

Supplemental Information

Molecular prosthetics for CFTR designed for anion selectivity outperform amphotericin B in cystic fibrosis airway epithelia

Authors: Jonnathan P. Marin-Toledo^{1‡}, Daniel M. Greenan^{1‡}, Nohemy Celis^{1‡}, Laura Haske¹, Agnieszka Lewandowska¹, Christopher K. Rakowski¹, Shashank Shastry², Bryan Gworek², Arun Maji¹, Kelsie J. Green¹, Michael J. Welsh^{3,4}, Ian M. Thornell³, Taras V. Pogorelov^{1,2,5,6,7,8} ✉ & Martin D. Burke^{1,7,8,9,10,11} ✉

Affiliations:

1 Department of Chemistry, University of Illinois Urbana-Champaign, Urbana, IL, USA

2 Center for Biophysics and Quantitative Biology, University of Illinois Urbana-Champaign, Urbana, IL, USA

3 Department of Internal Medicine, Pappajohn Biomedical Institute, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA, USA

4 Howard Hughes Medical Institute, Iowa City, IA, USA

5 Department of Chemical and Biomolecular Engineering, University of Illinois Urbana-Champaign, Urbana, IL, USA

6 National Center for Supercomputing Applications, University of Illinois Urbana-Champaign, Urbana, IL, USA

7 Cancer Center at Illinois, University of Illinois Urbana-Champaign, Urbana, IL, USA

8 Department of Biochemistry, University of Illinois Urbana-Champaign, Urbana, IL, USA

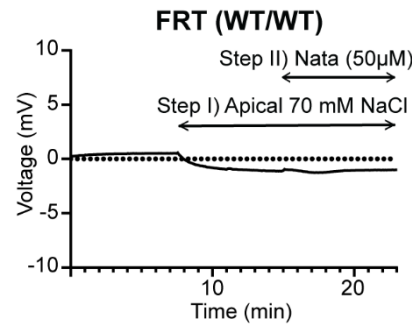
9 Molecule Maker Lab, Beckman Institute for Advanced Science and Technology, Urbana, IL, USA

10 Molecule Maker Lab Institute, Carl R. Woese Institute for Genomic Biology, Urbana, IL, USA

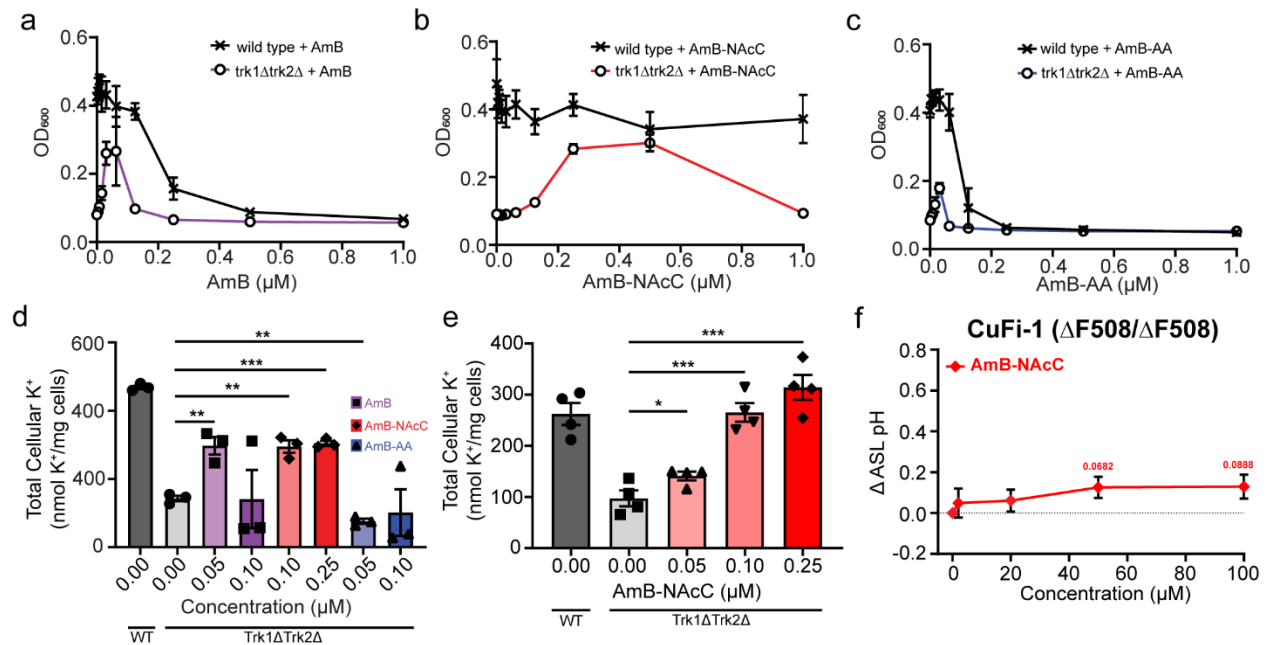
11 Carle Illinois College of Medicine, University of Illinois Urbana-Champaign, Urbana, IL, USA

‡These authors contributed equally to this work.

