Discovery and Characterization of a Peptoid with Antifungal Activity Against *Cryptococcus neoformans*

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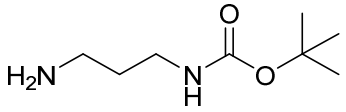
I. Synthesis and Screening Procedures

Materials and Methods

Chemicals for this project were purchased from Fisher Scientific (Waltham, MA), Alfa Aesar (Haverhill, MA), Amresco (Solon, OH), TCI America (Portland, OR), Anaspec (Fremont, CA), EMD Millipore (Billerica, MA), Peptides International (Louisville, KY), and Chem-Implex (Wood Dale, IL). Human red blood cells (hRBCs) and pooled human serum were acquired from Innovative Research (Novi, MI). Synthesis of N-(*tert*-butyoxycarbonyl)-cystamine, N-(tert-butoxycarbonyl)-1,4-diaminobutane, N-(tert-butoxycarbonyl)-1,2-diaminoethane, and the TentaGel immobilized PLAD branched disulfide linker, and the proof-of-concept 18 peptoid library were all synthesized as previously reported.[1] All mass spectra were acquired on either a Waters Synapt HDMS QToF with Ion Mobility or a Thermo Scientific LTQ XL Linear Ion Trap Mass Spectrometer and all NMR spectra were acquired on a JOEL ECA 500 NMR spectrometer. All images were acquired using a Leica M165FC stereomicroscope and images were analyzed using Adobe Photoshop and Microsoft Excel.

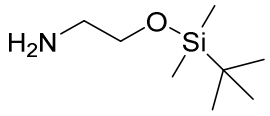
N-(*tert*-butoxycarbonyl)-1,3-diaminopropane



Concentrated hydrochloric acid (1.91 mL, 0.0229 mol) was added dropwise to 30 mL methanol (MeOH) and stirred for 15 minutes at room temperature (RT). Ice cold 1,3-diaminopropane (1.91 mL, 0.0229 mol) was added dropwise to the HCl/MeOH mixture and stirred for 15 minutes at RT. Deionized water (10 mL) was added dropwise to the solution at RT

and stirred for 30 minutes. Di-*tert*-butyl dicarbonate (7.90 mL, 0.0344 mol) was dissolved in 40 mL MeOH, stirred for 30 minutes on ice, added dropwise over 10 minutes to the diamine solution, and stirred at RT for 1 hour. The solvent was evaporated *in vacuo* and the resulting solid washed with diethyl ether (3x 30 mL). One M NaOH solution was added and the product was extracted 2x with CH_2Cl_2. Both organic layers were combined and washed 1x with a brine solution. The organic layer was then dried over $CaCl_2$ and concentrated *in vacuo* to yield a white solid (3.79 g, 95% yield). ESI $[M+H]^{+1}$ expected 175.14 Da, observed 175.21 Da. ^{1}H-NMR (500 MHz, CD_3CN) δ 5.52 (s, 1H), δ 3.06 (m, 2H), δ 2.60 (m, 2H), δ 1.78 (m, 2H), δ 1.50 (m, 2H), δ 1.40 (s, 9H).

Synthesis of *O*-(*tert*-butyldimethylsilyl)-ethanolamine

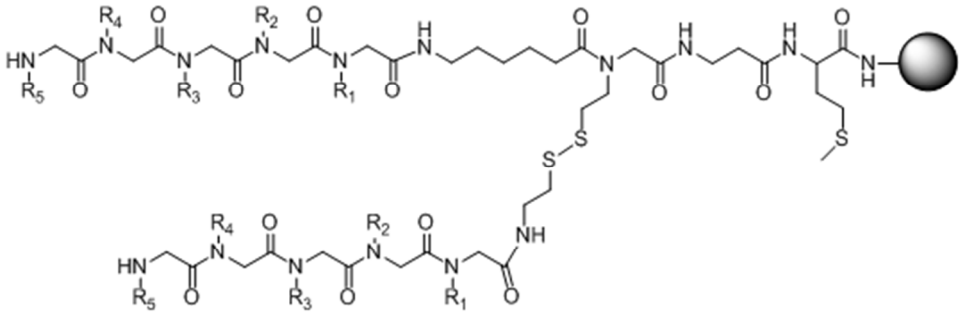


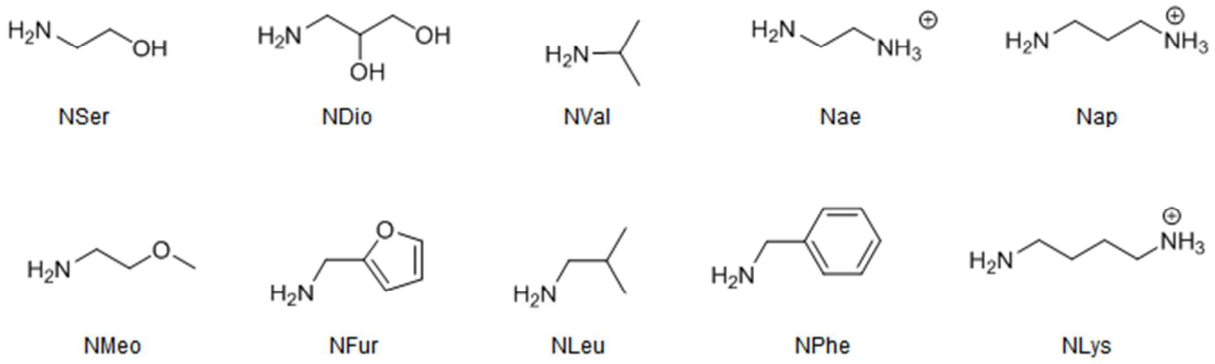
Synthesis of *O*-(*tert*-butyldimethylsilyl)-ethanolamine was done as previously described.[2] Briefly, ethanolamine (1.27 mL, 0.021 mol) and imidazole (2.72 g, 0.040 mol) were dissolved in 20 mL CH_2Cl_2. *Tert*-butyldimethylchlorosilane (TBDMS-Cl, 3.17 g, 0.021 mol) was dissolved in 10 mL CH_2Cl_2 and slowly added to the ethanolamine/imidazole mixture over 5 minutes. The reaction was stirred at RT for 1 hour. Deionized water (20 mL) was added, the organic layer collected, and the aqueous layer washed twice more with CH_2Cl_2. The combined organic layers were dried over $CaCl_2$ and concentrated *in vacuo* to yield a pale yellow oil (3.12 g; 84% yield). ESI $[M+H]^{+1}$ expected 176.15 Da, observed 176.19 Da. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.00 (s, 6H), δ 0.833 (s, 9H), δ 2.75 (t, 2H, J=5.15 Hz), δ 3.49 (s, 2H), δ 3.59 (t, 2H, J=5.15).

