, 7.23, 7.20, 7.19, 7.07, 6.30, 6.11, 5.84, 5.82, 5.81, 5.76, 5.72, 5.70, 5.58.

HRMS (ESI)
calculated for $C_{120}H_{215}NO_{19}Si_9$ (M + Na)$^+$: 2249.3710
found: 2249.3630

Heptaenone 2.27

Prior to the reaction, calcium carbonate was dried via storage under vacuum for a minimum of eight hours in the presence of phosphorus pentoxide desiccant. Also, 2.18 was azeotropically dried via coevaporation with benzene (3 x 25 mL) and left under vacuum for a minimum of eight hours. The resulting yellow solid (4.33 g, 1.94 mmol, 1 eq) was dissolved in THF (100 mL) at 23 °C and calcium carbonate (1.94 g, 19.4 mmol, 10 eq) was added. DDQ (0.660 g, 2.91 mmol, 1.5 eq) was added and an immediate color change to dark red was observed. This mixture was stirred
for 20 minutes and then quenched with the addition of 100 mL saturated aqueous sodium bicarbonate. The resulting emulsion was diluted with water (300 mL) and extracted with dichloromethane (10 x 250 mL). The combined organic extracts were washed with brine (1 x 150 mL) and concentrated in vacuo. Purification of the resulting residue by flash chromatography (SiO$_2$; hexanes:Et$_2$O 20:1 → 6:1) yielded 2.27 as a dark orange solid (2.06 g, 1.26 mmol, 65%).

![Chemical structure of 2.27](image)

2.27

P = Triethylsilyl

TLC (hexanes:Et$_2$O 2:1)

$R_f = 0.44$, visualized by eye

$^1$H NMR (500 MHz, acetone d-6)

$\delta$ 7.84 (dd, $J = 11$, 15.5 Hz, 1H), 7.13 (dd, $J = 11.5$, 14.5 Hz, 1H), 6.81 (dd, $J = 11$, 14.5 Hz, 1H), 6.62-6.55 (m, 2H), 6.49-6.14 (m, 7H), 6.05 (d, $J = 15.5$ Hz, 1H), 6.01 (app ddd, $J = 6$, 10, 16 Hz, 1H), 5.51 (dd, $J = 10$, 15 Hz, 1H), 5.42 (dd, $J = 1.5$, 17.5 Hz, 1H), 5.27 (dd, $J = 1.5$, 10.5 Hz, 1H), 4.68 (app d, $J = 5.5$ Hz, 2H), 4.50 (dd, $J = 5$, 10.5 Hz, 1H), 4.45 (dd, $J = 5.5$, 10 Hz, 1H), 4.28 (app t, $J = 10.5$ Hz, 1H), 4.13-4.08 (m, 2H), 3.96 (dd, $J = 3$, 9.5 Hz, 1H), 3.77 (t, $J = 10.5$ Hz, 1H), 3.72-3.64 (m, 4H), 3.20 (dd, $J = 11$, 12.5 Hz, 1H), 2.92 (s, 3H), 2.70 (dd, $J = 4$, 18 Hz, 1H), 2.62 (dd, $J = 10$, 18 Hz, 1H), 2.46 (app dd, $J = 9$, 16 Hz, 1H), 2.40 (t, $J = 10.5$ Hz, 1H), 2.30 (app t, $J = 11$ Hz, 1H), 2.13 (d, $J = 12$ Hz, 1H), 1.96-1.86 (m, 4H), 1.83-1.76 (m, 4H), 1.67 (app d, $J = 12.5$ Hz, 1H), 1.50 (app t, $J = 10.5$ Hz, 1H), 1.17 (d, $J = 6$ Hz, 3H), 1.15 (t, $J = 8$ Hz, 9H), 1.09-0.96 (m, 60H), 0.89 (q, $J = 8.5$ Hz, 6H), 0.78-0.54 (m, 36H)
$^{13}$C NMR (125 MHz, acetone $d$-6)
\[
\delta 199.3, 172.1, 169.9, 149.2, 143.7, 140.8, 140.2, 139.2, 138.3, 137.1, 133.2, 132.0, 131.6, 131.3, 130.7, 130.6, 130.5, 130.1, 118.6, 76.7, 74.1, 73.6, 71.5, 70.4, 68.9, 67.8, 67.0, 65.9, 57.9, 48.4, 47.1, 44.6, 43.5, 43.1, 40.9, 40.7, 39.9, 35.1, 27.7, 20.0, 19.7, 10.9, 7.66, 7.45, 7.44, 7.39, 7.21, 7.16, 6.98, 6.50, 6.06, 5.80, 5.77, 5.63, 5.54, 5.50.
\]

HRMS (ESI)
calculated for $C_{87}H_{164}O_{14}Si_7$ (M + Na)$^+$: 1652.0404
found: 1652.0461

**Allylic alcohol 2.28**

Prior to the reaction 2.27 was azeotropically dried via coevaporation with benzene (3 x 25 mL) and left under vacuum for a minimum of eight hours. The resulting solid (2.05 g, 1.26 mmol, 1 eq) was dissolved in THF:MeOH 3:1 (12 mL) the resulting solution was cooled to 0 °C and sodium borohydride (0.480 g, 12.6 mmol, 10 eq) was added. The solution was stirred for 30 minutes and a color change from dark red to light orange was observed during the course of the reaction. The reaction was then quenched at 0 °C by the addition of saturated aqueous ammonium chloride (100 mL). The resulting emulsion was diluted with diethyl ether (250 mL). The two layers were separated and the organic phase was washed with water (3 x 50 mL) and brine (1 x 50 mL). The combined aqueous washings were back-extracted with diethyl ether (1 x 100 mL) and the combined organic extracts were dried over sodium sulfate and concentrated in vacuo. Purification of the resulting residue by flash chromatography (SiO$_2$; hexanes:diethyl ether 20:1 → 4:1) furnished 2.28 as a pale orange solid (1.59 g, 0.974 mmol, > 20:1 dr, 77%).
TLC (hexanes:diethyl ether 2:1)
\[ R_f = 0.25, \text{stained by anisaldehyde} \]

$^1$H NMR (500 MHz, acetone $d$-6)
\[
\delta 6.52-6.13 \text{ (m, 13H)}, 5.98 \text{ (app ddd, } J = 6, 10.5, 16.5 \text{ Hz, 1H)}, 5.55 \text{ (dd, } J = 9.5, 15 \text{ Hz, 1H)}, 5.40 \text{ (dd, } J = 1.5, 16 \text{ Hz, 1H)}, 5.24 \text{ (dd, } J = 1, 6.5 \text{ Hz, 1H)}, 4.70 \text{ (app t, } J = 6.5 \text{ Hz, 1H)}, 4.63 \text{ (app t, } J = 5.5 \text{ Hz, 2H)}, 4.52 \text{ (bs, 1H)}, 4.44 \text{ (dt, } J = 4.5, 10.5 \text{ Hz, 1H)}, 4.24 \text{ (m, 1H)}, 4.14 \text{ (t, } J = 9.5 \text{ Hz, 1H)}, 4.06 \text{ (dq, } J = 3, 7.5 \text{ Hz, 1H)}, 4.04-3.99 \text{ (m, 1H)}, 3.95 \text{ (d, } J = 3.5 \text{ Hz, 1H)}, 3.86 \text{ (dd, } J = 2.5, 9 \text{ Hz, 1H)}, 3.73-3.70 \text{ (m, 2H)}, 3.65 \text{ (dd, } J = 4.5, 10.5 \text{ Hz 1H)}, 3.17 \text{ (s, 3H)}, 2.58 \text{ (app t, } J = 2 \text{ Hz, 1H)}, 2.44 \text{ (app dd, } J = 9, 15.5 \text{ Hz, 1H)}, 2.34 \text{ (t, } J = 10 \text{ Hz, 1H)}, 2.12 \text{ (dd, } J = 4.5, 12.5 \text{ Hz, 1H)} 2.07-2.05 \text{ (m, 2H)}, 2.05-1.99 \text{ (m, 1H)}, 1.97-1.59 \text{ (m, 10H)}, 1.52 \text{ (bs, 1H)} 1.18 \text{ (d, } J = 6 \text{ Hz, 3H)}, 1.08-0.95 \text{ (m, 69H)}, 0.78-0.59 \text{ (m, 42H)}. \]

$^{13}$C NMR (125 MHz, acetone $d$-6)
\[
\delta 172.8, 170.6, 139.0, 135.0, 134.9, 134.1, 133.5, 133.1, 133.0, 132.6, 131.8, 131.5, 130.8, 128.1, 126.0, 118.4, 101.4, 76.7, 74.0, 71.0, 69.0, 67.5, 67.4, 65.6, 57.7, 48.1, 47.8, 44.4, 43.5, 42.4, 41.3, 40.7, 35.6, 30.7, 29.2, 27.4, 19.8, 19.1, 7.62, 7.59, 7.48, 7.30, 7.27, 7.12, 6.38, 6.17, 5.89, 5.83, 5.79, 5.64. \]

HRMS (ESI)
\[
calculated for C$_{57}$H$_{166}$O$_{14}$Si$_7$ (M + Na)$^+$: 1654.0506
found: 1654.0493
Polyol 2.34

To a stirred solution of 2.28 (1.55 g, 0.949 mmol, 1 eq) in THF (7 mL) in a polypropylene vial at 0 °C was added a chilled (0 °C) solution of 70% HF/pyridine complex (10 mL) in THF:pyridine 5:3 (160 mL). The resulting solution was allowed to warm to 23 °C and stirred for 6 hours. The reaction was then cooled to 0 °C and quenched by the addition of saturated aqueous sodium bicarbonate (500 mL). The resulting yellow emulsion was extracted with CH$_2$Cl$_2$:MeOH 5:1 (5 x 200 mL). The combined organic extracts were washed with brine (1 x 100 mL), dried over sodium sulfate and concentrated in vacuo. Purification of the resulting residue by flash chromatography (C$_{18}$ bonded SiO$_2$; MeCN:H$_2$O 1:1) furnished 2.34 as an orange solid (0.442 g, 0.531 mmol, 56%).

TLC (CH$_2$Cl$_2$:MeOH 10:1)

$R_f = 0.25$, stained by anisaldehyde

HPLC

$tR = 18.90$ min; flow rate = 4 mL/min, gradient of 5 → 95% MeCN in 5 mM ammonium acetate over 25 min.

$^1$H NMR (500 MHz, CD$_3$OD)

$\delta$ 6.43-6.13 (m, 12H), 5.98 (app ddd, $J = 6, 10.5, 16$ Hz, 1H), 5.80 (dd, $J = 7.5, 15$ Hz, 1H), 5.47 (dd, $J = 9.5, 14$ Hz, 1H), 5.38 (dd, $J = 1.5, 16$ Hz, 1H), 5.24 (dd, $J = 1, 10.5$ Hz, 1H),
1H), 4.66 (d, J = 5 Hz, 2H), 4.48 (app dd, J = 7, 11.5 Hz, 1H), 4.20-4.12 (m, 3H), 3.99 (dq, J = 3.5, 10.5 Hz, 1H), 3.91 (dd, J = 8.5, 12.5 Hz, 1H), 3.71 (app t, J = 6 Hz, 1H), 3.53 (app d, J = 10 Hz, 1H), 3.26 (dd, J = 7.5, 9.5 Hz, 1H), 3.22 (dd, J = 3.5, 16.5 Hz, 1H), 2.11 (dd, J = 5, 13 Hz, 1H), 1.90-1.81 (m, 4H), 1.72-1.61 (m, 4H), 1.51-1.26 (m, 8H), 1.19 (d, J = 6.5 Hz, 3H), 1.11 (d, J = 6.5 Hz, 3H), 1.00 (d, J = 7.5 Hz, 3H).