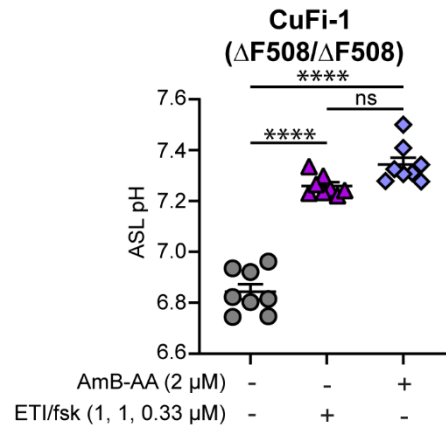
✉ Correspondence should be addressed to M.D.B. (mdburke@illinois.edu) and T.V.P. (pogorelo@illinois.edu)



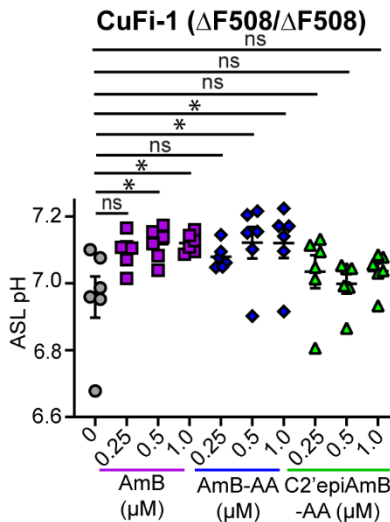
Supplementary Figure 1. Non-channel forming polyene macrolide, Natamycin, does not elicit voltage changes when added to Fischer Rat Thyroid (FRT) epithelia (WT/WT). Graphed representative traces of Ussing chamber current clamp ion-selectivity protocol prior to correcting for junction potentials. Step I) Apical media switch to 70 mM NaCl solution for paracellular ion movement determination. Step II) Addition of Natamycin bilaterally.



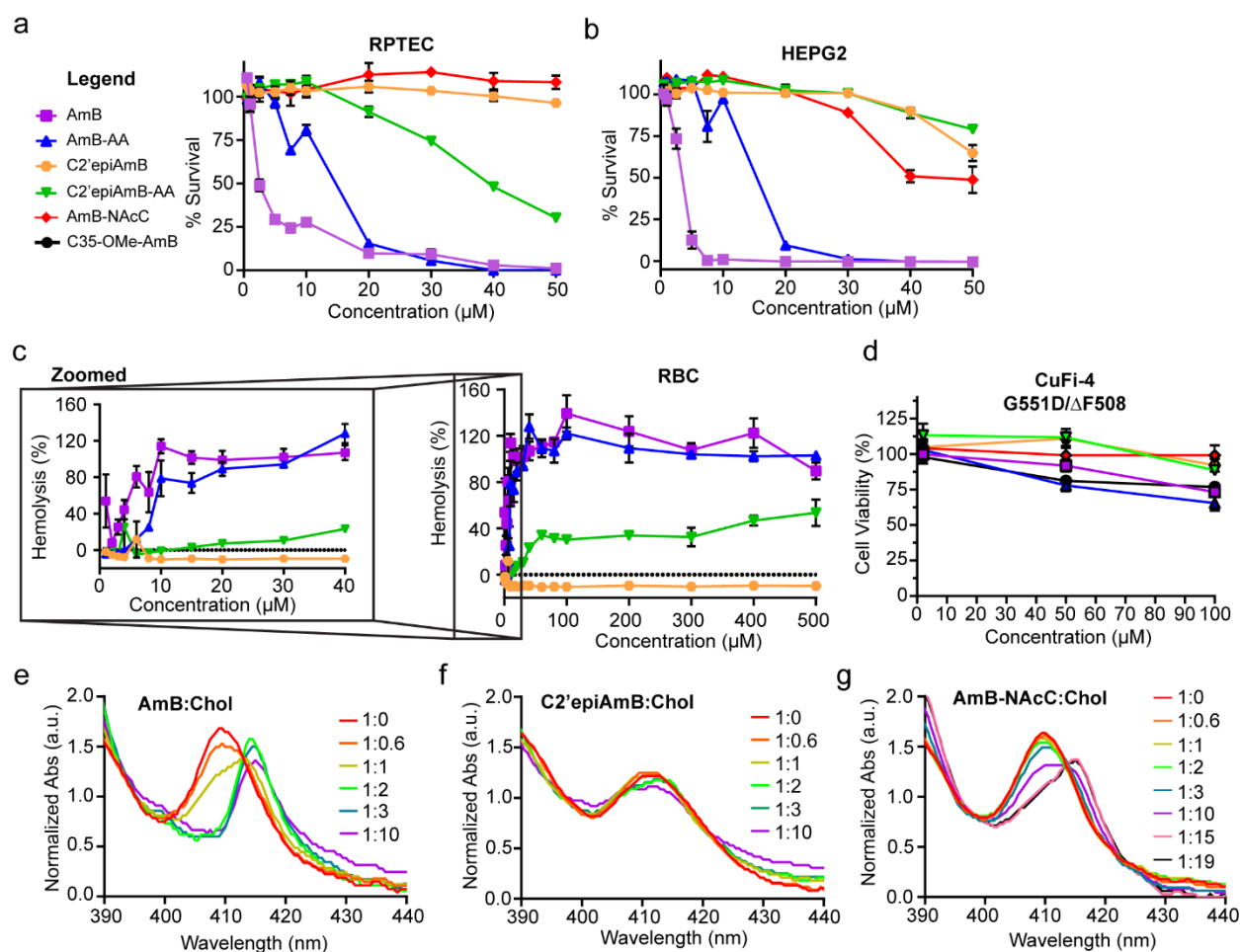
Supplementary Figure 2. AmB-NAcC hints at higher cation selectivity than AmB in physiological Assays. (a) Dose response of AmB in yeast rescue assay. (b) Dose response of AmB-NAcC in yeast rescue assay. (c) Dose response of AmB-AA in yeast rescue assay. (d) Total Cellular K⁺ levels of yeast for AmB, AmB-AA, AmB-NAcC via ICPMS (n = 3 per group). ** P<0.0048, *** P=0.0001. (e) Total Cellular K⁺ levels of trk1Δtrk2Δ with AmB-NAcC at varying doses (n = 4 per group). * P=0.048, *** P<0.0004. (f) ASL pH dose response for AmB-NAcC in CuFi-1 (ΔF508/ΔF508) epithelia (vehicle, n =5; all other doses, n = 6 per group). (d-f) Unpaired t-tests. Statistics utilized the Shapiro-Wilk test to check for normal distribution.



