Peptoid Library Agar Diffusion (PLAD) Assay for the High-Throughput Identification of Antimicrobial Peptoids

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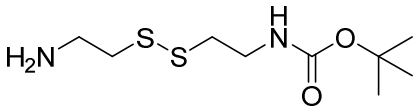
I. Synthesis and Screening Procedures

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). Non-pathogenic *E. coli* (ATCC 25290) were provided by Dr. Mary Farone in the Department of Biology at Middle Tennessee State University (MTSU). 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.

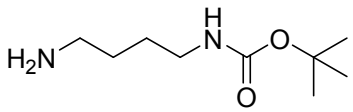
N-(*tert*-butoxycarbonyl)-cystamine



Cystamine dihydrochloride (4 g, 17.78 mmol) was dissolved in methanol (200 mL) and cooled to 0°C. Triethylamine (7.45 mL, 53.33 mmol) was added and stirred for 30 min. Boc-anhydride (4.05 mL, 17.78 mmol) was then added drop wise over 10 min and allowed to stir for 1 h. The solution was concentrated *in vacuo*, then washed with diethyl ether (3x 30mL). 1 M NaOH solution was added to the product and extracted 2x with CH_2Cl_2. Both organic layers were combined and washed 2x with H_2O. The organic layer was then dried over $CaCl_2$ and concentrated *in vacuo* to yield a white solid (3.9 g, 86% yield). ESI $[M+H]^{+1}$ expected 253.39

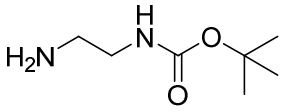
Da, observed 253.1 Da. ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9H), δ 2.77 (q, 4H, J=6.19 Hz), δ 3.02 (t, 4H, J=6.19 Hz), δ 3.45 (m, 2H), δ 5.02 (s, 1 H).

***N*-(*tert*-butoxycarbonyl)-1,4-diaminobutane**



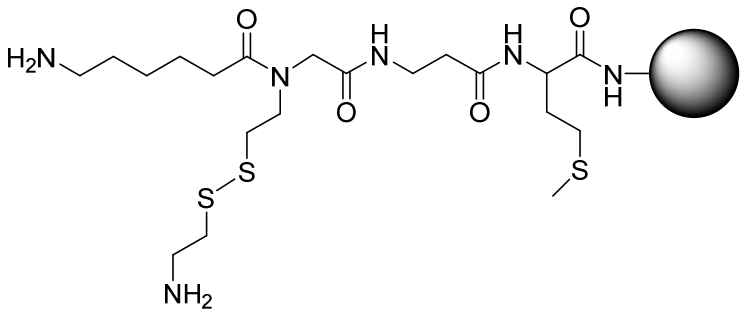
Concentrated HCl (2.85 mL, 34.09 mmol) was added to methanol (50 mL) and cooled to 0°C on ice. 1,4-Diaminobutane (3 g, 34.09 mmol) was added to the mixture and stirred for 20 min. Water (ddH_20; 7 mL) was added and stirred 30 minutes. Di-*tert*-butyl dicarbonate (11.72 mL, 51.11 mmol) in methanol (30 mL) was added drop wise over 10 min then stirred for 1 h. The solvent was evaporated *in vacuo* and the resulting solid washed with diethyl ether (3x 30 mL). 1 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 (4.81 g, 75% yield). ESI $[M+H]^{+1}$ expected 189.27 Da, observed 189.2 Da. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.61 (s, 1H), 3.13 (m, 2H), 2.17 (m, 2H), 1.50 (m, 6H), 1.44 (s, 9H).

***N*-(*tert*-butoxycarbonyl)-1,2-diaminoethane**



Concentrated HCl (3.89 mL, 46.6 mmol) was added to methanol (50 mL) and cooled to 0°C on ice. 1,2-Diaminoethane (2.8 g, 46.6 mmol) was added to the mixture and stirred for 20 min. Water (ddH_20; 8 mL) was added and stirred 30 minutes. Di-*tert*-butyl dicarbonate (16.08 mL, 70.0 mmol) in methanol (34 mL) was added drop wise over 10 min then stirred for 1 h. The solvent was evaporated *in vacuo* and the resulting solid washed with diethyl ether (3x 30 mL). 1 M NaOH solution was added and the product was extracted 2x with CH_2Cl_2. Both organic layers were combined and washed 1x with brine. The organic layer was then dried over $CaCl_2$ and concentrated *in vacuo* to yield a white solid (2.92 g, 39.2% yield). ESI $[M+H]^{+1}$ expected 161.22 Da, observed 161.1 Da. ^1H NMR (500 MHz, $CDCl_3$): δ 4.85 (s, 1H), 3.09 (m, 2H), 2.67 (q, 2H, J=6.30 Hz), 1.46 (m, 2H), 1.43 (s, 9H).

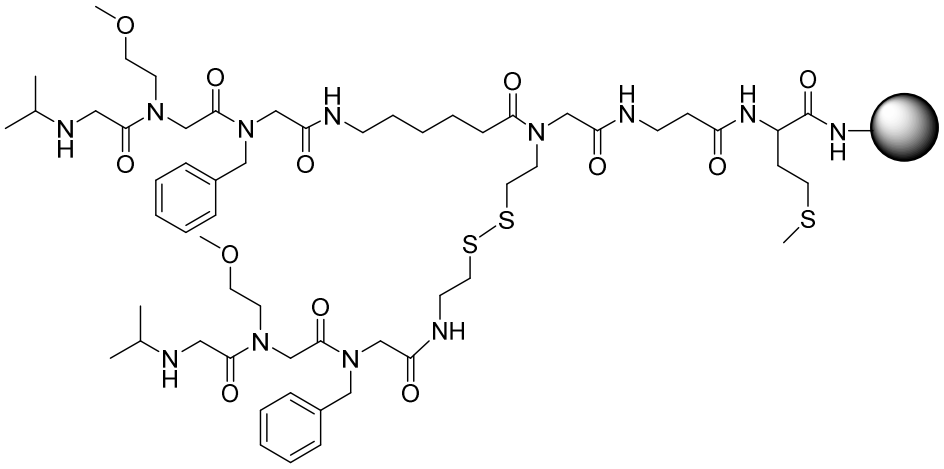
Branched Disulfide Linker Synthesis



500 mg of TentaGel macrobeads (0.25 mmol/g loading capacity) were swollen for 20 min in dimethylformamide (DMF). Fmoc-Met-OH (320 mg, 0.82 mmol, 7 eq.) was activated with

N,N,N′,N′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU; 330 mg, 0.82 mmol, 7eq.) for 10 min in 10 mL DMF with 5% N-methylmorpholine (NMM; v/v). This solution was then added to the TentaGel resin and allowed to react for 1 h with gentle rocking. Fmoc deprotection of the methionine was accomplished using 10 mL of 20% piperidine/DMF (v/v) solution for 10 min twice. Fmoc-β-Ala-OH (270 mg, 0.87 mmol, 7 eq.) was activated with HBTU (330 mg, 0.87 mmol, 7 eq.) for 10 min in 10 mL DMF with 5% NMM (v/v), added to the resin and allowed to react for 1 h with gentle rocking. Fmoc deprotection was again accomplished with 10 mL 20% piperidine/DMF (v/v) as done before. *N*-(*tert*-butoxycarbonyl)-cystamine was next incorporated using peptoid submonomer synthesis.[1] Briefly, bromoacetic acid (1.38 g; 10 mmol) in anhydrous DMF (5 mL) was mixed with diisopropylcarbodiimide (DIC; 2.5 mL; 16 mmol) in anhydrous DMF (5 mL) and added to the resin. The reaction was then microwaved in a 1000 kW commercial microwave at 10% power (100 kW) for 15 s twice and rocked gently for 15 min. *N*-(*tert*-butoxycarbonyl)-cystamine (550mg, 2.2 mmol, 17 eq.) was added to the resin in 8 mL anhydrous DMF, microwaved at 10% power for 15 seconds twice and rocked gently for 45 min. Fmoc-6-aminohexanoic acid (Fmoc-Aca-OH; 175mg, 0.50 mmol, 4 eq.) was activated with HBTU (187 mg, 0.50 mmol, 4 eq.) for 10 min in 10 mL DMF, added to the resin, and allowed to react for 1 h with gentle rocking. Boc group deprotection from the cystamine side chain was then done using 10 mL of a 95% TFA/2.5% H_2O/2.5% triisopropylsilane (TIS) mixture for 1 h followed by washing 5x with CH_2Cl_2 and 5x with DMF. Deprotection of the remaining Fmoc group was done with 20% piperidine/DMF (v/v) followed by washing 4x with DMF. All reactions were tested with a ninhydrin color test, and after each reaction the resin was washed 4x with DMF unless stated otherwise.

