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C3-OH of Amphotericin B Plays an Important Role in Ion Conductance

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Supporting Information

ABSTRACT: Amphotericin B (AmB) is the archetype for small molecules that form ion channels in living systems and has recently been shown to replace a missing protein ion transporter and thereby restore physiology in yeast. Molecular modeling studies predict that AmB self-assembles in lipid membranes with the polyol region lining a channel interior that funnels to its narrowest region at the C3-hydroxyl group. This model predicts that modification of this functional group would alter conductance of the AmB ion channel. To test this hypothesis, the C3-hydroxyl group was synthetically deleted, and the resulting derivative, C3deoxyAmB (C3deOAmB), was characterized using multidimensional NMR experiments and single ion channel electrophysiology recordings. C3deOAmB possesses the same macrocycle conformation as AmB and retains the capacity to form transmembrane ion channels, yet the conductance of the C3deOAmB channels is 3-fold lower than that of AmB channels. Thus, the C3-hydroxyl group plays an important role in AmB ion channel conductance, and synthetic modifications at this position may provide an opportunity for further tuning of channel functions.

The antifungal polyene macrolide natural product amphotericin B (AmB) is the archetypical small molecule capable of forming ion channels in living systems,[1] and we recently reported that this small molecule can functionally substitute for a missing protein ion transporter and thereby restore physiology in yeast.[2] To maximally harness this remarkable functional capacity, it is necessary to understand the molecular underpinnings that govern AmB ion channel formation, conductance, gating, and selectivity. However, despite more than half a century of research, the structure of the AmB ion channel remains unknown.

Modeling studies predict that AmB self-assembles into multimeric structures in which the polyol region lines a water-filled channel interior.[3] This channel is predicted to have a wide entrance near the C15 alcohol funneling to its narrowest region near the C3 alcohol (Figure 1).[4] This model therefore predicts that modifications at the C3 position would permit channel formation but alter ion conductance.

No derivatives with modifications at the polyol region of AmB have been studied using electrophysiological recordings, which are critical for characterizing differences in single ion channel conductances. This is likely in large part a consequence of the synthetic difficulties in obtaining such site-specifically modified AmB derivatives. Synthetic modification of the C13 hemiketal[5] and genetic manipulations of the producing organism[6] and related organisms[7] have yielded polyol modified derivatives. However, the impacts of these modifications on single ion channel conductance have not been reported.

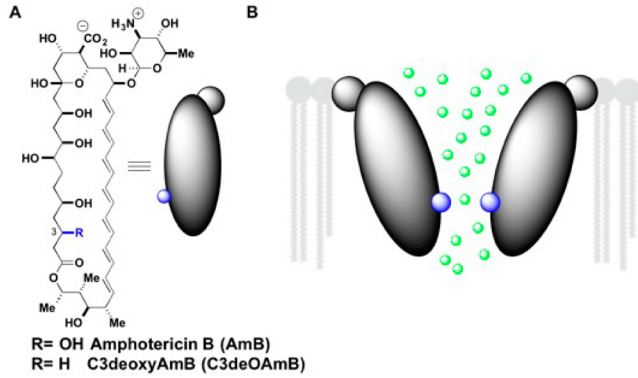

Figure 1. (A) AmB and C3deOAmB structures. (B) AmB ion channel model funneling to narrowest region at C3-OH (highlighted in blue).

To test the hypothesis that the C3-hydroxyl group plays an important role in ion channel conductance, we targeted its chemoselective deletion.[8] As one of 10 distinct hydroxyl groups appended to AmB, this represented a substantial synthetic challenge. We recognized, however, that the unique β-positioning of the C3 alcohol relative to the C1 carbonyl may provide an opportunity for selective elimination to form an alpha, beta-unsaturated macrolactone followed by chemoselective conjugate reduction.

This plan was ultimately reduced to an efficient 9-step synthesis as shown in Scheme 1. Starting with the natural product, a series of functional group protections delivered intermediate **1** (Scheme 1 and Supporting Information (SI)). Gratifyingly, exposure of **1** to NaHMDS at low temperatures chemoselectively eliminated the C3 *p*-methoxyphenylacetal, presumably via an E1cB type mechanism, yielding intermediate **2**. Subsequent site-selective Stryker reduction[9] of the carbonyl-

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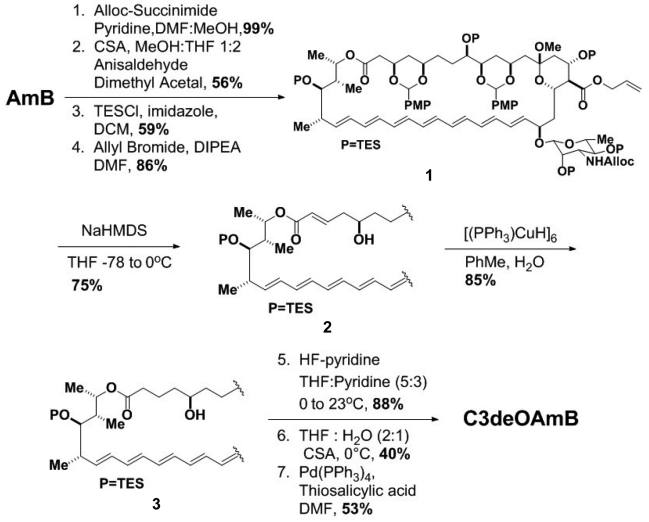
Scheme 1. Synthesis of C3deOAmB



conjugated C3, C4 double bond provided deoxygenated intermediate **3**. A final series of deprotections yielded the targeted single-atom-modified variant, C3deOAmB.