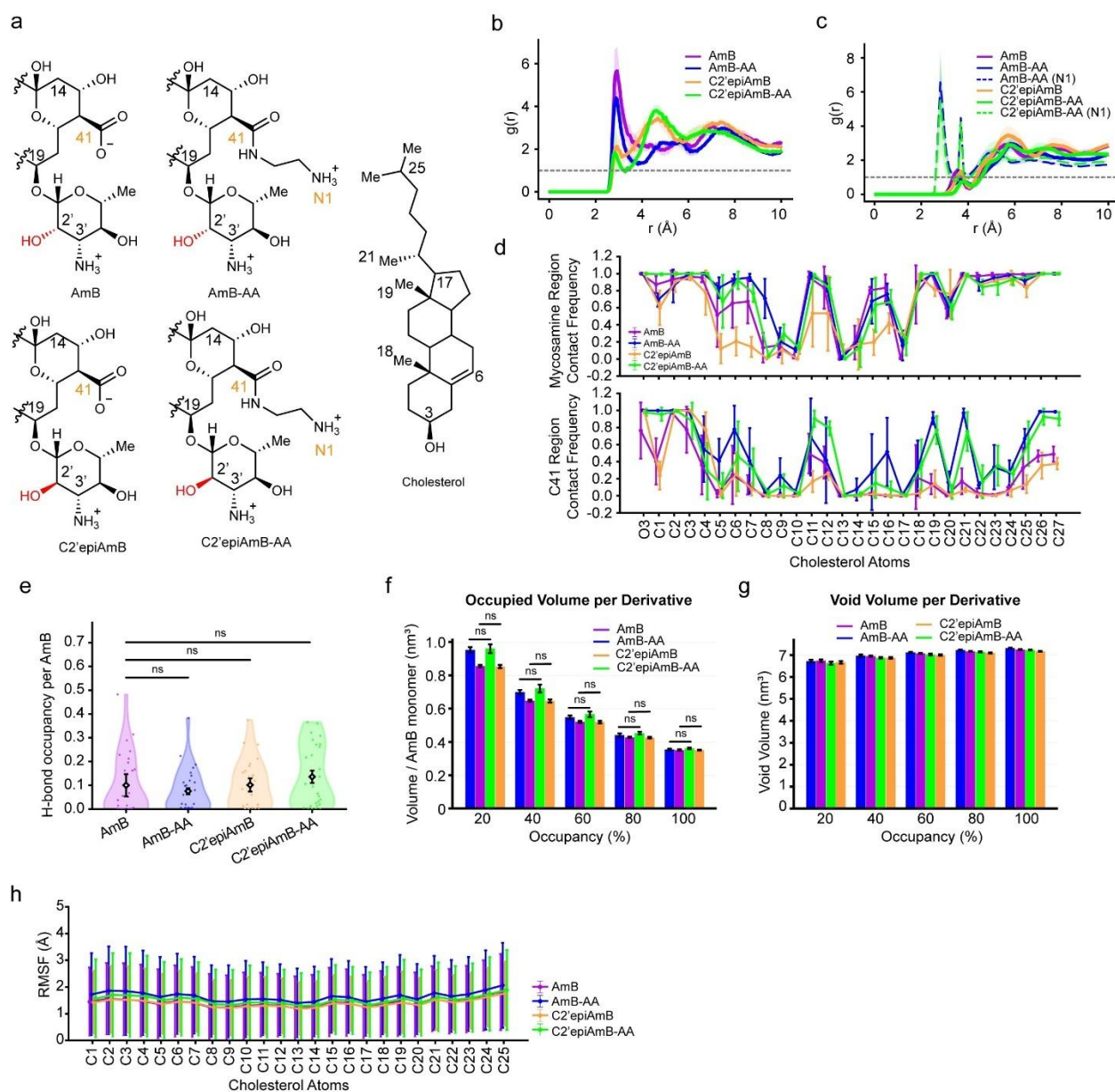
Supplementary Figure 3. AmB-AA results in a similar increase in ASL pH as current CFTR modulators. ASL pH measurements comparing the addition of 2 μ M AmB-AA to the addition of 1 μ M Elexacaftor, 1 μ M Tezacaftor, and 0.33 μ M Ivacaftor with 0.33 μ M forskolin to CuFi-1 (Δ F508/ Δ F508) epithelia (n = 8 per group). One-Way ANOVA with multiple comparisons. Statistics utilized the Sharpiro-Wilk test to check for normal distribution. ns, not Significant. **** P < 0.0001.