DTT/Cell Count Optimization

The ideal concentration of *C. neoformans* cells and of dithiothrietol (DTT) were determined experimentally. This optimization was done prior to the complete reducing reagent study published previously,[1] which is why DTT was the only reagent tested here. Briefly, an overnight culture of *C. neoformans* H99S in yeast extract-peptone-dextrose (YPD) broth was pelleted by centrifugation, the cells washed with PBS three times, and the average cell count determined with a hemocytometer. Varying concentrations of cells (1×10^6 cells/mL, 5×10^6 cells/mL, 1×10^7 cells/mL, and 5×10^7 cells/mL) were tested plus/minus 10 mM DTT (from a 100 mM stock). The proper concentrations of cells and DTT were added to 3 mL of YPD soft agar that had been maintained at 56°C in a water bath. The mixture was gently mixed and placed on a Petri dish containing hard YPD agar. The plates were incubated at 37°C for 24 hours prior to imaging on a Leica stereoscope. Luminosity values were obtained in triplicate from Adobe Photoshop to serve as measure of fungal lawn density. These values provided information regarding the optimum cell concentration to use in screening assays and the effect of DTT on the growth of *C. neoformans*.

Peptoid Library Synthesis





PLAD linker on TentaGel NH_2 Macrobeads (0.60 g; 0.27 mmol/g loading; Rapp Polymere) was first prepared as previously described.[1] Synthesis of the peptoid library proceeded following submonomer peptoid synthesis[3] and split- and-pool chemistry methods.[4-6] Ten different amines were used in the synthesis of this library; N-(tert-butoxycarbonyl)-1,2-diaminoethane, *N*-(*tert*-butoxycarbonyl)-1,3-diaminopropane, N-(tert-butoxycarbonyl)-1,4-diaminobutane, furfurylamine, methoxyethylamine, 2,2-dimethyl-1,3-dioxolane-4-methanamine, *O*-(*tert*-butyldimethylsilyl)-ethanolamine, isobutylamine, isopropylamine, and benzylamine. In a synthesis column with fritted filter, the resin containing the PLAD linker was equilibrated in anhydrous dimtheylformamide (DMF) for approximately 5 min. followed

by the addition of bromoacetic acid (0.432 g in 1.5 mL anhydrous DMF) and diisopropylcarbodiimide (DIC 0.75 mL in 0.75 mL anhydrous DMF). The resin was microwaved in a commercial microwave for 30 s at 10% power (100 kW) and rocked gently at RT for 15 min. Solution was removed by aspiration and the resin was washed with DMF three times and a ninhydrin test performed to confirm coupling.

The resin was then divided by transferring approximately 60 mg of resin to each of ten vials. A solution of each of the amines listed above (2 M in 2 mL of anhydrous DMF) was then added to each vial and the mixtures microwaved for 30 s at 10% power (100 kW) and rocked gently for 15 min. The contents of each vial was then transferred back to the larger parent vial, filtered, and washed with DMF several times. A ninhydrin test was used to confirm coupling of submonomers. The process of coupling bromoacetic acid, splitting, coupling amines, and pooling back together was repeated four more times to give a pentamer peptoid library. The library was then treated with 95:2.5:2.5 trifluoroacetic acid (TFA):triisopropylsilane:water to remove the Boc, TBDMS, and acetal protecting groups from submonomer side chains. The solution was removed by aspiration and washed 3x with CH_2Cl_2, 3x with DMF, and 3x again with CH_2Cl_2.

Quality control was done on the library by sequencing ten random beads by tandem mass spectrometry (MS/MS) as follows. Single beads from the library were isolated randomly, treated with 10 mM DTT for 30 min, and washed with water to remove the β-strand peptoid from the PLAD linker. Cyanogen bromide (CNBr; 50 μL; 40 mg/mL) in 0.1 M HCl in 80:20 acetonitrile:water was added and allowed to react in the dark for 24-hours to cleave the compound from the bead at the C-terminal methionine. The resulting solution was concentrated

in vacuo and the compound resuspended in 100 μL of 80:20 acetonitrile:water with 0.05% TFA and analyzed via linear and tandem mass spectrometry.

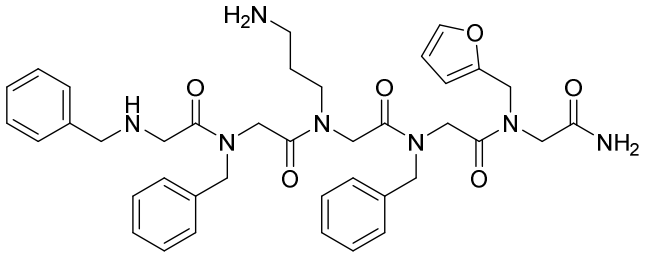
PLAD Screening Against *Cryptococcus neoformans*

The general screening procedure previously described[1] was adapted for screening against *Cryptococcus neoformans* Individual Petri dishes of hard YPD were used to support a mixture of YPD soft agar (3 mL; maintained at 56˚C in water bath), 6 mg library resin (6 mg), DTT (10 mM), and $1x10^6$ cells mL^{-1} of *C. neoformans* H99S (serotype A, lab strain).[7] Library resin was prepared by aliquoting, equilibrating in water for 18 hours, and suspending in phosphate buffered saline (PBS; pH 7.4; 500 μL) prior to addition to the soft agar. Preparation of *C. neoformans* cells was done from an overnight culture of the organism in YPD which was pelleted by centrifugation and washed with PBS the day of plating. Cells were diluted 1:200 with PBS to count the cell density with a hemocytometer. This concentration was used to determine the appropriate amount of concentrated cells to add to the soft agar.

Each component was added to the soft agar under sterile conditions and then to a plate of hard YPD agar. After allowing the soft agar mixture to solidify, the plates were incubated at 37°C for 24-hours and analyzed. Analysis of plates involved observation and imaging under a Leica stereoscope and subsequent measurement of zones of inhibition for beads displaying antimicrobial activity. A zone of inhibition is defined as a uniform area around a bead with no growth. Beads with a significant zone of inhibition, termed "hits," were then removed from the plate manually for further analysis by MS.

Hits were isolated in individual tubes and boiled in 1% sodium dodecylsulfate (SDS; 500 μL) for 1 hour to remove residual media and fungal debris. SDS was removed from the beads and the beads were washed with water five times. The beads were then treated with DTT (500 μL; 10 mM) in 80:20 acetonitrile:water for 30 minutes to assure that all β-strand peptoids were removed prior to MS analysis. Once again, the beads were washed five times with water. A solution of CNBr (50 μL; 40 mg/mL) in 0.1 M HCl in 80:20 acetonitrile:water was added and allowed to react in the dark for 24 hours. The resulting solution was concentrated *in vacuo* and the compound resuspended in 100 μL of 80:20 acetonitrile:water with 0.05% TFA and analyzed via linear and tandem mass spectrometry. Peptoid sequences were determined by analyzing the MS/MS spectra for the predictable fragment masses of the submonomers.