\(^{13}\)C NMR (125 MHz, pyridine d-5:CD\textsubscript{3}OD 10:1)
\[ \delta 173.0, 171.4, 140.1, 135.4, 134.4, 134.2, 134.1, 133.6, 133.2, 133.1, 132.9, 132.8, 132.7, 132.4, 131.8, 128.5, 101.7, 77.7, 75.2, 74.5, 71.5, 70.5, 67.9, 67.7, 67.6, 67.4, 66.7, 66.5, 65.1, 57.0, 44.6, 43.5, 43.1, 42.9, 42.3, 41.3, 41.2, 38.9, 36.0, 33.9, 32.1, 30.5, 29.0, 24.7, 23.9, 23.1, 18.6, 18.5, 17.5, 13.9, 12.2, 10.9. \]

HRMS (ESI)
calculated for C\textsubscript{45}H\textsubscript{68}O\textsubscript{12} (M + Na): 855.4507
found: 855.4482

Amphoteronolide B allyl ester 2.30
To a stirred solution of 2.34 (10 mg, 0.012 mmol, 1 eq) in THF:H\textsubscript{2}O 2:1 (1.2 mL) at 23 °C was added camphorsulfonic acid (0.6 mg, 0.003 mmol, 0.25 eq). The solution was stirred for 5 hours and was then diluted with THF (2 mL) and quenched by addition of solid sodium bicarbonate. The mixture was stirred vigorously for five minutes and the solids were removed by filtration through a pad of Celite. The filtrate was concentrated \textit{in vacuo} and the resulting yellow solid was dissolved in THF (1.5 mL) and purified by prep RP-HPLC (Waters SunFire Prep C\textsubscript{18} OBD 5 micron 30 x 150 mm; 300 µL injection volume, 25 mL/min flow rate, MeCN:H\textsubscript{2}O 1:19 → 19:1,
over 25 minutes) to yield amphoteronolide B allyl ester 2.30 as a yellow powder (8 mg, 0.0098 mmol, 81%).

HPLC

tR = 28.6 min; flow rate = 25 mL/min, gradient of 5 → 95% MeCN in H2O over 25 min.

1H NMR (500 MHz, pyridine d-5:CD3OD 10:1)

δ 6.83 (dd, J = 11, 15 Hz, 1H, H-22), 6.77 (dd, J = 11, 15 Hz, 1H, H-24), 6.69 (dd, J19,20 = 9.5 Hz, J20,21 = 15.5 Hz, 1H, H-20), 6.61 (dd, J = 14.5, 15 Hz, 1H, H-26), 6.54-6.35 (m, 9H), 6.04-5.96 (app ddd, J42,43 = 6.5, J43,44cis = 11 Hz, J43,44trans = 17 Hz, 1H, H-43), 5.80 (app d, J36,37 = 1.9 Hz, 1H, H-37), 5.55 (dd, J32,33 = 15 Hz, J33,34 = 10.1 Hz, 1H, H-33), 5.42 (dd, J44cis,44trans = 1.5 Hz, J43,44trans = 17.5 Hz, 1H, H-44trans), 5.30 (app t, J16,17 = 10.6 Hz, J17,18e = 0.9 Hz, J17,18a = 8.7 Hz, 1H, H-17), 5.14 (dd, J44cis,44trans = 1.5 Hz, J43,44cis = 10.5 Hz, 1H, H-44cis), 5.06 (app dt, J14e,15 = 3.5 Hz, J14a,15 = 11.0 Hz, J15,16 = 10.7 Hz, 1H, H-15), 4.87 (t, J10a,11 = 3.1 Hz, J10e,11 = 10.3 Hz, J11,12a = 3.4 Hz, J11,12e = 11.3 Hz, 1H, H-11), 4.80-4.73 (m, J18e,19 = 6.0 Hz, J19,20 = 8.8 Hz, 3H, H-19, H-42(2)), 4.64 (app t, J2a,3 = 4.7 Hz, J2c,3 = 9.2 Hz, J3,4a = 3.9 Hz, J3,4e = 10.1 Hz, 1H, H-3), 4.13 (app t, J4a,5 = 4.4 Hz, J4e,5 = 9.5 Hz, J5,6a = 5.4 Hz, J5,6c = 10.3 Hz, 1H, H-5), 4.04 (app d, J5,9 = 3.0 Hz, J9,10a = 3.3 Hz, J9,10e = 10.7 Hz, 1H, H-9), 3.61 (app d, J7c,8 = 2.8 Hz, J7a,8 = 11.2 Hz, J8,9 = 3.0 Hz, 1H, H-8), 3.43 (app d, J34,35 = 9.8 Hz, J35,36 = 2.6 Hz, 1H, H-35), 2.85 (t, J15,16 = 10.7 Hz, J16,17 = 10.6 Hz, 1H, H-16), 2.66 (m, J33,34 = 10.1 Hz, J34,35 = 9.8 Hz, 1H, H-34), 2.61 (dd, J2a,2e = 17 Hz J2c,3 = 9.2 Hz, 1H, H-2e), 2.51 (dd, J14a,14e = 12 Hz J14e,15 = 3.5 Hz, 1H, H-14e), 2.46 (dd, J2a,2e = 16.5, J2a,3 = 4.7, 1H, H-2a), 2.41 (dd, J17,18e = 0.9 Hz, J18a,18e = 14 Hz, J18e,19 = 6.0 Hz, 1H, H-18e), 2.37 (m, J7c,8 = 2.8 Hz, 1H, H-7e), 2.19-2.15 (m, J9,10e = 10.7 Hz, J10e,11 = 10.3 Hz, J17,18a = 8.7 Hz, 2H, H-10e, H18a), 2.10 (m, J35,36 = 2.6 Hz, J36,37 = 1.9 Hz, 1H, H-36), 2.00-1.95 (m, J5,6c = 10.3 Hz, J11,12e = 11.3 Hz, 2H, H-6e, H-12e), 1.85-1.80 (m, J3,4e = 10.1 Hz, J4e,5 = 9.5 Hz, J7a,8 = 11.2 Hz, J14e,15 = 3.5 Hz, 3H, H-
4e, H-7a, H-14e), 1.78-1.70 (m, \( J_{5,6a} = 5.4 \) Hz, \( J_{11,12a} = 3.4 \) Hz, 2H, H-6a, H-12a), 1.61 (app t, \( J_{3,4a} = 3.9 \) Hz, \( J_{4a,5} = 4.4 \) Hz, 1H, H-4a), 1.56 (app d, \( J_{9,10a} = 3.3 \) Hz, \( J_{10a,10e} = 14.5 \) Hz, \( J_{10a,11} = 3.1 \) Hz, 1H, H-10a), 1.46 (d, \( J_{37,38} = 6.5 \) Hz, 3H, H-38), 1.31 (d, \( J_{34,40} = 6 \) Hz, 3H, H-40), 1.25 (d, \( J_{36,39} = 7 \) Hz, 3H, H-39).

\[ ^{13}C \text{ NMR (150 MHz, pyridine } d-5:CD_{3}OD, 10:1) \]
\[ \delta 173.5, 172.2, 140.9, 137.5, 135.0, 134.8, 134.7, 134.1, 133.7, 133.5, 133.4, 133.3, 133.2, 133.1, 132.9, 132.8, 129.1, 118.0, 98.6, 78.8, 76.3, 75.2, 72.2, 71.1, 70.4, 69.7, 68.5, 68.1, 67.0, 66.7, 65.4, 59.0, 47.6, 45.9, 45.1, 43.8, 43.0, 42.8, 41.1, 41.0, 39.4, 36.6, 31.8, 30.2, 29.5, 26.0, 24.4, 23.5, 19.1, 17.4, 12.9. \]

HRMS (ESI)

calculated for C_{44}H_{66}O_{14} (M+Na)^{+}: 841.4350
found: 841.4369

**Amphoteronolide B 2.2**

Prior to the reaction, 2.30 was azeotropically dried via coevaporation with acetonitrile (3 x 5 mL) and left under vacuum for a minimum of eight hours. The resulting yellow solid (20 mg, 0.024 mmol, 1 eq) was dissolved in THF (1.5 mL) at 23 °C and thiosalicylic acid (20 mg, 0.12 mmol, 5 eq). Palladium tetrakis(triphenylphosphine) (27 mg, 0.024 mmol, 1 eq) was added and the solution was stirred for 13 hours, during which time the formation of a precipitate was observed. The reaction was then concentrated in vacuo and the residue was triturated with cold (0 °C) diethyl ether (5 x 5 mL). The yellow solid was then dissolved in DMSO (1.5 mL) and purified by preparative RP-HPLC (Waters SunFire Prep C_{18} OBD 5 micron 30 x 150 mm; 250 µL injection volume, 25 mL/min flow rate, 5 → 75% MeCN in 10 mM NH_{4}OAc over 25 minutes) to afford amphoteronolide B 2.2 (9 mg, 0.012 mmol, 50%) as a yellow powder.
HPLC

tR: 21.6 min; flow rate = 25 mL/min, gradient of 0 → 75% MeCN in 10 mM ammonium acetate over 25 min.

^1^H NMR (pyridine d-5:CD_{3}OD 10:1)

δ 6.76-6.67 (m, 2H), 6.59-6.55 (m, 2H), 6.45-6.35 (m, 10H), 5.72 (app d, J = 5 Hz, 1H), 5.16 (app t, J = 10 Hz, 1H), 4.92 (bs, 1H), 4.78 (bs, 2H), 4.57 (app t, J = 10 Hz, 1H), 4.05 (app t, 9.5 Hz, 1H), 3.95 (app d, J = 10.5 Hz, 1H), 3.65 (app d, J = 4 Hz, 1H), 3.52 (app d, J = 10.5 Hz, 1H), 3.41 (app d, J = 9 Hz, 1H), 2.64 (bs, 2H), 2.56 (dd, J = 10, 16.5 Hz, 1H), 2.43-2.40 (m, 2H), 2.24 (bs, 2H), 2.10-2.05 (m, 2H), 1.95-1.90 (m, 2H), 1.79-1.65 (m, 5H), 1.58 (app d, J = 13.5 Hz, 1H), 1.51 (app d, J = 11 Hz, 1H), 1.41 (d, J = 6.5 Hz, 3H), 1.28 (d, J = 6.5 Hz, 3H), 1.21 (d, J = 7 Hz, 3H).

HRMS

Calculated for C_{41}H_{62}O_{14} (M+Na)^+: 801.4037

found: 801.4039
To a stirred solution of 2.21 (500 mg, 0.219 mmol) in THF (7 mL) in a polypropylene vial at 0 °C was added chilled (0 °C) 70% HF/pyridine complex (2.2 mL, 77 mmol, 350 eq) diluted with THF:pyridine 5:3 (40 mL). The solution was allowed to warm to 25 °C and stirred for 6.5 hours. The solution was subsequently cooled to 0 °C and quenched with the addition of saturated aqueous sodium bicarbonate (100 mL). The resulting yellow emulsion was extracted with CH$_2$Cl$_2$:MeOH 5:1 (5 x 100 mL). The combined organic extracts were washed with brine (1 x 25 mL), dried over sodium sulfate and concentrated in vacuo. Purification of the resulting...
residue by flash chromatography (SiO$_2$; DCM:MeOH 100:1 → 15:1) afforded 2.35 as a yellow solid (202 mg, 0.160 mmol, 73%).

TLC (DCM:MeOH 10:1)

$R_f = 0.5$, stained by anisaldehyde

HPLC

$t_R = 23.40$ min; flow rate = 4 mL/min, gradient of 5 → 95% MeCN in 5 mM ammonium acetate over 25 min.