Supplementary Figure 4. ASL pH measurements following addition of 0.25 μ M, 0.50 μ M, and 1 μ M of AmB, AmB-AA, and C2'epiAmB-AA to CuFi-1 (Δ F508/ Δ F508) epithelia (n = 6 per group). One-Way ANOVA with multiple comparisons. Statistics utilized the Sharpiro-Wilk test to check for normal distribution. ns, not Significant. * P < 0.021.

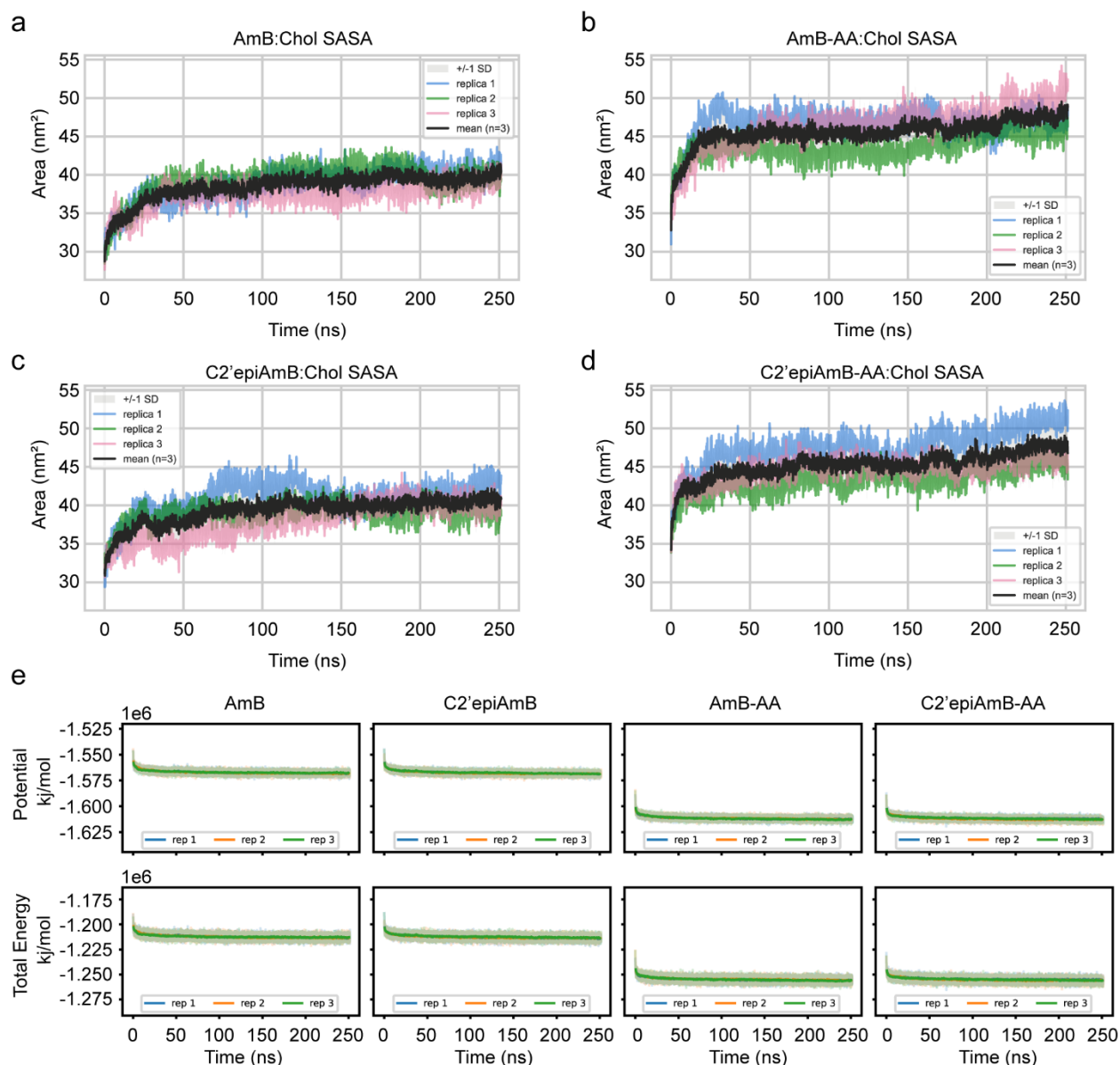


Supplementary Figure 5. Toxicity profile of AmB-derivatives in cholesterol containing cell lines. (a) RPTEC (primary renal proximal tubule epithelial cells; n=3 biological replicates per group) toxicity plot of AmB, AmB-AA, C2'epiAmB, C2'epiAmB-AA, AmB-NAcC. (b) HEPG2 (human liver cells; n=3 biological replicates per group) toxicity plot of AmB, AmB-AA, C2'epiAmB, C2'epiAmB-AA, and AmB-NAcC. (c) Hemolysis Plot of AmB, AmB-AA, C2'epiAmB, and C2'epiAmB-AA (n = 3 technical replicates per group). (d) CuFi-4 Toxicity of AmB, AmB-AA, C2'epiAmB, C2'epiAmB-AA, AmB-NAcC, and C35-OMeAmB at 2, 50, and 100μM (n = 12 per group for AmB, AmB-AA, C2'epiAmB-AA, and AmB-NAcC doses; n = 6 for C2'epiAmB and C35-OMeAmB doses.). (e) AmB:Cholesterol UV-Vis binding plot. (f) C2'epiAmB:Cholesterol UV-Vis binding plot. (g) AmB-NAcC:Cholesterol UV-Vis binding plot.