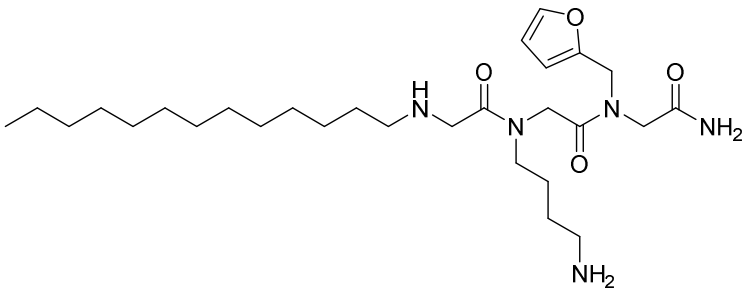
Synthesis of AEC89



To 370 mg of deprotected Rink Amide was added bromoacetic acid (0.834 g, 6 mmol) in anhydrous DMF (3 mL) and DIC (1.5 mL, 9.6 mmol) in anhydrous DMF (1.5 mL). The mixture was microwaved for a total of 30 seconds at 10% power and then gently rocked for 15 minutes. The mixture was aspirated and washed 3x with DMF. Furfurylamine (1.11 mL, 12 mmol) in anhydrous DMF (4.89 mL) was then added and mixture was again microwaved at 10% for a total of 30 seconds, rocked for 15 minutes, and washed 3x with DMF. The bromoacetic acid and DIC

step was repeated. Benzylamine (1.31 mL, 12 mmol) in anhydrous DMF (4.69 mL) was added, microwaved, and reacted for 15 minutes. The mixture was aspirated and washed 3x with DMF. The bromoacetic acid and DIC step was repeated. N-(*tert*-butoxycarbonyl)-1,3-diaminopropane (2.09 g, 12 mmol) in anhydrous DMF (6 mL) was added, microwaved, rocked for 15 minutes, and washed 3x with DMF. The bromoacetic acid and DIC step and benzylamine step were repeated two more times. The resin was washed 3x with CH_2Cl_2 and allowed to then equilibrate in CH_2Cl_2 for 10 minutes. Removal of the peptoid from rink was accomplished by gently rocking the resin in 95% TFA (14.25 mL), 2.5% TIS (0.375 mL), and 2.5% H_2O (0.375 mL) for 1 hour. The solution was then filtered to separate the compound from the resin and TFA was bubbled off. The remaining compound was suspended in 1:1 acetonitrile:H_2O (10 mL) and purified via RP-HPLC. Concentration of the purified product *in vacuo* provided a white powder (16.3 mg; 16.8% yield). Purity of product confirmed by HPLC to be >99%. ESI $[M+H]^{+1}$ expected 710.37 Da, observed 710.61 Da.

Synthesis of AEC5



To 500 mg of deprotected Rink Amide resin (0.37 mmol/g) was added bromoacetic acid (0.834 g, 6.0 mmol) in anhydrous DMF (3 mL) and DIC (1.5 mL, 9.6 mmol) in anhydrous DMF (1.5 mL). The mixture was microwaved for 30 s at 10% power (100 kW) and gently rocked for

15 min. The solution was aspirated and the resin washed 3x with DMF. Furfurylamine (1.11 mL, 12 mmol) in anhydrous DMF (4.89 mL) was then added to the resin and the mixture was again microwaved at 10% power for 30 s, rocked for 15 min, and washed 3x with DMF. The bromoacetic acid and DIC step was repeated. N-(*tert*-butoxycarbonyl)-1,4-diaminobutane (2.26 g, 12 mmol) in anhydrous DMF (6 mL) was added, microwaved, and rocked for 15 min. The mixture was aspirated and washed 3x with DMF. The bromoacetic acid and DIC step was again repeated. Aminotridecane (2.40 g, 12 mmol) in anhydrous DMF (6 mL) was added, microwaved, rocked for 15 min, and washed 3x with DMF. The resin was washed 3x with CH_2Cl_2 and allowed to then equilibrate in CH_2Cl_2 for 10 minutes. Removal of the peptoid from the resin and deprotection of the Boc protecting group was accomplished by treating the resin in 95% TFA (14.25 mL), 2.5% TIS (0.375 mL), and 2.5% H_2O (0.375 mL) for 1 hour. The solution was then filtered to separate the compound from the resin and TFA evaporated by bubbling with air. The remaining oily mixture was resuspended in 1:1 acetonitrile:H_2O (10 mL) containing 0.05% TFA and purified via reverse phase high performance liquid chromatography (RP-HPLC; C18 column; water/acetonitrile gradient containing 0.05% TFA). Concentration of the purified product *in vacuo* provided a white powder (39.1 mg; 40.5% yield). Purity of product confirmed by HPLC to be >99%. ESI $[M+H]^{+1}$ expected 522.40 Da, observed 522.53 Da. ^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.41 (s, 1H), 7.34 (d, J = 10.6 Hz, 1H), 6.37 (dd, J = 6.9, 3.2 Hz, 1H), 6.32 (s, 1H), 6.28 (d, J = 6.2 Hz, 1H), 4.70 (s, 1H), 4.64 (s, 2H), 4.51 (d, J = 9.6 Hz, 1H), 4.48 (s, 2H), 4.19 (d, J = 17.8 Hz, 1H), 4.07 (d, J = 7.0 Hz, 2H), 3.99 (d, J = 15.7 Hz, 1H), 3.77 (s, 1H), 3.30 (m, 2H), 2.93 (m, 4H), 1.53 (m, 6H), 1.16 (m, 20H), 0.74 (t, J = 6.7 Hz, 3H). ^{13}C NMR (D2O, 126 MHz) δ 172.60, 170.05, 166.40, 148.54, 143.68, 110.70, 109.77, 48.95,

48.12, 47.99, 47.70, 47.42, 47.19, 45.14, 43.55, 39.10, 31.42, 29.06, 28.77, 28.37, 25.80, 25.57, 25.26, 24.51, 24.05, 23.60, 22.23, 13.56.