Synthesis of PLAD Linked Test Peptoid

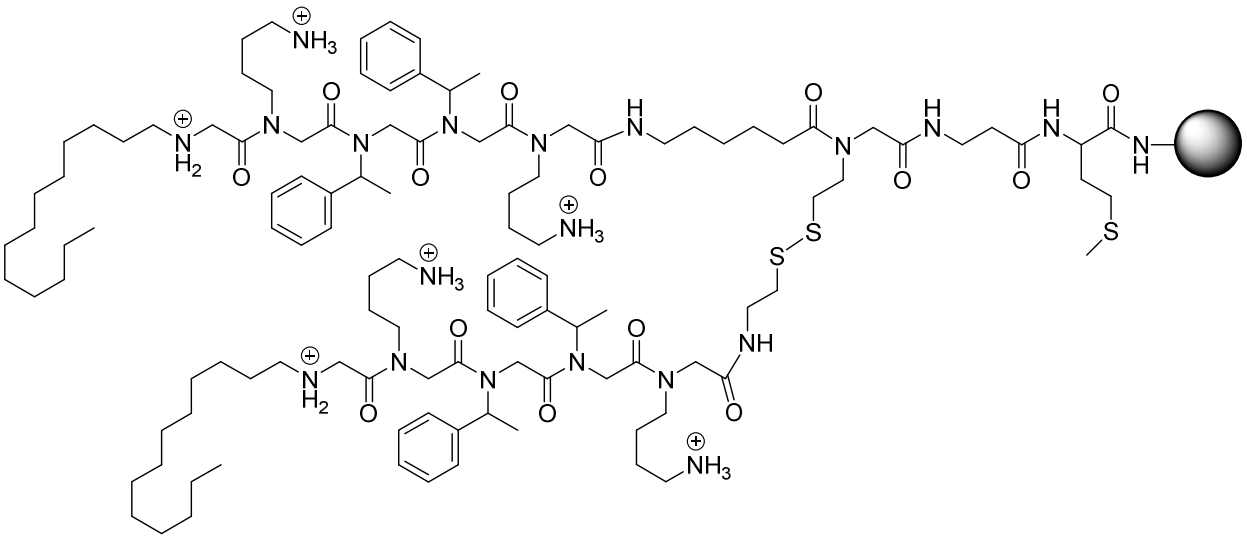


Solutions of bromoacetic acid (0.417 g; 3 mmol) in anhydrous DMF (1.5 mL) and DIC (0.75 mL; 4.8 mmol) in anhydrous DMF (1.5 mL) were combined with 50 mg of resin immobilized PLAD linker. The suspended resin was microwaved twice at 10% power (100 kW) for 15 seconds, then rocked gently for 15 minutes. After the prescribed time, the mixture was aspirated and washed four times with DMF. Benzylamine (0.643 g; 6 mmol) in anhydrous DMF (3.0 mL) was added to the resin and microwaved twice at 10% power for 15 seconds followed by gentle rocking for 15 minutes. The suspension was aspirated and washed 4x with DMF. The previously described bromoacetic acid and DIC reaction was repeated. 2-methoxyethylamine (0.451 g; 6 mmol) in anhydrous DMF (3.0 mL) was added, microwaved, and rocked for 15 minutes. The suspension was then aspirated and washed 4x with DMF. Again, the bromoacetic acid and DIC coupling was repeated. Isopropylamine (0.355 g; 6 mmol) in anhydrous DMF (3.0 mL) was added to the resin, microwaved, and rocked for 15 minutes. The mixture was aspirated and washed 4x with DMF. All reactions were tested using a ninhydrin color test.

The complete test peptoid was analyzed by treating a small aliquot of resin with 75 µL of cyanogen bromide (CNBr; 40 mg/mL) in 80:20 acetonitrile (ACN):water containing 0.1 M HCl

for 18 h. This solution was then removed *in vacuo* and the cleaved peptoid resuspended in 200 µL of 80:20 ACN:water containing 0.05% TFA and analyzed by MS. The β-strand of the test peptoid was analyzed by treating a small aliquot of resin with 500 µL of tris(2-carboxyethyl)phosphine (TCEP; 1 mM) in water for 1 h at room temperature. The resulting supernatant was then analyzed by MS. The α-strand of the peptoid was analyzed by washing the TCEP treated aliquot of resin 3x with water and subsequently treating with 75 µL CNBr (40 mg/mL) in 80:20 ACN:water containing 0.1 M HCl for 18 h. This solution was then removed *in vacuo* and the cleaved peptoid resuspended in 200 µL of 80:20 acetonitrile:water containing 0.05% TFA and analyzed by MS and MS/MS.

Synthesis of PLAD Linked C13$_{4mer}$



To 150 mg of resin immobilized branched disulfide linker was added bromoacetic acid (0.414 g; 3 mmol) in anhydrous DMF (1.5 mL) and DIC (0.75 mL; 4.8 mmol) in anhydrous DMF (1.5 mL). This mixture was microwaved 2x at 10% power (100 kW) for 15 seconds,

rocked gently for 15 minutes, and washed 4x with DMF. *N*-(*tert*-butoxycarbonyl)-1,4-diaminobutane (300 mg, 1.59 mmol) in anhydrous DMF (3 mL) was then added, microwaved 2x at 10% power for 15 seconds, and reacted for 30 minutes, followed by washing 4x with DMF. The bromoacetic acid and DIC step was then repeated as described above. Phenylethylamine (2 M) in anhydrous DMF (3 mL) was added, microwaved, and allowed to react for 30 minutes, after which it was washed with 4x with DMF. These procedures were then repeated with phenylethylamine and *N*-(*tert*-butoxycarbonyl)-1,4-diaminobutane again, respectively. After an additional bromoacetic acid/DIC coupling step, 1-aminotridecane (2 M) in anhydrous DMF (3 mL) was added to the resin, microwaved, and rocked gently for 50 minutes. Resin was then washed 4x with CH_2Cl_2 and 4x with DMF. Deprotection of remaining Boc groups was then accomplished using 8 mL of a 95% TFA/2.5% H_2O/2.5% TIS solution for 1 hr. Resin was then washed 5x with CH_2Cl_2 and 5x with DMF. All reactions were tested using a ninhydrin color test.

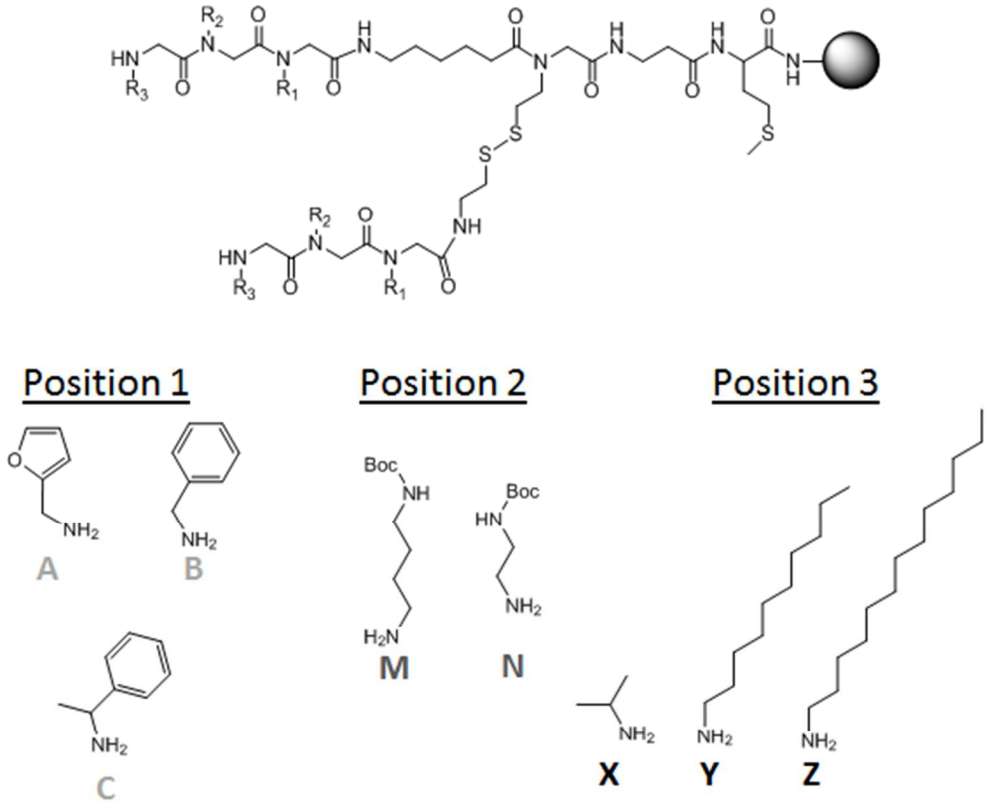
The complete $C13_{4mer}$ was analyzed by treating a small aliquot of resin with 75 µL of cyanogen bromide (CNBr; 40 mg/mL) in 80:20 ACN:water containing 0.1 M HCl for 18 h. This solution was then removed *in vacuo* and the cleaved peptoid resuspended in 200 µL of 80:20 acetonitrile:water containing 0.05% TFA and analyzed by MS. The β-strand of the test peptoid was analyzed by treating a small aliquot of resin with 500 µL of tris(2-carboxyethyl)phosphine (TCEP; 1 mM) in water for 1 h at room temperature. The resulting supernatant was then analyzed by MS.

Reducing Reagent Optimization

Solid lysogeny broth (LB) was autoclaved at 121°C and agar plates (10 mL) were poured and kept at room temperature overnight to dry them of excess condensation. The solid agar was plated first to serve as a support to be overlaid with soft agar, allowing for a smooth, thin layer for the peptoid modified TentaGel resin to be dispersed in. Overnight culture was prepared in LB broth (5 mL) by inoculating with ATCC 25922 *E. coli* frozen stock and incubating at 37°C for 20 h. TentaGel beads functionalized with PLAD linked $C13_{4mer}$ were washed 2x with H_2O then allowed to equilibrate overnight in H_2O. Soft agar for overlay was heated to 100°C for 30 minutes and cooled to 47°C, which kept it liquid. Compound beads were then equilibrated in 500 µL phosphate-buffer saline (PBS; pH 7.2), for each plate, for 30 minutes. Soft agar (3 mL), 75 µL of E. coli overnight culture, and PLAD linked $C13_{4mer}$ beads in PBS solution (500 µL) were then combined, and inverted 6-7 times gently to avoid air bubbles. This mixture, serving as the negative control with no reducing reagent, was then poured onto a hard agar plate and spread evenly into a thin layer by manual agitation. Dithiothreitol (DTT), 2-mercaptoethanol (BME), and tris(2-carboxyethyl)phosphine (TCEP) were then tested at varying concentrations to determine effectiveness at releasing the peptoid β-strand from the bead while maintaining good bacterial growth. Stock solutions of 100 mmol/L DTT, BME, and TCEP were prepared in PBS (pH 7.2). Each reducing agent was tested as described above by addition of the appropriate amount of stock reagent to separate individual plates. Final concentrations of reducing reagent in the soft agar overlay mixture 2, 4, 10, and 14 mmol/L for all three reducing agent. All plates were then allowed to solidify and incubated at 37°C for 18 hours. Zones of inhibition, defined as the distance between the edge of a bead and the beginning of bacterial growth near that bead, were measured using a Leica M165FC microscope. Images were also analyzed by Adobe

Photoshop to gain a measure of bacterial lawn density by measuring the light reflected off of the bacterial lawn when illuminated at an angle. Both sets of analyses, zone of inhibition measurements and bacterial lawn density measurements were performed on the same plates; bacterial lawn density was measured in areas of the plate where beads were not found.

