A Simple Strategy for Taming Membrane-Disrupting Antibiotics

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Supporting information

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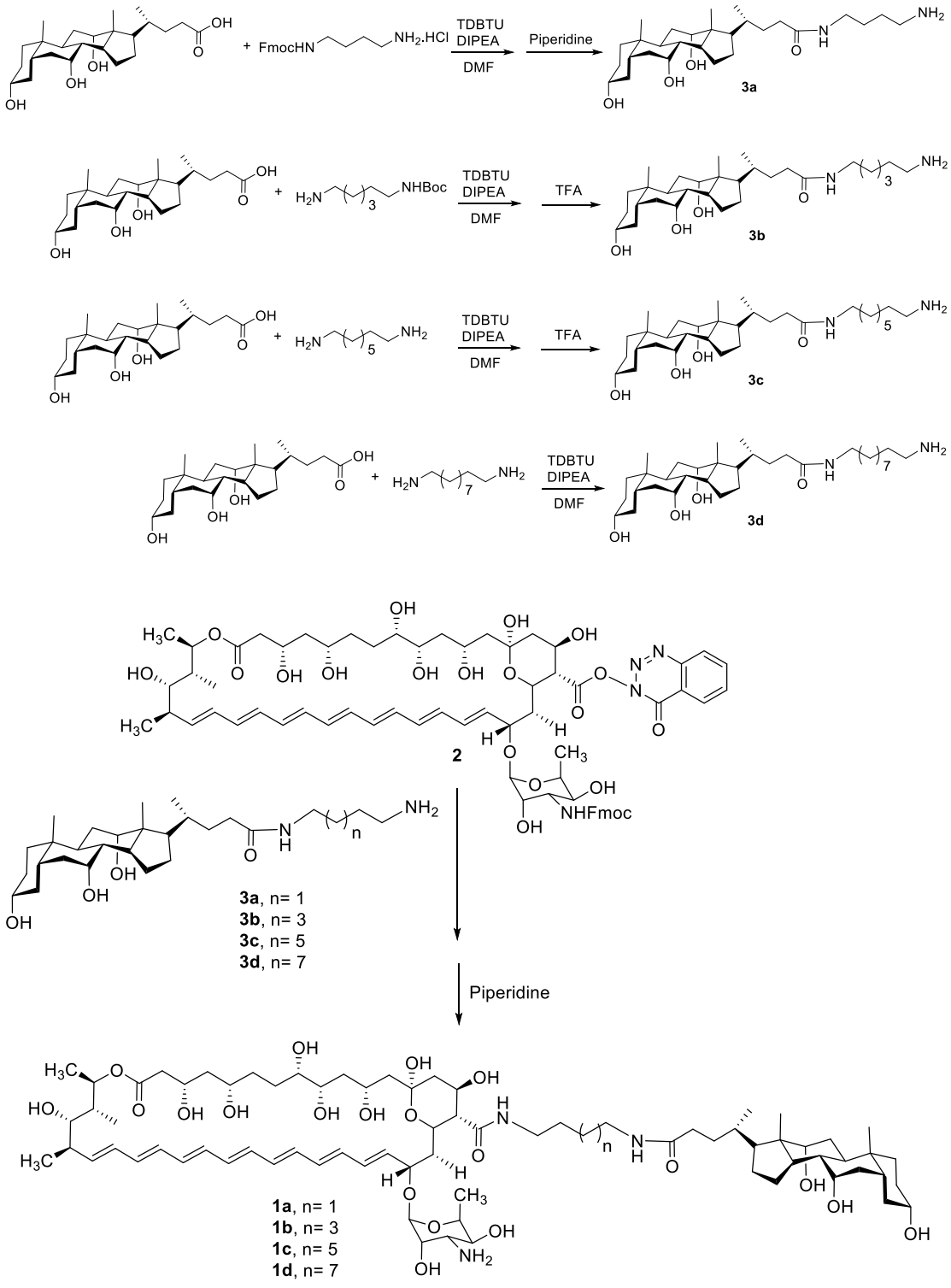
1. General information

Medium pressure liquid chromatography was performed using an Isolera One system equipped with a dual wave-length UV detector from Biotage and Biotage SNAP Ultra columns. For purification via preparative TLC, silica gel plates (EMD, 1 mm, 20 × 20 cm) were used, which contained a fluorescence indicator F254. Column chromatography was carried out using silica gel 60, EMD Milipore. All solvents were purchased from EMD Millipore Corporation and used as obtained. Deionized water was purified by a Millipore Milli-Q filtering system equipped with one carbon and two ion-exchange stages. All mass spectral measurements were performed by Apex-ultra 70 hybrid fourier transform mass spectrometer (Bruker Daltonics) at Rutgers University-Newark. All NMR spectra were recorded on a Bruker Avance 500 MHz instrument. Residual solvent signals were used as a reference. All UV measurements were performed on a Cary 300 Bio spectrometer from Varian.