Submonomer		^{1}H (ppm)	^{13}C (ppm)
1 Ntri	C=O		166.40
	CHα	3.77	47.19
2 Nlys	C=O		170.05
	CHα	3.99, 4.19	48.95
3 Nfur	C=O		172.60
	CHα	3.30	47.99
	Furan	6.32, 6.37, 7.41	109.77, 110.70, 143.68, 148.54

Minimum Inhibitory Concentration Testing

The minimum inhibitory concentration (MIC) for AEC89 was determined against *C. neoformans* H99S (serotype A lab strain). The MIC for AEC5 was determined against *C. neoformans* H99S, two clinical strains of serotype A, serotype D lab strain (ATCC 24067), and two clinical strains of serotype D. All MIC assays were carried out following CLSI guidelines.[8] Briefly, the organism to be tested was streaked from frozen stock for isolation and incubated for 96 hours. A 5 mL solution of 0.85% saline was inoculated with 3-4 colonies of organism of interest. The inoculated solution was vortexed for 1.5 minutes. The optical density at 530 nm was acquired on a spectrophotometer and adjusted through addition of saline or cells to obtain an OD_{530nm} of 0.15 to 0.2. A 1:100 solution of Roswell Park Memorial Institute (RPMI) media containing 3-morpholinopropane sulfonic acid (MOPS; 0.1 M) and cells was made by adding 0.100 mL of saline incubated with cells to 9.9 mL of RMPI. The solution was vortexed and used to make a 1:20 solution by adding and vortexing 0.5 mL of the 1:100 solution to 9.5 mL of RPMI + 0.1 M MOPS. This was repeated twice to make a total of 20 mL of 1:20 RPMI + cells.)

Two-fold serial dilutions (0.700 mL total volume) for either AEC5 or AEC89 in water were prepared using the 1:20 solution of RPMI/MOPS + cells. On a 96-well plate, compound dilutions were placed in 200 μL in triplicate. RPMI/MOPS alone, 1:20 RPMI/MOPS + cells solution, and RPMI/MOPS + cells treated with amphotericin B (2 μg/mL) were included as controls on each plate. Note that complete inhibition of *C. neoformans* was observed in response to amphotericin B for each assay completed, given that the concentration used was above the reported MIC for this microorganism.[9] The plate was incubated at 35°C for 72 h. For plate analysis, 20 μL PrestoBlue was added to each well and the plate incubated for 8 hours and analyzed with a M5 plate reader (Ex. 555 nm; Em. 585 nm). All triplicate assays were repeated three times on different days. The MIC values for known antifungal agents fluconazole and amphotericin B were also determined against *C. neoformans* H99S and shown to be similar to reported values[9] in our hands (**Figure S4**).

Fungicidal vs. Fungistatic Assay

An MIC plate of AEC5 was prepared against *C. neoformans* H99S as described above. Upon completion of the 72 h incubation, cell/RPMI/drug triplicates were collected from each of the three wells into a sterile 1.5 mL microcentrifuge tube. A total of nine concentrations (2-fold serial dilutions from 200.0 μg/mL to 0.781 μg/mL) were collected as well as a control sample that contained no drug. In order to remove the drug compound from the solutions, the cells were collected by centrifugation (2000 rpm; 5 min). The resulting supernatant was decanted, the cells resuspended in 1 mL of 1X PBS, and the cells again collected by centrifugation (2000 rpm; 5 min). After decanting the supernatant, 100 μL of fresh PBS was used to resuspend the cells with gentle shaking.

The cell solutions were transferred to a 96-well plate and serially diluted 10-fold in PBS four times to create cell dilutions from 10^0 to 10^{-4}. This assured that cell colonies could be accurately counted, even at high concentrations of viable cells. Aliquots (5 μL) of each drug concentration and cell dilution were spotted onto YPD agar plates that had been dried briefly in a 37°C incubator to ensure that the deposited solutions of cells + PBS would not run together on the surface of the agar. Three spotted plates were generated with each plate containing three concentrations of cells + PBS solution and one control row that was diluted to the same factor as the sample rows. Spots were dried briefly and the plates were incubated at 37°C for 24 h. The plates were removed, photographed, and the individual colonies counted to provide an understanding of the fungicidal vs. fungistatic properties of AEC5.

Mammalian Cytotoxicity Testing

NIH/3T3 mouse fibroblasts, HepG2 hepatocarcinoma cells, and HPL1A human peripheral lung epithelial cells were grown in DMEM containing 10% FBS at 37 ˚C and 5% CO_2 atmosphere. For cytotoxicity testing, cells were seeded into 96-well plates in phenol red free DMEM containing 10% FBS and incubated for 3 h to allow cell attachment. Cells were then treated with varying concentrations of peptoid (2-fold serial dilution in PBS from 800-3.125 μg/mL). PBS alone and 1% Triton X-100 served as negative and positive controls, respectively. After 48 h incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 20 μL; 5 mg/mL) was added to each well and the plate incubated for 3.5 h. Media was removed from each well and 100 μL of DMSO was added and the plate incubated at 37 ˚C for 15 min. The absorbance at 570 nm was then read on a spectrophotometer, percent inhibition determined,

and IC_{50} calculated using GraFit. All triplicate assays were repeated three times on different days.

Hemolytic Activity

The hemolytic activity of AEC5 against human red blood cells (hRBCs) was done as previously described.[10] Briefly, hRBCs were washed 3x with PBS (11.8 mM phosphate; 140.4 mM NaCl; pH 7.4) by centrifugation (10 min. at 1000 xg). Cell were resuspended in PBS and 100 µL aliquots placed into individual wells of a 96-well plate. Peptoid solutions (2-fold serial dilution in PBS; 800-3.125 µg/mL final) were added to the wells. A negative control was prepared by adding PBS with no peptoid to the hRBCs and a positive control was prepared by adding 1% Triton X-100 to the hRBCs. The plate was incubated at 37 ˚C for 1 h, centrifuged at 1000 x g for 10 min, and 5 µL of supernatant transferred into 95 µL of PBS to a new 96-well plate. Released hemoglobin was measured by reading the absorbance at 405 nm on a spectrophotometer. All triplicate assays were repeated three times on different days. Percent hemolysis was calculated as follows;

$$\% \text{ hemolysis} = \frac{(OD_{405nm} \text{ sample} - OD_{405nm} \text{ neg. control})}{(OD_{405nm} \text{ pos. control} - OD_{405nm} \text{ sample})} \times 100$$

The Hill Slope (H) and HC_{50} was determined using GraFit and HC_{10} was calculated from HC_{50} using the following equation;

$$HC_{10} = HC_{50} [10\%/(100\%-10\%)]^{1/H}$$

Physiochemical Property Predictions

Several physiochemical properties were predicted using ChemAxon's MarvinSketch Calculator Plugins.[11] This program was used to calculate the octanol-water partition coefficient (log*P*), distribution coefficient at pH 7.4 (log$D_{7.4}$), and water solubility coefficient (log*S*), isoelectric point, and 2D polar surface area (PSA) for AEC5, amphotericin B, fluconazole, flucytosine, daptomycin, pexiganan, and colistin. The pKa values for the two amino groups of AEC5 were also calculated and the predominant microspecies of AEC5 at pH 7.4 was determined.