Proof-of-Concept Library Synthesis



To 100 mg of resin modified with branched disulfide linker was equilibrated in anhydrous DMF and bromoacetic acid (0.414 g; 3 mmol) in anhydrous DMF (1.5 mL) and DIC (0.75 mL; 4.8 mmol) in anhydrous DMF (1.5 mL) were added. This mixture was microwaved 2x at 10% power (100 kW) for 15 seconds, rocked gently for 30 minutes, and washed 4x with DMF. Anhydrous DMF (3 mL) was then added and the resin was split into three vials (1 mL

each). DMF was removed from each vial and to the first was added 2 M furfurylamine in anhydrous DMF (2 mL), to the second 2 M benzylamine in anhydrous DMF (2 mL), and to the third 2 M phenylethylamine in anhydrous DMF (2 mL). All three vials were then microwaved 2x at 10% power (100 kW) for 15 seconds and rocked gently for 30 minutes. The resin from the three vials was then pooled together and washed 4x with DMF and equilibrated in anhydrous DMF. Bromoacetic/DIC coupling was then done for 30 minutes and the resin was washed 4x with DMF. Anhydrous DMF (2 mL) was added, and the resin was split into two vials (1 mL each). DMF was removed from both vials and to the first vial *N*-(*tert*-butoxycarbonyl)-1,4-diaminobutane (700 mg, 1.85 M) in anhydrous DMF (2 mL) was added, and to the second vial *N*-(*tert*-butoxycarbonyl)-1,2-diaminoethane (550 mg, 1.80 M) in anhydrous DMF (2 mL) was added. Both vials were microwaved 2x at 10% power (100 kW) for 15 seconds and rocked gently for 30 minutes. The two vials were combined and washed 4x with DMF, then equilibrated in anhydrous DMF. Bromoacetic/DIC coupling was then done for 30 minutes and the resin was washed with DMF 4x. Anhydrous DMF (3 mL) was then added to the resin and split into three separate vials. The DMF was removed and to the first vial 2 M isopropylamine in anhydrous DMF (2 mL) was added, to the second vial 2 M 1-aminodecane in anhydrous DMF (2 mL) was added, and to the third vial 2 M 1-aminotridecane in anhydrous DMF (2 mL) was added. These amine coupling vials were microwaved 2x at 10% power (100 kW) for 15 seconds and rocked gently for 30 minutes. Vials were then pooled and washed 4x with DMF and 4x with CH_2Cl_2. Deprotection of Boc groups was accomplished using 8 mL 95% TFA/2.5% H_2O/2.5% TIS solution for 1 hour. Resin was washed 5x with CH_2Cl_2 followed by 5x with DMF. This semi-combinatorial synthesis resulted in 18 unique peptoid sequences immobilized on the PLAD

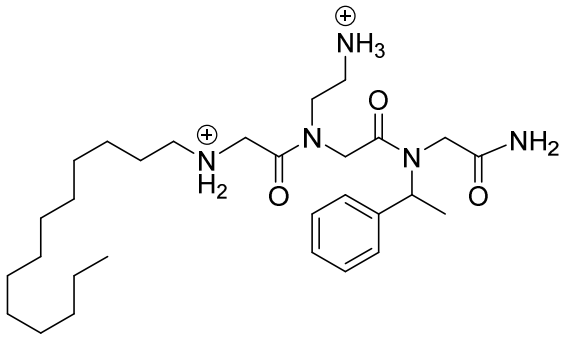
linker system for proof-of-concept testing. Ninhydrin tests were done following each successive bromoacetic acid step and pooling of amines to show confirm successful coupling.

Proof-of-Concept Library Screening

Solid lysogeny broth (LB) was autoclaved at 121°C and agar plates (10 mL) were poured and kept at room temperature overnight to dry them of excess condensation. The solid agar was plated first to serve as a support to be overlaid with soft agar, allowing for a smooth, thin layer for the peptoid modified TentaGel resin to be dispersed in. Overnight culture was prepared in LB broth (5 mL) by inoculating with ATCC 25922 *E. coli* frozen stock and incubating at 37°C for 20 h. Three aliquots of resin (4 mg) functionalized with PLAD linked proof-of-concept library were washed 2x with H_2O then allowed to equilibrate overnight in H_2O. Soft agar for overlay was heated to 100°C for 30 minutes and cooled to 47°C, which kept it liquid. The resin aliquots were then equilibrated in 500 µL phosphate-buffer saline (PBS; pH 7.2) for 30 minutes. Soft agar (3 mL), 75 µL of E. coli overnight culture, 500 µL of TCEP (100 mM stock; 14 mM final) and resin in PBS (500 µL) were then combined, and inverted 6-7 times gently to avoid air bubbles. This mixture was then poured onto a hard agar plate and spread evenly into a thin layer by manual agitation. All plates were then allowed to solidify and incubated at 37°C for 18 hours. Zones of inhibition, defined as the distance between the edge of a bead and the beginning of bacterial growth near that bead, were measured using a Leica M165FC microscope. Hits, defined as beads with a measurable zone of inhibition, were isolated manually with surgical tweezers and placed into individual tubes. These beads were boiled in 1% sodium dodecylsulfate (SDS) for 1 hour and washed 4x with water. The alpha strand of the peptoid was cleaved from the bead using cyanogen bromide (50 µL; 40 mg/mL) in 80:20 acetonitrile:water

containing 0.1 M HCl for 18 hours in the dark. This solution was then removed *in vacuo* and the cleaved peptoid resuspended in 80:20 acetonitrile:water containing 0.05% TFA. MS and MS/MS analysis was then done as previously described to identify the structure of the unknown peptoid. In total 34 hits were identified (24% hit rate) and 31 sequences were successfully obtained by MS and MS/MS.

Synthesis of K15 Peptoid



Rink Amide resin (0.101 g; 0.38 mmol/g loading) was swollen in DMF for 20 minutes. After removing the DMF, 20% piperidine in DMF (5 mL) was added to the resin and rocked for 30 minutes. The piperidine solution was drained, and the beads were washed 3x with DMF. The piperidine deprotection step was once more repeated, and the resin washed 3x with DMF. Solutions of bromoacetic acid (0.417 g; 3 mmol) in anhydrous DMF (1.5 mL) and DIC (0.75 mL; 4.8 mmol) in anhydrous DMF (1.5 mL) were combined with the deprotected Rink Amide beads. The resin was microwaved twice at 10% power (100 kW) for 15 seconds, then rocked gently for 15 minutes; after which, the mixture was aspirated and washed four times with DMF. 2-phenylethylamine (0.364 g; 3 mmol) in anhydrous DMF (3.0 mL) was added to the resin and microwaved twice at 10% power for 15 seconds followed by gentle rocking for 30 minutes. The

suspension was aspirated and washed 4x with DMF. The previously descried bromoacetic acid and DIC reaction was repeated. Boc-ethylene diamine (0.486 g; 3 mmol) in anhydrous DMF (3.0 mL) was added, microwaved, and rocked for 30 minutes. The suspension was then aspirated and washed 4x with DMF. Again, the bromoacetic acid and DIC coupling was repeated. Tridecylamine (0.598 g; 3 mmol) in anhydrous DMF (3.0 mL) was added to the resin, microwaved, and rocked for 40 minutes. The mixture was aspirated and washed 4x with DMF and 4x with CH_2Cl_2. A ninhydrin test was performed on a small sample after every coupling. The tripeptoid was cleaved from the resin by treating 2x with TFA:water:TIS (95:2.5:2.5) for 1 h each. TFA was removed by bubbling with air and residual substance resuspended in 1:1 water:acetonitrile containing 0.05% TFA. K15 was then purified by reverse phase HPLC using a C18 column and a gradient of water with 0.05% TFA to acetonitrile with 0.05% TFA. The identity of the compound was confirmed by MS and the solvent removed under vacuum to provide pure K15 (17 mg; 17% yield).

K15 MIC Testing in ESKAPE Pathogens

Peptoid K15 was analyzed via a traditional broth minimum inhibitor concentration (MIC) assay against seven different ESKAPE pathogens (*Acinetobacter baumanii,* ATCC 19606; *Enterococcus faecalis,* ATCC 29212; *Enterococcus faecium,* ATCC 19434; *Escherichia coli,* ATCC 25922; *Klebsiella pneumoniae,* ATCC 700603; *Pseudomonas aeruginosa,* ATCC 27853; *Staphylococcus aureus,* ATCC 29213). For each of the bacterial strains screened in the ESKAPE panel, 1-3 isolated colonies were collected from a TSA plate by a flame sanitized wire loop and resuspended in 5 mL of TSB. The solutions were incubated at 37ºC for 18-24 hours. After the growth period, the turbidity was measured at 600 nm and adjusted to an optical density

of 0.08-0.13 by diluting with TSB for an approximate concentration of $1x10^8$ CFU/mL. Once the desired OD was achieved, 20 µL of the bacteria suspension were diluted 1:20 in 380 µL Cation Adjusted Mueller-Hinton broth (CAMHB) for a final concentration of $5x10^6$ CFU/mL.

4 µL of a 10 mM stock of K15 were diluted in 356 µL CAMHB for each bacterial strain assayed (a total of 28 µL stock in 2.478 mL broth for ESKAPE panel). 180 µL of this solution were delivered to three wells. For each dilution to be studied, 90 µL of the 100 µM solution were withdrawn and delivered to 90 µL of broth This 1:2 serial dilution was continued to give final K15 concentrations of 100, 50, 25, 12.5, 6.3, 3.1, and 1.6 µM. 90 µL of the final triplicate set being removed such that each well has a volume of 90 µL. A negative control containing 90 µL of broth with no K15 was also prepared. 10 µL of the 1:20 diluted bacteria were added to each well for a total volume of 100 µL. 100 µL of broth were delivered to a well in triplicate to serve as a media control. A tetracycline control was used, composed of 4 µL 2 mg/mL antibiotic in 356 µL broth with 40 µL bacteria. 100 µL of this solution were delivered to each of three wells.

