Supporting Information

Design and synthesis of unprecedented cyclic γ-AApeptides for antimicrobial development

Haifan Wu,[1] Youhong Niu,[1] Shruti Padhee,[1] Rongsheng E Wang,[1] Yaqiong Li,[1] Qiao Qiao,[1] Ge Bai,[1] Chuanhai Cao,[2] and Jianfeng Cai[1,*]
[1]Department of Chemistry, University of South Florida, 4202 E. Fowler Ave., Tampa, FL 33620; and
[2]USF College of Pharmacy, University of South Florida, 4202 E. Fowler Ave., Tampa, FL 33620

jianfengcai@usf.edu

1. General experimental methods.

α-amino acid esters and Knorr resin (0.66 mmol/g, 200-400 mesh) were provided by Chem-Impex International, Inc. All other reagents and solvents were purchased from either Sigma-Aldrich or Fisher Scientific. The γ-AApeptide building block was synthesized following previously reported procedure. [1] NMR spectra of the γ-AApeptide building block were obtained on a Varian Inova 400 instrument. Cyclic γ-AApeptides were prepared on a Rink amide resin in peptide synthesis vessels on a Burrell Wrist-Action shaker. The cyclic γ-AApeptides were analyzed and purified on an analytical and a preparative Waters HPLC system, respectively, and then dried on a Labcono lyophilizer. Molecular weights of cyclic γ-AApeptides were identified on a Bruker AutoFlex MALDI-TOF mass spectrometer.

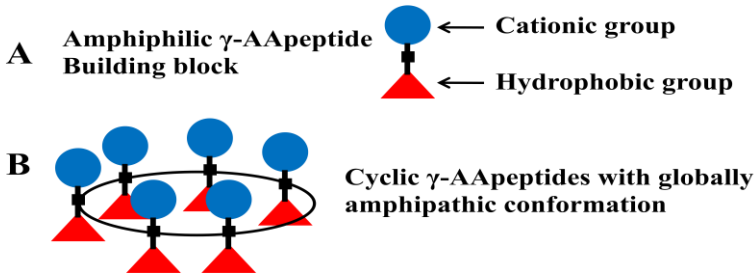


Figure S1. Illustration of cyclic antimicrobial γ-AApeptide design. **A**, Basic representation of the amphiphilic γ-AApeptide building block; **B**, Amphipathic cyclic γ-AApeptide with globally amphipathic conformation.

2. Synthesis[1-3] and characterization of the γ-AApeptide building blocks.

The γ-AApeptide building blocks (Figure S2a) were synthesized following previously reported procedure. [1-3] The characterization of building blocks **2** has been reported. [3] The synthesis of building block **1** and **3** is shown in Figure S2b.

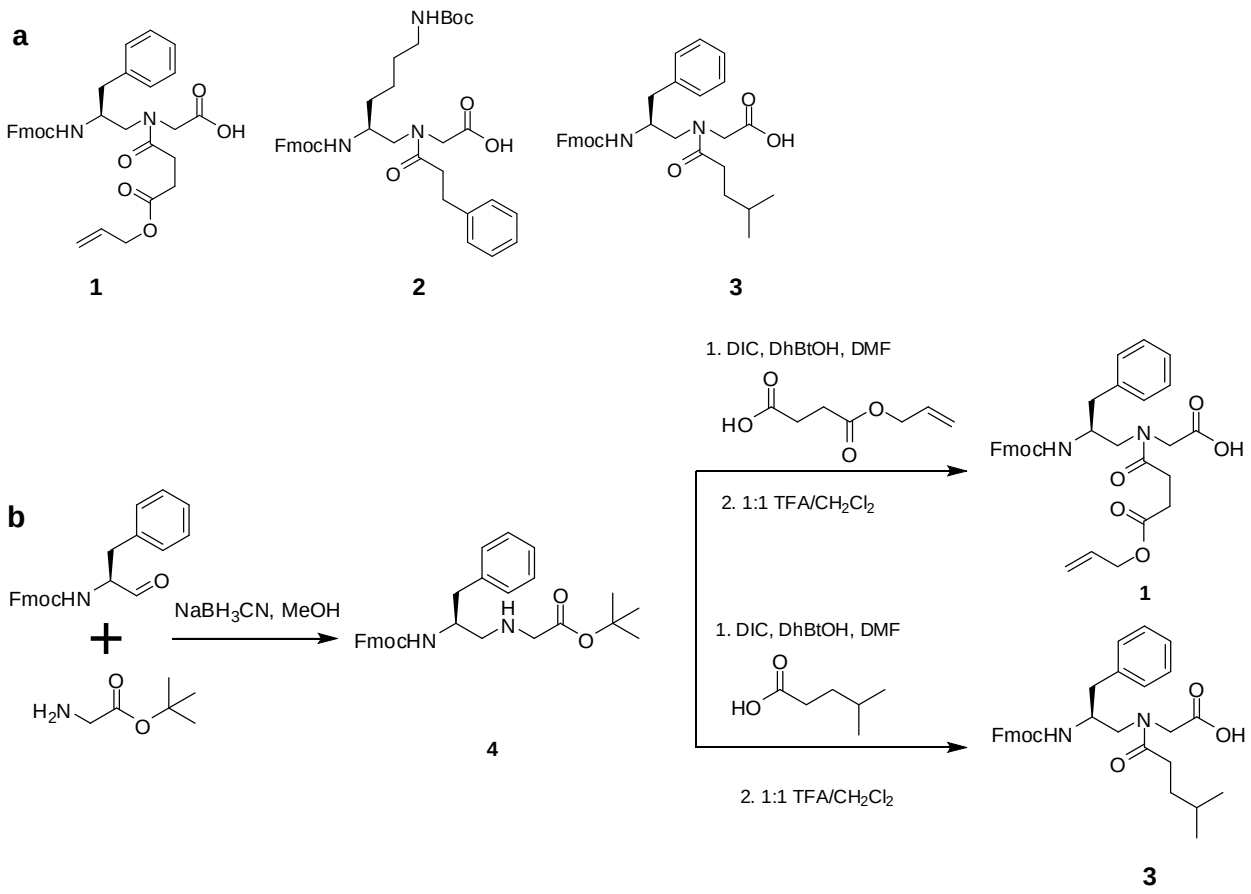


Figure S2. γ-AApeptide building blocks used in the preparation of cyclic γ-AApeptides. **a**, The structures of building blocks; **b**, the synthesis of building block **1** and **3**.

Compound **1**. Yield 60 % (two steps from **4**). ^{1}H NMR (DMSO-d_6, 400MHz) δ (two rotamers) 7.88 (d, 2H), 7.62-7.57 (m, 2H), 7.42-7.29 (m, 4H), 7.28-7.15 (m, 5H), 5.93-5.83 (m, 1H), 5.31-5.25 (m, 1H), 5.19-5.15 (m, 1H), 4.52-4.49 (m, 2H), 4.21-4.03 (m, 4H), 3.88 (d, 2H), 3.63-3.35 (m, 2H), 3.10-2.47 (m, 6H). ^{13}C NMR (DMSO-d_6, 100MHz) δ 171.8, 171.7, 171.2, 171.0, 170.6, 155.6, 155.6, 143.8, 143.7, 143.7, 143.7, 140.6, 140.6, 138.7, 138.6, 132.6, 132.6, 129.0, 128.0, 127.9, 127.5, 126.9, 126.0, 125.8, 125.0, 125.0, 120.0, 117.4, 117.4, 65.3, 64.2, 64.2, 51.7, 51.4, 46.6, 46.5, 37.3, 28.9, 28.8, 27.4, 27.1. HR-ESI: $[M+H]^+$ cacl: 571.2439, found: 571.2410.