Serum Stability Testing

The stability of AEC5 in human serum was determined as done previously.[12] Briefly, AEC5 (8 μL of 50 mg/mL stock; 0.1 mg/mL final concentration) was added to 25% pooled human serum in PBS (4 mL total; pH. 7.4) pre-warmed to 37 ˚C. Aliquots (0.5 mL) were removed at varying time points (0, 45, 90, 150, and 240 min) and added to tubes containing trichloroacetic acid (12.5 μL of 100%; 2.5% final) to precipitate serum proteins. Samples were cooled on ice 5 min and centrifuged 5 min at 17,000 xg, and analyzed by RP-HPLC. Concentration of non-degraded AEC5 was determined by calculating the area under the curve. Stability assays were completed in duplicate on two different days.

Supporting Figures

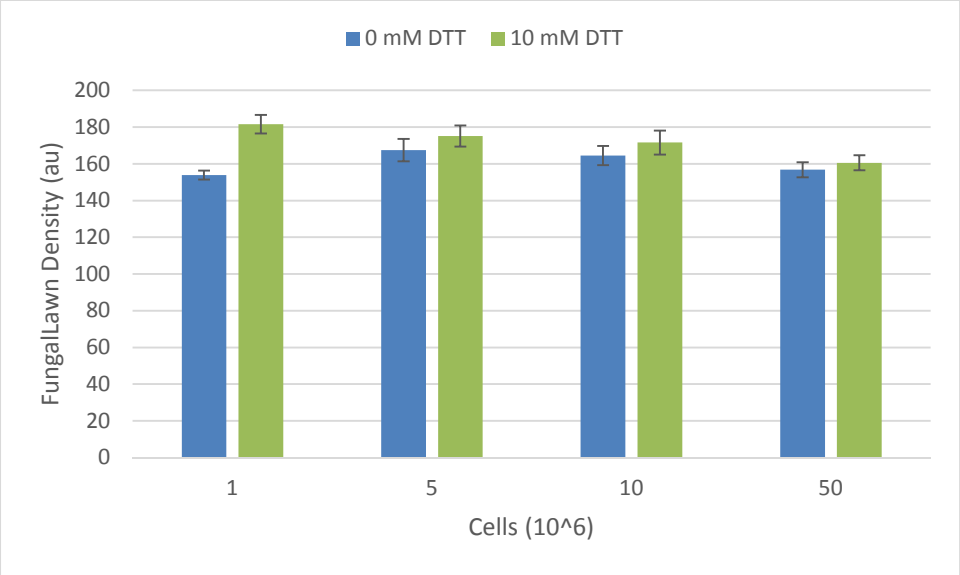


Figure S1. Results of experimental optimization of DTT and cell concentrations for the PLAD assay identification of antimicrobial peptoids towards *C. neoformans* H99S. Error bars represent standard deviation.

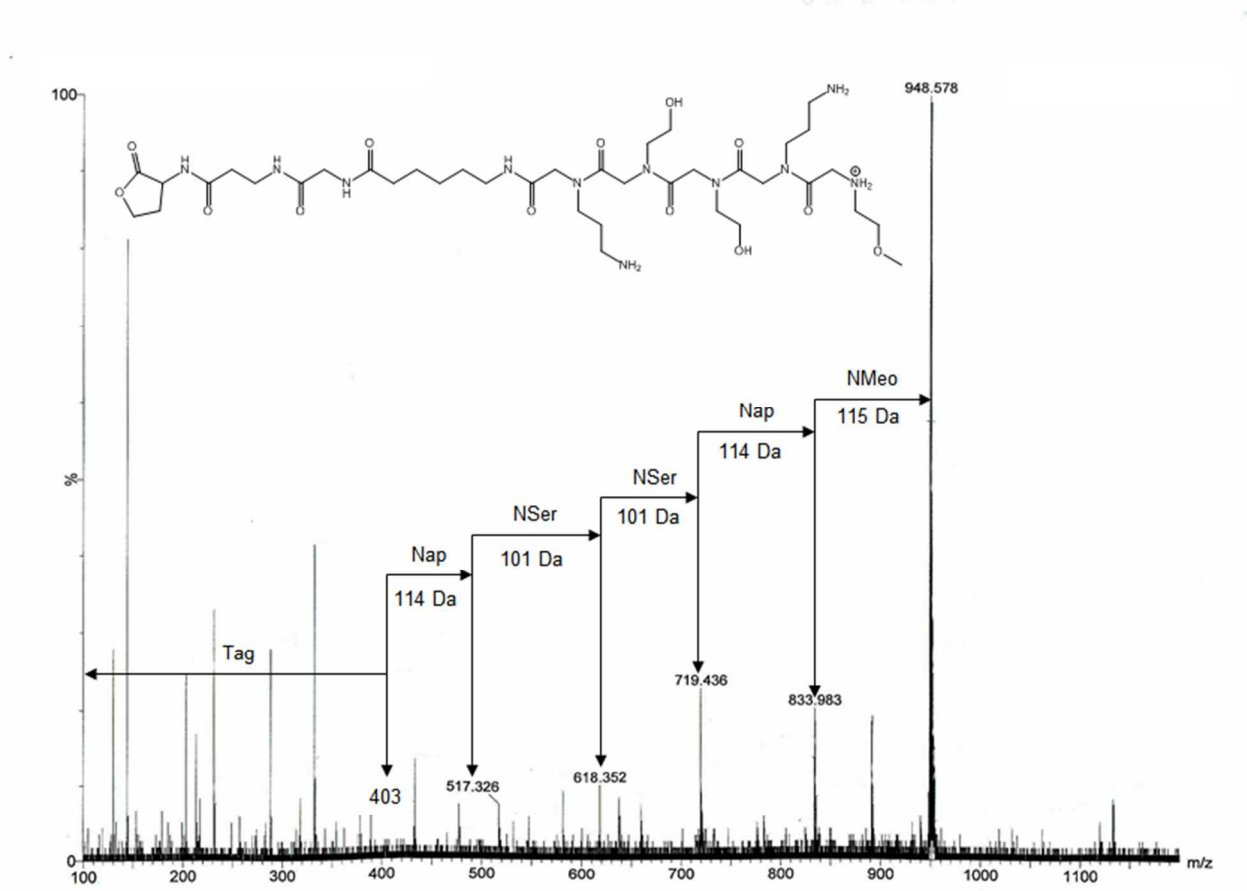


Figure S2. Tandem mass spectrometry (MS/MS) sequencing of a peptoid randomly selected from the first generation antifungal library, confirming library quality and ability to sequence the *alpha*-strand.