2. Experimental procedures

2.1 Synthetic procedures



***N*-Cholyl-butanediamine 3a**, (*N*,*N*,*N*',*N*'-Tetramethyl-*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)uranium tetrafluoroborate) (210 mg, 0.6 mmol) was added directly to a stirred solution that was made from cholic acid (245 mg, 0.6 mmol), 8 mL of DMF and 400 μL of *N*,*N*-diisopropylethylamine. The resulting mixture was stirred for 20 min at room temperature. Then Fmoc-1,4-diaminobutane (CHEM-IMPEX INT'L INC.) (208 mg, 0.53 mmol) was added and stirring continued for 4 h. After the reaction was completed (monitored by TLC, ($CHCl_3$/MeOH/H_2O, 60/10/1, v/v/v, Rf 0.60), the product mixture was added, dropwise, into 200 mL of 5% $NaHCO_3$ and the precipitate collected by filtration, and washed 3 times with de-ionized water. The resulting solid was further purified by column chromatography using silica gel and $CHCl_3$/MeOH/H_2O (60/10/1, R_f = 0.60, v/v/v) as the eluent to give 360 mg (97%) of the Fmoc-protected intermediate. This intermediate was then dissolved in 4 mL of DMF and 2 mL piperdine and the mixture stirred for 20 min. After the reaction was completed, the mixture was added dropwise into 200 mL diethyl ether, the precipitate collected by filtration. The product proved to be pure enough for subsequent coupling and was used directly, without further purification. ^{1}H NMR (500 MHz, MeOD) δ 3.96 (s, 1H), 3.80 (s, 1H), 3.44 – 3.34 (m, 1H), 3.22 – 3.13 (m, 2H), 2.66 (t, *J* = 6.9 Hz, 2H), 2.45 – 0.95 (m, 31H), 0.94 (d, *J* = 17.7 Hz, 3H), 0.72 (s, 3H).

***N*-Cholyl-butanediamine 3b,** To the stirred solution of 326.4 mg (0.8 mmol) cholic acid in 5 ml DMF was added 286.2 mg (0.8 mmol) TDBTU (N,N,N',N'-Tetramethyl-O-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)uronium tetrafluoroborate) and 100 μL DIPEA (N,N-diisopropylethylamine). The mixture was stirred at room temperature for 10 min. Then 172.8 mg (0.8mmol) Boc-1,6-hexanediamine was added into the reaction mixture and stirred for 4 h at room temperature. After the reaction was completed (monitored by TLC), the mixture was dropped into 200 mL 5% $NaHCO_3$, and the precipitate collected by filtration. The solid was further purified by chromatography using $CHCl_3$: MeOH : H_2O = 80:10:1 as the eluent to give 402 mg (87%) of the Boc-protected intermediate. This intermediate was then dissolved in 4 mL of CH_2Cl_2 and 1 mL of CF_3COOH and the mixture stirred for 1.5 h. After the reaction was completed, the solvent was removed under reduced pressure. The product proved to be pure enough for subsequent coupling and was used directly, without further purification. ^{1}H NMR (500 MHz, MeOD) δ 3.95 (s, 1H), 3.82 (s, 1H), 3.43 – 3.34 (m, 1H), 3.20 – 3.12 (m, 2H), 2.65 (t, *J* = 6.9 Hz, 2H), 2.45 – 0.95 (m, 35H), 0.94 (s, 3H), 0.72 (s, 3H).

***N*-Cholyl-octanediamine 3c**, (*N*,*N*,*N*',*N*'-Tetramethyl-*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)uranium tetrafluoroborate) (180 mg, 0.52 mmol) was added directly to a stirred solution that was made from cholic acid (215 mg, 0.52 mmol), 8 mL of DMF, 4 ml of $CHCl_3$ and 300 μL of *N*,*N*-diisopropylethylamine. After stirring the resulting mixture for 20 min at room temperature, it was added dropwise into solution made from 1,8-octanediamine (750 mg, 5.2 mmol), 5 mL of DMF and 5 mL of $CHCl_3$. The reaction mixture was stirred at room temperature for an additional 4 h. After the reaction was completed [as judged by monitoring the formation of product by TLC ($CHCl_3$/MeOH/H_2O; 60/10/1, v/v/v)], the product mixture was added, dropwise, into 200 mL of 5% $NaHCO_3$ and the precipitate collected by filtration. The solid was then washed 3 times with de-ionized water and further purified by column chromatography using $CHCl_3$/MeOH/H_2O (40/10/1, v/v/v) to $CHCl_3$/MeOH/NH_4OH (40/10/1, R_f = 0.55, v/v/v) as the eluent to give 276 mg (98%) of **3c** having ^{1}H NMR (500 MHz, MeOD) δ 3.96 (s, 1H), 3.81 (s, 1H), 3.44 – 3.35 (m, 1H), 3.23 – 3.10 (m, 2H), 2.73 – 2.57 (m, 2H), 2.37 – 0.98 (m, 39H), 0.92 (s, 3H), 0.72 (s, 3H).

***N*-Cholyl-decanediamine 3d**, (*N*,*N*,*N*',*N*'-Tetramethyl-*O*-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)uranium tetrafluoroborate) (215 mg, 0.52 mmol) was added directly to a stirred solution that was made from cholic acid (215 mg, 0.52 mmol), 8 mL of DMF and 200 μL of *N*,*N*-diisopropylethylamine. The resulting mixture was stirred for 20 min at room temperature. Then 1,10-decanediamine (900 mg, 5.2 mmol) was added and stirring continued for an additional 4 h. After the reaction was completed [as judged by monitoring the formation of product by TLC ($CHCl_3$/MeOH/H_2O; 60/10/1, v/v/v)], the mixture was added, dropwise, into 200 mL of 5% $NaHCO_3$ that was maintained at 60 ^oC, and the precipitate collected by filtration. The solid was then washed 3 times with de-ionized water. The resulting solid was further purified by column chromatography using silica gel using $CHCl_3$/MeOH/H_2O (40/10/1, v/v/v) to $CHCl_3$/MeOH/NH_4OH (50/10/1, R_f = 0.45, v/v/v) as the eluent to give 285 mg (yield: 96%) of **3d** having ^{1}H NMR (500 MHz, MeOD) δ 3.96 (s, 1H), 3.80 (s, 1H), 3.36 (d, *J* = 11.2 Hz, 1H), 3.24 – 3.04 (m, 2H), 2.80 – 2.57 (m, 2H), 2.43 – 0.96 (m, 43H), 0.92 (s, 3H), 0.72 (s, 3H).