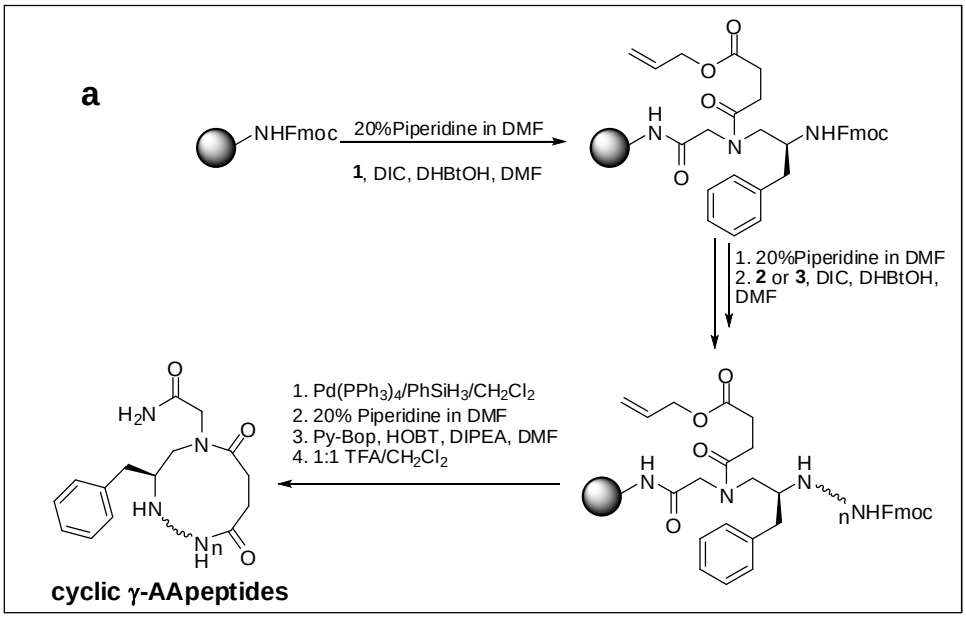
Compound **3**. Yield 60 %. ^{1}H NMR (DMSO-d_6, 400 MHz) δ (two rotamers) 7.83 (d, J = 7.6 Hz, 2H), 7.58-7.53 (m, 2H), 7.36-7.26 (m, 5H), 7.24-7.09 (m, 5H), 4.13-4.06 (m, 3H), 3.96-3.74 (m, 4H), 3.51-3.46 (m, 1H), 3.40-3.32 (m, 1H), 3.38-3.11 (m, 1H), 2.78-2.72 (m, 1H), 2.67-2.56 (m, 1H), 2.32-2.06 (m, 2H), 1.47-1.36 (m, 1H), 1.33-1.24 (m, 2H), 0.80-0.71 (m, 6H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 173.7, 156.1, 144.2, 141.1, 139.5, 139.1, 129.53, 129.46, 128.4, 128.0, 127.4, 126.5, 126.3, 125.7, 125.6, 125.5, 120.5, 120.5, 65.8, 65.8, 51.5, 51.0, 47.0, 38.3, 34.3, 34.1, 30.9, 30.9, 27.6, 27.5, 22.8, 22.7. HR-ESI: $[M+H]^+$ cacl: 529.2697, found: 529.2700.

Compound **4**. Yield 82 %. ^{1}H NMR ($CDCl_3$, 400MHz) δ 7.72 (d, J = 8 Hz, 2H), 7.49-7.45 (m, 2H), 7.38-7.34 (m, 2H), 7.26-7.14 (m, 7H), 6.18-6.14 (m, 1H), 4.26-4.16 (m, 3H), 4.09-4.06 (m, 1H), 3.78-3.68 (m,

2H), 3.42-3.37 (m, 1H), 3.17-3.15 (m, 1H), 2.99-2.94 (m, 1H), 2.86-2.81 (m, 1H), 1.39 (s, 9H). ^{13}C NMR ($CDCl_3$, 100MHz) δ 165.2, 165.1, 162.1, 161.7, 156.9, 156.8, 143.9, 143.6, 141.2, 141.1, 136.0, 129.0, 128.8, 127.7, 127.6, 127.1, 125.2, 119.8, 84.8, 67.3, 50.6, 49.8, 47.8, 46.8, 38.6, 36.9, 36.8, 27.8, 27.7. HR-ESI: $[M+H]^+$ cacl: 487.2591, found: 487.2565.

3. Solid phase synthesis, purification and characterization of cyclic γ-AApeptides.

Cyclic γ-AApeptides were prepared on a Rink amide resin in peptide synthesis vessels, on a Burrell Wrist-Action shaker, following the standard Fmoc chemistry protocol of solid phase peptide synthesis. Synthesized γ-AApeptide building blocks were used (Figure S3a). Each coupling cycle included a Fmoc deprotection using 20% Piperidine in DMF, and 8 h coupling of 1.5 equiv of γ-AApeptide building blocks in the presence of 4 equiv of DIC (diisopropylcarbodiimide) /DhbtOH (3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine) in DMF. The cyclization was achieved on resin via the γ-AApeptide building block **1**. Briefly, **1** was first attached to the solid support, followed by standard Fmoc solid phase synthesis. After desired sequences were assembled, the allyl group was removed by treatment of $Pd(PPh_3)_4$ (0.2 equiv.) /$PhSiH_3$ (10 equiv.) /CH_2Cl_2 for 2h (repeated two times). The deprotection of Fmoc group was then carried out on the N-terminus. The intramolecular cyclization was accomplished using PyBop/HOBt/DIEA/DMF. Next, the resin was transferred into 4 mL vials and cyclic γ-AApeptides were cleaved from solid support in 50:48:2 TFA/CH_2Cl_2/triisopropylsilane overnight. Then solvent was evaporated and the residues were analyzed and purified on an analytical (1 mL/min) and a preparative Waters (20 ml/min) HPLC systems, respectively, using 5% to 100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 40 min, followed by 100% solvent B over 10 min. The HPLC traces were detected at 215 nm. The desired fractions were eluted as single peaks at > 95% purity with yields of 6-10% (based on loading of the resin, see Figure S3b for sequences.). They were collected and lyophilized. The molecular weights of cyclic γ-AApeptides (Table S1) were obtained on a Bruker AutoFlex MALDI-TOF mass spectrometer using α–cyano-4-hydroxy-cinnamic acid.