$^1$H NMR (500 MHz, CD$_3$OD)

$\delta$ 7.79 (d, $J = 7.5$ Hz, 2H), 7.68 (dd, $J = 4.5$, 7.5 Hz, 2H), 7.39 (t, $J = 7$ Hz, 2H), 7.31 (t, $J = 8$ Hz, 2H), 6.41-6.14 (m, 12H), 5.86 (dd, $J = 6.5$, 14 Hz, 1H), 5.55 (dd, $J = 9$, 14 Hz, 1H), 5.14 (bs, 1H), 4.82 (s, 1H), 4.63 (t, $J = 7$ Hz, 1H), 4.36 (app d, $J = 7$ Hz, 2H), 4.23 (t, $J = 7$ Hz, 1H), 4.15-4.11 (m, 1H), 3.97-3.93 (m, 2H), 3.86 (dt, $J = 5.5$, 10 Hz, 1H), 3.80 (t, $J = 9.5$ Hz, 1H), 3.75 (d, $J = 9$ Hz, 1H), 3.72-3.68 (m, 1H), 3.64 (dd, $J = 2.5$, 9 Hz, 1H), 3.62 (app d, $J = 10.5$ Hz, 1H), 3.36-3.31 (m, 1H), 3.23 (app d, $J = 9.5$ Hz, 1H), 3.18 (s, 3H), 2.38 (app dd, $J = 7.5$, 15.5 Hz, 1H), 2.29 (dd, $J = 9$, 16.5 Hz, 1H), 2.23 (dd, $J = 3$, 16.5 Hz, 1H), 2.15 (dd, $J = 5$, 13 Hz, 1H), 1.86-1.83 (m, 1H), 1.80-1.75 (m, 1H), 1.69-1.54 (m, 8H), 1.50-1.39 (m, 9H), 1.30 (d, $J = 5.5$ Hz, 3H), 1.20 (d, $J = 6.5$ Hz, 3H), 1.11 (d, $J = 6.5$ Hz, 3H), 1.01 (d, $J = 7.5$, 3H).

$^{13}$C NMR (125 MHz, CD$_3$OD)

$\delta$ 172.6, 158.7, 145.4, 145.3, 142.6, 137.4, 135.0, 134.5, 134.1, 134.0, 133.9, 133.4, 133.2, 132.7, 128.8, 128.2, 126.3, 120.9, 102.3, 98.6, 79.0, 75.8, 75.4, 75.1, 74.6, 72.3,
71.9, 71.5, 71.2, 68.8, 68.3, 67.9, 66.8, 58.3, 46.2, 44.7, 43.3, 43.1, 41.5, 36.0, 35.3, 30.4, 18.9, 18.3, 17.9, 12.2.

HRMS (ESI)

calculated for $C_{63}H_{86}INO_{17} \text{(M + Na)}^+$: 1278.4838

found: 1278.4817

41-methyl 2.24

Prior to the reaction, 2.35 was azeotropically dried via coevaporation with acetonitrile (3 x 10 mL) and left under vacuum for a minimum of eight hours. The resulting yellow solid (50 mg, 0.0398 mmol, 1 eq) was dissolved in DMSO (1.3 mL) at 23 °C and sodium borohydride (7.5 mg, 0.199 mmol, 5 eq) was added. The solution was then stirred for 8.5 hours and subsequently quenched with the addition of saturated aqueous sodium bicarbonate (1 mL). The resulting emulsion was diluted with water (25 mL) and extracted with CH$_2$Cl$_2$:MeOH 5:1 (5 x 10 mL). The combined organic phases were washed with brine (1 x 20 mL), dried over sodium sulfate and concentrated in vacuo. Purification of the residue by flash chromatography (SiO$_2$; DCM:MeOH 20:1 → 10:1) yielded 2.24 as a yellow solid (26 mg 0.023 mmol, 58%).
TLC (CH₂Cl₂:MeOH 10:1)
  
  \( R_f = 0.5 \), stained by anisaldehyde

HPLC

  \( t_R = 22.59 \text{ min}; \) flow rate = 4 mL/min, gradient of 5 \( \rightarrow \) 95% MeCN in 5 mM ammonium acetate over 25 min.

\(^1\)H NMR (500 MHz, CD₃OD)

\[ \delta \ 7.79 \ (d, \ J = 7.5 \text{ Hz}, 2H), \ 7.68 \ (t, \ J = 6.5 \text{ Hz}, 2H), \ 7.39 \ (t, \ J = 7 \text{ Hz}, 2H), \ 7.31 \ (t, \ J = 7.5 \text{ Hz}, 2H), \ 6.44-6.15 \ (m, 12H), \ 5.83 \ (dd, \ J = 8, 15 \text{ Hz}, 1H), \ 5.44 \ (dd, \ J = 10, 14 \text{ Hz}, 1H), \ 5.28 \ (bs, 1H), \ 4.61 \ (s, 1H), \ 4.49 \ (dt, \ J = 2.5, 8.5 \text{ Hz}, 1H), \ 4.40-4.33 \ (m, 2H), \ 4.23 \ (t, \ J = 7 \text{ Hz}, 1H), \ 4.18-4.13 \ (m, 1H), \ 3.94-3.89 \ (m, 3H), \ 3.81 \ (d, \ J = 3 \text{ Hz}, 1H), \ 3.72-3.67 \ (m, 2H), \ 3.65-3.62 \ (m, 1H), \ 3.59-3.52 \ (m, 4H), \ 3.27 \ (dd, \ J = 6, 8 \text{ Hz}, 1H), \ 3.22 \ (dd, \ J = 1.5, 12.5 \text{ Hz}, 1H), \ 3.16 \ (s, 3H), \ 2.38 \ (app dd, \ J = 9.5, 16 \text{ Hz}, 1H), \ 2.29 \ (dd, \ J = 9, 16.5 \text{ Hz}, 1H), \ 2.22 \ (dd, \ J = 3, 16.5 \text{ Hz}, 1H), \ 2.09 \ (dd, \ J = 9, 13.5 \text{ Hz}, 1H), \ 2.04-1.97 \ (m, 2H), \ 1.90-1.81 \ (m, 2H), \ 1.72-1.65 \ (m, 2H), \ 1.55-1.39 \ (m, 8H), \ 1.28 \ (d, \ J = 5.5 \text{ Hz}, 3H), \ 1.19 \ (d, \ J = 6 \text{ Hz}, 3H), \ 1.11 \ (d, \ J = 6 \text{ Hz}, 3H), \ 1.02 \ (d, \ J = 6.5 \text{ Hz}, 3H), \ 1.01 \ (d, \ J = 7 \text{ Hz}, 3H). \]

\(^{13}\)C NMR (125 MHz, CD₃OD)

\[ \delta \ 171.5, \ 157.6, \ 144.2, \ 144.1, \ 141.4, \ 136.2, \ 135.8, \ 133.9, \ 133.5, \ 133.4, \ 133.3, \ 133.0, \ 132.6, \ 132.4, \ 132.0, \ 130.9, \ 127.6, \ 127.0, \ 125.1, \ 125.0, \ 119.8, \ 107.7, \ 101.3, \ 98.4, \ 76.4, \ 74.7, \ 73.7, \ 71.4, \ 71.2, \ 70.6, \ 70.3, \ 70.0, \ 69.7, \ 69.1, \ 67.7, \ 67.5, \ 67.1, \ 66.7, \ 57.1, \ 43.6, \ 42.7, \ 42.0, \ 41.8, \ 41.4, \ 40.2, \ 35.0, \ 29.6, \ 28.9, \ 17.8, \ 17.0, \ 16.2, \ 12.1, \ 11.1. \]

HRMS (ESI)

  calculated for C₆₅H₈₇NO₁₇ (M + Na)⁺: \ 1152.5872
N-Fmoc-41-methyl amphotericin B 2.29

To a stirred solution of 2.24 (9 mg, 0.008 mmol) in THF:H$_2$O 2:1 (1 mL) at 23 °C was added camphorsulfonic acid (0.6 mg, 0.002 mmol, 0.25 eq). The solution was stirred for 30 minutes and was then diluted with THF (1 mL) and quenched by the addition of solid sodium bicarbonate. The solids were removed by filtration through a pad of Celite and the filtrate was concentrated in vacuo to give 2.29 as a yellow solid (9 mg, 0.008 mmol, ~100%). This material was used in the next step without further purification. Alternatively, 2.29 was purified by preparative RP-HPLC (Waters SunFire Prep C$_{18}$ OBD 5 micron 30 x 150 mm; 300 µL injection volume, 25 mL/min flow rate 1:19 → 19:1 MeCN:10mM NH$_4$OAc, over 25 minutes) for use in NMR studies.
HPLC

tR = 25.1 minutes; flow rate = 25 mL/min, gradient of 5 → 95% MeCN in 5 mM ammonium acetate over 25 min.

\(^1\)H NMR (600 MHz, pyridine \(d\)-5:CD\(_3\)OD 10:1)

\(\delta\) 7.85 (d, \(J = 7.2\) Hz, 2H), 7.71 (dd, \(J = 6, 8.5, 2\) Hz, 2H), 7.41 (t, \(J = 7.2\) Hz, 2H), 6.74 (m, 2H), 6.60-6.34 (m, 11H), 5.77 (app d, \(J_{36,37} = 1.8\) Hz, 1H, H-37), 5.54 (dd, \(J_{32,33} = 15\) Hz \(J_{33,34} = 10.0\), 1H, H-33), 4.97 (app s, \(J_{1,2} = 1.6\) Hz, 1H, H-1'), 4.82-4.78 (m, \(J_{10c,11} = 10.4\) Hz, \(J_{11,12c} = 11.0\) Hz, \(J_{18a,19} = 2.5\) Hz, \(J_{18c,19} = 4.6\) Hz, \(J_{19,20} = 8.0\) Hz, 2H, H-11, H-19), 4.62 (app t, \(J_{2a,3} = 1.8\) Hz, \(J_{2e,3} = 9.1\) Hz, \(J_{3,4a} = 2.0\) Hz, \(J_{3,4e} = 10.1\) Hz, 1H, H-3), 4.51-4.43 (m, \(J_{16,17} = 10.4\) Hz, \(J_{17,18a} = 7.5\) Hz, 3H, H-17, H-1''(2)), 4.39 (app d, \(J_{1,2} = 1.6\) Hz, 1H, H-2'), 4.34 (dd, \(J_{2,3} = 3\) Hz, \(J_{3,4} = 9.7\) Hz, 1H, H-3'), 4.29 (t, \(J = 7.2\) Hz, 1H, H-2''), 4.12-4.07 (m, \(J_{4a,5} = 1.0\) Hz, \(J_{4e,5} = 9.6\) Hz, \(J_{5,6a} = 1.8\) Hz, \(J_{5,6e} = 10.7\) Hz, \(J_{14a,15} = 2.6\) Hz, \(J_{14a,15} = 11.8\) Hz, \(J_{15,16} = 11.2\) Hz, 2H, H-5, H-15), 4.02-3.99 (m, \(J_{8,9} = 3.4\) Hz, \(J_{9,10a} = 2.6\) Hz, \(J_{9,10a} = 10.7\) Hz, \(J_{3,4} = 9.7\) Hz, \(J_{4,5} = 9.1\) Hz, 2H, H-9, H-4'), 3.73 (app dd, \(J_{4',5'} = 9.1\) Hz, \(J_{5',6'} = 6.6\) Hz, 1H, H-5'), 3.58 (app d, \(J_{7e,8} = 2.5\) Hz, \(J_{7a,8} = 10.6\) Hz, \(J_{8,9} = 3.4\) Hz, 1H, H-8), 3.43 (app d, \(J_{34,35} = 9.6\) Hz, \(J_{35,36} = 2.5\) Hz, 1H, H-35), 2.67 (app dd, \(J_{33,34} = 10.0\) Hz, \(J_{34,35} = 9.6\) Hz, 1H, H-34), 2.60 (dd, \(J_{2a,2e} = 16.8\) Hz, \(J_{2e,3} = 9.1\) Hz, 1H, H-2e), 2.46 (app dd, \(J_{18a,18c} = 16.8\) Hz, \(J_{18c,19} = 4.6\) Hz, 1H, H-18e), 2.44-2.40 (m, \(J_{2a,3} = 1.8\) Hz, \(J_{14c,15} = 2.6\) Hz, 2H, H-2a, H-14e), 2.36-2.33 (m, \(J_{6c,7e} = 13.4\) Hz, \(J_{7e,8} = 2.5\) Hz, 1H, H-7e), 2.17-2.07 (m, \(J_{9,10c} = 10.7\) Hz, \(J_{10c,11} = 10.4\) Hz, \(J_{35,36} = 2.5\) Hz, \(J_{36,37} = 1.8\) Hz, 2H, H-10e, H-36), 2.02-1.90 (m, \(J_{5,6c} = 10.7\) Hz, \(J_{6c,7a} = 4.4\) Hz, \(J_{6c,7e} = 13.4\) Hz, \(J_{11,12c} = 11.0\) Hz, \(J_{17,18a} = 7.5\) Hz, \(J_{18a,19} = 2.5\) Hz, 3H, H-6e, H-12e, H-18a), 1.81-1.76 (m, \(J_{3,4e} = 10.1\) Hz,
Hz, $J_{4e,5} = 9.6$ Hz, $J_{6e,7a} = 4.4$ Hz, $J_{6a,7a} = 12.7$ Hz, $J_{7a,8} = 10.6$ Hz, 3H, H-4e, H-7a, H12a), 1.73-1.69 (m, $J_{5,6a} = 1.8$ Hz, $J_{6a,7a} = 12.7$ Hz, $J_{14a,15} = 11.8$ Hz, 2H, H-6a, H-14a), 1.62-1.60 (m, $J_{3,4a} = 2.0$ Hz, $J_{4a,5} = 1.0$ Hz, $J_{15,16} = 11.2$ Hz, $J_{16,17} = 10.4$ Hz, 2H, H-4a, H-16), 1.56 (d, $J_{5',6'} = 6.6$ Hz, 3H, H-6'), 1.55-1.52 (m, $J_{3,4a} = 2.0$ Hz, $J_{4a,5} = 1.0$ Hz, $J_{15,16} = 11.2$ Hz, $J_{16,17} = 10.4$ Hz, 2H, H-4a, H-16), 1.23 (d, $J_{16,41} = 5.2$ Hz, 3H, 3H, H-41).