The prepared plates were incubated for another 18-24 hours. Their respective absorbance at 600 nm was analyzed on a SpectraMax M5 Plate Reader. 10 µL of PrestoBlue were added to each well and allowed to incubate for an hour. Absorbance at 555, 570, and 585 nm was analyzed to determine viable cells having survived treatment by the antimicrobial compound. This assay, which utilizes triplicates of each K15 concentration, was ran in duplicate or triplicate for each microorganism tested on different days.

II. Supporting Figures

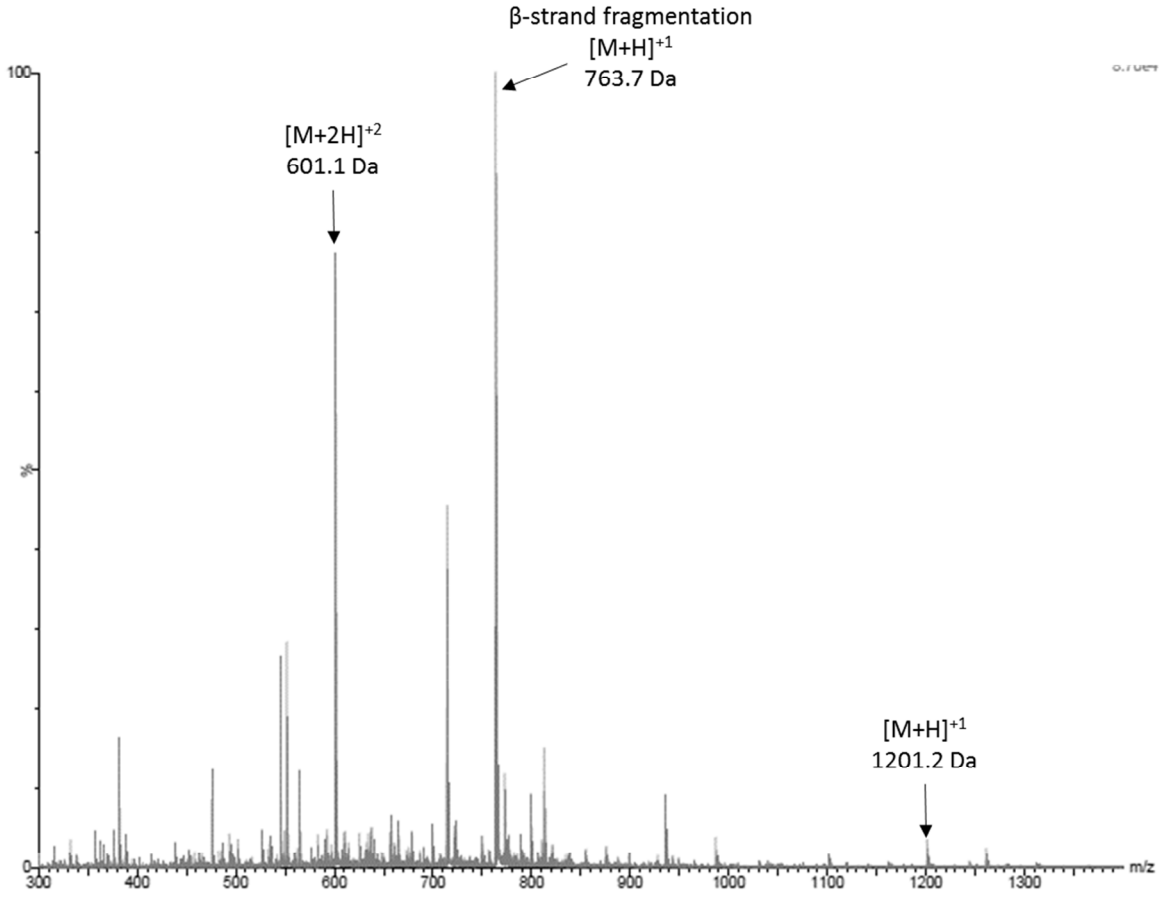


Figure S1. Linear MS of complete test peptoid, showing the desired compound at 1201.2 Da (M+H) and 601.1 Da (M+2H). The disulfide bond is easily fragmented during MS analysis, giving the complete compound minus the β-strand at 763.7 Da (M+H).

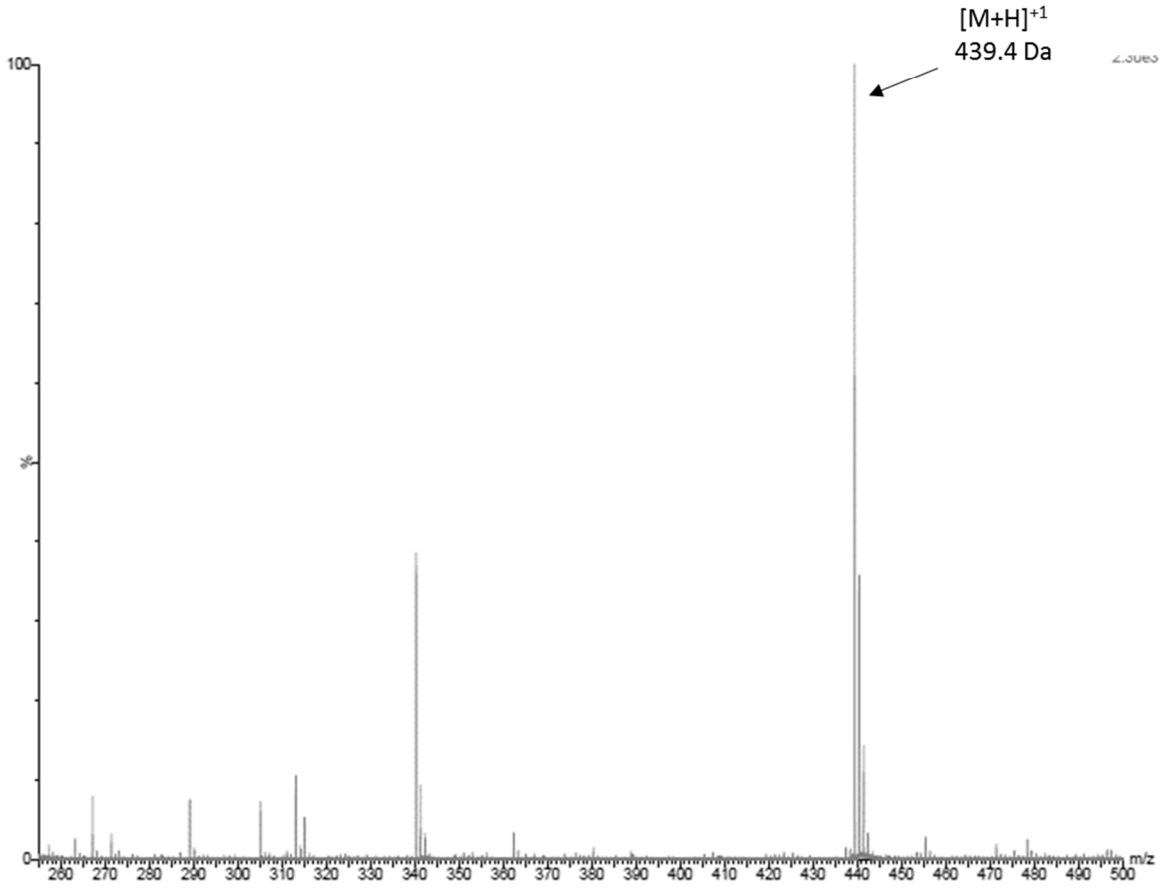


Figure S2. Linear MS of the β-strand of the test peptoid at 439.4 Da (M+H), released from the PLAD linker by treatment with TCEP.

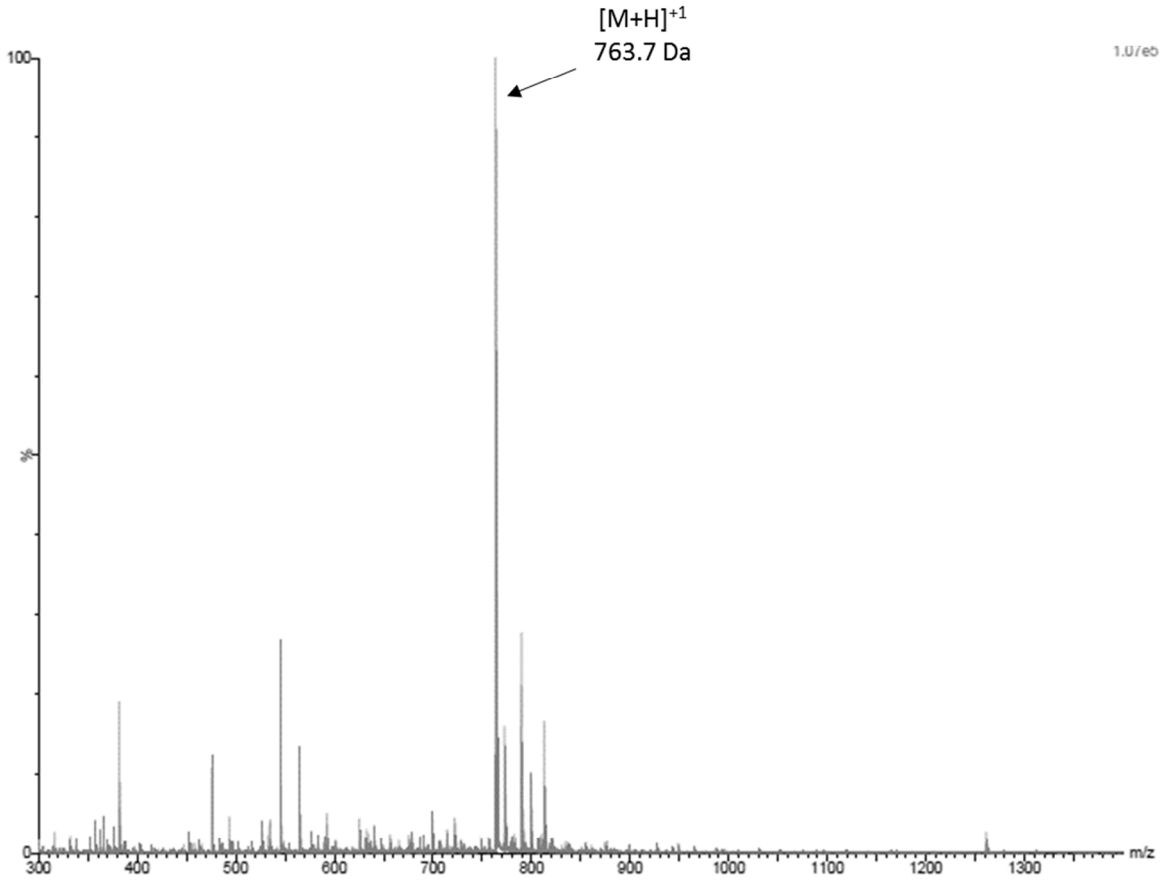


Figure S3. Linear MS of the α-strand of the test peptoid at 763.7 Da (M+H) after β-strand cleavage by TCEP.