Amphotericin B conjugate 1a. To a solution that was made from 30 mg (23 μmol) of N-Fmoc Amhotericin B carbamate-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl) ester (**2**), 12.0 mg (25 μmol) of amine **3a** and 2 mL of anhydrous DMF was added 50 μL of N,N-diisopropylethylamine.[1] The resulting mixture was stirred in closed flask for 5 h at room temperature. After the starting material (**2**) completely disappeared (monitored by TLC, CHCl3/MeOH/H2O, 50/10/1, v/v/v), 0.5 mL of piperidine was added and the mixture allowed to stir for an additional 15 min. This mixture was then added dropwise into 100 mL of cold diethyl ether. The resulting solid was collected by centrifugation and the crude product purified by flash chromatogtaphy using ($CHCl_3$/MeOH/H_2O, 60/20/3, v/v/v) as eluent. The product was further purified by preparative thin layer chromatography ($CHCl_3$/MeOH/H_2O, 10/5/1, v/v/v) to give 26 mg (80%) of conjugate **1a** (R_f 0.78) having ^{1}H-NMR (500 MHz, CD_3OD:$CHCl_3$ = 2:1, ppm): δ 6.62 – 5.89 (m, 13H), 5.42 – 5.32 (m, 2H), 4.56 – 4.36 (m, 3H), 4.29 – 4.13 (m, 2H), 3.95 (s, 1H), 3.87 – 3.77 (m, 2H), 3.73 (t, *J* = 10.7 Hz, 1H), 3.64 (d, *J* = 12.8 Hz, 1H), 3.40 – 3.32 (s, 1H), 3.29 – 3.07 (m, 8H), 2.57 – 0.94 (m, 65H), 0.91 (s, 3H), 0.71 (s, 3H), HR-ESI MS: for $C_{75}H_{121}N_3O_{20}$, (M+H) calculated: 1384.8616; found: 1384.8633.

Amphotericin B conjugate 1b. To a solution that was made from 38 mg (29 μmol) of N-Fmoc Amhotericin B carbamate-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl) ester (**2**), 30 mg (59 μmol) of amine **3b** and 2 mL of anhydrous DMF was added 50 μL of N,N-diisopropylethylamine. The resulting mixture was stirred in closed flask for 5 h at room temperature. After the starting material (**2**) completely disappeared (monitored by TLC, $CHCl_3$/MeOH/H_2O, 50/10/1, v/v/v), 0.5 mL of piperidine was added and the mixture allowed to stir for an additional 15 min. This mixture was then added dropwise into 100 mL of cold diethyl ether. The resulting solid was collected by centrifugation and the crude product purified by flash chromatogtaphy using ($CHCl_3$/MeOH/H_2O, 60/20/3, v/v/v) as the eluent. The product was further purified by preparative thin layer chromatography ($CHCl_3$/MeOH/H_2O, 10/5/1, v/v/v) to give 30 mg (72%) of conjugate **1b** (R_f 0.78) having ^{1}H NMR (500 MHz, CD_3OD:$CHCl_3$ = 2:1, ppm) δ 6.60 – 5.97 (m, 14H), 5.55 – 5.21 (m, 2H), 4.53 – 4.30 (m, 5H), 4.30 – 4.07 (m, 2H), 3.93 (s, 1H), 3.79 (s, 2H), 3.72 (t, J = 11.3 Hz, 1H), 3.62 (d, J = 9.0 Hz, 1H), 3.48 – 3.35 (m, 1H), 3.28 – 2.99 (m, 8H), 2.62 – 0.93 (m, 67H),

0.89 (s, 3H), 0.68 (s, 3H). HR-ESI MS: for $C_{77}H_{125}N_3O_{20}$, (M+H) calculated: 1412.8929; found: 1412.8925.

Amphotericin B conjugate 1c. To a solution that was made from 25.8 mg (20 μmol) of N-Fmoc Amhotericin B carbamate-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl) ester (**2**), 22 mg (40 μmol) of amine **3c** and 2 mL of anhydrous DMF was added 50 μL of N,N-diisopropylethylamine.[1] The resulting mixture was stirred in closed flask for 5 h. After the starting material (**2**) completely disappeared (monitored by TLC, $CHCl_3$/MeOH/H_2O, 50/10/1, v/v/v), 0.5 mL of piperidine was added and the mixture stirred for an additional 15 min. The product mixture was then added dropwise into 100 mL of cold diethyl ether. The resulting solid was collected by centrifugation and the crude product purified by flash chromatogtaphy using ($CHCl_3$/MeOH/H_2O, 60/20/3, v/v/v) as the eluent. The product was further purified by preparative thin layer chromatography ($CHCl_3$/MeOH/H_2O, 12/5/1, v/v/v) to give 21.6 mg (75%) of conjugate **1c** (R_f 0.72) having ^{1}H-NMR (500 MHz, CD_3OD:$CHCl_3$ = 2:1, ppm): δ 6.59 – 5.94 (m, 13H), 5.53 – 5.22 (m, 2H), 4.56 – 4.34 (m, 3H), 4.31 – 4.21 (m, 1H), 4.17 (t, *J* = 9.9 Hz, 1H), 3.95 (s, 1H), 3.82 (dd, *J* = 20.3, 2.1 Hz, 2H), 3.73 (t, *J* = 9.5 Hz, 1H), 3.64 (d, *J* = 9.9 Hz, 1H), 3.45 – 3.31 (m, 1H), 3.27 – 3.01 (m, 8H), 2.72 – 0.93 (m, 73H), 0.91 (s, 3H), 0.70 (s, 3H). HR-ESI MS: for $C_{79}H_{129}N_3O_{20}$, (M+H) calculated: 1440.9242; found: 1440.9268.