$^{13}$C NMR (150 MHz, pyridine $d$-5:CD$_3$OD 10:1)

$\delta$ 172.2, 158.0, 145.1, 144.9, 142.0, 141.9, 138.2, 137.6, 134.9, 134.8, 134.5, 134.1, 133.7, 133.5, 133.4, 133.2, 132.9, 130.3, 128.3, 127.8, 126.1, 123.6, 126.0, 120.7, 108.6, 99.6, 98.3, 78.8, 78.1, 76.3, 75.3, 75.0, 72.2, 71.9, 71.5, 70.4, 69.7, 69.5, 68.5, 68.5, 67.6, 67.1, 58.7, 48.1, 45.1, 44.2, 43.1, 41.1, 36.6, 30.3, 19.1, 18.8, 17.5, 14.1, 12.8.

HRMS (ESI)

calculated for C$_{62}$H$_{85}$NO$_{17}$ (M + Na)$^+$: 1138.5715

found: 1138.5734

C(41)-Methyl amphotericin B 2.4

To a stirred solution of 2.24 (9 mg, 0.008 mmol) in DMSO:MeOH 15:2 (380 µL) was added piperidine (0.02 mmol, 2 µL, 2 eq). The solution was stirred for 3 hours and was then diluted with THF (1 mL) and purified by prep RP-HPLC (Waters SunFire Prep C$_{18}$ OBD 5 micron 30 x 150 mm; 300 µL injection volume, 25 mL/min flow rate 1:19 → 19:1 MeCN:10mM NH$_4$OAc, over 25 minutes) to afford C(41)-methyl amphotericin B (4) as a yellow powder (4 mg, 0.0045 mmol, 56% over 2 steps).

1 Two of the Fmoc protons were obscured by the pyridine solvent peak.
HPLC

tR = 21.7 minutes; flow rate = 25 mL/min, gradient of 5 → 95% MeCN in 5 mM ammonium acetate over 25 min.

\(^1\)H NMR (500 MHz, pyridine d-5:CD\(_2\)OD 10:1)

δ 6.78-6.71 (m, 2H), 6.58-6.37 (m, 9H), 6.43-6.35 (m, 2H), 5.77 (app d, J = 5.5 Hz, 1H), 5.54 (dd, J = 10, 15 Hz, 1H), 4.95 (s, 1H), 4.81-4.77 (m, 2H), 4.61 (dt, J = 3, 12.5 Hz, 1H), 4.45-4.40 (m, 2H), 4.35 (d, J = 2.5 Hz, 1H), 4.11-4.03 (m, 2H), 4.00 (app d, J = 11 Hz, 1H), 3.77 (t, J = 9 Hz, 1H), 3.66-3.58 (m, 2H), 3.57 (app d, J = 11 Hz, 1H) 3.42 (app d, J = 9.5 Hz, 1H), 2.66 (app dd, J = 7, 9.5 Hz, 1H), 2.60 (dd, J = 9.5, 16.5 Hz, 1H), 2.49 (dd, J = 5.5, 14.5 Hz, 1H), 2.41 (app dd, J = 7.5, 12 Hz, 2H), 2.39-2.35 (m, 1H), 2.10 (app dd, J = 7.5, 15.5, 2H), 1.95-1.88 (m, 3H), 1.81-1.72 (m, 3H), 1.70-1.67 (m, 2H), 1.66-1.59 (m, 2H), 1.52 (d, J = 6 Hz, 3H), 1.44 (d, J = 6.5 Hz, 3H), 1.30 (d, J = 6.5 Hz, 3H), 1.24 (d, J = 7 Hz, 3H), 1.22 (d, J = 6.5 Hz, 3H).

HRMS (ESI)

calculated for C\(_{47}\)H\(_{75}\)NO\(_{15}\) (M + Na): 894.5191
found: 894.5182
Part 2. NMR Studies

Selection of compounds for NMR analysis. MeAmdeB 2.3 was used directly for NMR studies. Compounds 2.1, 2.2, and 2.4 were not amenable to high resolution NMR analysis due to their poor solubilities in appropriate NMR solvents. However, it has been demonstrated that AmB derivatives having covalent modifications of the carboxylic acid or amine functional groups have the same ground state conformation as judged by NMR or X-ray crystallographic analysis, respectively. Therefore, we chose suitably protected analogs AmB N-acyl methyl ester 2.27 AmdeB allyl ester 2.30, and N-Fmoc MeAmB 2.29 for the conformational analysis of 2.1, 2.2, and 2.4, respectively.

gCOSY NMR spectra. 500 MHz and 600 MHz gCOSY NMR spectra were acquired at 30 °C with 2048 points, 256 or 512 increments and 1, 4, or 8 transients. Spectra were processed on a SUN Microsystems SPARCstation Ultra 5 computer using Varian VNMR software, version 6.1, revision C, with zero-filling to 4096 x 4096 and sine bell apodization such that sb = at/2 and sb1 = ni/(2*sw1).
H-H NOESY NMR spectra. Samples for NOESY NMR experiments were prepared in an Innovative Technologies, Inc. glove box using a NMR tube sealed with a PTFE screw cap. Sealed ampules of pyridine $d$-5 and CD$_3$OD with 0.03% tetramethylsilane were used as solvents for these experiments. 600 MHz NOESY spectra were acquired at 30 °C with 2048 points, 256 increments, 8 transients per increment, $\tau_{\text{mix}} = 0.7$ s, and an interscan delay (d1) of 3*T1 (standard T1 relaxation experiments were performed for each compound). Spectra were processed using nmrPipe$^{28}$ as follows: 1) 4 points back prediction, 2) 90° shifted sinebell apodization, 3) zero-filling to 8192 points, 4) Fourier transformation and phasing, 5) linear prediction to 512 points, 6) 90° shifted sinebell apodization, 7) zero-filling to 2048 points, and 8) Fourier transformation and phasing. The Sparky program,$^{29}$ version 3.113 was used for peak-picking and integration of crosspeaks.

Phase-sensitive COSY (COSYPS) NMR spectra. 500 MHz COSYPS spectra were acquired at 30 °C with 2048 points, 256 increments, and 4 transients per increment. 600 MHz COSYPS spectra were acquired at 30 °C with 2458 points, 308 increments and 8 transients per increment. All COSYPS spectra were acquired with sufficient interscan delay to allow for full spin-relaxation (d1 = 23.2 seconds, as determined by T1 relaxation experiments, was sufficient for all compounds).

Gradient HMBC NMR spectrum. A gradient HMBC spectrum of 2.27 was acquired at 23 °C with 2048 points, 280 increments, and 128 transients. Parameters for C-H coupling were set such that $j_{1xh} = 140$ Hz and $j_{nxh} = 8$ Hz. The spectrum was processed on a SUN Microsystems SPARCstation Ultra 5 computer using Varian VNMR software, version 6.1 revision C, with zero-filling to 1024 points in the indirect dimension and sinebell apodization such that $sb = at/2$ and $sb1 = ni/(2*sw1)$.

COSYPS processing and $J_H-H$ determination. Raw COSYPS data were processed as described by Bax and coworkers$^{20}$ to produce a diagonal-suppressed spectrum and a diagonal-only spectrum (Representative spectra for AmdeB allyl ester 2.30 are shown on page 36). Amplitude-constrained multiplet evaluation (ACME),$^3$ was used to determine $J_H-H$ coupling constants.

Figure 2.7. Diagonal-only COSYPS spectrum of Amde B allyl ester 2.30. Adapted with permission from Palacios, D.S.; Anderson, T.M.; Burke, M.D. *J. Am. Chem. Soc.* **2007**, *129*, 13804-13805. Copyright 2007 American Chemical Society.
Crosspeak Fitting. The ACME method for determining $J$ values from COSYPS spectra is described in detail by Delaglio et al. $^3$ Briefly, the ACME program can accurately integrate the crosspeaks of a COSYPS spectrum provided the experiment is run with interscan delay time sufficient for the spins to fully relax. ACME integrates selected peaks from the diagonal-only spectrum and the resulting integration values are used to integrate crosspeaks in the diagonal-suppressed spectrum. The figure on this page shows the results of fitting three peaks from the COSYPS diagonal of AmdeB allyl ester 2.30. Panels A, B, and C contain; A) the selected region of the spectrum (with peaks for fitting labeled 1 and 2), B) simulated peaks calculated in the fitting process, C) the residual between the experimental and calculated peaks. Therefore, in an accurate simulation, no residual is present for the selected peaks. Through this process, the average integration of selected peaks may be used to integrate crosspeaks from the diagonal-suppressed spectrum.

Figure 2.8. ACME results for fitting the AmdeB allyl ester 2.30 diagonal. A) The selected spectral region with peaks for fitting labeled 1 and 2. B) Simulated peaks calculated in fitting process. C) Residual between panels A) and B). Adapted with permission from Palacios, D.S.; Anderson, T.M.; Burke, M.D. *J. Am. Chem. Soc.* **2007**, *129*, 13804-13805. Copyright 2007 American Chemical Society.
The fine structure of crosspeaks in a COSYPS spectrum can be used to obtain coupling constants in a manner analogous to obtaining coupling constants from a one-dimensional multiplet. In a two-dimensional crosspeak, the spacing between antiphase portions of the crosspeak correspond to the $J$ value for the associated spins. The ACME method employs the reference integration from the diagonal in a peak-fitting algorithm that integrates selected crosspeaks and calculates coupling constants. The figure on this page shows the results from the fitting algorithm applied to the cross-peak corresponding to protons H-18a and H-17 in AmdeB allyl ester 2.30. Panel A again contains the selected peak from the spectrum. Panel B depicts the simulated crosspeak, and Panel C displays the residual. The lack of any significant residual is consistent with accurate reproduction of the multiplet fine structure in the fitting process. Similar analysis for each crosspeak in the spectra of compounds 2.27, 2.3, 2.29 and 2.30 was used to derive the coupling constants from which dihedral constraints were calculated.

**Figure 2.9.** Calculation of $^3J$ for H-18a(pseudoaxial) and H-17 for AmdeB allyl ester 2.30. ACME accurately reproduces the fine structure of the multiplet with no residual between the experimental and calculated peaks. Adapted with permission from Palacios, D.S.; Anderson, T.M.; Burke, M.D. *J. Am. Chem. Soc.* 2007, 129, 13804-13805. Copyright 2007 American Chemical Society.
Dihedral angles were calculated from H-H $^3J$ values according to Altona’s extended Karplus equation\textsuperscript{18} using the “HLA (4 substituents)” setting in the MestReJ software.\textsuperscript{30} However, each $^3J$ gives rise to 4 possible solutions to the Karplus equation. Methods for choosing the appropriate value are well-precedented. For example, in the context of conformational analysis of the erythronolide B lactone, Aurichio\textsuperscript{31} and Egan\textsuperscript{32} chose dihedral values consistent with NOE data and the erythronolide B crystal structure, respectively. For our analyses, we chose angles consistent with both NOESY data and the AmB crystal structure.\textsuperscript{5} In some cases, two solutions to the Karplus equation were consistent with both NOESY and crystal structure data, and both solutions were included. The selected dihedral angles (± 30°) were used as constraints in Monte Carlo conformational searches (see Part 3).