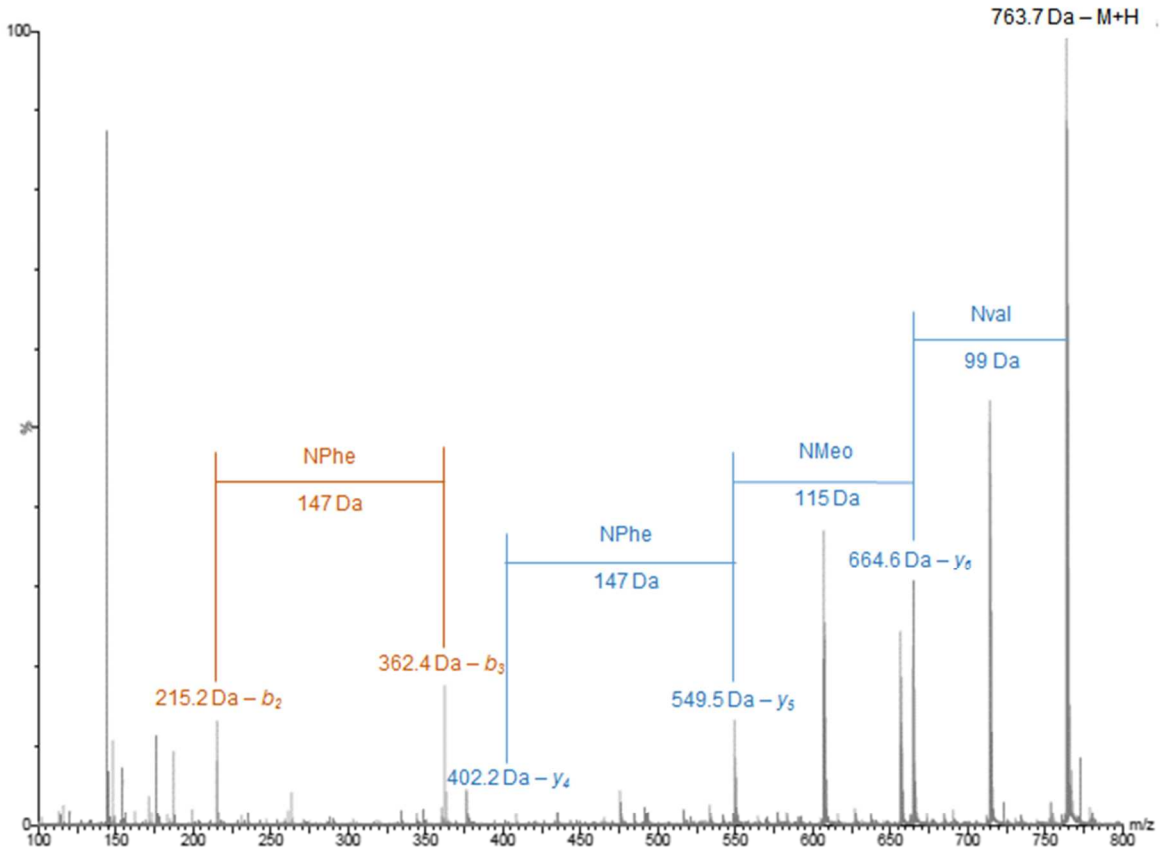


Figure S4. Tandem MS of the α-strand of the test peptoid after β-strand cleavage by TCEP. The sequence of this compound (NVal-NMeo-NPhe-PLAD linker) can be confirmed using both *y* ions (shown in blue) and *b* ions (shown in orange).

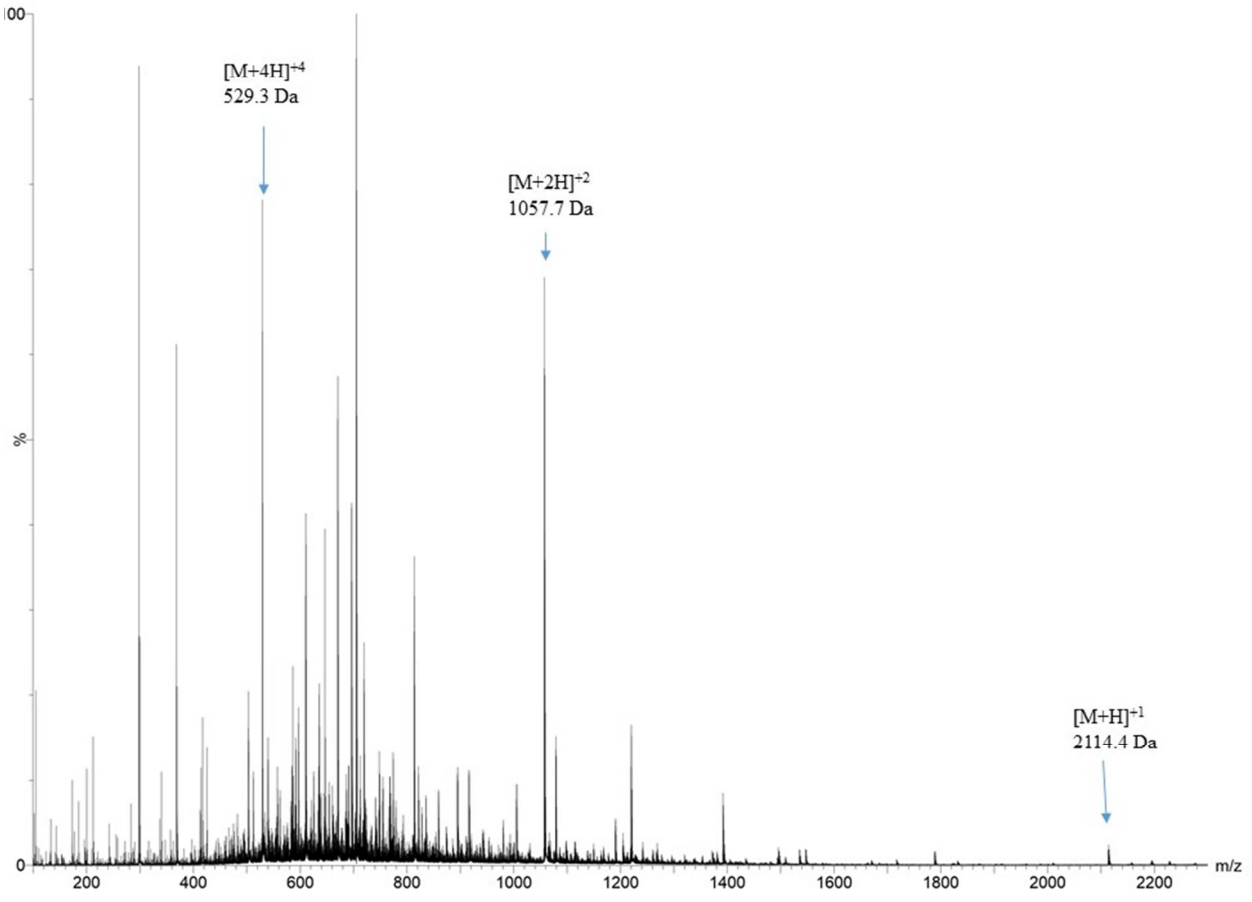


Figure S5. Linear MS of the complete $C13_{4mer,}$ containing both the α and β-strands of the peptoid.