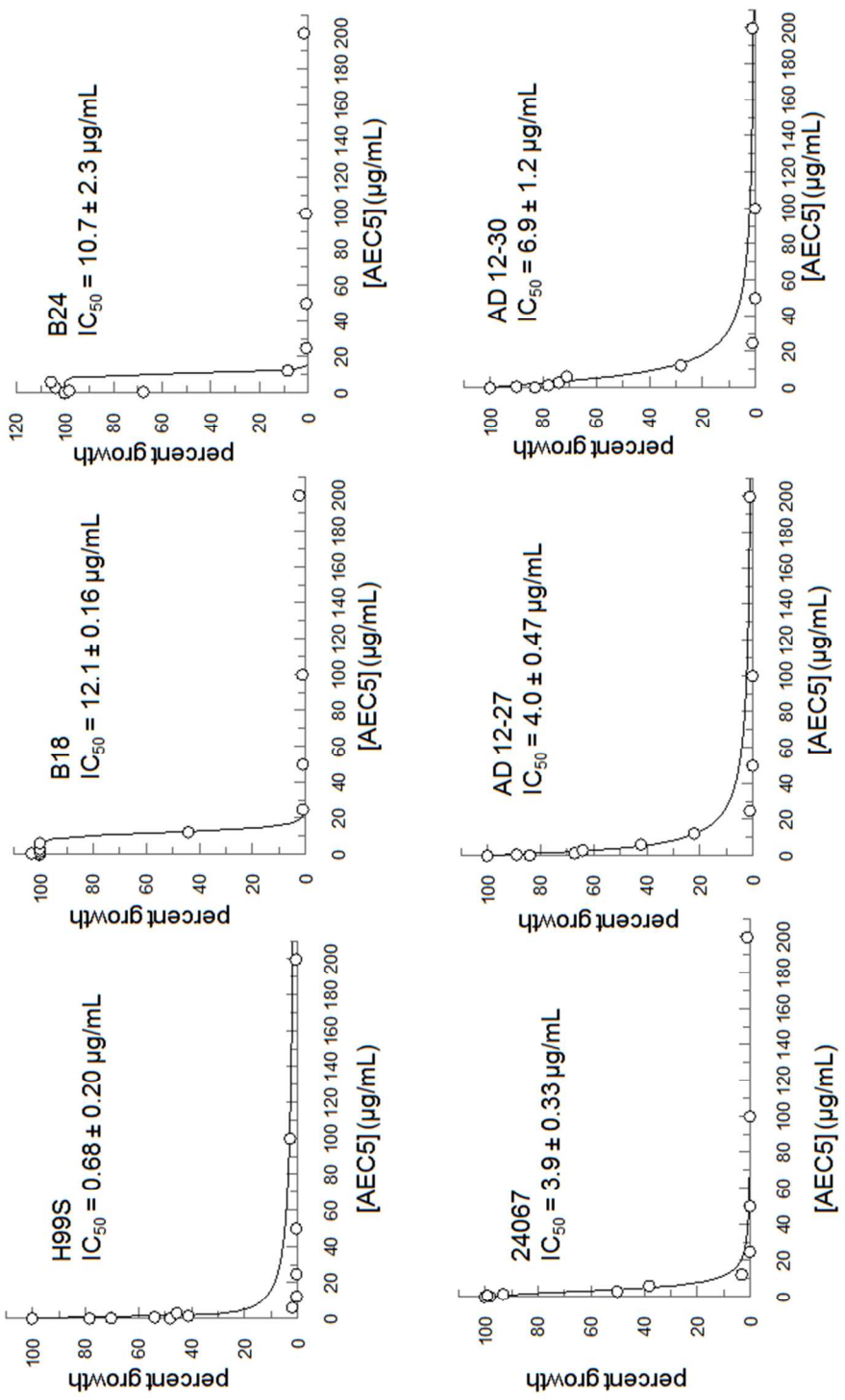


Figure S3. Inhibitory concentration 50% (IC_{50}) curves of AEC5 against the serotypes and strains of *C. neoformans* tested. Reported error values are in the form of standard deviation.

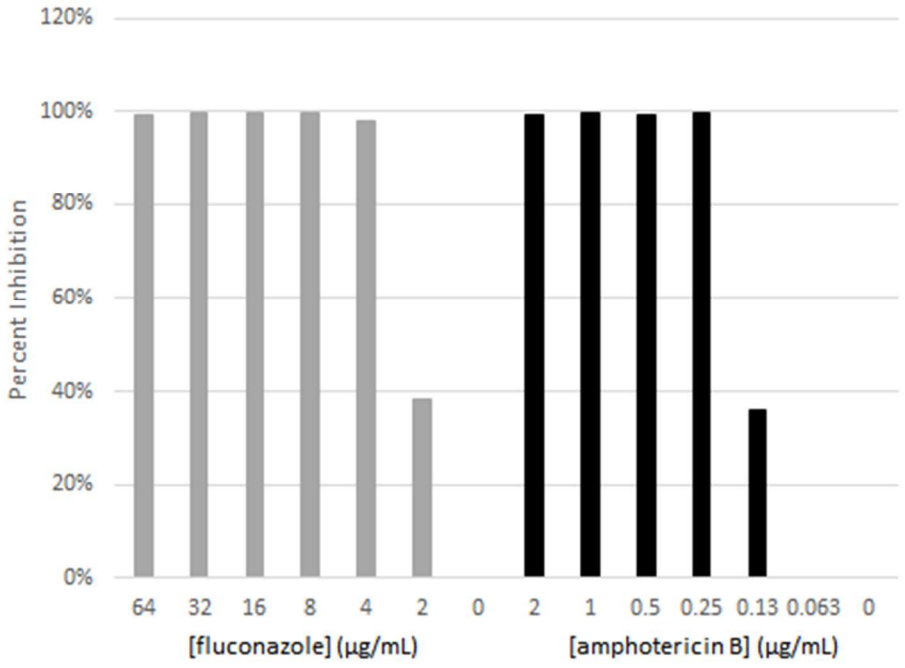


Figure S4. Control testing of two well characterized antifungal agents, fluconazole and amphotericin B, against *C. neoformans* H99S.