Amphotericin B conjugate 1d. To a solution that was made from 30 mg (23 μmol) of N-Fmoc Amhotericin B carbamate, (3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl) ester (**2**), 20 mg (35 μmol) of amine **3d** and 2 mL of anhydrous DMF was added 50 μL of N,N-diisopropylethylamine.[1] The resulting mixture was stirred in closed flask for 5 h. After the starting material (**2**) completely disappeared (monitored by TLC, $CHCl_3$/MeOH/H_2O, 50/10/1, v/v/v), 0.5 mL of piperidine was added and the mixture stirred for an additional 15 min. The product mixture was added dropwise into 100 mL of cold diethyl ether. The resulting solid was collected by centrifugation and the crude product purified by flash chromatogtaphy using ($CHCl_3$/MeOH/H_2O, 40/10/1, v/v/v) as the eluent. The product was further purified by preparative thin layer chromatography ($CHCl_3$/MeOH/H_2O, 60/20/3, v/v/v) to give 24.2 mg (71%) of conjugate **1d** (R_f 0.6) having ^{1}H-NMR (500 MHz, CD_3OD:$CDCl_3$ = 2:1, ppm): δ 6.55 – 5.94 (m, 12H), 5.52 – 5.14 (m, 2H), 4.55 – 4.27 (m,

3H), 4.22 (td, *J* = 10.9, 4.7 Hz, 1H), 4.13 (t, *J* = 10.1 Hz, 1H), 3.94 (s, 1H), 3.86 – 3.77 (m, 1H), 3.72 (t, *J* = 9.9 Hz, 1H), 3.62 (d, *J* = 10.7 Hz, 1H), 3.45 – 3.34 (m, 1H), 3.26 – 3.01 (m, 8H), 2.63 – 0.91 (m, 77H), 0.89 (s, 3H), 0.68 (s, 3H). HR-ESI MS: for $C_{81}H_{133}N_3O_{20}$, (M+H) calculated: 1468.9555; found: 1468.9536.

2.2 Critical Aggregation Concentration, Hemolytic Activity and Cytotoxicity Measurements.

Determination of Critical Aggregation Concentration (cac) of AmB, **1a**, **1b**, **1c** and **1d.**

Solutions of AmB, **1a**, **1b**, **1c** and **1d** in DMSO (Sigma-Aldrich) were prepared having a concentration of 1.00 mmol/L. Aliquots (1-10 μL) were then introduced into test tubes containing 0.750 mL to 5.88 mL of phosphate-buffered saline (PBS), pH 7.4 at 37°C to give concentrations ranging from 0.17 to 13.1 μM. After vortex mixing for 10 s, the solution was transferred to a 1.60 mL UV cuvette that was maintained at 37°C. The UV spectrum was then recorded in the range of 250-550 nm. The absorbance at 409 nm was plotted as a function of the reciprocal value of the concentration of AmB and the critical aggregation concentration of **1a**, **1b**, **1c** and **1d** was then determined, graphically, using methods previously described.[1]

Hemolysis measurements. Sheep Red Blood Cells, 10%, in saline (Innovative Research, Novi, MI), were diluted saline (pH 7.4) to a concentration of 4%, which corresponds to 4 x 10^7 cells/mL. All measurements were carried out in duplicate. Solutions of Amphotericin B (Sigma-Aldrich) were prepared by adding 10 μL of a DMSO solution containing a given concentration of the antibiotic (i.e., ranging from 0.2 mM to 100 mM) to 490 μL of PBS. After the resulting solution was vortex mixed for 30 s it was incubated at 37°C for 15 min. The resulting solution of antibiotic (500 μL) was then mixed with 500 μL of the 4% erythrocyte dispersion at 37°C in a 1 mL plastic centrifuge vial to give a dispersion that was vortex mixed at slow rate for 5 s. After 1 h of incubation at 37°C, all samples were centrifuged (1500 g, Eppendorf centrifuge 5415C, 5000 rpm) at rt for 6 min. The supernatant (~ 0.7 mL) from each plastic vial was carefully separated from sediment using a disposable glass pipette and quickly transferred to test tube. A volume of 100 μL of this supernatant was

then transferred to a UV cell containing 500 µL of PBS that was maintained at 37°C. The UV spectrum was then scanned (450-650 nm) and the absorbance at 575 nm used to determine the extent of hemolysis. The extent of 0% and 100% of hemolysis was obtained from A_{575} values of experiments that were run in absence of AmB (control, $[A_{575,}]_o$) and in presence, 100 µM AmB ($[A_{575}]_{max}$), respectively. The concentrations of AmB, **1a**, **1b**, **1c** and **1d** that resulted in 50% release of hemoglobin, {[A575]max -[A575]o}/2, which are our EH_{50} values, were determined graphically.