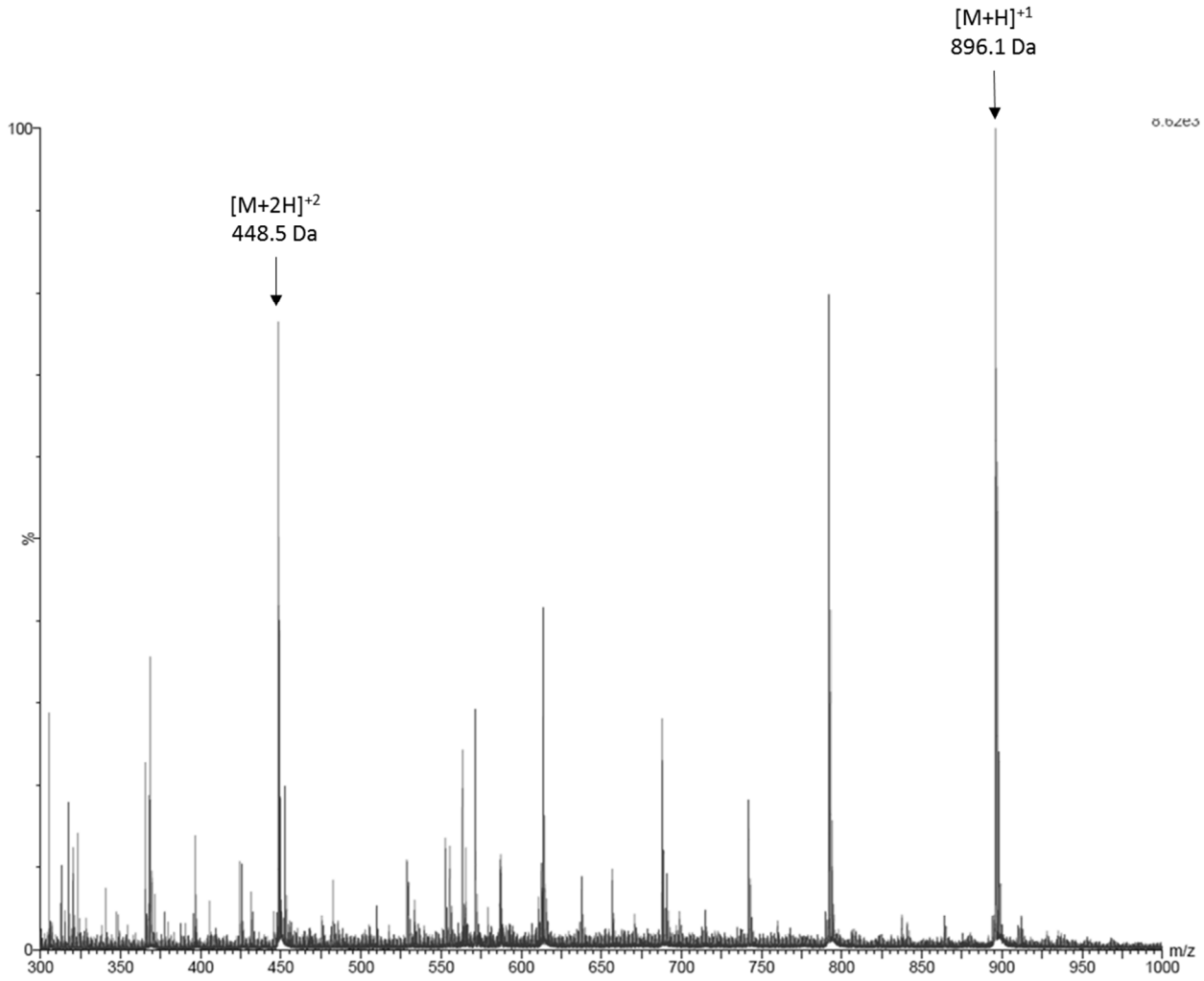


Figure S6. Linear MS of the β-strand of the PLAD linked $C13_{4mer}$, released using 1 mM TCEP.

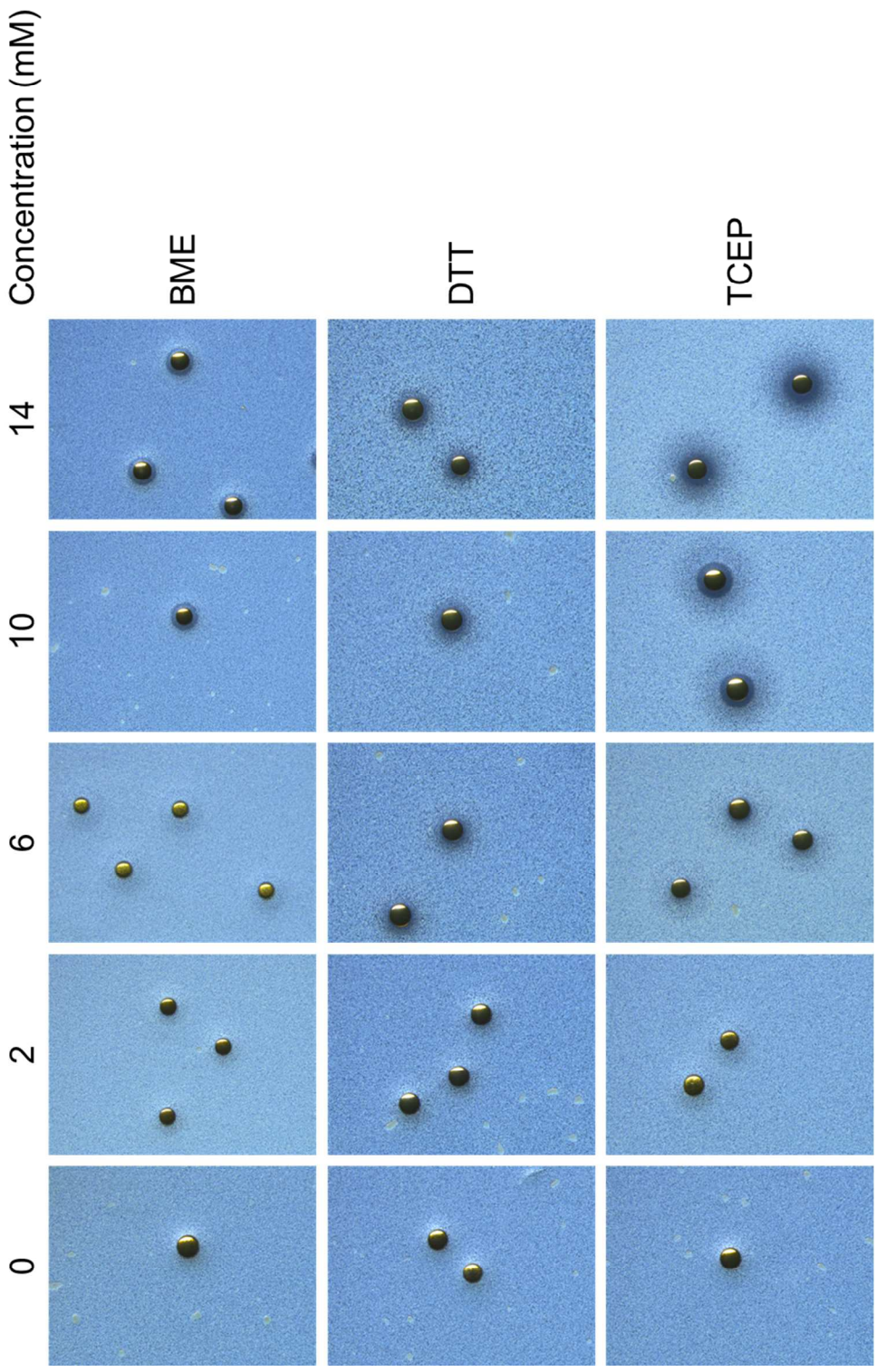


Figure S7. Cleaving reagent comparison and concentration optimization. Three different reducing reagents were tested at several different concentrations to determine the most suitable reducing reagent for screening. β-mercaptoethanol (BME); dithiothreitol (DTT); tris(2-carboxyethyl)phosphine (TCEP)

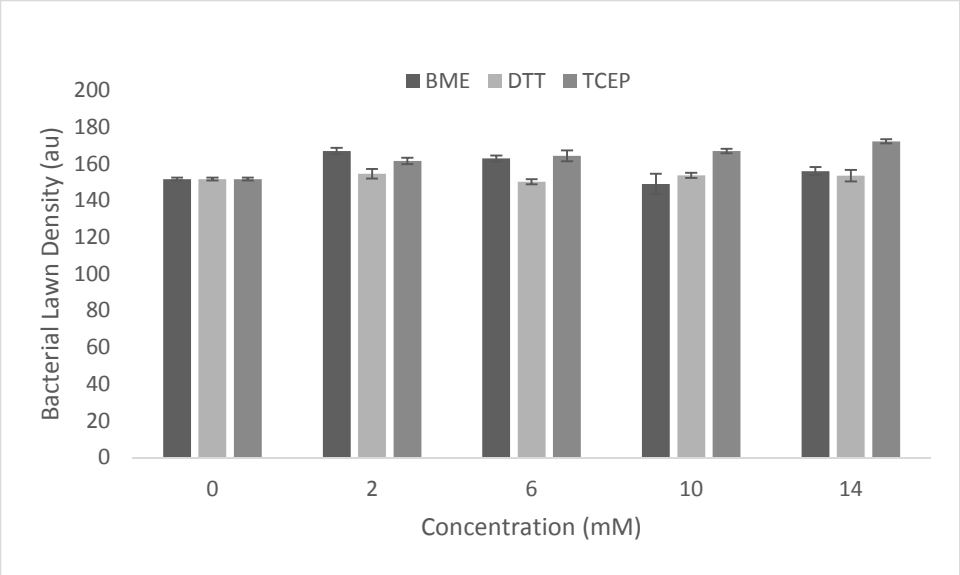


Figure S8. Cleaving reagent effect on bacterial lawn density, showing no appreciable decrease in bacterial lawn density with increasing reducing reagent concentration.

	C	Position	N	
Sequence	**1**	**2**	**3**	**MS MW (Da)**
KJF1	NFur	Nae	NVal	739
KJF2	NFur	Nae	NDec	837
KJF3	NFur	Nae	NTri	879
KJF4	NFur	NLys	NVal	767
KJF5	NFur	NLys	NDec	865
KJF6	NFur	NLys	NTri	907
KJF7	NPhe	Nae	NVal	749
KJF8	NPhe	Nae	NDec	847
KJF9	NPhe	Nae	NTri	889
KJF10	NPhe	NLys	NVal	777
KJF11	NPhe	NLys	NDec	875
KJF12	NPhe	NLys	NTri	917
KJF13	NPea	Nae	NVal	763
KJF14	NPea	Nae	NDec	861
KJF15	NPea	Nae	NTri	903
KJF16	NPea	NLys	NVal	791
KJF17	NPea	NLys	NDec	889
KJF18	NPea	NLys	NTri	931

Figure S9. The identity, sequence, and molecular weight of each compound in the proof-of-concept library.