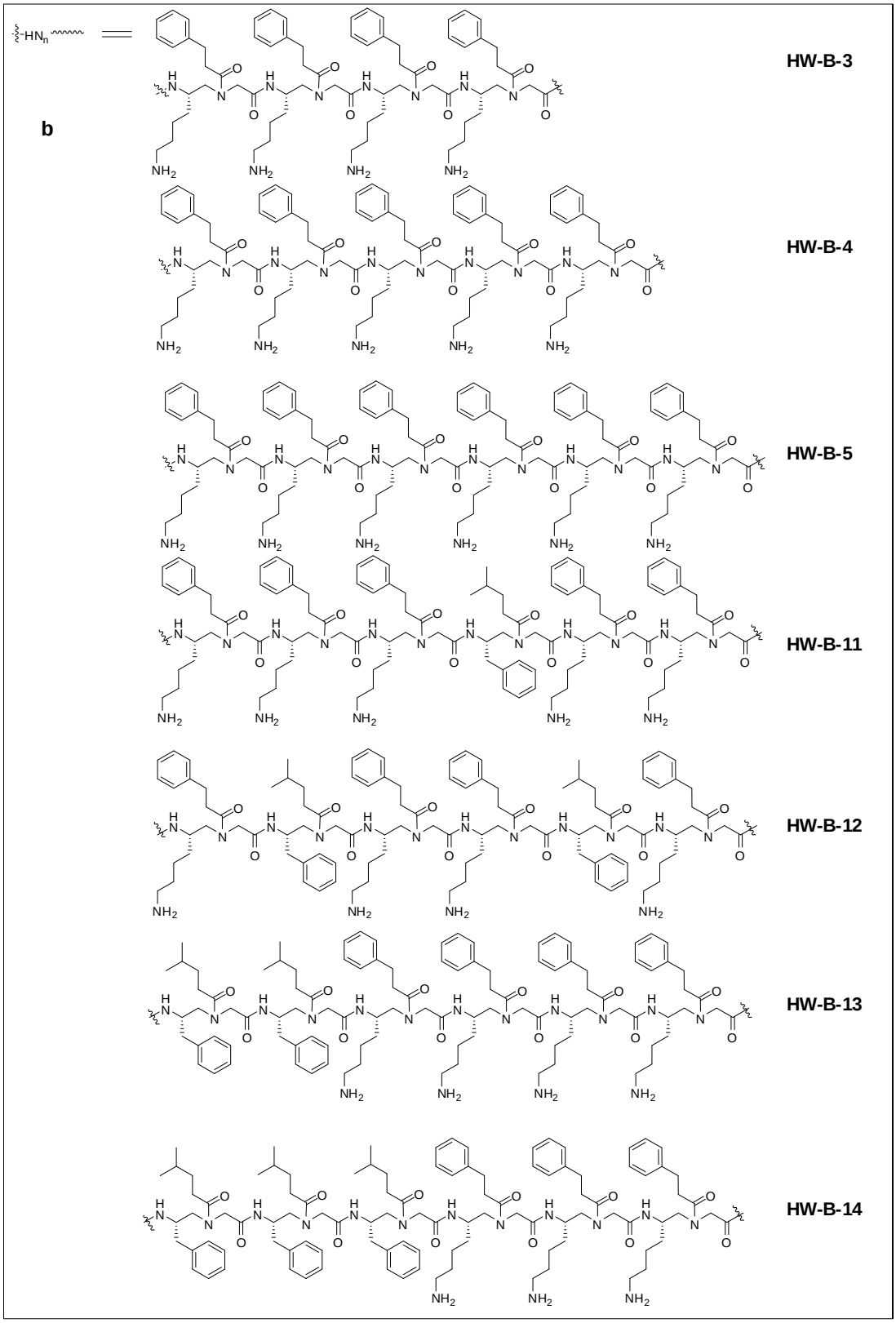
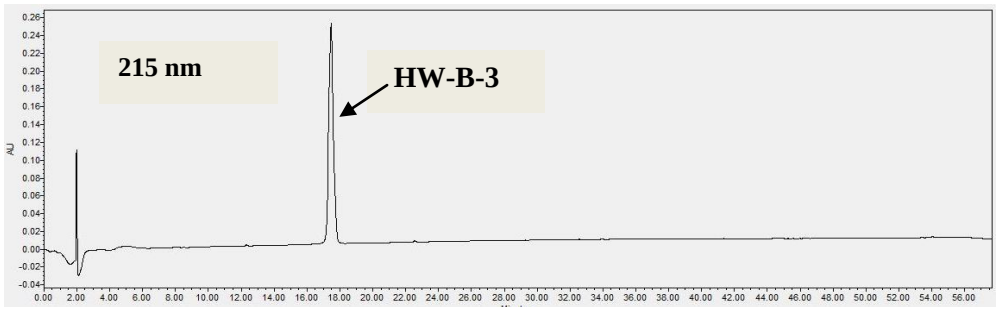


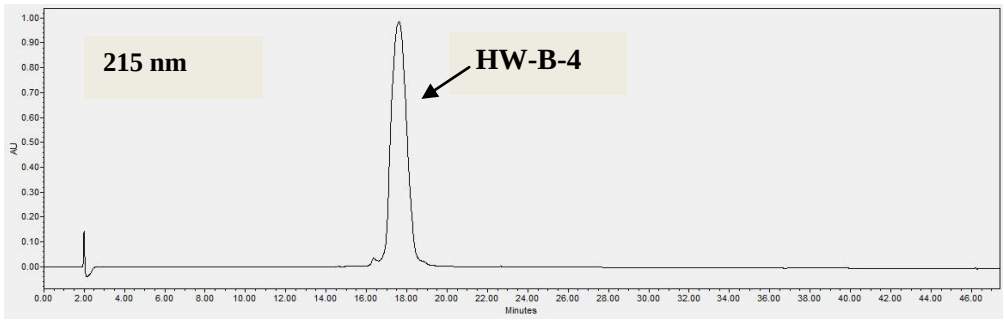
Figure S3. a, the route for the synthesis of cyclic γ-AApeptides; **b**, the structures of synthesized cyclic γ-AApeptides.

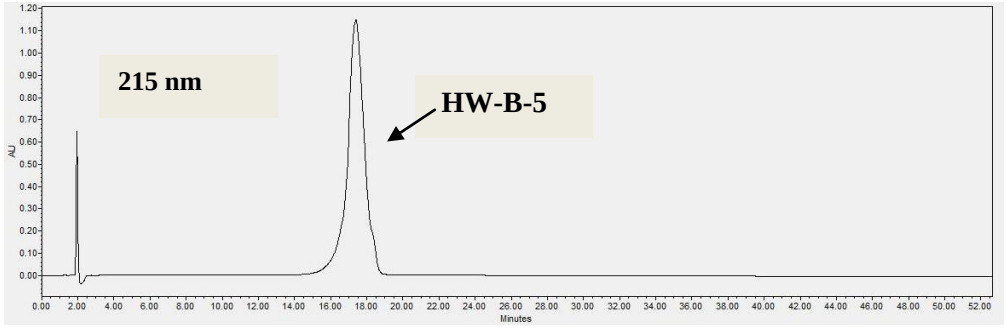
Table S1. MALDI analysis of cyclic γ-AApeptides.

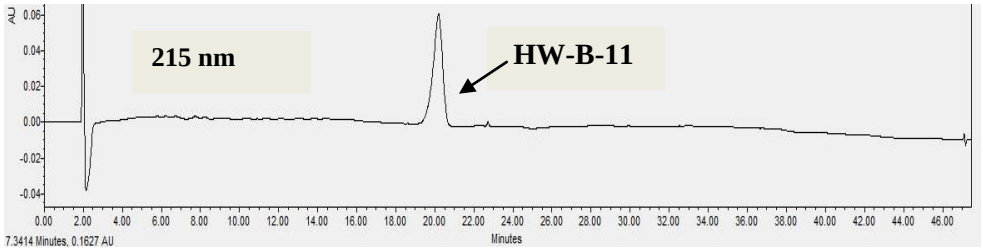
Cyclic γ-AApeptides	Yield (based on loading of the resin)	molecular weight (Actual)	molecular weight (found)
HW-B-3	10.5%	1501.2	1053.1 ($M+H^+$)
HW-B-4	8.6%	1805.1	1806.6 ($M+H^+$)
HW-B-5	6.2%	2108.3	2109.6 ($M+H^+$)
HW-B-11	6.8%	2093.3	2147.6 ($M+3NH_4^+$)
HW-B-12	6.5%	2078.3	2079.9 ($M+H^+$)
HW-B-13	6.0%	2078.3	2079.0 ($M+H^+$)
HW-B-14	6.4%	2063.3	2117.3 ($M+3NH_4^+$)