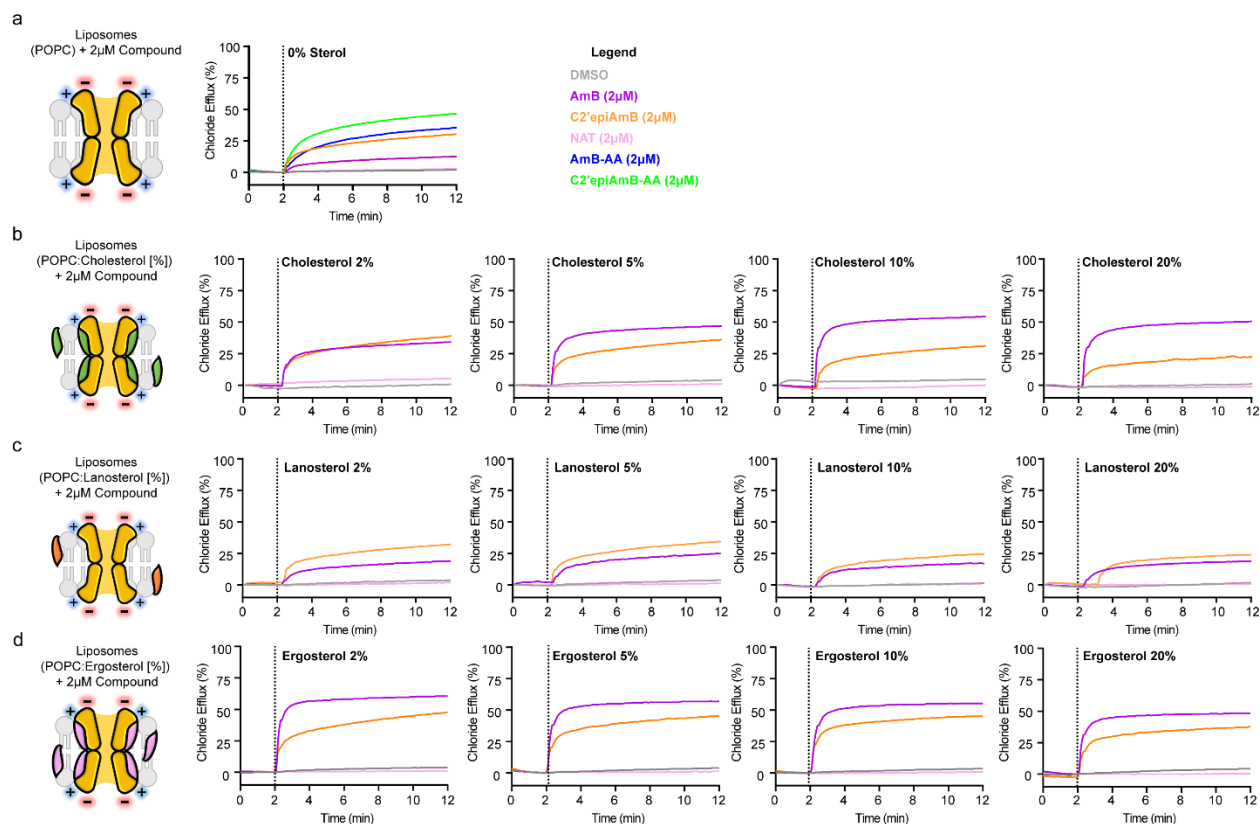


Supplementary Figure 6. AmB derivatives compatible with the local cholesterol environment of the experimentally derived sponge scaffold shows consistency with sterol-sponge mechanism. (a) Chemical structures of amphotericin B (AmB), AmB-AA, C2'epiAmB, C2'epiAmB-AA and cholesterol. The C2'-hydroxyl of the mycosamine is highlighted in red and the C41 carboxylate/carboxamide site in yellow. (b) Radial distribution function, $g(r)$, between the C2'-hydroxyl oxygen of each derivative and the cholesterol 3-hydroxyl oxygen (O3). C2'epiAmB derivatives lose the close-range contacts characteristic of hydrogen bonding. (c) $g(r)$ between the C41 region and cholesterol O3; the reference atom is C41 for all derivatives and the terminal N1 amine of the ethylenediamine side chain for AmB-AA and C2'epiAmB-AA. Both ethylenediamine-modified derivatives display close-range N1-O3 contacts. Shaded regions in (b,c) indicate standard deviation across replicates. (d) Per-atom contact frequency between

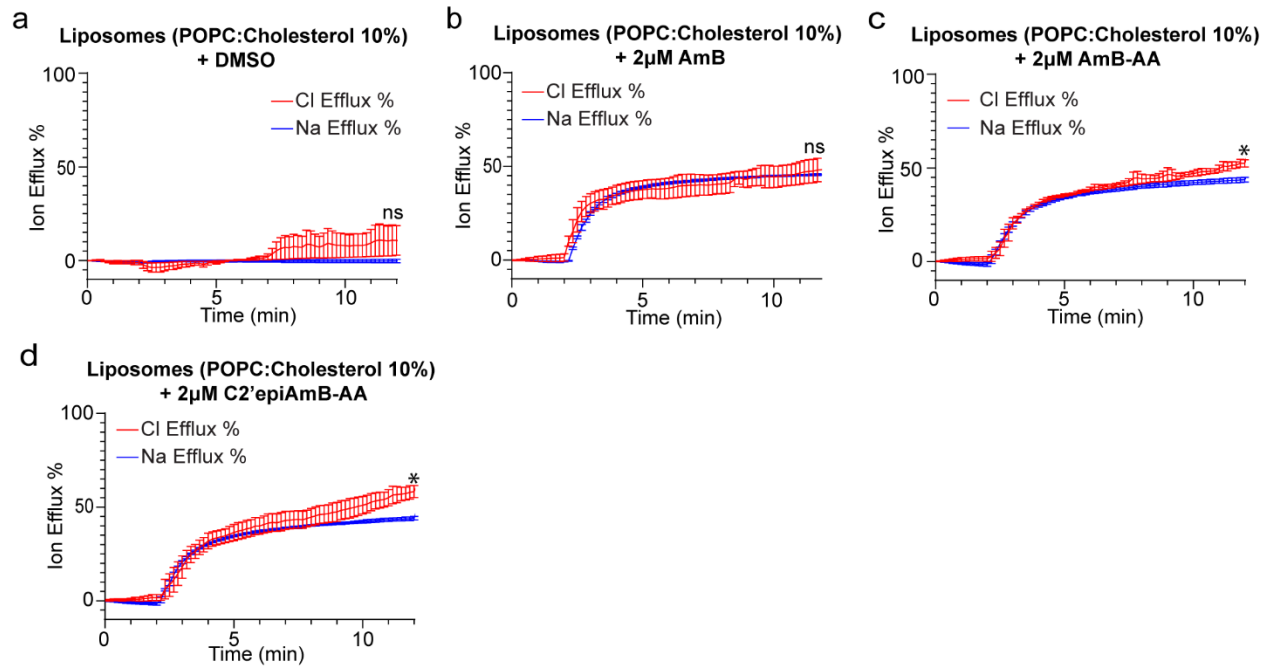
cholesterol (O3, C1–C27) and the mycosamine region (top; C2'-hydroxyl and C3'-amine) or C41 region (bottom; heavy atoms from C41 to terminal N1) of each derivative. **(e)** Hydrogen-bond occupancy with cholesterol, the fraction of frames in which a given AmB forms at least one hydrogen bond with a cholesterol. **(f)** Occupied volume analysis captures the conformational freedom of the C41 modification and mycosamine of each derivative within the sponge. Occupancy (%) denotes the fraction of simulation time during which the same voxels remain occupied; 100 corresponds to macrolide atoms restrained in the MD simulations. Standard deviation bars show replicate level uncertainty. AmB and C2'epiAmB are directly comparable, as are AmB-AA and C2'epiAmB-AA. **(g)** Void volume quantifies the change in volume available to accommodate a sterol, with occupancy (%) here denoting the fraction of simulation time a voxel remains unoccupied. **(h)** Root mean square fluctuation (RMSF) of each cholesterol carbon (C1–C25), showing comparable conformational freedom across all four derivatives. Error bars in (d,h) indicate standard deviation across replicates. Significance in (e–g) was assessed by Welch's t-test between AmB derivatives (ns, $P > 0.05$).