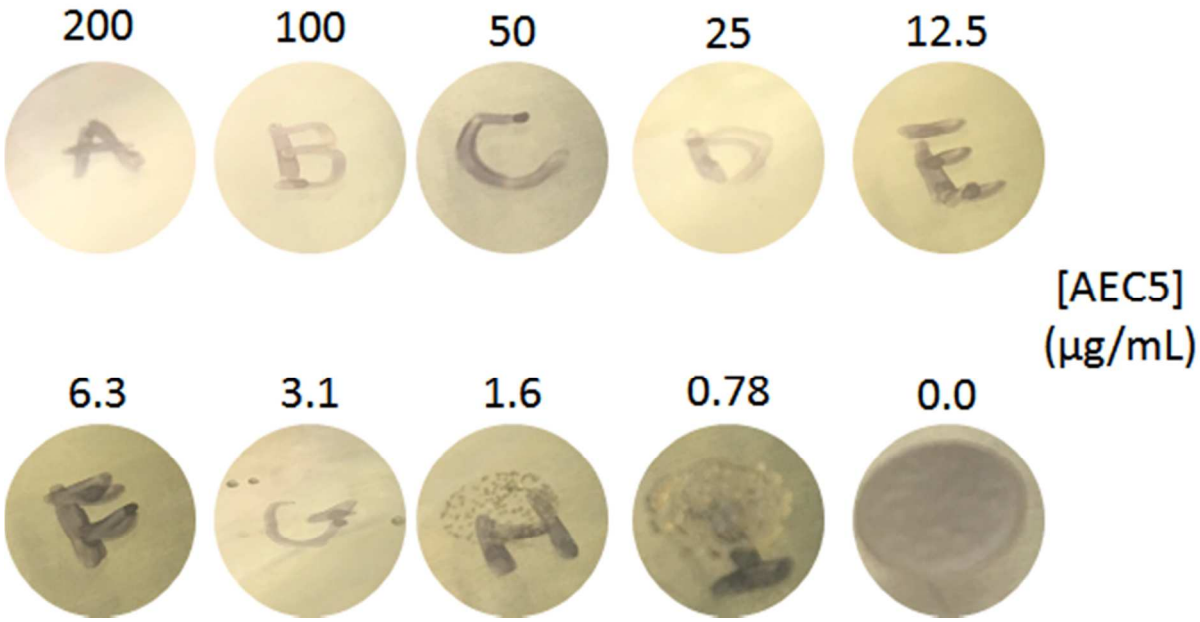


Figure S5. Determining the fungicidal activity of AEC5. Images of spotted plates after washing to remove drug from cells treated with varying concentrations of AEC5 for 72 h. No colonies observed down to 6.3 µg/mL, 2 colonies observed at 3.1 µg/mL, and multiple colonies observed at concentrations below 3.1 µg/mL.

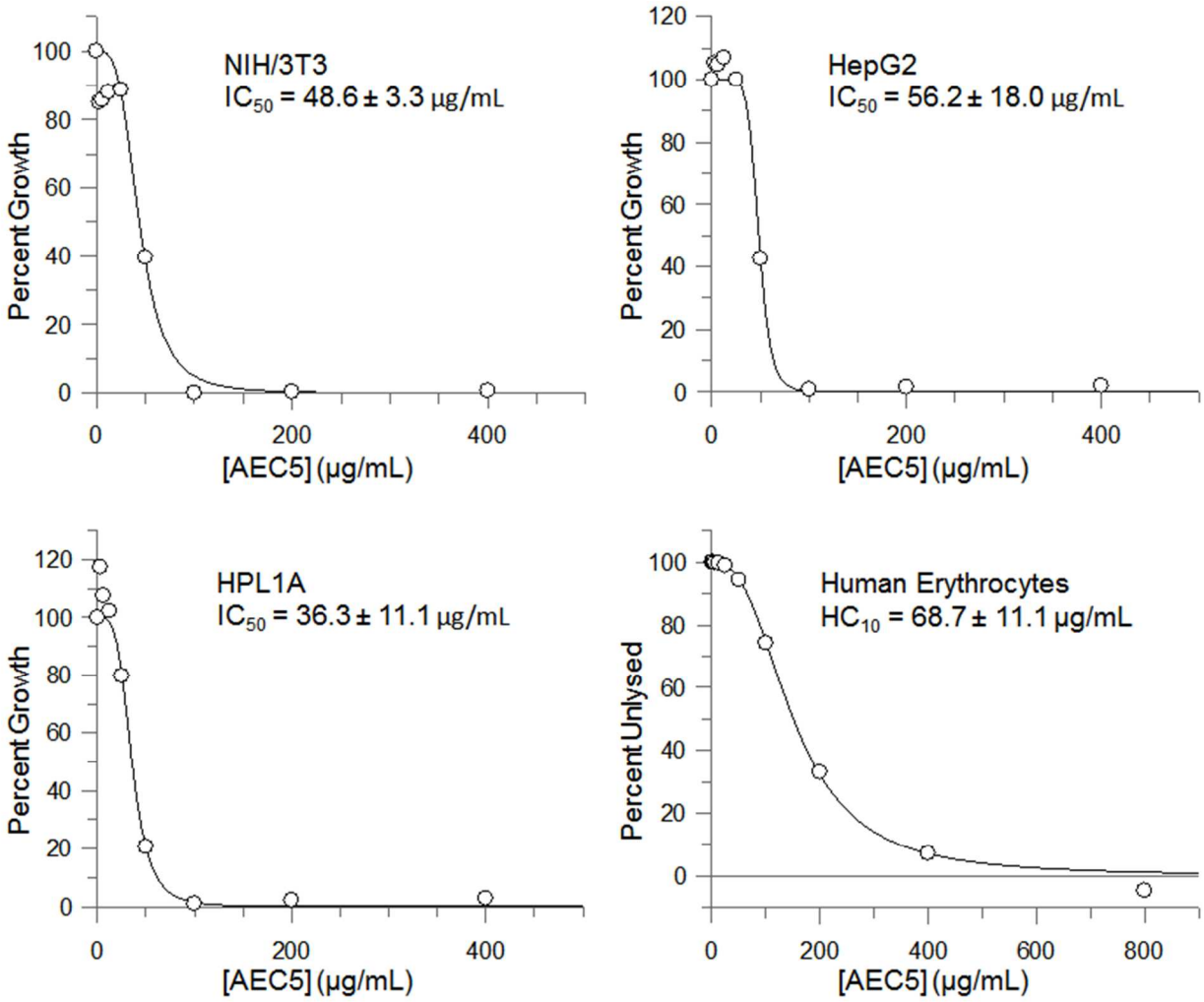


Figure S6. Hemolytic activity 10% (HC_{10}) and inhibitory concentration 50% (IC_{50}) curves of AEC5 against several mammalian cell types. Reported error values are in the form of standard deviation.