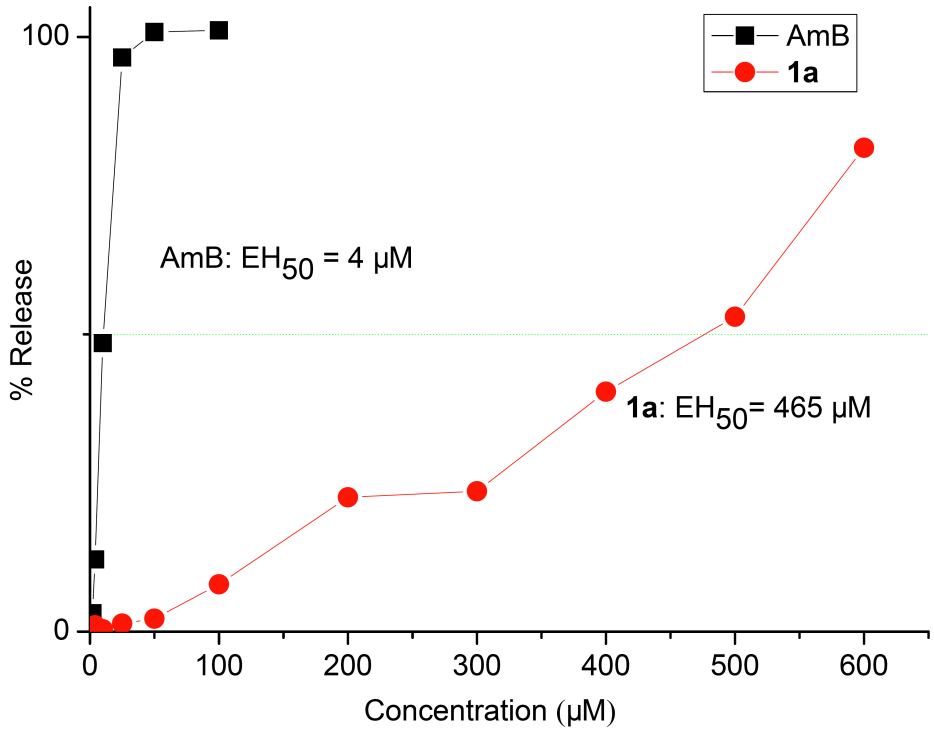


Figure SI-1. Plot of percent release of hemoglobin from sheep red blood cells as a function of concentration of AmB (□) and **1a** (●) at 37 °C in PBS, pH 7.4.

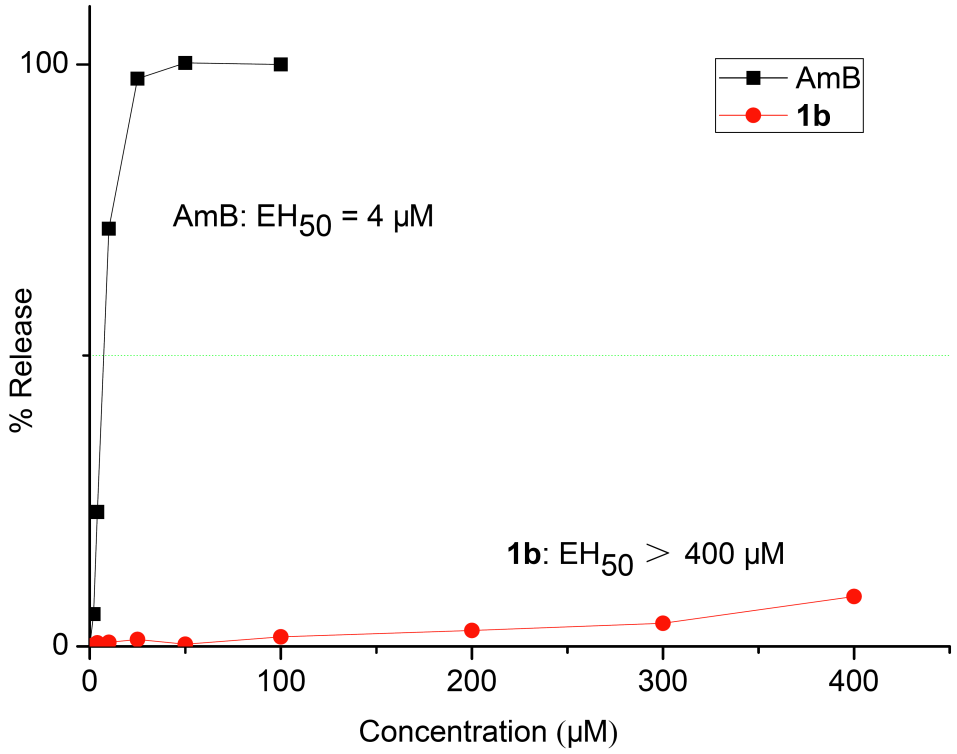


Figure SI-2. Plot of percent release of hemoglobin from sheep red blood cells as a function of concentration of AmB (□) and **1b** (●) at 37 °C in PBS, pH 7.4.

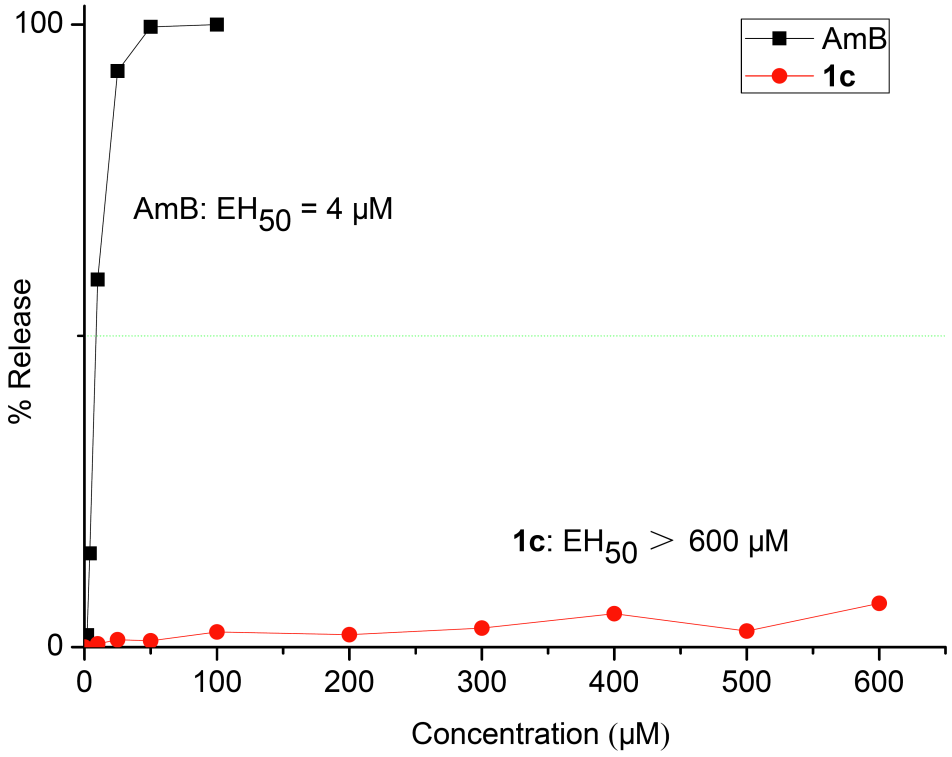


Figure SI-3. Plot of percent release of hemoglobin from sheep red blood cells as a function of concentration of AmB (□) and **1c** (●) at 37 °C in PBS, pH 7.4.