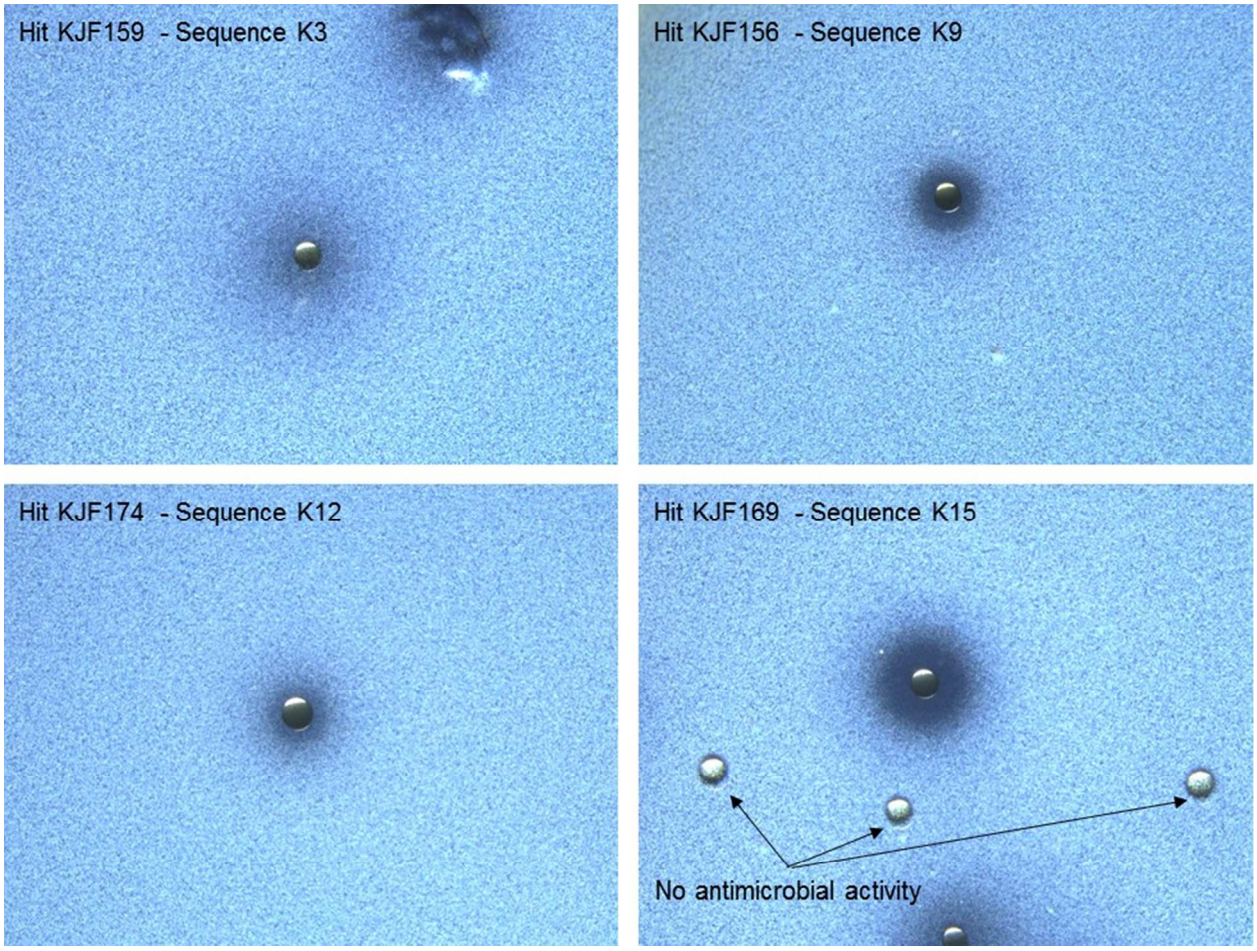


Figure S10. Representative images from screening of the PLAD linked proof-of-concept library. Easily visible zones of inhibition are observed around beads releasing peptoids with varying degrees of antimicrobial activity. Beads releasing peptoids from the library with no antimicrobial activity can be observed in the lower right image.

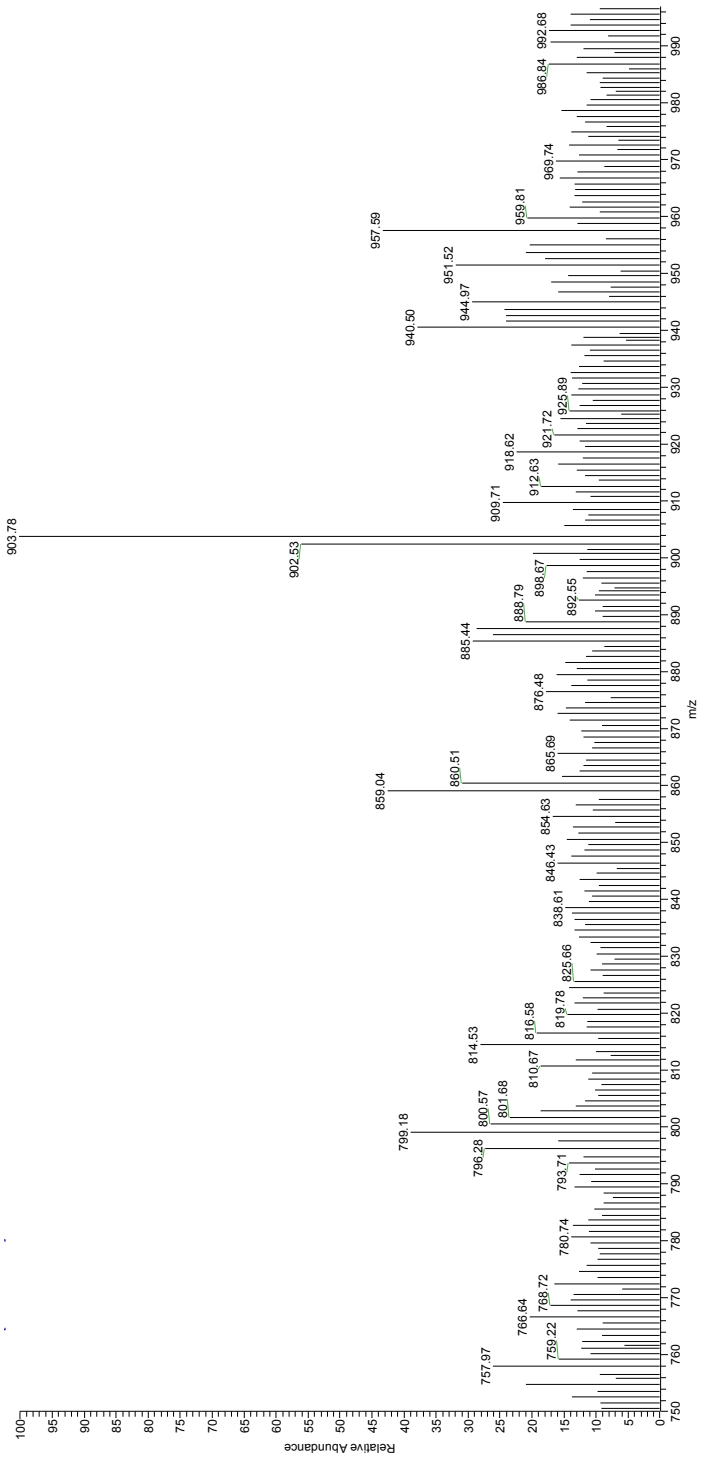


Figure S11. Linear MS of the α-strand of a peptoid hit identified from the proof-of-concept library screening. With a molecular weight of 903 da, this hit was identified as sequence K15.

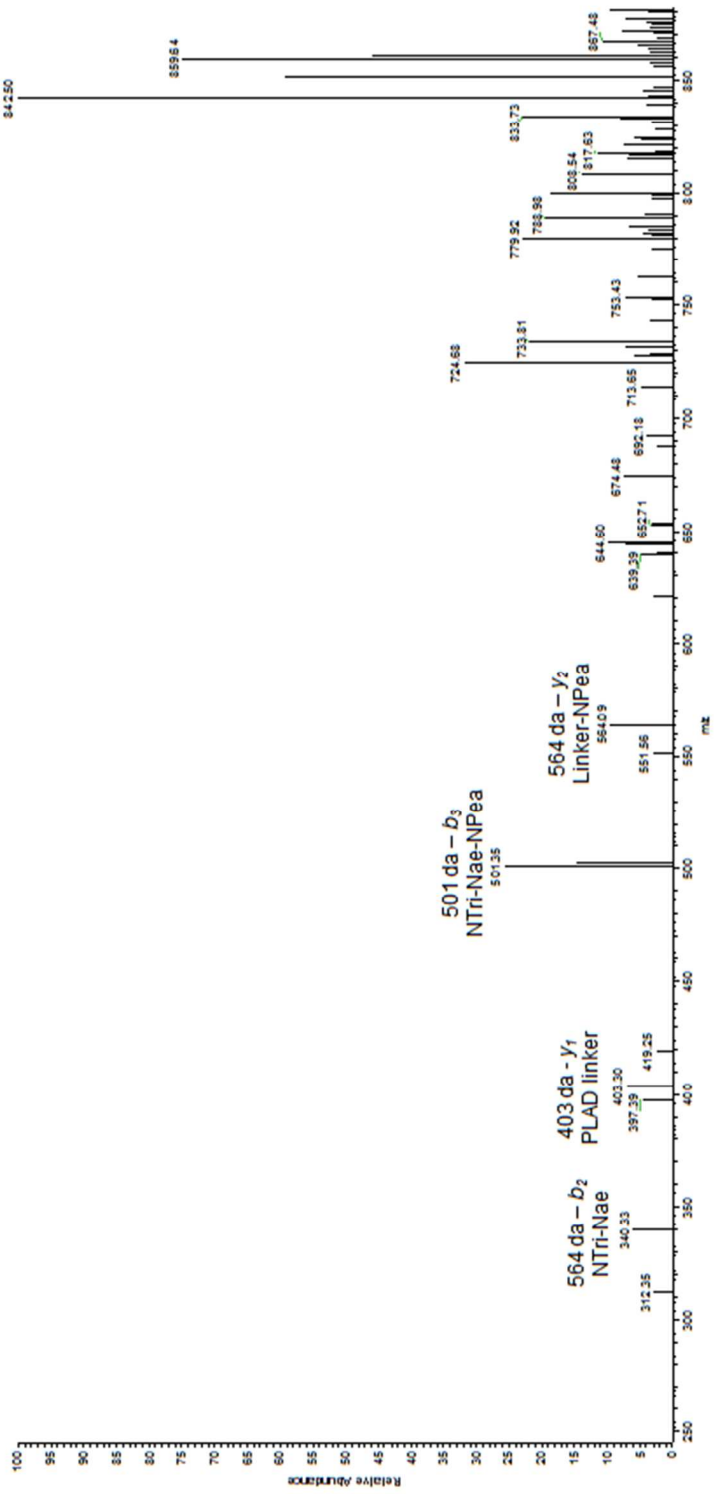


Figure S12. Tandem MS of the α-strand of a peptoid hit identified as K15 during linear MS analysis. The *y* and *b* ions successfully identified are shown and confirm the sequence of this peptoid as K15.