**Part 3. Energy Minimization Calculations**

**NMR-Restrained Model Structures.** Monte Carlo conformational searches were performed using the Molecular Operating Environment program (MOE), Version 2006.08,\textsuperscript{33} with the empirical MMFF94x force field and a Born solvation model with no distance cutoffs for non-bonded interactions. Initial atomic coordinates and structure files were generated from the AmB crystal structure using MOE. NMR-derived distance and dihedral constraints were set with a weighting factor of 200. 3500 random conformations were generated and minimized with Gaussian distribution of dihedrals biased towards multiples of 30°, dihedral minimization (RMS = 100), 0.001 Cartesian minimization RMS gradient, 0.0001 Cartesian perturbation, 0.1 RMS tolerance, a maximum of 2000 energy minimization steps for each minimization, a failure limit of 5000, no chiral inversion, no rotation about π bonds or amide bonds, and an energy cutoff of 5 kcal/mol. Force field partial charges were calculated before each minimization. Default values were used for all other parameters.

Consistent with protein structural analysis techniques,\textsuperscript{34} each H-H dihedral was constrained to the selected value ± 30°. When two solutions to the Karplus equation were selected, both values (± 30°) were allowed. The table on page 40 lists the dihedral constraints used in the conformational searching. Consistent with the standard convention for dihedral angles, values for the dihedral, $\theta$, were defined over the range $-180° < \theta \leq 180°$. Consistent with the known trans-
configuration of the seven double bonds of the polyene moiety, the π-bonds were constrained to 180 ± 10°.

Interproton distances were constrained for proton pairs exhibiting NOE correlations, with the lower limit set at 1.8 Å (twice the hydrogen van der Waals radius), and the upper limit set at 5.0 Å. The table on page 41 lists the NOE correlations used for conformational searching, and the figures on pages 42-45 depict these correlations (red lines indicate NOE correlations) Notably, all four compounds contain a diagnostic series transannular NOEs between protons of the polyol and those of the polyene.

The Monte Carlo conformational search explores conformational space by randomly perturbing all dihedral angles in the molecule and then minimizing the resulting structures (taking into account the restraints as described above). MOE repeated this process 3500 times, and the lowest energy conformation of each compound was used in rigid root mean square (RMS) atom alignment (see below).
**Figure 2.10.** Dihedral constraints used in conformational searches using MOE. Also shown are the coupling constants (calculated by ACME) from which the dihedral constraints were derived. Adapted with permission from Palacios, D.S.; Anderson, T.M.; Burke, M.D. *J. Am. Chem. Soc.* 2007, 129, 13804-13805. Copyright 2007 American Chemical Society.

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\(^{a}\) Associated cross peak was either too close to the diagonal or too weak to achieve an acceptable peak fit.

\(^{b}\) The generalized Karplus equation was utilized to determine dihedral constraint (Karplus, M.; *J. Phys. Chem.* 1959, 30, 11-15.)
Figure 2.11. Observed NOE correlations used to derive distance constraints in conformational searches using MOE. Adapted with permission from Palacios, D.S.; Anderson, T.M.; Burke, M.D. J. Am. Chem. Soc. 2007, 129, 13804-13805. Copyright 2007 American Chemical Society.

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Figure 2.12. NOE correlations for N-acyl AmB Methyl ester 2.27. For clarity, appendages other than protons have been removed from the macrolide skeleton. Selected carbon atoms are numbered. Adapted with permission from Palacios, D.S.; Anderson, T.M.; Burke, M.D. J. Am. Chem. Soc. 2007, 129, 13804-13805. Copyright 2007 American Chemical Society.
Figure 2.13. NOE correlations for MeAmdeB 2.3. For clarity, appendages other than protons have been removed from the macrolide skeleton. Selected carbon atoms are numbered. Adapted with permission from Palacios, D.S.; Anderson, T.M.; Burke, M.D. *J. Am. Chem. Soc.* **2007**, *129*, 13804-13805. Copyright 2007 American Chemical Society.
Figure 2.14. NOE correlations for AmdeB allyl ester 2.30. For clarity, appendages other than protons have been removed from the macrolide skeleton. Selected carbon atoms are numbered. Adapted with permission from Palacios, D.S.; Anderson, T.M.; Burke, M.D. J. Am. Chem. Soc. 2007, 129, 13804-13805. Copyright 2007 American Chemical Society.
Figure 2.15. NOE correlations for N-Fmoc MeAmB 2.29. For clarity, appendages other than protons have been removed from the macrolide skeleton. Selected carbon atoms are numbered. Adapted with permission from Palacios, D.S.; Anderson, T.M.; Burke, M.D. *J. Am. Chem. Soc.* **2007**, *129*, 13804-13805. Copyright 2007 American Chemical Society.
Rigid RMS atom alignment for NMR-restrained model structures. Only the atoms of the macrolactone ring and the cyclic hemiketal were used for RMS alignment. All other atoms were deleted from the lowest-energy conformers of 2.27, 2, 20, and 22, and the resulting skeletons representing the ground-state conformations of AmB 2.1, MeAmB 2.4, AmdeB 2.2, and MeAmdeB 2.3, respectively were saved as MDL MOL files (*.mol) and imported into the Cerius² program, Version 4.11, with no energy minimization or calculation of charges. Rigid RMS atom alignment revealed RMSD = 0.081 Å for the four structures. The aligned structures were saved as PDB files (*.pdb), and the overlay image (Figure 2.2) was generated using VMD.³⁶

2-10 REFERENCES


33 Molecular Operating Environment, version 2006.08; Chemical Computing Group: Montreal, Quebec, Canada.
Chapter 3

Functional Group Deletions Reveal the Mycosamine-Dependent Sterol Binding of Amphotericin B

Aiming to definitively test the three specific hypotheses for the mechanistic role(s) played by the C(41) carboxylate and C(19) mycosamine, a series of biological and biophysical assays were developed in which to study our functional group deficient derivatives. The experiments focused on four key functional properties: antifungal activity, ion channel formation, membrane binding and membrane embedded sterol binding. The results of these investigations conclusively demonstrated that, in stark contrast to the leading model for AmB’s mechanism of action, oxygenation at C(41) is not required for potent antifungal or ion channel activity. Alternatively, mycosamine was shown to be absolutely required for both biological activity and the capacity to form ion channels. Furthermore, using an isothermal titration calorimetry (ITC) based assay, we obtained the first conclusive evidence for a direct AmB-ergosterol interaction, thereby resolving the long-standing debate regarding the nature of AmB’s sterol dependency. Moreover, we found that mycosamine, but not the carboxylate, is absolutely required to promote this binding event. These results therefore established a new functional role for a glycoside appended to a small molecule natural product, i.e. the mediation of a functionally vital small molecule-small molecule interaction. Based upon these results, and related studies with another natural product, natamycin, we proposed a novel, potentially general, two mechanism model to account for AmB’s potent antifungal activity. Ian Dailey performed the experiments shown in Figures 3.10, 3.11, 3.12 and 3.13 and contributed to the development of the ITC sterol binding assays. David M. Seibert assisted with the MIC assays shown in Figure 3.3 and helped with the development of the SEC-based liposome binding assay in Figure 3.8. Brandon C. Wilcock prepared additional amphoteronolide B for these experiments. Portions of this chapter were adapted from Palacios, D.S.; Dailey, I.; Seibert, D.M.; Wilcock, B.C.; Burke, M.D. Proc. Natl. Acad. Sci. USA 2011, 108, 6733-6738.
Chapter 2 presented the synthesis and three-dimensional characterization of MeAmB, AmdeB and MeAmdeB. This represented the successful completion of the first two modules of the functional group deletion algorithm presented in Chapter 1. Thus, we turned our attention to the final component, the biophysical and biological investigation of AmdeB, MeAmdeB, and MeAmB. As discussed in Chapter 1, many investigations have probed the function(s) of the C(41) carboxylate and C(19) mycosamine but the role(s) of these two chemical groups have yet to be definitively established. Therefore, we designed a series of assays to unambiguously evaluate the three hypotheses shown in Figure 3.1 using MeAmB, AmdeB and MeAmdeB as mechanistic probes. The development, application and results of these assays will be discussed in the succeeding sections.

*Figure 3.1* Three proposals for the role of the C(41) carboxylate and C(19) mycosamine in the antifungal mechanism of AmB. As is discussed in more depth in Chapter 1, it has been hypothesized that the acid and mycosamine A) form an intermolecular salt-bridge/hydrogen bond that stabilizes the AmB ion channel, B) interact electrostatically with the zwitterionic phospholipid headgroup and C) promote sterol binding by hydrogen bonding to the sterol 3β hydroxyl group. Adapted from Palacios, et al *Proc. Natl. Acad. Sci. USA* 2011, 108, 6733-6738 Copyright 2011, National Academy of Sciences.
3-2 EXTINCTION COEFFICIENT DETERMINATION

The syntheses of MeAmB, AmdeB and MeAmdeB are not trivial, requiring multiple HPLC purification steps and up to two weeks to fully complete. With this in mind, the conservation of these molecules in the course of assay development and execution was very important. Accordingly, we determined the extinction coefficient of AmB, MeAmB, AmdeB and MeAmdeB and then split the compounds up into approximately 100 microgram quantities. The extinction coefficient for each molecule is shown in Figure 3.2. Interestingly, we found that the identity of the chemical groups at C(41) and C(19) had a large impact on the extinction coefficient of the polyene, but this effect is difficult to rationalize. Prior to running an experiment, an appropriate vial(s) was removed from the freezer, and the compound was quantified spectrophotometrically after being dissolved in DMSO. By creating a depository of multiple samples, the danger of catastrophic loss was precluded, and potentially harmful freeze-thaw cycles were also avoided.

![Chemical structures of AmB, MeAmB, AmdeB, and MeAmdeB](image)

*Figure 3.2. The experimentally determined extinction coefficients of AmB, MeAmB, AmdeB, and MeAmdeB.*

3-3 ANTIFUNGAL ACTIVITY

Prior to pursuing any biophysical experiments, we wanted to first determine the fungicidal properties of HPLC purified MeAmB, AmdeB and MeAmdeB. As discussed in Chapter 2, the biological activities of these molecules had been minimally investigated prior to
Towards this end, we employed a disk diffusion assay\(^2\) for the qualitative evaluation of antimicrobial activity against *S. cerevisiae*. The results of this experiment are shown in Figure 3-3. Confirming the validity of the assay, AmB potently inhibits the growth of *S. cerevisiae* as demonstrated by the zone of growth inhibition around the paper disk. Next, neither aglycone displayed any antifungal activity, results consistent with the proposed critical role(s) of mycosamine. However, counter to the leading models, MeAmB displayed roughly equipotent antifungal activity to the natural product in this assay. A subsequent broth mircodilution minimum inhibitory concentration (MIC) assay,\(^3\) confirmed the results of the disk diffusion assay, and established that AmB and MeAmB are indeed quantitatively equipotent against both *S. cerevisiae* and the opportunistic pathogen *C. albicans*. This assay also showed that, remarkably, the two molecules lacking mycosamine remained completely inactive even at concentrations 100-200 fold higher than the MIC of AmB. Thus, the mycosamine appendage, but not oxidation at C(41), is strictly required for antifungal activity. With this data in hand, we set out to systematically evaluate the role(s) of the C(41) carboxylate and C(19) mycosamine in each of the three proposed mechanisms shown in Figure 3.1, beginning with ion channel formation.