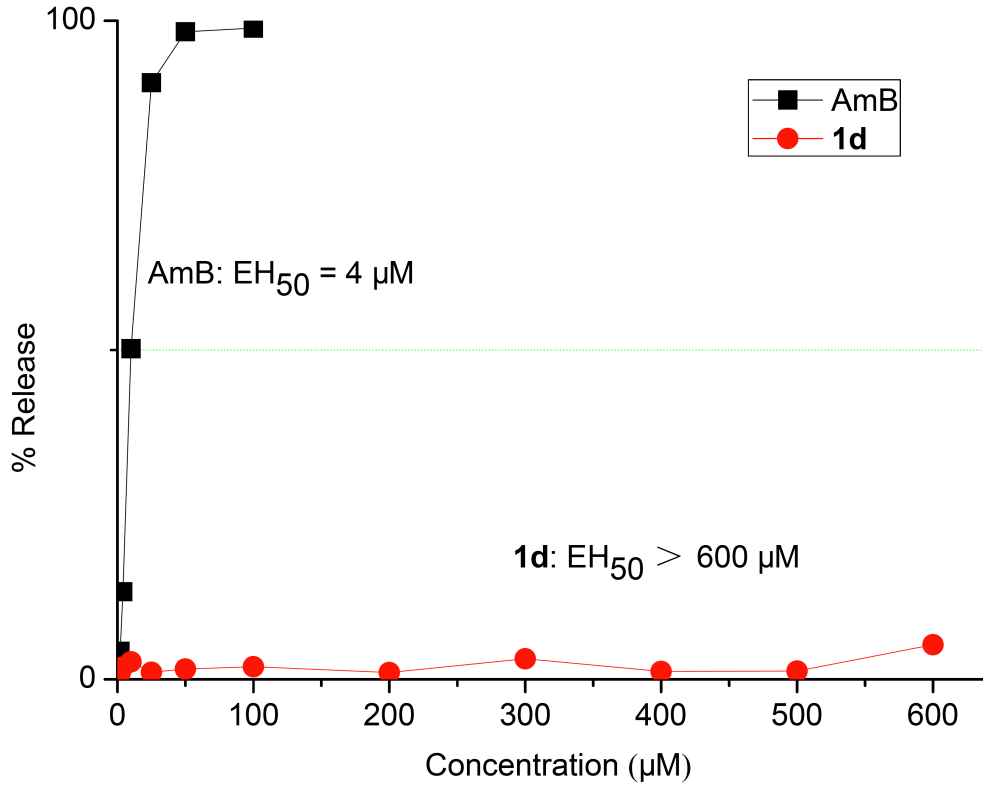


Figure SI-4. Plot of percent release of hemoglobin from sheep red blood cells as a function of concentration of AmB (□) and **1d** (●) at 37 °C in PBS, pH 7.4.

Cell Culture of Human embryonic kidney HEK293:

Materials

Dimethyl sulfoxide (DMSO) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Thermo Fisher Scientific Inc. Fetal Bovine Serum (FBS) was purchased from Corning. Penicillin-Streptomycin was purchased from Sigma-Aldrich. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from EMD Millipore.

Cell Culture

Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

Anti-Proliferation Assay

HEK293T cells were seeded in 96-well plates at a density of 10, 000 cells/well and incubated overnight. Prior to treatment, constructs were dissolved in DMSO to obtain 21 mg/mL aliquoted stock solutions. Appropriate volumes of these stock solutions were added to DMEM media so the final concentration of DMSO was equal to 1%. After removal of cell media, 200 μL of treatment solutions were added to each well and incubated at 37 °C for 72 h. After treatment, the media was removed and the cells were washed with 100 μL of complete DMEM. Next, 100 μL of complete DMEM was added to each well. Cell viability was determined using the colorimetric MTT assay, in which 10 μL of a 5 mg/mL MTT stock solution was added to the treated cells and incubated for 2 h at 37 °C. The resulting formazan crystals were solubilized in 200 μL of DMSO. Absorbance was measured at 580 nm using an Infinite 200 PRO microplate reader (Tecan). Cell viability was calculated against control cells treated with complete medium.

Determination of MIC and MFC Values. Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) are the lowest concentrations that are required for completely inhibiting growth, and killing at least 99% of the fungi, respectively. Specific procedures that were used were similar to those previously described (ref 1).

Table SI-1. Antifungal activities

MFC (μg/mL)[a]					
Microbe	AmB	**1a**	**1b**	**1c**	**1d**
C. albicans	1	1	8	2	---
C. glabrata	1	2	16	---	---
C. neoformans	0.5	2	4	2	>16
C. gatti	0.5	2	1	2	>16

[a]MFC values are the lowest concentrations required for killing at least 99% of the fungi.