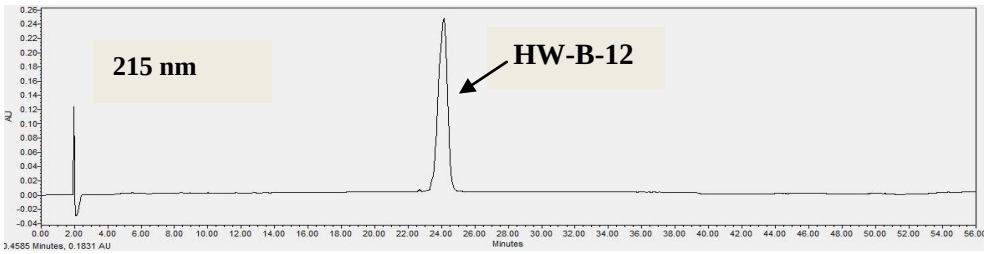
HPLC traces

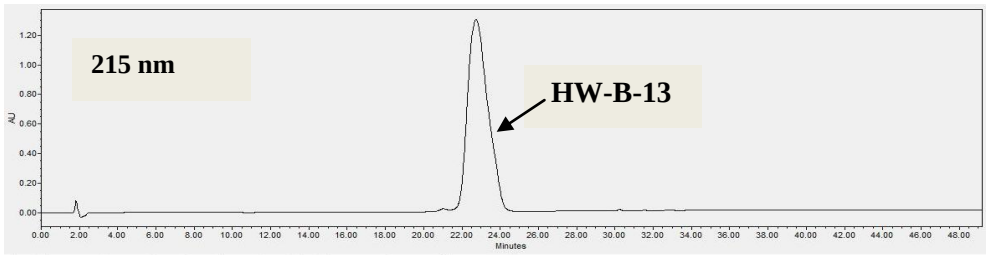


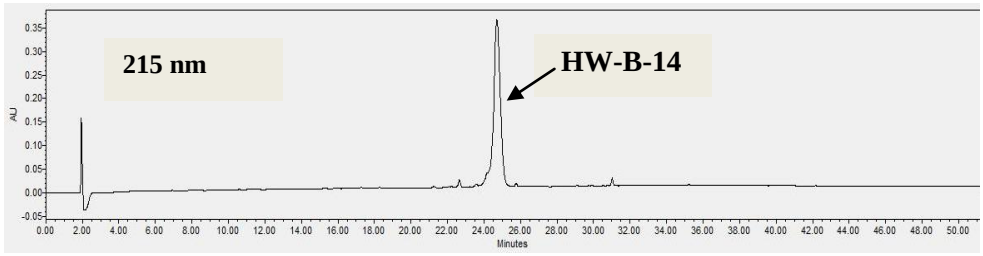












4. Antimicrobial assays

The microbial organisms used were *B. subtilis* (BR151), multi-drug resistant *S. epidermidis* (RP62A), *C. albicans* (ATCC 10231), Vancomcin-resistant *E. faecalis* (ATCC 700802), Methicillin-resistant *S. aureus* (ATCC 33592), *K. pneumoniae* (ATCC 13383), multi-drug resistant *P. aeruginosa* ATCC 27853. The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits the growth of bacteria in 24 h. The highest concentration tested for antimicrobial activity was 50 μg/mL. The antimicrobial activities of the cyclic γ-AApeptides were determined in a sterile 96 -well plates by broth micro-dilution method. Bacterial cells [4] and fungi [5] were grown overnight at 37 ℃ in 5 mL medium, after which a bacterial suspension (approximately 10^6 CFU/mL) or fungal suspension *Candida albicans* (ATCC 10231) (approximately 10^3 CFU/mL) in Luria broth or trypticase soy was prepared. Aliquots of 50 μL bacterial or fungal suspension were added to 50 μL of medium containing the cyclic γ-AApeptides

for a total volume of 100 μL in each well. The cyclic γ-AApeptides were prepared in PBS buffer in 2 – fold serial dilutions, with the final concentration range of 0.5 to 50 μg/mL. Plates were then incubated at 37 ℃ for 24 h (for bacteria) or 48h (for *Candida albicans* (ATCC 10231). The lowest concentration at which complete inhibition of bacterial growth (determined by a lack of turbidity) is observed throughout the incubation time is defined as the minimum inhibitory concentration (MIC). The experiments were carried out independently three times in duplicates.

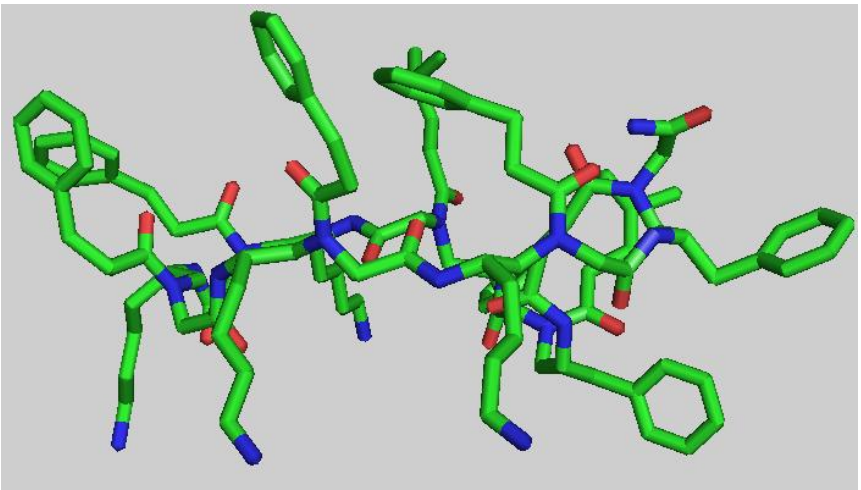


Figure S4. The energy-minimized structure of HW-B-13. The computer modeling was carried out using ChemBioOffice MM2 energy minimization.

5. Lipid depolarization[6-8]

The Lipid depolarization of the bacterial cell membrane was conducted using the membrane potential sensitive dye 3, 5'-dipropylthiacarbocyanine iodide ($DiSC_3$-5) that distributes between the cells and the medium depending on the membrane potential gradient. *S. aureus* (ATCC 33592) cells were grown in Luria broth and Trypticase soy broth medium respectively to a mid –logarithmic phase (OD_{600}=0.5-0.6). The bacterial cells were then collected by centrifugation at 3000 rpm for 10 min and then washed once with buffer (5mM HEPES and 5mM Glucose,pH 7.2). The cells were re-suspended to OD_{600} =0.05 with 100 mM KCl, 2 μM $DiSC_3$-5,5mM HEPES and 5 mM Glucose and were incubated for 30 min at 37 ℃ for maximal dye uptake and fluorescence self-quenching. This bacterial suspension (90 μL) and 10 μL of compound stock solutions or control drug solution were added to white flat bottomed polypropylene 96-well plate (Costar) and incubated at 37 ℃ for 30 min. The fluorescence reading was monitored using the microplate reader (Biotek) at an excitation wavelength of622 nm and an emission wavelength of 670 nm); the fluorescence increased due to the disruption of cytoplasmic membrane. Valinomycin (final concentration 250 μg/mL) was used as a positive control, and the blank with only cells and dye was used as the background.

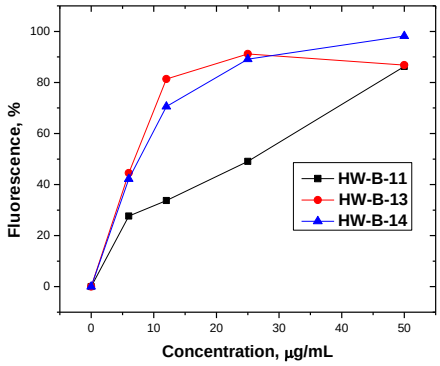


Figure S5. Depolarization of the cytoplasmic membrane of *S. aureus* by cyclic γ-AApeptides.

6. Hemolysis assay

Freshly drawn human red blood cells (hRBC's) with additive K_2 EDTA (spray-dried) was washed with PBS buffer several times and centrifuged at 1000 g for 10 min until a clear supernatant was observed. The hRBC's were resuspended in 1 × PBS to get a 5% v/v suspension. Two fold serial dilutions of γ-AApeptides dissolved in 1 × PBS from 250 μg/ml to1.6 μg/ml were added to a sterile 96-well plate to make up to a total volume of 50 μL in each well. Then 50 μL of 5% v/v hRBC solution was added to make up a total volume of 100 μL in each well. The 0% hemolysis point and 100% hemolysis point were determined in 1 × PBS and 0.2% Triton-X-100, respectively. [4] The plate was then incubated at 37 ^{0}C for 1 h and centrifuged at 3500 rpm for 10 min. The supernatant (30 μL) was diluted with 100 μL of 1 × PBS and absorption was detected by measuring the optical density at 360 nm by Biotek Synergy HT microtiter plate reader. % hemolysis was determined by the following equation:

$$\% \text{ hemolysis} = (Abs_{sample} - Abs_{PBS})/(Abs_{Triton} - Abs_{PBS}) \times 100$$

H_{10} is the concentration of cyclic γ-AApeptide at which 10% hemolysis was observed. H_{50} is the concentration of cyclic γ-AApeptide at which 50% hemolysis was observed. The highest concentration tested in the hemolytic assay was 500 μg/mL.