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<th>MeAmdeB 3.3</th>
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<tr>
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<td>&gt;50</td>
<td>&gt;50</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
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**Figure 3.3** Antifungal activity of AmB and the three functional group deficient derivatives against *S. cerevisiae* in a disk diffusion assay and *S. cerevisiae* and *C. albicans* in an MIC broth microdilution assay. The MIC is defined as the lowest concentration of compound that completely inhibits yeast growth. Adapted from Palacios, et al. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 6733-6738 Copyright 2011, National Academy of Sciences.
To test whether the ring of polar interactions shown in Figure 3.1A is required for ion channel formation, we first performed a potassium efflux assay from live *S. cerevisiae* cells.\(^4\) This assay employed a commercially available valiomycin-based potassium selective ion probe to detect the cellular efflux of this ion. As shown in Figure 3.4A, both AmB and MeAmB rapidly caused the loss of intracellular potassium, whereas the two aglycones showed no activity beyond the DMSO vehicle. In an attempt to more generally detect the electrochemical disruption of *S. cerevisiae* by AmB and MeAmB, we also tried experiments using the fluorescent potentiometric probe 3,3′-dipropylthiacarbocyanine iodide (diS-C\(_3\)(5)).\(^5\) This emissive dye is sensitive to the electrochemical potential of live cells and, promisingly, it has been effective for studying the selective permeabilization of prokaryotic cell membranes by small molecules.\(^6\) In addition, diS-C\(_3\)(5) was reported to be compatible with *S. cerevisiae*,\(^7\) but, despite this potential, we were unable to develop a reliable assay for cellular depolarization with diS-C\(_3\)(5).

**Figure 3.4.** Potassium efflux from **A.** *S. cerevisiae* and **B.** 10 percent ergosterol EYPC liposomes demonstrate the membrane permeabilizing activity of AmB and MeAmB but not AmdeB or MeAmdeB. The concentration of each molecule is 3 µM in the *S. cerevisiae* assay and 1 µM in the EYPC assay. Adapted from Palacios, et al *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 6733-6738 Copyright 2011, National Academy of Sciences.

With the fluorescence-based assay abandoned, we returned to the potentiometric potassium efflux platform. To remove potentially complicating factors present with yeast, such as cellular proteins and the β-1,3 glucan cell wall, we also performed this assay using 200 nm egg yolk phosphatidylcholine (EYPC) liposomes loaded with 10 percent ergosterol (Figure
Consistent with the results of the *S. cerevisiae* potassium efflux assay, AmB and MeAmB displayed potent membrane permeabilizing activity while AmdeB and MeAmdeB failed to elicit detectable levels of ionic flux. While the potentiometric experiments were extremely useful and convenient for showing the inability of AmdeB and MeAmdeB to permeabilize membranes, the resolution of this assay is insufficient to distinguish between gross membrane disruption and discrete ion channel formation. Thus, a possible and valid interpretation of the efflux data is that MeAmB is not actually forming ion channels, but is rather acting as a detergent to non-specifically damage phospholipid bilayers. Single AmB ion channels have been observed previously using voltage clamp planar lipid bilayer technology, so we decided to acquire this technology for a definitive clarification of the potential role of the salt bridge in ion channel formation.

The planar lipid bilayer (also referred to as a black lipid membrane or BLM) is a commonly employed electrophysiological technique to separate two aqueous compartments by a small patch of membrane. This technology is convenient for the study of channel forming species such as AmB because both aqueous chambers are easily accessible and can be manipulated to suit the experiment but are electrically isolated by the lipid bilayer. The original and still ubiquitously employed method for forming planar lipid bilayers is the painting technique developed by Muller in 1962. This procedure employs a solution of lipids in a non-polar solvent, usually *n*-decane, which is successively applied to a small (about 100 microns) hole in a non-polar substrate using a glass applicator. From this “painting” process, lipid bilayers spontaneously form across the hole, and the integrity of these newly formed bilayers can be investigated using the electrical components of the instrument. One of the drawbacks of planar lipid bilayers is their physical fragility. These bilayers rarely persist for more than one hour under standard experimental conditions, and relatively small mechanical disruptions (such as bumping into the instrument) are sufficient to rupture the bilayer.

The other key component of the planar lipid bilayer system is the voltage clamp, which is required to investigate the formation and electrophysiological properties of ion channels. This technology has proven to be a robust tool for electrophysiology research since it was first introduced in the late 1940’s for studies of the giant squid axon. The essential function of the voltage clamp is to control the membrane potential (*i.e.* “clamping”), with the magnitude of the membrane potential being chosen by the operator to suit the experiment. When an ion channel
forms in the planar lipid bilayer and ions flow through the newly formed pore, the membrane potential is altered because of the direct, Ohmic relationship between current and voltage. The voltage clamp then precisely applies amperage to the system to counteract the current resulting from electrolyte flow through the ion channel and thereby return the membrane voltage to the preset resting value. Thus, it is this negative feedback that is key to the function of the voltage clamp, and the method by which it can precisely provide the electrophysiological characteristics of protein or small molecule ion channels. The exquisite sensitivity of the voltage clamp (detection in the picoamp range) system requires that these experiments be housed in a faraday cage to exclude extraneous electrical signals.

Applying this technology to the study of AmB, MeAmB, AmdeB and MeAmdeB required that significant technical hurdles be overcome. After initially building the instrument in Roger Adams Lab, a stable baseline for an unmodified lipid bilayer could not be achieved. While a membrane could be formed, as indicated by the characteristic changes in capacitance and current consistent with bilayer formation that detected by the instrument, the baseline appeared to have a sinusoidal noise associated with it. This roll in the baseline was suggestive of a repetitive mechanical vibration, and we suspected that the sound of the vacuum pumps and/or fume hoods was the source of the unstable baseline. However, turning off all of the vacuum pumps and fume hoods in the room did not correct the rolling baseline. Following this, we suspected that the instrument was picking up motions of the building through the bench it was placed on. With this working hypothesis, we had a winch installed that could suspend the instrument from the ceiling on a concrete slab, but the unstable baseline persisted. Upon the failure of the winch setup, we discovered that, directly above where the instrument was located, were the pumps to run the fume hoods present in Roger Adams Lab and this was the source of the mechanical noise that produced the unstable baseline. Thus, Roger Adams Lab proved to be unsuitable for this very sensitive equipment, and so the instrument was disassembled and moved to a temporary location in the basement of the Institute for Genomic Biology. After being moved, the system was rebuilt and planar lipid bilayers with a flat, stable baseline could be reproducibly formed.
The initial experiments with AmB utilized the standard experimental setup, consisting of a Delrin® cup with a precisely drilled hole that supports the planar lipid bilayer. As shown in Figure 3-5A, the cup conveniently fits into a complementary cell and it can also be used multiple times without physical degradation of the hole, providing highly reproducible bilayer formation. It is this ease of use that makes this particular setup advantageous, but it turned out to not be compatible for experiments with AmB. The poor solubility of AmB prevented us from effectively cleaning out the Delrin® cup, and the AmB contaminated cups could not be used for further experimentation. We tried to use harsh cleaners and/or detergents to remove the AmB, but these treatments physically degraded the aperture, making the cups useless. The solution we devised for this problem is shown in Figure 3-5B. This setup uses a home built Delrin® cell with a small “V” cut out of a partition that separates two chambers. A small, thin (125 µM) sheet of Teflon® with an approximately 100 µM hole burned into it is placed over this cut and is adhered to the walls of the partition with vacuum grease. We could readily and reproducibly form planar lipid bilayers using this setup and, at the conclusion of an experiment, the Teflon® sheet was simply discarded, obviating the threat of compound carryover.

**Figure 3.5.** A. Photograph of the conventional setup for planar lipid bilayer experiments. The Delrin® cup with a 200 µM hole drilled into it is placed into the larger, back chamber of the cell (The hole is too small to be visible in the picture). Once a bilayer is formed the front (cis) and back (trans) aqueous solutions are electrically isolated by the phospholipid bilayer. B. Picture of the home-built Delrin® cell used for the AmB experiments. The notch in the partition where the Teflon® sheet is placed is visible (The Teflon® sheet is not shown).
Once the system had been fully setup and validated we were able to study the channel forming capacity of AmB, MeAmB, AmdeB and MeAmdeB. For these studies, we used a mixture of pig brain phosphatidylcholine and pig brain phosphatidylethanolamine to form the planar bilayers, because this particular lipid mixture was used in prior studies with AmB. Representative results from these experiments are shown in Figure 3-6. We routinely observed single ion channel formation for AmB and MeAmB, but channel activity was never observed for either of the two aglycones. The concentrations 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 ion channels were observed at concentrations between 0.5 and 5 nM, while MeAmB displayed single channel activity between 30 and 80 nM. When the concentration of MeAmdeB was raised to 100 nm in an attempt to see channel activity, we...
instead only observed gross membrane destruction, as evidenced by an abrupt change from zero current to an offscale reading.

Based upon the results described in this section, we have conclusively demonstrated that the predicted ring of charged interactions shown in Figure 3.1A that were proposed to be critical for ion channel formation and antifungal activity is not required for either. Conversely, a different and vital role for the mycosamine appendage in ion channel formation is apparent from the total lack of channel activity observed for AmdeB and MeAmdeB. Intriguingly, variations were seen between the electrophysiological characteristics of AmB and MeAmB during the course of the planar lipid bilayer studies, as can be clearly viewed in Figure 3-6. Furthermore, the permeabilizing activities of these two compounds against S. cerevisiae are also visibly distinct (Figure 3-5A). The differences in ion channel behavior between AmB and MeAmB were not investigated in a quantitative fashion, however, and this remains an interesting preliminary observation that merits a more extensive inquiry.

3-5 PHOSPHOLIPID MEMBRANE BINDING

One possible explanation for the lack of channel and biological activity of AmdeB and MeAmdeB is that these molecules are not capable of inserting into phospholipid bilayers. This

![Figure 3.7](image.png)

**Figure 3.7.** A. Binding of AmB, MeAmB, AmdeB and MeAmdeB to live S. cerevisiae cells. B. When the experiment was run in the absence of S. cerevisiae greater than 90% of each compound was recovered. Adapted from Palacios, et al *Proc. Natl. Acad. Sci. USA* 2011, 108, 6733-6738 Copyright 2011, National Academy of Sciences.

would be consistent with the hypothesis that electrostatic interactions between mycosamine and
zwitterionic phospholipid headgroups anchor AmB into the bilayer membrane (Figure 3-1B). To test this proposal, we first analyzed the binding of AmB, MeAmB, AmdeB and MeAmdeB to live *S. cerevisiae* cells. The degree of binding was analyzed by incubating the cells separately with each molecule, followed by centrifugation of the yeast and spectrophotometric quantification of the amount of compound remaining in the supernatant. As shown in Figure 3-7A, all four compounds readily bind *S. cerevisiae* by this centrifugation-based assay. To ensure that the compounds were not simply adhering to the walls of the polyethylene microcentrifuge tubes or decomposing during the course of the experiment, we also performed this experiment in the absence of *S. cerevisiae* and found that greater than ninety percent of each compound was recovered in this control experiment (Figure 3-7B).