Supplementary Figure 7. Solvent accessible surface area calculations. (a-d) Computed SASA values for the internal core of each AmB:Chol derivative sponge, containing 27 AmD molecules, shows solvent equilibration with C41 and mycosamine regions occur in the first 20% of the simulation. There is a ~17% increase in solvent accessible surface area of AmB-AA and C2'epiAmB-AA sponges at equilibrium. This can be attributed to the ethylenediamine modification at the C41 carbon. **(e)** Internal and potential energies are converged before 20% of simulation time.



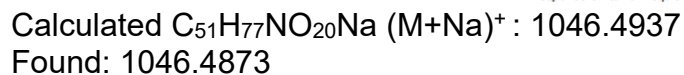
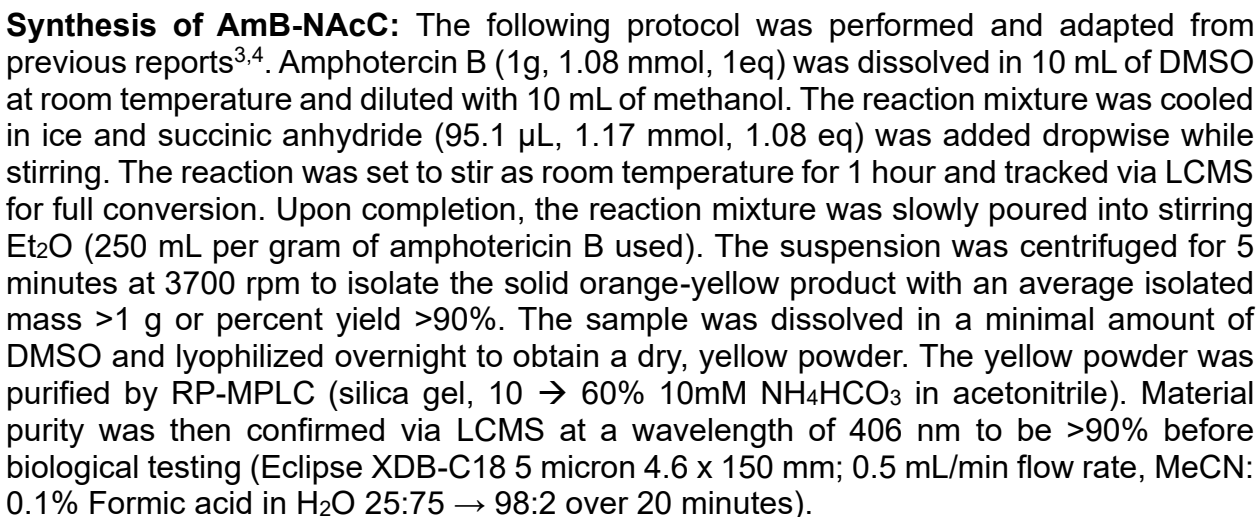
Supplementary Figure 8. POPC Liposomes shows AmB channel formation in non-sterol containing membranes (a) Chloride efflux of DMSO, AmB, C2'epiAmB, Natamycin, AmB-AA, and C2'epiAmB-AA (2 μ M) in sterol free liposomes. (b) DMSO, AmB, C2'epiAmB, and Natamycin (2 μ M) in cholesterol containing liposomes (2, 5, 10, and 20%). (c) Chloride efflux of DMSO, AmB, C2'epiAmB, and Natamycin (2 μ M) in lanosterol containing liposomes (2, 5, 10, and 20%). (d) Chloride efflux of DMSO, AmB, C2'epiAmB, and Natamycin (2 μ M) in ergosterol containing liposomes (2, 5, 10, and 20%).

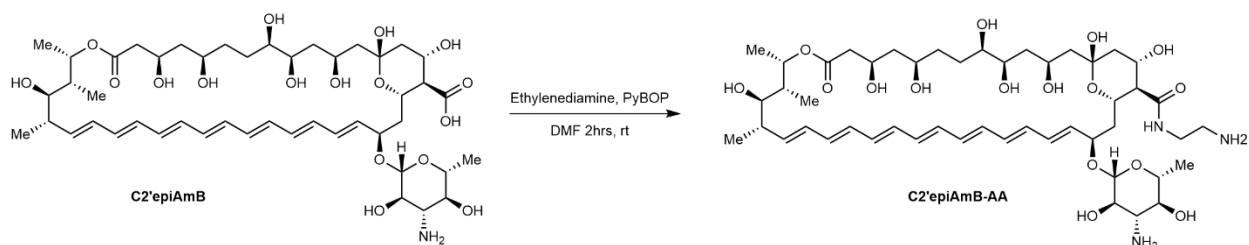


Supplementary Figure 9. Sodium and chloride efflux of (a) DMSO, (b) AmB, (c) AmB-AA (* $P=0.021$), and (d) C2'epiAmB-AA (2 μ M) in 10% cholesterol containing liposomes (* $P=0.013$; $n = 3$ per group). Unpaired t-test at 12 minutes between Cl Efflux % and Na Efflux %. Statistics utilized the Shapiro-Wilk test to check for normal distribution.