7. Fluorescence microscopy

A double staining method with DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride, Sigma, >98%) and PI (Propidium iodide, Sigma) as fluorophores was used to visualize and differentiate the viable from the dead *B. subtilis* cells. DAPI as a double stranded DNA binding dye, stains all bacterial cells irrespective of their viability, whereas Ethidium derivatives such as propidium iodide (PI) is capable of passing through only damaged cell membranes and intercalating with the nucleic acids of injured and dead cells to form a bright red fluorescent complex. [9] The cells were first stained with PI and then with DAPI. Bacterial cells were grown until they reached mid-logarithmic phase and then they (~2 × 10^6 cells) were incubated with the cyclic γ-AApeptide HW-B-13 at the concentration of 2 × MIC (10 μg/mL) for 2 h. Then the cells were pelleted by centrifugation at 3000 g for 15 min in an Eppendorf microcentrifuge. The supernatant was then decanted and the cells were washed with 1 × PBS for several times and then incubated with PI (5 μg/mL) in dark for 15 min at 0 ^{o}C. The excess PI was removed by washing the cells with 1 × PBS for several times. Then the cells were incubated with DAPI (10 μg/mL in water) for 15 min

in dark at 0 °C. The DAPI solution was removed and cells were washed with 1 × PBS for several times. Controls without the addition of HW-B-13 were performed following the exactly same procedure for bacteria. The bacterial cells were then examined by using the Zeiss Axio Imager Z1optical microscope with an oil-immersion objective (100 ×). [10-12]

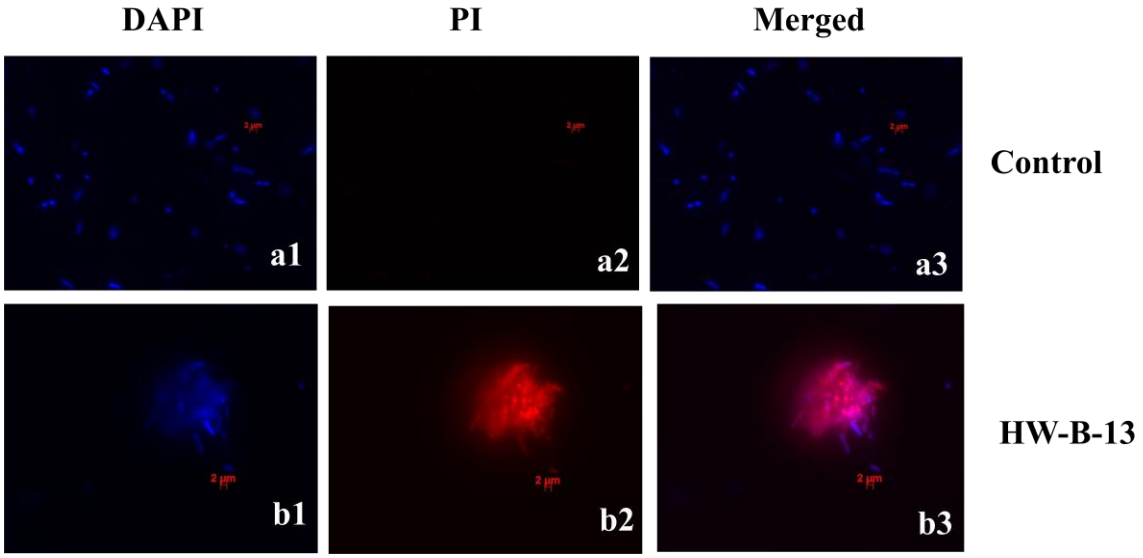


Figure S6. Fluorescence micrographs of *B. subtilis* treated with 5 μg/ml cyclic γ-AApeptide HW-B-13 for 2 h. **a1**, control, no treatment, DAPI stained; **a2**, control, no treatment, PI stained; **a3**, control, no treatment, the merged view. **b1**, HW-B-13 treatment, DAPI stained; **b2**, HW-B-13 treatment, PI stained; **b3**, HW-B-13 treatment, the merged view. Scale bar: 2 μm.

8. MTT cytotoxicity assay

N2a APP cells were used to access the cytotoxicity of cyclic γ-AApeptides towards mammalian cells. Typically, stock concentration of the drug (1 mg/ml) was diluted in media to make different concentrations in 96-well plates, and then incubated at 37 ℃. Following that, N2a APP cells were seeded to 1×10^4 cells/well in 100μl media in another 96-well plate. After incubation for 12 hours, 100 μl of different concentrations of the drug were added and the plate was incubated for another 36 hours. The media in 96-well plate was removed and washed with fresh media once, and 110 μl MTT reagent was added. The mixture was incubated for another 4 hours, after which 100 μl of pre-warmed solubilization solution was added. The plate was then incubated at 37 ℃ for 12 h, and the absorbance at 550 nm was read. Percentage of cell viability was calculated based on the following equation:

cell viability % = $(A/A_{control}) \times \%$

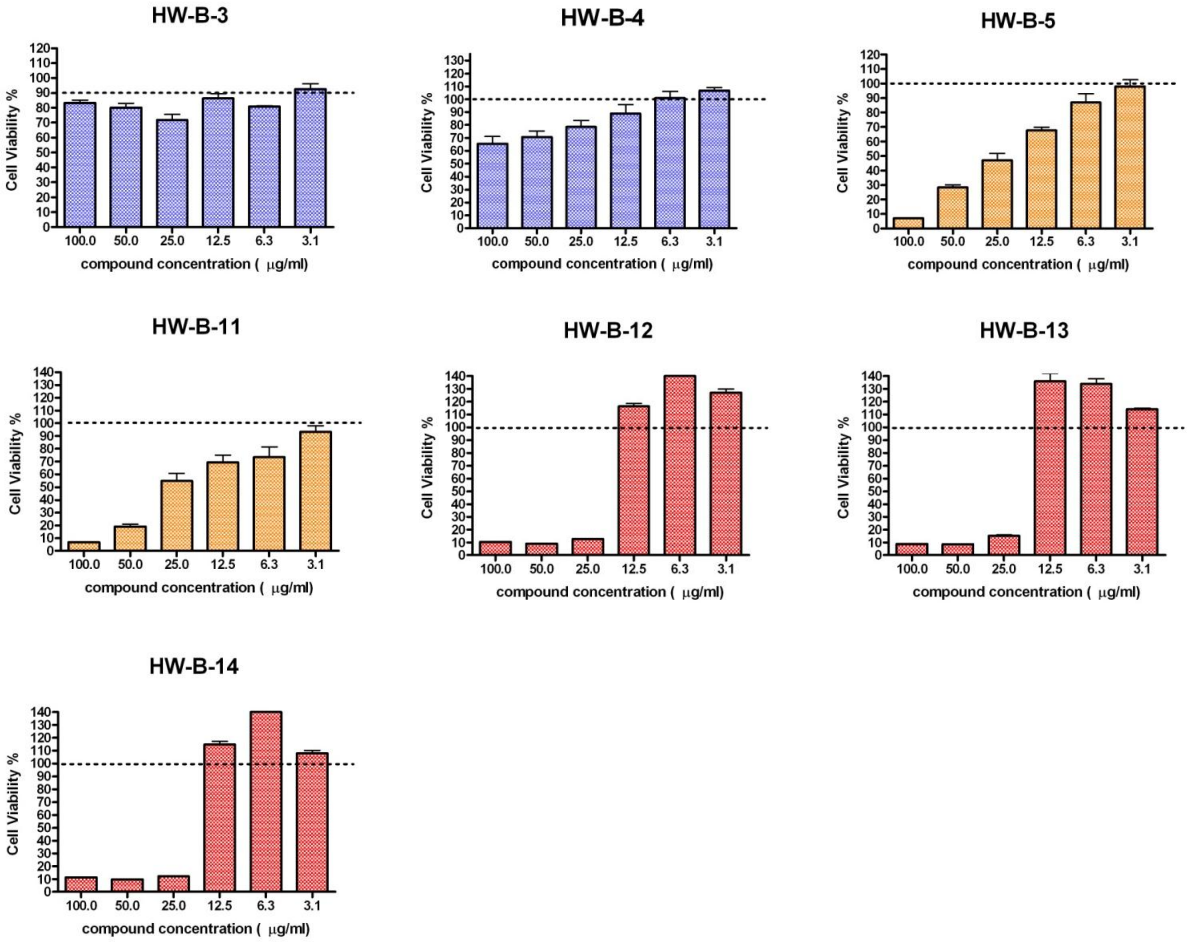
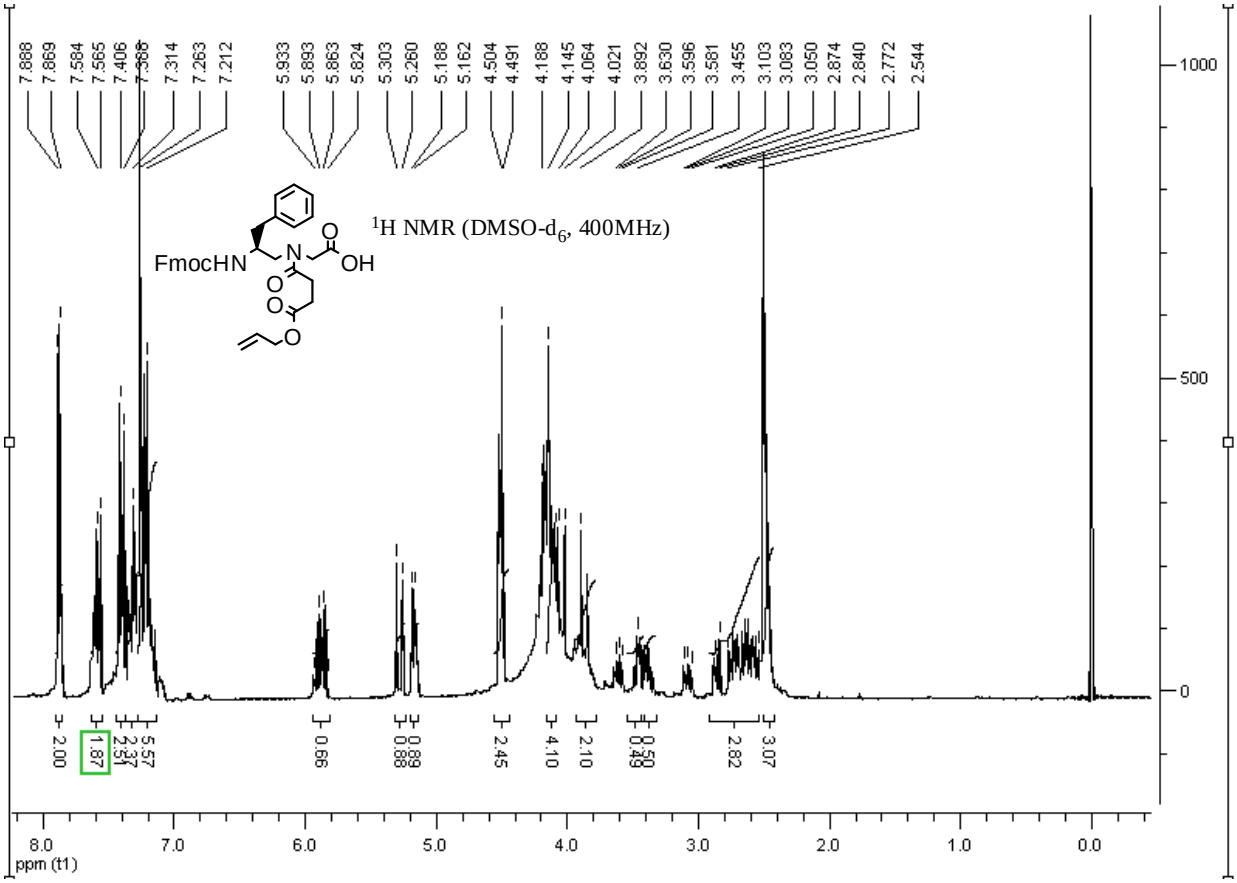
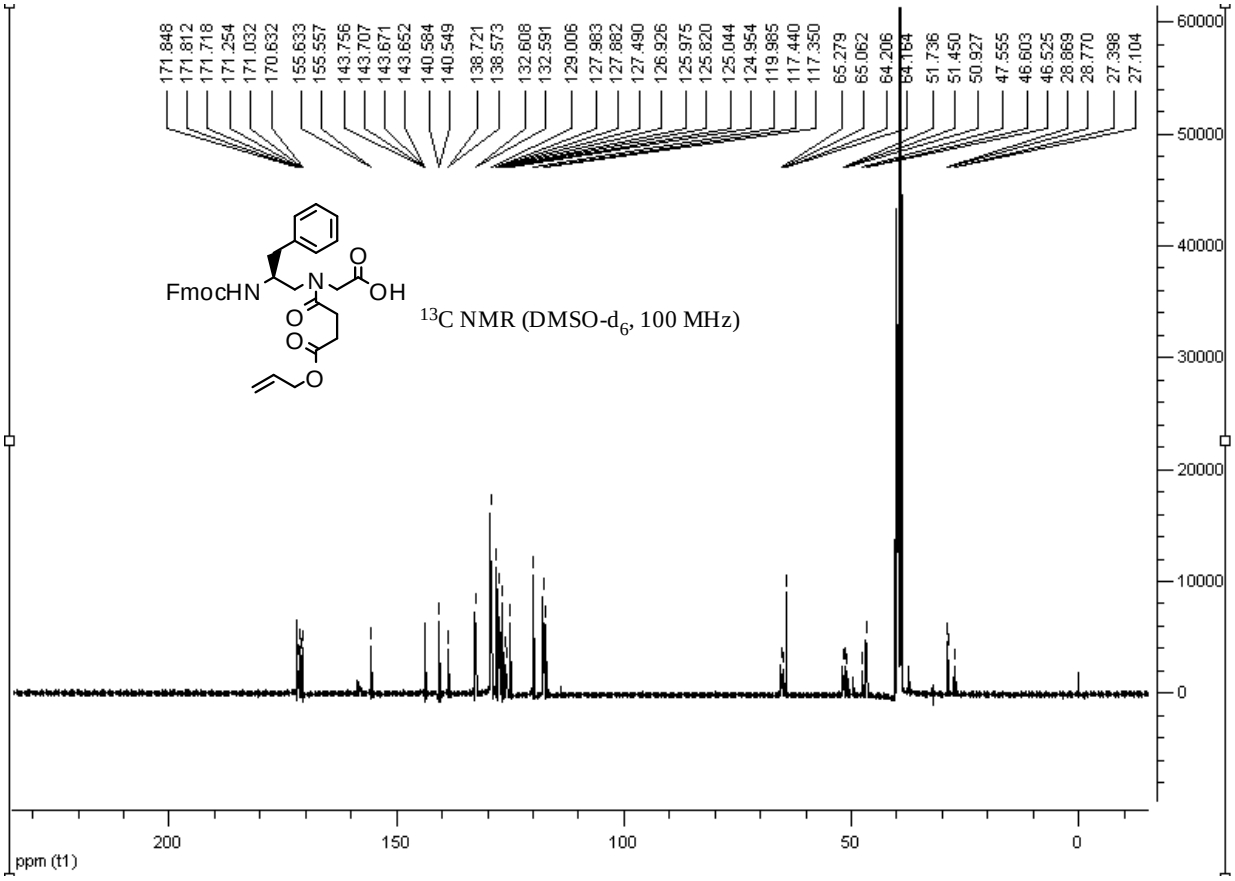


Figure S7. MTT cytotoxicity assay of N2a/APP cells treated with different concentrations of **cyclic γ-AApeptides,** respectively.