Ergosterol containing EYPC liposomes were next utilized to determine the ability of these four compounds to partition directly into naked phospholipid bilayers. The partitioning of AmB into sterol-containing and sterol-free liposomes has previously been determined, but these studies relied on changes in measureable physiochemical properties (such as the electronic absorption spectrum of AmB) to measure lipid binding. These methods therefore rely on assumptions regarding the origins of the observed physiochemical changes, for example that the change in $\lambda_{\text{max}}$ is directly correlated to lipid binding. In contrast, physically separating bound and unbound molecules, as was done with the *S. cerevisiae* assay, is advantageous because no such assumptions have to be made, since the amount of bound or unbound compound is directly quantified. Accordingly, we first attempted to directly translate the *S. cerevisiae* experiment to 200 nm EYPC liposomes, but under the intense centrifugation conditions required to pellet these liposomes (70,000 g for at least an hour) over fifty percent of the AmB also precipitated in the liposome-free control experiments. To combat this issue, we used the dibrominated lipid 1-oleoyl-2-(9,10-dibromostearoyl)-sn-glycero-3-phosphocholine (OPBC), which, due to the increased density of the dibrominated acyl chain, can be precipitated using milder centrifugation conditions. Even under these more gentle conditions, though, a significant degree of AmB precipitation was still observed, which we attributed to the known propensity of AmB to form large, multi-molecular aggregates in aqueous solution. Aiming to circumvent the continued precipitation of AmB, we developed a size exclusion chromatography (SEC) based assay to measure membrane partitioning. In this experiment, after incubating each small molecule with a suspension of liposomes, the mixture was purified by SEC and any unbound small molecules...
were retained on the column while the liposomes rapidly elute and were collected. The amount of drug inserted into the bilayer could then be directly measured by dissolving the post-column liposome suspension in an organic solvent and spectrophotometrically quantifying the now solution-phase drug. As shown in Figure 3-8A, all four compounds bound equally to the ergosterol containing model membranes, confirming the results of the S. cerevisiae binding assay. We also determined that these molecules effectively partition into sterol-free liposomes (Figure 3-8B). Importantly, when AmB, MeAmB, AmdeB and MeAmdeB were independently loaded onto the SEC column in the absence of liposomes, less than ten percent of each compound eluted at the same retention time as the EYPC liposomes (Figure 3-8C).

The finding that AmB and the functional group deficient derivatives readily bind phospholipid bilayers has two important implications. One, it has been proposed that AmB alternatively exerts its antifungal activity via autooxidation of the polyene subunit, an event that triggers a radical chain process which chemically damages cellular membranes. However, given our result with the aglycones, which both readily bind S. cerevisiae and retain the
putatively redox active polyene but yet are completely lacking in antifungal activity, strongly argues against the autooxidation mechanism as the main source of AmB’s antifungal activity. In addition, the hypothetical polar interactions between the C(41) carboxylate and/or mycosamine and the charged phospholipid headgroups are not required for AmB to effectively partition into sterol containing or sterol free membranes. The insertion of AmB and the functional group deficient derivatives into phospholipid bilayers is instead, similar to many antimicrobial peptides and detergents, most likely driven primarily by the hydrophobic effect. \(^{27}\)

3-6 STEROL BINDING

Definitively testing the hypothesis that the C(41) carboxylate and/or C(19) mycosamine are required bind membrane sterols (Figure 3-1C) has proven to be very difficult. Whether or not AmB even directly binds sterols, or if AmB is dependent on sterol-mediated global changes in membrane properties remains an issue of significant debate in the literature (See Chapter 1 for a more in depth discussion). Recently, an isothermal titration calorimetry (ITC) based assay was developed and used to test the sterol binding ability of natamycin, \(^{28}\) a mycosamine containing polyene macrolide related to AmB. We predicted that this highly sensitive membrane embedded sterol binding assay, in combination with our functional group deficient derivatives, could be used to finally resolve this long-standing question. After adapting this assay for our own purposes, we initially looked at the interaction between AmB and three different 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC) liposome systems. The three model membranes we used were sterol-free, ten percent lanosterol and ten percent ergosterol (Figure 3.9). We chose to

![Figure 3.9](image-url)

**Figure 3.9.** The structures of ergosterol, cholesterol and lanosterol. Ergosterol is the main sterol found in fungal cell membranes, cholesterol is the major mammalian sterol and lanosterol is the biosynthetic progenitor to both molecules.
also use lanosterol-loaded liposomes because, importantly, it has been shown in micropipette aspiration and solid-state NMR experiments that ergosterol and lanosterol have very similar macroscopic and molecular effects on POPC bilayers.\textsuperscript{29} Thus, if the dominant interaction between AmB and membrane sterols is the sterol-induced differential preorganization of the membrane, the ITC data for ergosterol and lanosterol would be expected to also be similar.

\begin{figure}
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\begin{tabular}{ccc}
\hline
 & time (min) & time (min) & time (min) \\
\hline
\textbf{A} & \textbf{B} & \textbf{C} \\
\hline
\textbf{AmB (-) sterol} & \textbf{AmB (+) lanosterol} & \textbf{AmB (+) ergosterol} \\
\hline
\end{tabular}
\end{figure}

\begin{itemize}
\item \textbf{D} \quad \textbf{(-) sterol} \quad \textbf{(+)} \textbf{lano} \quad \textbf{(+)} \textbf{erg} \quad \Delta \textbf{h} \textbf{(\mu cal)} \\
\textbf{AmB} \quad -18.1 \pm 4.1 \quad -14.0 \pm 6.0 \quad -37.3 \pm 4.4 \quad -19.3 \pm 6.0 \quad P = 0.0008
\end{itemize}

The results of these experiments are shown in Figure 3-10. The titration of AmB with sterol-free POPC liposomes (Figure 3-10A) produced a very small net exotherm, and a similar small heat was also observed when titrating AmB with 10 percent lanosterol containing liposomes (Figure 3-10B). Conversely, when 10 percent ergosterol containing liposomes were added to a solution of AmB, we observed a marked increase in the net exotherm, consistent with
a direct AmB-ergosterol binding interaction (Figures 3-10C and 3-10D). Further supporting this conclusion is the lanosterol experiment, which clearly demonstrates that sterol dependent global membrane properties do not lead to an increase in the net exotherm beyond sterol-free membranes. Therefore, these results provide the first conclusive evidence that AmB and ergosterol participate in a direct small molecule-small molecule binding interaction.

These ITC experiments have therefore finally resolved the long-standing debate between the indirect sterol binding hypothesis and the direct sterol binding hypothesis. The definitive clarification of this lasting issue highlights the power of the synthesis-enabled functional group deletion strategy to reveal the function of complex small molecules. Thus, this experimental strategy holds much promise as a general approach for probing other interesting mechanistic questions.30
Figure 3.11. A. Representative thermograms of AmB titrated with sterol-free and ergosterol-loaded liposomes. B. Representative thermograms of MeAmB titrated with sterol-free and ergosterol-loaded liposomes. C. Summary of data, showing the increase in heats between sterol-free and ergosterol containing liposomes. Adapted from Palacios, et al Proc. Natl. Acad. Sci. USA 2011, 108, 6733-6738 Copyright 2011, National Academy of Sciences.

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<th>$\Delta \sum_{i=1}^{N} h_i (\mu cal)$</th>
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<td>-68.1 ± 6.9</td>
<td>-25.0 ± 10 P = 0.0003</td>
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</tbody>
</table>

Figure 3.12. A. Representative thermograms of AmdeB titrated with sterol-free and ergosterol-loaded liposomes. B. Representative thermograms of MeAmdeB titrated with sterol-free and ergosterol-containing liposomes. C. Summary of data showing that the net exotherm did not change in the presence of ergosterol. Adapted from Palacios, et al Proc. Natl. Acad. Sci. USA 2011, 108, 6733-6738 Copyright 2011, National Academy of Sciences.

<table>
<thead>
<tr>
<th></th>
<th>(-) erg</th>
<th>(+) erg</th>
<th>$\Delta \sum_{i=1}^{N} h_i (\mu cal)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmdeB</td>
<td>-9.3 ± 3.8</td>
<td>-9.5 ± 2.0</td>
<td>-0.2 ± 4.3</td>
</tr>
<tr>
<td>MeAmdeB</td>
<td>-10.9 ± 5.0</td>
<td>-6.2 ± 2.1</td>
<td>-4.7 ± 5.4</td>
</tr>
</tbody>
</table>
Following the establishment of the AmB-ergosterol interaction, we moved forward to look at the sterol binding capacity of the functional group deficient derivatives to probe the role of the carboxylate and mycosamine in sterol binding (Figures 3-11 and 3-12). First, we repeated the ergosterol binding experiment with AmB and EYPC liposomes with and without 10 percent ergosterol. We changed to EYPC lipids at this point because the binding and efflux assays were performed using this liposome system. The ITC experiment with the EYPC liposomes gave similar results for AmB (Figure 3-11A) as for the POPC liposomes, so we then assayed the sterol-binding capacity of MeAmB. As shown in Figure 3-11B, changing from sterol-free to 10 percent ergosterol liposomes also produced a dramatic increase in the net exotherm for MeAmB, in a manner analogous to AmB. For MeAmB, the observed heats in microcals were significantly greater for both the sterol free and ergosterol containing liposomes than the corresponding values for AmB, but the difference in these heats is statistically the same for both AmB and MeAmB two compounds (Figure 3-11C). Therefore, based upon this ITC data, oxidation at C(41) is not required to bind ergosterol.

Subsequent examination of the ergosterol binding ability of AmdeB and MeAmdeB produced strikingly different results. For example, unlike the previous three ITC experiments, titrating AmdeB with ten percent ergosterol loaded liposomes did not produce an increase in the net exotherm relative to the sterol free system (Figure 3-12A). Similarly, no differences between the two exotherms were observed in the titration experiments with MeAmdeB (Figure 3-12B). Indeed, for both AmdeB and MeAmdeB, the numerical difference between the evolved heat for sterol free and ten percent ergosterol liposomes is within experimental error of zero (Figure 3-12C). Thus, the results with the aglycones clearly demonstrate that mycosamine is absolutely required to bind ergosterol, and this represents a previously unknown role for a glycoside appended to a small molecule natural product, i.e. the mediation of a functionally vital small molecule-small molecule binding interaction.31

The demonstration that AmB directly interacts with ergosterol led us to question if AmB also binds cholesterol, the main sterol found in mammalian cells, in a mycosamine dependent fashion. For the initial cholesterol ITC experiments, we used the same experimental protocol that had proven to be successful in establishing the AmB-ergosterol interaction, but we did not observe any cholesterol dependent changes in the net exotherm under these conditions. We attributed this effect to a possible lowered affinity of AmB for cholesterol, and so we altered the
experimental conditions in an attempt to record this binding event. We tripled the amount of AmB in the experiment (from 50 to 150 μM). We also increased the concentration of the liposomes injected into the AmB solution from 8 to 12 mM, to add more cholesterol to the system. Under these new, augmented conditions, we were able to observe the mycosamine-dependent binding of AmB to cholesterol (Figure 3-13).

**Figure 3.13.** A. Representative thermograms of AmB titrated with sterol-free and cholesterol-loaded liposomes under the more sensitive conditions. B. Representative thermograms of AmdeB titrated with sterol-free and cholesterol-containing liposomes under the more sensitive conditions. C. Summary of data showing the net increase in the exotherm in the presence of cholesterol for AmB but not AmdeB. Adapted from Palacios, et al *Proc. Natl. Acad. Sci. USA* 2011, 108, 6733-6738 Copyright 2011, National Academy of Sciences.
The results described in this chapter clearly and conclusively demonstrate that mycosamine is required for 1) binding sterols, 2) forming ion channels and 3) killing yeast. This suggests an intimate and causative link between sterol-dependent ion channel formation and antifungal activity. That is, of the four compounds tested, only those that could both bind ergosterol and form ion channels (AmB and MeAmB) also had the capacity to inhibit the growth of yeast. Accordingly, these experimental observations can be used to formulate two distinct hypotheses to explain the source of AmB’s antifungal activity. One, sterol binding is necessary for ion channel formation, but it is only ion channel activity that is responsible for killing yeast, and two, channel formation is one of multiple sterol-binding dependent mechanisms of action. Of these two mechanistic scenarios, we strongly favor the latter interpretation, for the following reasons. First, other mycosamine bearing polyene macrolides have been described that do not have the ability to form ion channels but display significant antifungal activity. Recently, a mechanistic investigation of the non-channel forming macrolide natamycin (Figure 3.14) led to the conclusion that this molecule has a different mechanism of action than AmB, and kills yeast cells by sequestering ergosterol and preventing the participation of this molecule in vital cellular events. For the purposes of direct comparison with our AmB results, we performed MIC, S. cerevisiae potassium efflux and ITC binding assays with natamycin, the results of these experiments are shown in Figure 3.14. In addition, an earlier examination of the relative

![Figure 3.14. Biophysical and biological Studies with the polyene macrolide natamycin (also called pimaricin). A. Structure of natamycin. The mycosamine and acid are conserved but the molecule is significantly shorter than AmB. B. Natamycin does not elicit the efflux of potassium from S. cerevisiae cells. C. Natamycin is actively fungicidal against S. cerevisiae and C. albicans, though it is not as active as AmB or MeAmB. D. Natamycin binds ergosterol as demonstrated by the ITC binding assay. Adapted from Palacios, et al Proc. Natl. Acad. Sci. USA 2011, 108, 6733-6738 Copyright 2011, National Academy of Sciences.](image-url)
antifungal and membrane permeabilizing activities of AmB and the related natural product nystatin found that these properties were disconnected in the yeast *C. albicans*.\textsuperscript{34} In this study, nystatin was more effective than AmB at increasing the membrane permeability of *C. albicans*, but was a less potent antifungal agent. A disassociation between membrane permeabilization and cell death has also been described for some AmB-resistant *C. albicans* mutants.\textsuperscript{35} In these mutants, AmB induced ionic efflux did not necessarily also lead to cell death.