3. NMR Spectra of Compounds.

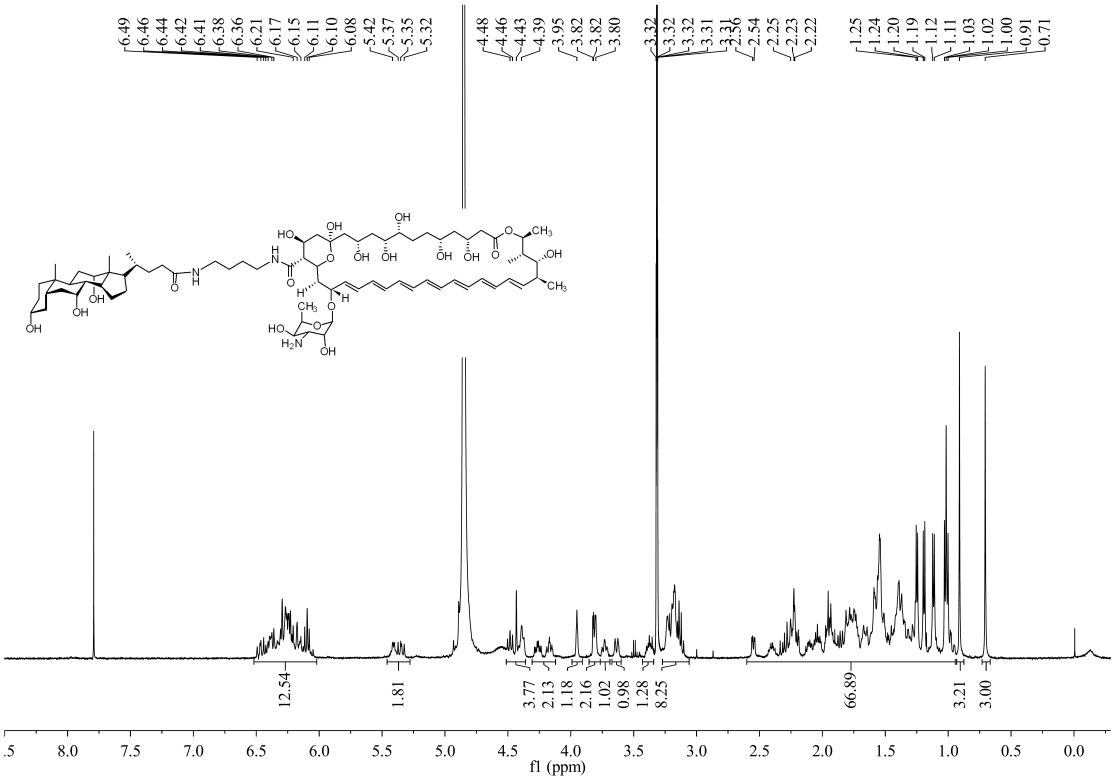


Figure SI-5: ^{1}H NMR spectrum of **1a**

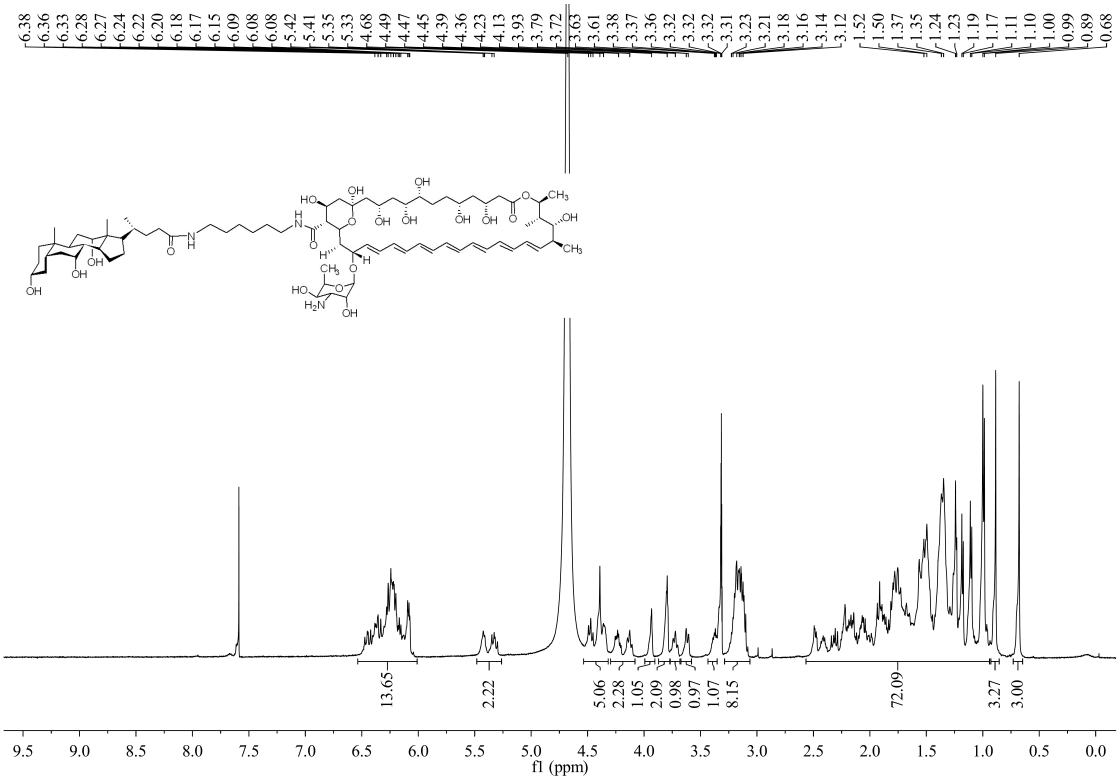


Figure SI-6: ^{1}H NMR spectrum of **1b**

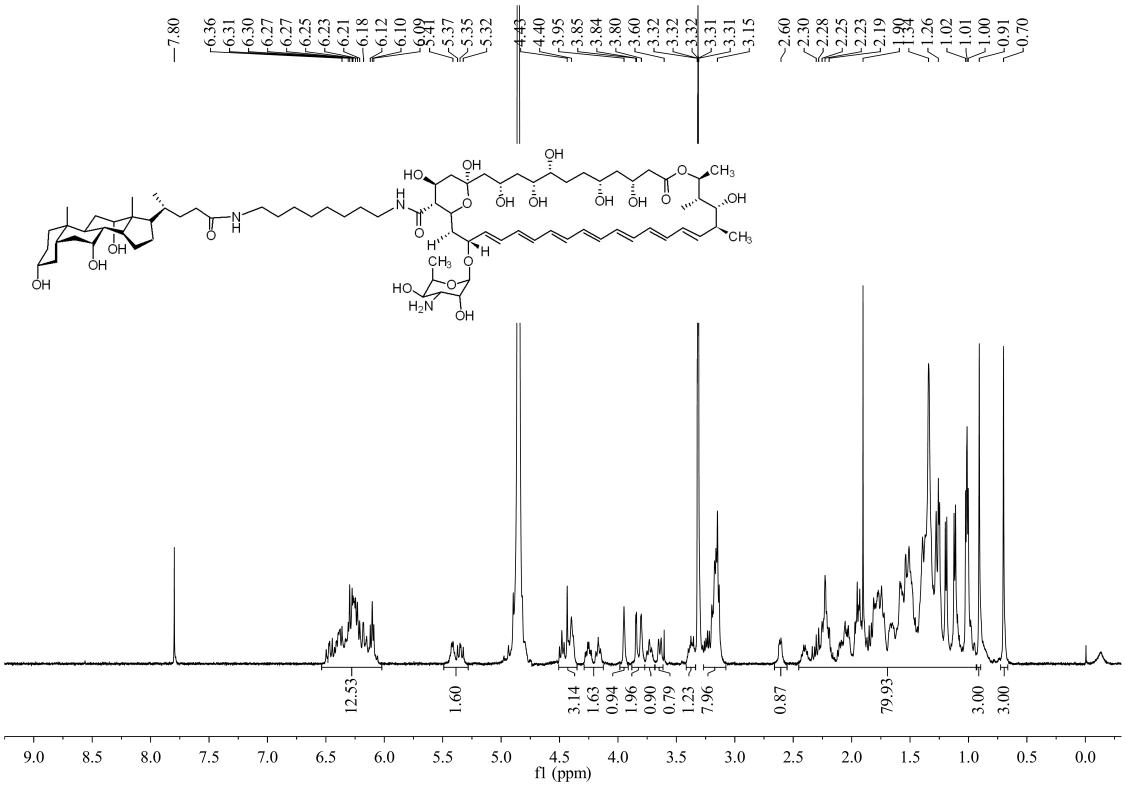


Figure SI-7: ^{1}H NMR spectrum of **1c**