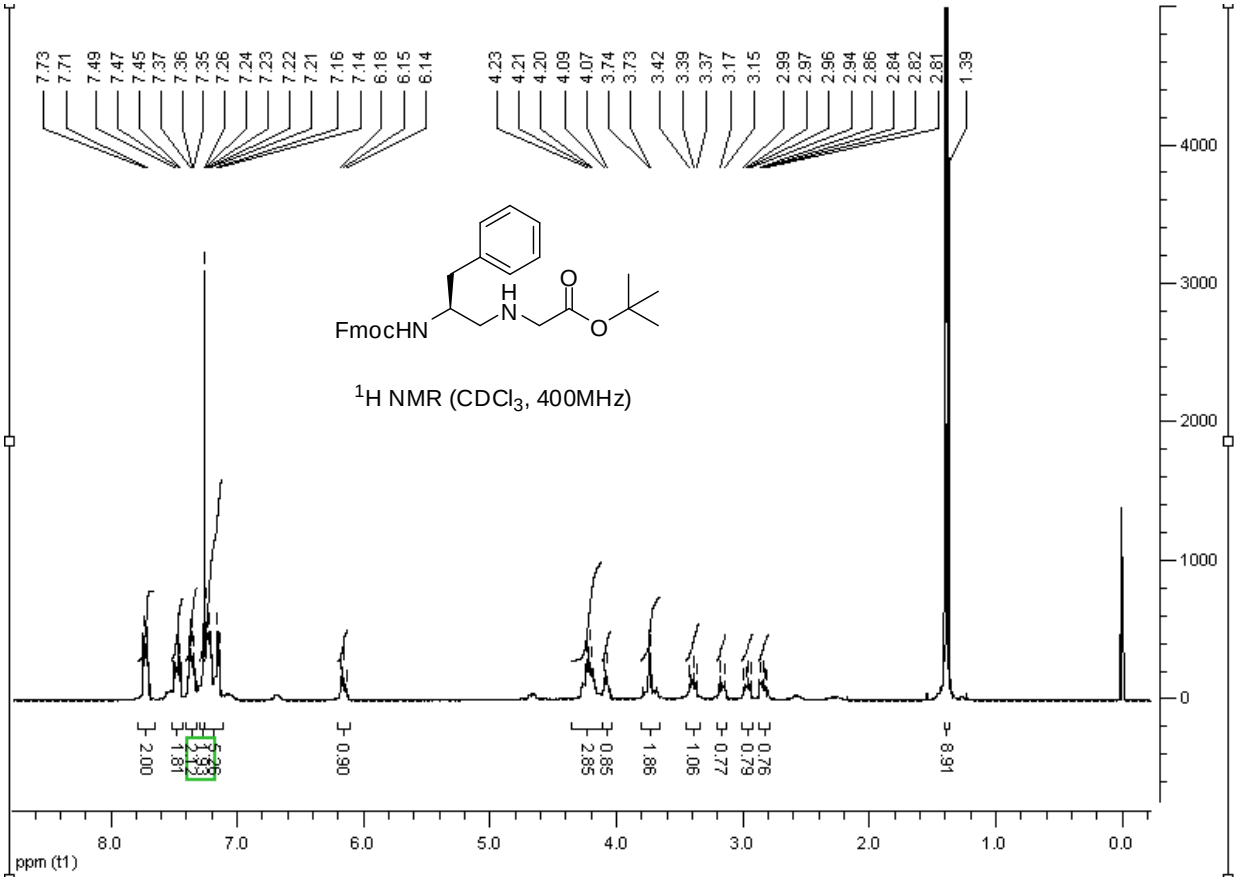
1. Y. Niu, Y. Hu, X. Li, J. Chen and J. Cai, *New Journal of Chemistry*, 2011, 35, 542-545.
2. Y. Niu, A. J. Jones, H. Wu, G. Varani and J. Cai, *Org Biomol Chem*, 2011, 9, 6604-6609.
3. Y. Niu, S. Padhee, H. Wu, G. Bai, L. Harrington, W. N. Burda, L. N. Shaw, C. Cao and J. Cai, *Chemical communications (Cambridge, England)*, 2011, 47, 12197-12199.
4. J. A. Patch and A. E. Barron, *Journal of the American Chemical Society*, 2003, 125, 12092-12093.
5. A. J. Karlsson, W. C. Pomerantz, B. Weisblum, S. H. Gellman and S. P. Palecek, *Journal of the American Chemical Society*, 2006, 128, 12630-12631.
6. M. Wu, E. Maier, R. Benz and R. E. W. Hancock, *Biochemistry*, 1999, 38, 7235-7242.
7. S. Choi, A. Isaacs, D. Clements, D. Liu, H. Kim, R. W. Scott, J. D. Winkler and W. F. DeGrado, *Proceedings of the National Academy of Sciences of the United States of America*, 2009, 106, 6968-6973.
8. C. L. Friedrich, D. Moyles, T. J. Beveridge and R. E. Hancock, *Antimicrob Agents Chemother*, 2000, 44, 2086-2092.
9. T. Matsunaga, M. Okochi and S. Nakasono, *Analytical chemistry*, 1995, 67, 4487-4490.

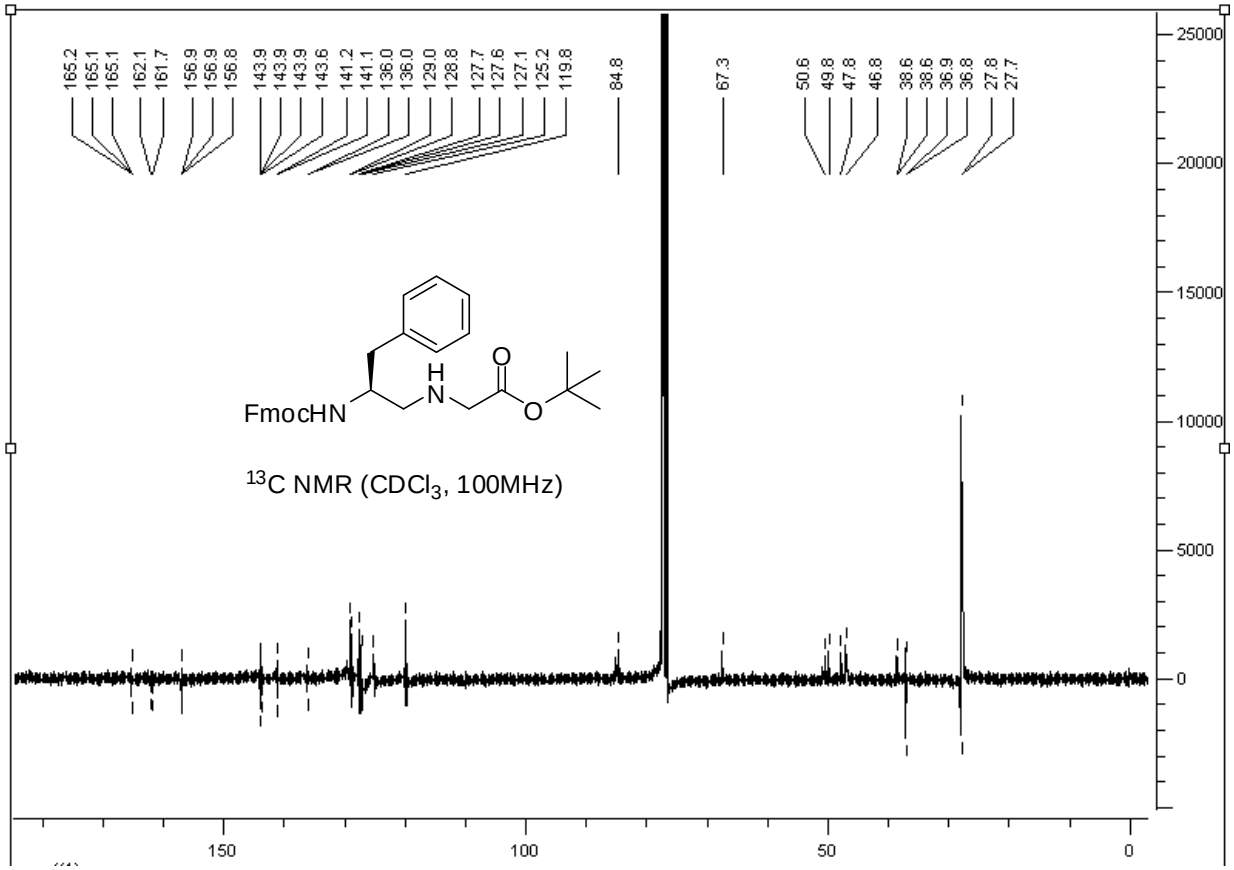
10. Y. Niu, S. Padhee, H. Wu, G. Bai, L. Harrington, W. N. Burda, L. N. Shaw, C. Cao and J. Cai, *Chemical communications (Cambridge, England)*, 2011, 47, 12197-12199.
11. S. Padhee, Y. Hu, Y. Niu, G. Bai, H. Wu, F. Costanza, L. West, L. Harrington, L. N. Shaw, C. Cao and J. Cai, *Chemical communications (Cambridge, England)*, 2011, 47, 9729-9731.
12. S. C. Williams, Y. Hong, D. C. A. Danavall, M. H. Howard-Jones, D. Gibson, M. E. Frischer and P. G. Verity, *Journal of Microbiological Methods*, 1998, 32, 225-236.