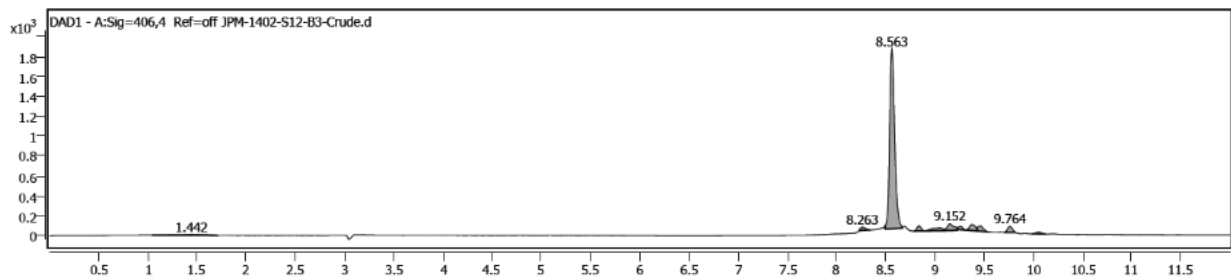
Chemical reaction scheme showing the conversion of Amphotericin B (AmB) to AmB-AA. The reaction conditions are Ethylenediamine, PyBOP, DMF, 2 hrs, rt. The structure of AmB-AA shows the addition of an aminoethyl group to the C3 position of the deoxy sugar moiety.

Chromatogram showing detector response over time. The x-axis is labeled "Response Units vs. Acquisition Time (min)" and ranges from 1 to 18 minutes. The y-axis is labeled "x10³" and ranges from 0 to 1.8. A major peak is labeled at 7.863 minutes. Other smaller peaks are labeled at 6.209, 9.211, 10.033, 11.604, 13.870, and 15.198 minutes. The plot title is "OAD1 - A:Sig=406,4 Ref=off LAH-F2.d".





Synthesis of C2'epiAmB-AA: The following protocol was followed and adapted from previous reports⁵: Dissolve 100 mg (103.89 μmol , 1 eq) of **C2'epiAmB** in 519 μL of DMF (200 mM). To the mixture add 296 μL (350 mM) of Et₃N and stir for 5 minutes at 25 °C. To the mixture 270.31 mg (519.43 μmol , 5 eq) of PyBop was added to the solution and stirred for an additional 5 minutes at 25 °C. The solution was also sonicated until all materials were fully dissolved. To this reaction mixture 41.67 μL (623.32 μmol , 6 eq) of diethyleneamine was added and the reaction mixture was allowed to stir at room temperature for 30 minutes. Upon completion, the reaction mixture was slowly poured into stirring Et₂O (25 mL), the mixture was centrifuged at 3800 RCF for 5 minutes at 0 °C. The supernatant was decanted and to the remaining black/orange solid 25 mL of EtOAc was added and mixture was sonicated before being centrifuged at 3800 RCF for 5 minutes at 0 °C. The supernatant was decanted and solid dried under vacuum to afford the desired product as a yellow/orange solid with an average isolated mass >98 mg or percent yield >93%. For pure material (>90%), the resulting material was dissolved in DMSO and purified via RP-HPLC (Waters SunFire Prep C18 OBD 5 micron 30 x 150 mm; 25 mL/min flow rate, MeCN: 2 mM NH₄HCO₃ in H₂O 1:19 $\rightarrow$ 19:1 over 30 minutes). Material purity was then confirmed via LCMS at a wavelength of 406nm following the protocol shown for **C2'epiAmB**.



Calculated for C₄₉H₇₉N₃O₁₆ (M+H)⁺: 966.5539
 Found: 966.5713

References:

1. Tevyashova, A. N. *et al.* Discovery of amphotamide, a drug candidate for the second generation of polyene antibiotics. *ACS Infect. Dis.* **6**, 2029–2044 (2020).

2. Maji, A. *et al.* Tuning sterol extraction kinetics yields a renal-sparing polyene antifungal. *Nature* **623**, 1079–1085 (2023).
3. Nicolaou, K. C. *et al.* Chemistry of amphotericin B. Degradation studies and preparation of amphoteronolide B. *J. Am. Chem. Soc.* **110**, 4660–4672 (1988).
4. Mazerski, J., Bolard, J. & Borowski, E. Effect of the modifications of ionizable groups of amphotericin B on its ability to form complexes with sterols in hydroalcoholic media. *Biochim. Biophys. Acta Biomembr.* **1236**, 170–176 (1995).
5. Zhang, J., Su, Y. A. N., Burke, M. D., Maji, A. & Nian, Y. Hybrid amide derivatives of amphotericin b. *Patent* (2021).