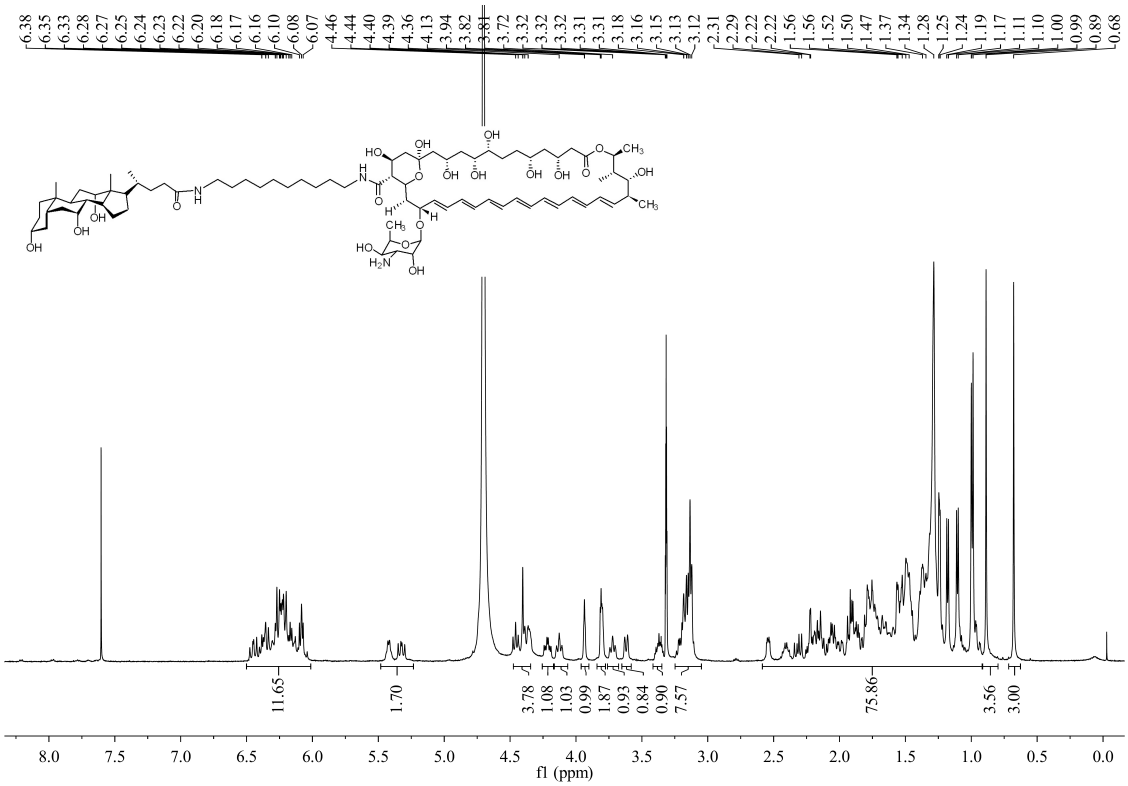


Figure SI-8: ^{1}H NMR spectrum of **1d**

References.

1. Janout, V., Schell, W. A., Thevenin, D., Yu, Y., Perfect, J. R., and Regen, S. L. (2015) Taming amphotericin b. *Bioconjugate Chem.*, *26*, 2021-2024.