Analysis	AEC5	Amphotericin B	Fluconazole	Flucytosine	Daptomycin	Pexiganan	Colistin
log*S*	-2.55	-2.40	-2.90	-1.31	0.00	2.13	0.00
log*P*	2.61	-0.04	0.56	-0.95	-6.79	-25.12	-8.1
log*D*							
pH 1.5	-3.67	-3.05	-0.48	-0.95	-9.76	-25.24	-23.26
pH 5.0	-3.55	-2.31	0.56	-0.95	-12.72	-23.32	-23.20
pH 6.5	-2.51	-2.30	0.56	-0.96	-17.01	-21.18	-21.78
pH 7.4	-1.18	-2.31	0.56	-1.01	-18.9	-18.97	-18.57
Isoelectric Point	12.68	6.41	7.53	NA	3.21	10.07	10.42
2D polar surface area ($Å^2$)	134.9	319.6	81.7	67.5	702	963.9	490.7
pKa							
N-terminus	8.74						
Sidechain	9.94						
at pH 7.4	95.7% doubly protonated						

Table S1. Physicochemical properties of AEC5 and several commercial antifungal agents and antimicrobial peptides as calculated by ChemAxon's MarvinSketch Calculator Plugins.

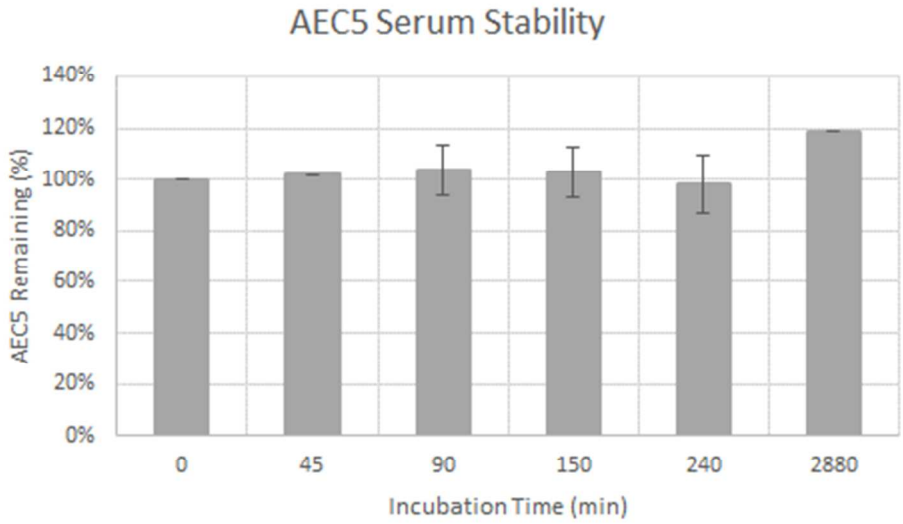


Figure S7. Stability of AEC5 in human serum, analyzed after incubation for various time points. Data show AEC5 to be completely stable in 25% pooled human serum out to 48 h. Error bars represent standard deviation.

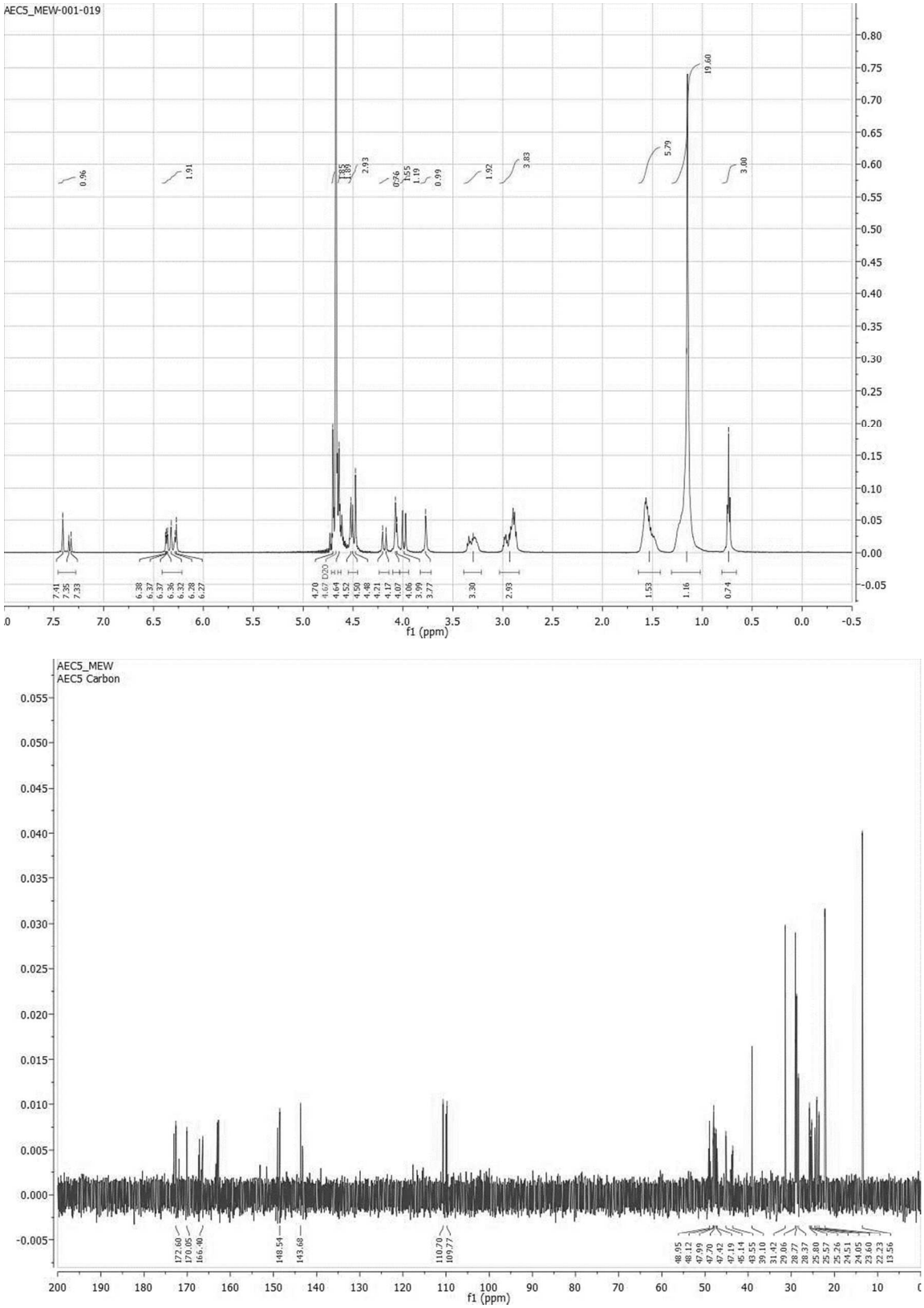


Figure S8. ^{1}HNMR and ^{13}CNMR for AEC5.

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