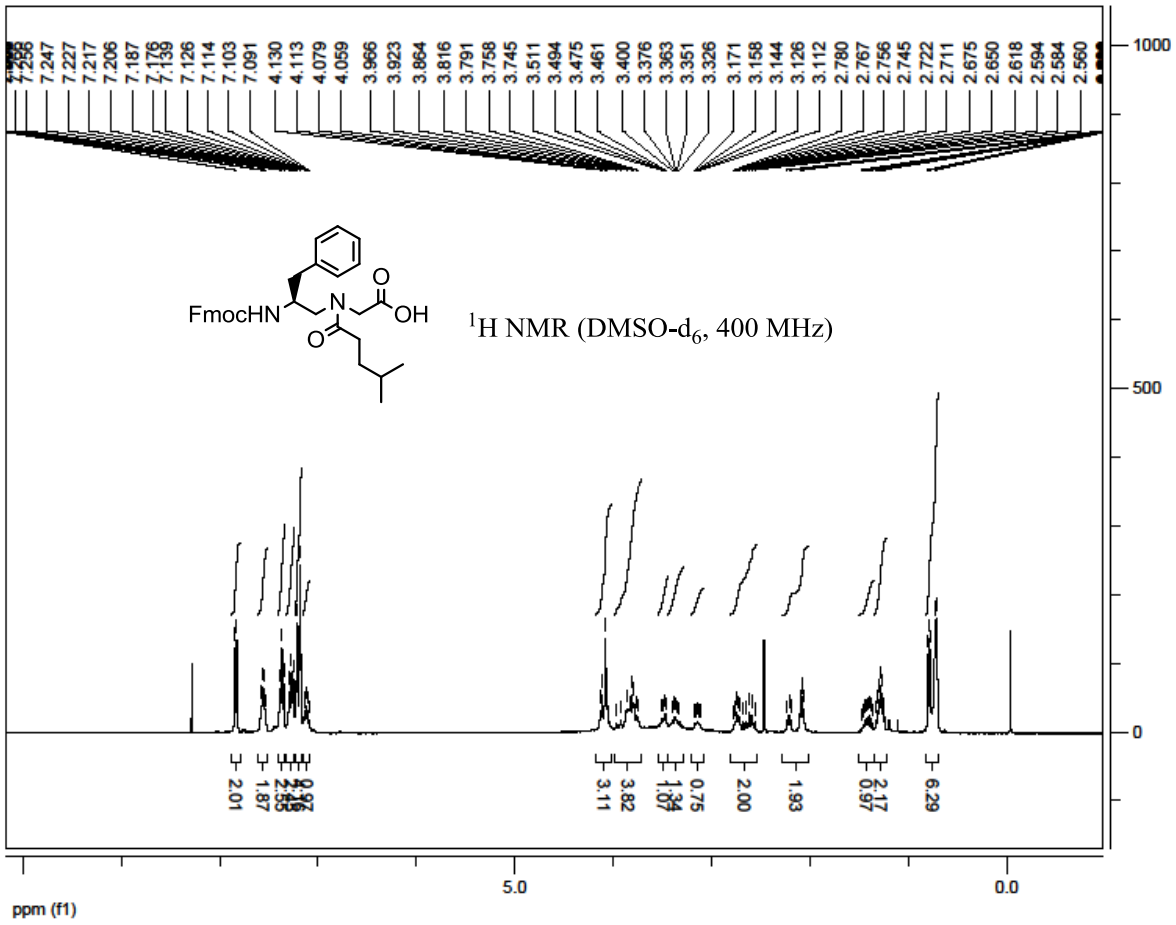

7.888
7.869
7.584
7.565
7.406
7.388
7.314
7.263
7.212
5.933
5.893
5.863
5.824
5.303
5.260
5.188
5.162
4.504
4.491
4.188
4.145
4.064
4.021
3.892
3.630
3.596
3.581
3.455
3.103
3.083
3.050
2.874
2.840
2.772
2.544
FmocHN
N
O
OH
O
O
O
1H NMR (DMSO-d6, 400MHz)
2.00
1.87
2.31
5.57
0.66
0.88
2.45
4.10
2.10
0.50
2.82
3.07
1000
500
0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm (t1)

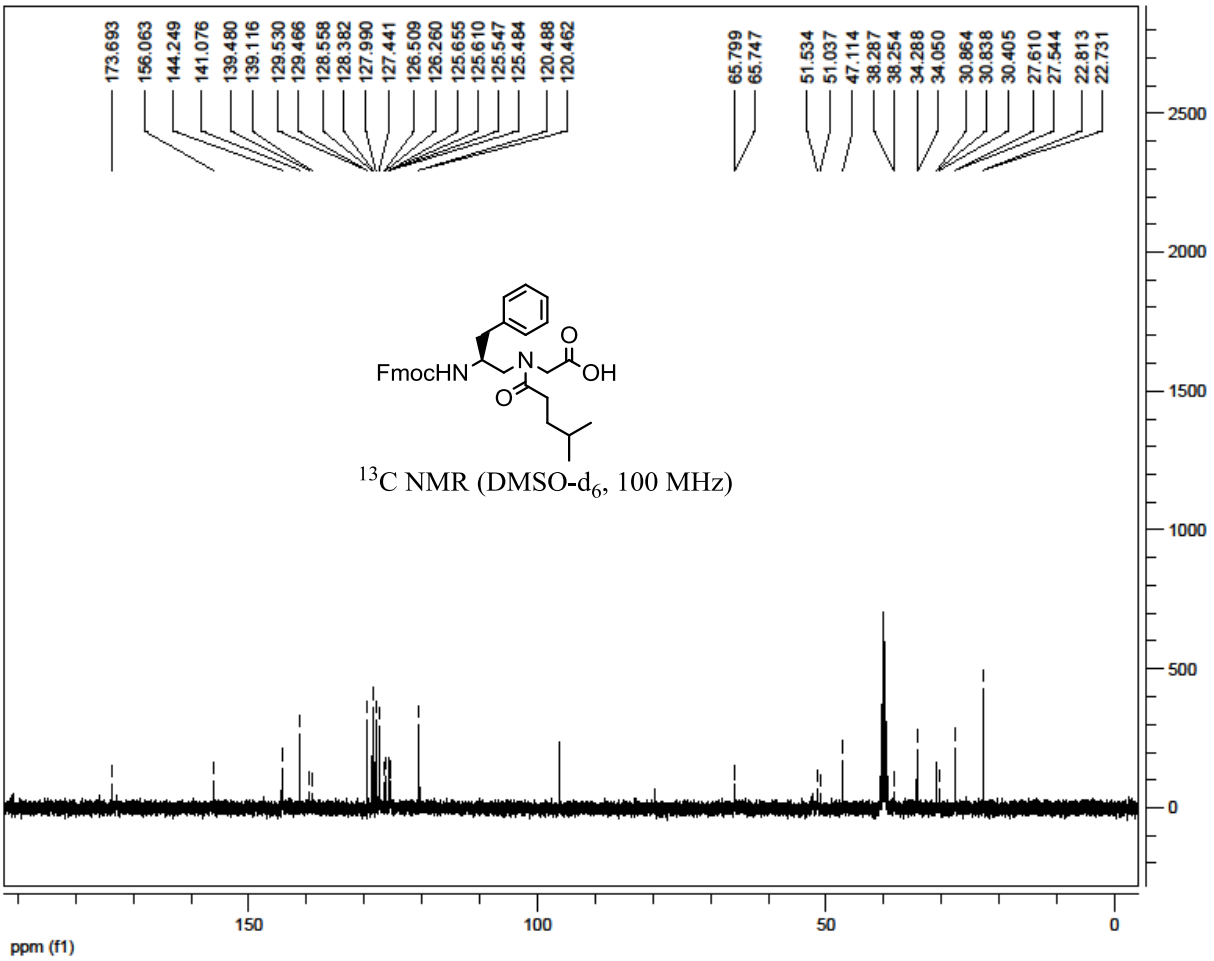


^{13}C NMR (DMSO-d$_6$, 100 MHz)






FmocHN
N
O
OH
O
^{1}H NMR (DMSO-d$_6$, 400 MHz)
1000
500
0
2.01
1.87
3.11
3.82
0.75
2.00
1.93
2.17
0.97
6.29
5.0
0.0
ppm (f1)



^{13}C NMR (DMSO-d$_6$, 100 MHz)