We herein propose a unifying model that is consistent with all this data. We propose that AmB kills yeast cells through two, complementary mechanisms of action.\textsuperscript{36} The first mechanism, shared in common with natamycin, is the binding and sequestering of ergosterol from vital physiological functions, and the second mode of action is the formation of a supramolecular transmembrane AmB ion channel that disrupts the cellular electrochemical gradient. This two mechanism model may prove to be generally applicable to explain the biological activity of the entire class of antifungal polyene macrolides, and requires further exploration.

**Figure 3.15.** A proposed general model for the mechanism of action of antifungal polyene macrolides, this model proposes two complementary mechanisms: 1) The mycosamine-mediated binding of ergosterol, preventing the sterol from participating in vital cellular physiology and 2) Sterol and mycosamine dependent ion channel formation. Adapted from Palacios, et al *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 6733-6738 Copyright 2011, National Academy of Sciences.
It is interesting to note that a similar two-mechanism model is hypothesized to be operative for the antibacterial lantibiotic nisin.\textsuperscript{37} Nisin is a peptidic natural product widely used industrially to prevent the spoilage of food and it exerts its antibiotic activity by forming pores in bacterial membranes in a lipid II dependent fashion.\textsuperscript{38} In addition, much like natamycin, the related, shorter lantibiotic mutacin cannot form pores because of its smaller size,\textsuperscript{39} but retains antibacterial activity by binding to lipid II. As a final striking overlap between the small molecule and peptide systems, both of the channel forming molecules, AmB and nisin, are more potent against their respective microorganism targets than their non-channel forming relatives, natamycin and mutacin. Thus, the polyene macrolides and lantibiotics appear to have convergently evolved a similar dual mechanism to achieve highly potent, difficult to resist antimicrobial activity. More recent studies with natamycin were accompanied by similar speculations.\textsuperscript{40}

3-8 THESIS SUMMARY

This thesis describes the articulation and initial application of the functional group deletion strategy as applied to the clinically vital antimycotic amphotericin B. This strategy is composed of three experimental modules: the synthesis of functional group knockouts, determination of the ground state conformation of the derivatives, and biophysical and biological investigation into the functional consequences of these deletions. The first chemical moieties that were marked for deletion were the C(41) carboxylate and C(19) mycosamine, both of which had been proposed to be critical for the antifungal activity of AmB. Three synthetic derivatives were therefore targeted: C(41)-Methyl amphotericin B, amphoteronolide B and C(41)-methyl amphoteronolide B. To access these molecules, a flexible degradative synthesis from the natural product was developed and executed to provide greater than 10 mg of each compound and in greater than ninety percent purity after preparative HPLC purification. The highlights of these syntheses are the modular strategy that maximizes the number of common synthetic intermediates and reaction conditions, and the selective removal of the acid and mycosamine under mild, high yielding conditions, thereby enabling excellent synthetic throughput and efficiency. Next, extensive two-dimensional NMR experiments with AmB, MeAmB, AmdeB and MeAmdeB were used to calculate the ground state conformation of these four molecules. It was determined that the conformation of the macrolactone was completely conserved in the
functional deficient derivatives, facilitating interpretation of the biophysical and biological experiments.

The third element of the functional group deletion strategy was then enacted, the biophysical and biological investigation of the functional group deficient derivatives. Through a series of assays to test biological activity, ion channel formation, membrane binding and membrane embedded sterol binding, we gained key insights into AmB’s molecular mechanisms of action. For example, we found that, contradictory to the leading hypothesis for AmB’s mechanism of action, oxygenation at C(41) is not required for potent ion channel or antifungal activity. In addition, we also discovered that the C(41) carboxylate is not required for membrane binding or ergosterol binding. However, experiments with AmdeB and MeAmdeB demonstrated the absolute requirement of mycosamine for biological activity, ion channel formation and sterol binding. Based upon these results and parallel studies with natamycin, we proposed a novel, potentially general model in which AmB exhibits two, distinct mechanisms of antifungal activity: sterol sequestration and sterol-dependent ion channel formation.

Collectively, the studies described in this thesis significantly advance the mechanistic understanding of this fascinating natural product. With these results as a foundation, it is possible that further mechanistic elucidation of AmB could yield a strategy to rationally design a less toxic analogue of this medically indispensable antifungal agent. In addition, the prospect of molecular prosthetics, small molecule mimics of protein function, is further strengthened by the more refined comprehension of this prototypical small molecule ion channel. Finally, this thesis is a powerful demonstration for the capacity of the functional group deletion strategy and synthetic organic chemistry to illuminate even the most elusive aspects of small molecule function.
3-9 EXPERIMENTAL SECTION

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\textsubscript{18} 30 x 150 mm column, as described previously. Ergosterol was 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 in Chapter 2.

General procedure for extinction coefficient determination. Several milligrams of dried compound were massed in a tared vial using a Mettler Toledo MT5 microbalance. This sample was then dissolved in DMSO to create a concentrated stock solution. From this concentrated stock, a 1 mM solution in DMSO was prepared and this 1 mM solution was used to prepare the following serial dilution in DMSO: 800 \textmu M, 500 \textmu M, 300 \textmu M, 100 \textmu M and 50 \textmu M. Then, each of the serial dilution solutions (including the 1 mM solution) was added to 450 \textmu L of methanol. The methanol samples were prepared in triplicate and analyzed by UV/Vis spectroscopy. The average absorbance and standard deviation for each sample was then plotted against concentration. The data was fitted with a linear least squares fit using Excel and the slope of the fitted line was used as the extinction coefficient.\textsuperscript{41}

Antifungal Assays

Growth Conditions for \textit{S. cerevisiae}.

\textit{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.

**Disk Diffusion Assay.**

Protocols for disk diffusion assays were adapted from the Clinical and Laboratory Standards Institute document M2-A8. *S. cerevisiae* were streaked on YPD agar plates with an inoculating loop and incubated at 30 °C until individual colonies could be identified by eye (~ 24 h). Five individual colonies were transferred from the agar plate with a sterile pipette tip to liquid YPD medium. The liquid culture was incubated overnight at 30 °C in a shaker incubator (200 rpm). The saturated cell culture was diluted with YPD medium to an OD₆₀₀ of 0.1 (~ 5 x 10⁶ cfu/mL) as measured on a Shimadzu PharmaSpec UV-1700 UV/Visible spectrophotometer. This culture was used to inoculate an YPD plate by streaking the entire plate with a sterile cotton tip applicator three times, turning the plate approximately 60 °C after each application and finishing by swabbing the rim of the agar. The plate was allowed to dry for approximately 2 to 3 minutes before application of paper disks impregnated with compounds 3.1-3.4. The disks were prepared in the following manner: 10 microliters of a 4 mg/ml solution of each compound in DMSO was added to a sterile 9 mm disk of Whatman 4 filter paper. Controls were prepared in a similar manner using only DMSO. The disks were then placed on the agar and gently pressed with forceps. All disks, including DMSO controls, were added within 15 minutes of inoculation. After disks were added to the plate the plate was inverted and incubated at 30 °C for 48 hours prior to assessment. Those compounds which showed a visible zone of growth inhibition were judged to be active. This experiment was repeated and yielded the same results. The assay for *C. albicans* was performed similarly except the liquid and solid cultures were incubated at 37 °C instead of 30 °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. 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\textsubscript{600} of 0.10 (\(~5 \times 10^6\) cfu/mL) as measured by a Shimadzu (Kyoto, Japan) PharmaSpec UV-1700 UV/Vis spectrophotometer. The yeast suspension 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 (\textit{S. cerevisiae}) or 37 °C (\textit{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.

**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 (\textit{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 \textit{S. cerevisiae}.**

The protocol to determine potassium efflux from \textit{S. cerevisiae} was adapted from a similar
experiment utilizing \textit{C. albicans}.\textsuperscript{4} An overnight culture of \textit{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\textsubscript{600} of 1.5 (~1x10\textsuperscript{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.

\textbf{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:

\[
S = 100 \left( \frac{[K^+]\text{final}}{[K^+]\text{initial}} - 1 \right)
\]

Each concentration data point was then normalized by entering into the above formula as “[K]\text{final}” before plotting as a function of time.

\textbf{Efflux from 10% ergosterol LUVs.}

\textbf{LUV Preparation.}

Egg yolk phosphatidylcholine (EYPC) was obtained as a 10 mg/mL solution in CHCl\textsubscript{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\textsubscript{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
l 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. 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 N₂. 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 H₂SO₄. 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 $10400 \text{ L mol}^{-1} \text{ cm}^{-1}$ at the $\text{UV}_{\text{max}}$ 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*.

**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\(^43\) 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\textsuperscript{17} 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.

\textbf{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.\textsuperscript{9,44} 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.

\textbf{Yeast Binding Assay}

\textbf{Determination of Binding.}

The yeast binding assay was adapted from the report by Kobayashi and coworkers that demonstrated binding of AmB to intact \textit{S. cerevisiae} cells.\textsuperscript{19} 10 mL of an overnight culture of \textit{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.\cite{24} 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 coefficient of each compound.\cite{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.

**LUV Binding Assay**

**Preparation of LUVs.**

LUVs were prepared as described in the liposome efflux studies 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 above.

**LUV Binding.**

The partitioning of AmB into both sterol-containing\cite{45,46,47} and sterol-free\cite{48,49,50} 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. 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.

**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
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, 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. The overall heat
evolved during the experiment was calculated using the following formula:

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

Where \( i = \) injection number, \( n = \) total number of injections, \( \Delta h^i_{\text{injection}} = \) heat of the \( i^{th} \) injection, \( \Delta h^n_{\text{injection}} = \) the heat of the final injection of the experiment.

**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.

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\textsubscript{2}O rather than pure DMSO as for AmB and its derivatives. The MIC values for natamycin represent the average of two independent runs.

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\textsubscript{2}O rather than pure DMSO as for AmB and its derivatives.

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\textsuperscript{-4} mmol of natamycin was added to a 4 mL vial as a solution in 85:15 DMSO:H\textsubscript{2}O. 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\textsubscript{2}O and then 2.178 mL of K buffer was added to give the 50 µM solution used in the titration experiment.
The extinction coefficient for AmB was previously determined to be 161,000 L mol$^{-1}$ cm$^{-1}$:


According to Ohm’s Law, current and voltage are directly related: $I = (V/R)$


We used the Planar Lipid Bilayer Workstation available from Warner Instruments.

The design of this cell was adapted from: Mayer, M.; Kriebel, J.K.; Tosteson M.T.; Whitesides G.M. Biophys. J. 2003, 85, 2684-2695.

The hole was burned into the Teflon® using a high voltage spark. This method is described in: Hartshorne R.; Tamkun, M.; Montal, M. The Reconstituted Sodium Channel from Brain In Ion Channel Reconstitution Miller C. ed.; Plenum Press, New York, pp 349-350.


41 The value obtained for AmB in this manner agrees with the previously reported value; see McNamara, C.M.; Box, S.; Crawforth, J.M.; Hickman, B.S.; Norwood, T.J.; Rawlings, B.J. J. Chem. Soc. Perkin Trans. I 1998, 83-87.