It was unclear at the outset whether this functional group deletion would cause changes in macrocycle conformation, which in turn would complicate the analysis of voltage clamp electrophysiological recordings of the corresponding ion channels. Specifically, in the crystal structure of a derivative of AmB, the C3 hydroxyl group is involved in a hydrogen-bonding network that includes both the C1 carbonyl and C5 hydroxyl group.[10] Disruption of such a hydrogen-bonding network might result in a change in macrocycle shape. To test this, we independently determined the ground-state conformations of both AmB and C3deOAmB using stochastic conformation generation methods constrained by extensive NOESY and phase-sensitive COSY NMR data processed using amplitude-constrained multiplet evaluation.[11] Optimization allowed us to perform these experiments without the use of solubilizing protective groups.

We determined the lowest energy conformations of the AmB and C3deOAmB structures using the LowModeMD conformational search method[12] with NOESY-based internuclear distance restraints and phase-sensitive COSY-based dihedral restraints.[11] As shown in Figure 2, the structure-based

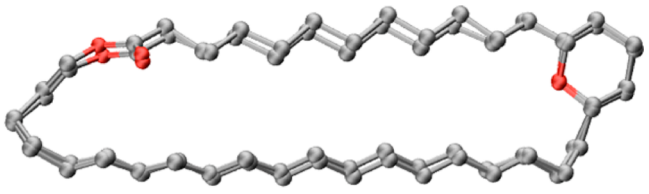


Figure 2. Overlay of the lowest energy conformations of AmB and C3deOAmB. The heavy atom RMSD is 0.33 ± 0.03 Å.

alignment of the lowest energy conformation of the C3deOAmB skeleton with the lowest energy conformer of AmB exhibits a heavy atom RMSD of 0.33 ± 0.03 Å, which is comparable to the precision of each structure. Thus, within the uncertainty of this NMR-based structure determination, the ground-state conformations of these two compounds are identical.

Further supporting this conclusion, both the ^{13}C and ^{1}H chemical shifts show excellent agreement between AmB and C3deOAmB, with the exception of the modified C3 site and its immediate neighbors, for which the chemical shifts change due to well understood electronegativity effects (Figures S6–S9).[13]

Finally, we tested the key hypothesis that removal of the C3 hydroxyl group would impact ion channel conductance. Specifically, HPLC-purified AmB and C3deOAmB were compared head-to-head in voltage-clamp single ion channel recording studies. Planar lipid bilayers were formed from a 70:30 ratio of DPhPC lipids to ergosterol at pH = 7.0. AmB or C3deOAmB were then added to both sides of the membrane as a solution in DMSO and channel activity was investigated. Shown in Figure 3A are representative single ion channel traces

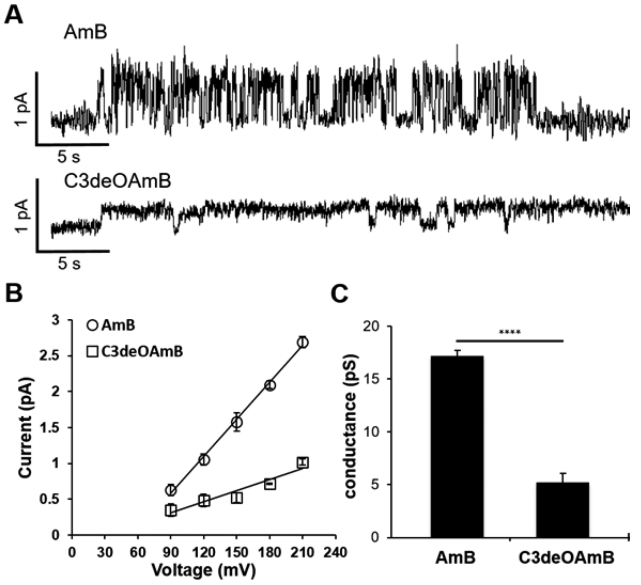


Figure 3. (A) Single ion channel activity of AmB (top) and C-3deOAmB (bottom) in planar lipids bilayers. DPhPC lipids with 30% ergosterol. One M KCl pH = 7.0, 5 mM HEPES + 150 mV. (B) Current vs voltage response of AmB and C3deOAmB from 90 to 210 mV in 30 mV increments. Two M KCl, pH = 7.0, 5 mM HEPES.[14] AmB r^2 = 0.9969. C3deOAmB r^2 = 0.9174. (C) Bar plot illustrating the differences in ion channel conductance between AmB and C3deOAmB. **** $p < 0.001$. Graphs depict means ± SD.

of both AmB (top) and C3deOAmB (bottom) (Figures S1–S2). The conductance of each molecule was determined by measuring the current of the single channels formed from each molecule at a range of voltages (Figure 3B). AmB has a single ion channel conductance of 17.2 ± 0.5 pS. Under identical conditions C3deOAmB also forms single ion channels, however, the conductance significantly decreases to 5.2 ± 0.9 pS, a 3-fold reduction (Figure 3C). Preliminary experiments in liposomes also support differences in ion selectivity between AmB and C3deOAmB channels (see SI).

Thus, deletion of a single atom at the C3 position of AmB is synthetically accessible, does not cause a change in macrocycle conformation, and permits the retention of channel-forming activity yet changes the ion channel conductance by 3-fold. These findings are consistent with structural[4] and theoretical[4d] models for the AmB ion channel that place the C3 alcohol in the path of ion conductance. They also suggest that other modifications at the C3-position, many of which should be accessible from intermediate **2**, might enable further under-

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standing and/or targeted optimization of this archetypical small molecule-based ion channel.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b05766.

Detailed synthesis, spectral data, and electrophysiological protocols (PDF)

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Notes

The authors declare no competing financial interest.

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