			C	Position	N
Hit Identifier	MS MW (Da)	Sequence	1	2	3
KJF150	931	K18	NPea	NLys	NTri
KJF151	903	K15	NPea	Nae	NTri
KJF154	917	K12	NPhe	NLys	NTri
KJF155	931	K18	NPea	NLys	NTri
KJF156	889	K9	NPhe	Nae	NTri
KJF157	917	K12	NPhe	NLys	NTri
KJF158	889	K9	NPhe	Nae	NTri
KJF159	879	K3	NFur	Nae	NTri
KJF160	931	K18	NPea	NLys	NTri
KJF161	917	K12	NPhe	NLys	NTri
KJF162	931	K18	NPea	NLys	NTri
KJF163	889	K9	NPhe	Nae	NTri
KJF164	903	K15	NPea	Nae	NTri
KJF165	931	K18	NPea	NLys	NTri
KJF166	903	K15	NPea	Nae	NTri
KJF167	917	K12	NPhe	NLys	NTri
KJF168	889	K9	NPhe	Nae	NTri
KJF169	903	K15	NPea	Nae	NTri
KJF170	917	K12	NPhe	NLys	NTri
KJF171	879	K3	NFur	Nae	NTri
KJF172	917	K12	NPhe	NLys	NTri
KJF173	917	K12	NPhe	NLys	NTri
KJF174	917	K12	NPhe	NLys	NTri
KJF175	889	K9	NPhe	Nae	NTri
KJF176	889	K9	NPhe	Nae	NTri
KJF177	931	K18	NPea	NLys	NTri
KJF178	889	K9	NPhe	Nae	NTri
KJF179	903	K15	NPea	Nae	NTri
KJF180	889	K9	NPhe	Nae	NTri
KJF181	931	K18	NPea	NLys	NTri
KJF183	931	K18	NPea	NLys	NTri

Figure S13. The identity and sequences of proof-of-concept peptoid library hits against *E. coli* ATCC 25922.

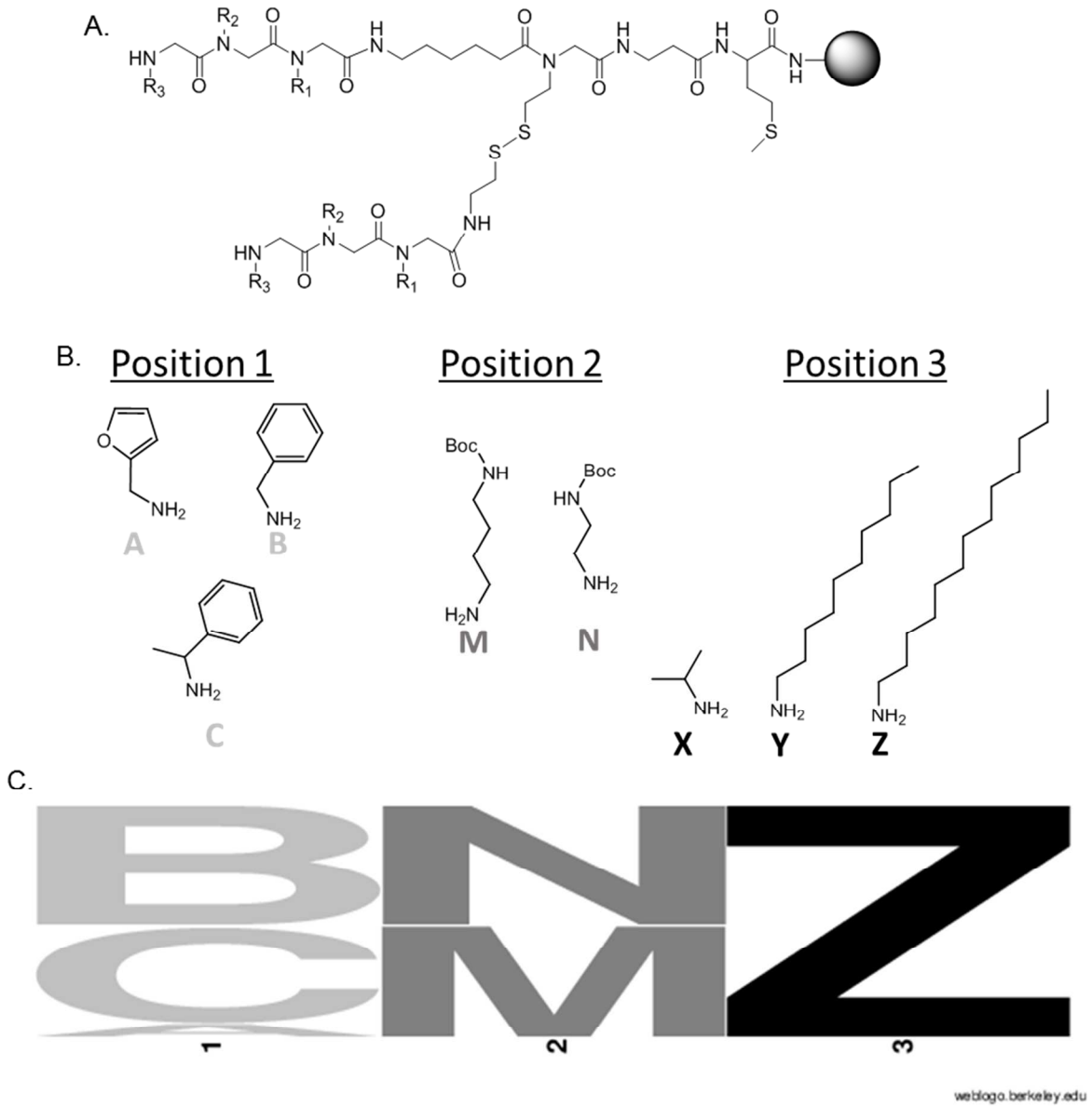


Figure S14. (**A**) General structure of the proof-of-concept PLAD linked library. (**B**) Amine submonomers incorporated into each of the positions in the library. (**C**) Homology chart from hits identified from screening of the proof-of-concept library indicating the prevalence of each submonomer at each position.

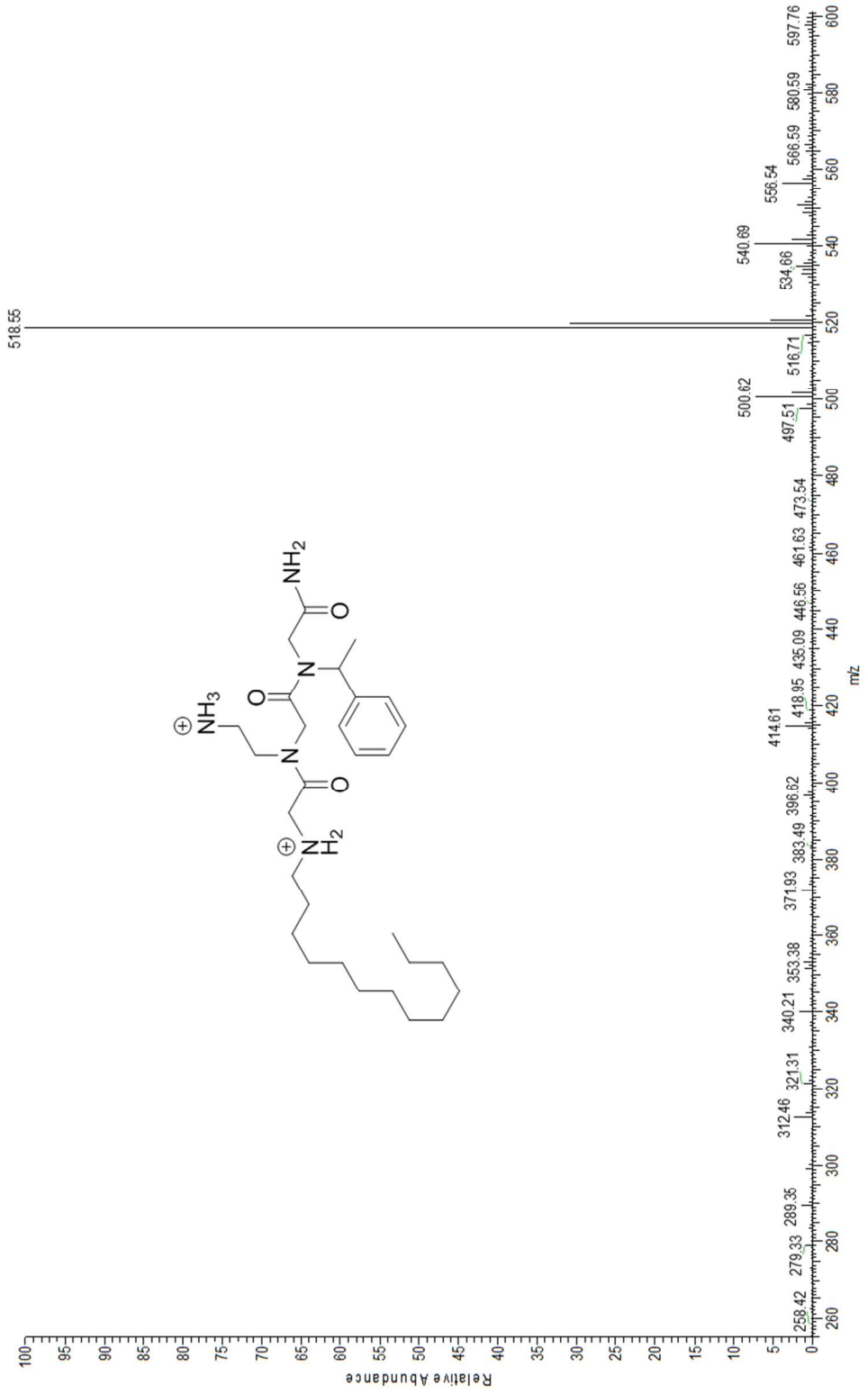


Figure S15. Structure and linear MS of K15

III. References

1. Zuckermann, R. N.; Kerr, J. M.; Kent, S. B. H.; Moos, W. H., Efficient method for the preparation of peptoids [oligo(N-substituted glycines)] by submonomer solid-phase synthesis. *Journal of the American Chemical Society* **1992,** 114, (26), 10646-10647.