Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Non-Hemolytic α-AApeptides as Antimicrobial Peptidomimetics

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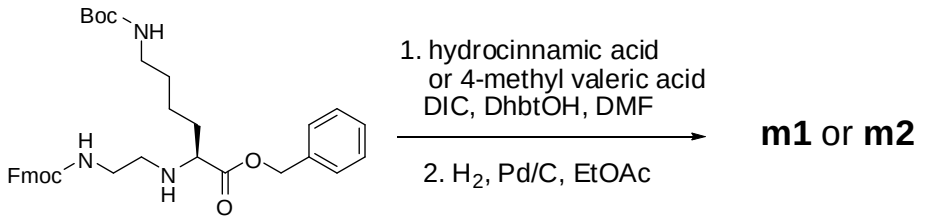
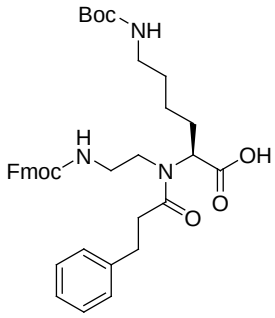
1. General experimental methods. α-amino acid esters and Knorr resin (0.66 mmol/g, 200-400 mesh) were provided by Chem-Impex International, Inc. All other reagents and solvents were purchased from either Sigma-Aldrich or Fisher Scientific. Magainin **7** and control peptide **8** were prepared and purified by USF peptide facility and used without further purification. α-AApeptide building blocks were synthesized following previously reported procedure. [1] NMR spectra of α-AApeptide building blocks were obtained on a Varian Inova 400 instrument. AApeptides were prepared on Knorr resin in peptide synthesis vessels on a Burrell Wrist-Action shaker. The AApeptides were analyzed and purified on an analytical and a preparative Waters HPLC systems, respectively, and then dried by a Labcono lyophilizer. Molecular weights of AApeptides were identified on a Bruker AutoFlex MALDI-TOF mass spectrometer. Circular Dichroism (CD) experiments were carried out on an Aviv 215 circular dichroism spectrometer.

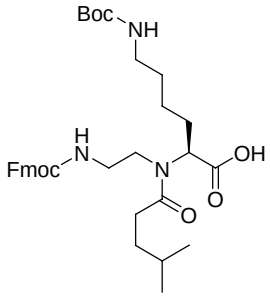
2. Synthesis[1] and characterization of α-AApeptide building blocks.



α-AApeptide building blocks were synthesized following previously reported procedure. [1]



Compound **m1**. Yield 59% (two steps). ^{1}H NMR ($CDCl_3$, 400MHz) δ = 7.75 (2H, m), 7.55 (2H,m), 7.38 (2H), 7.29-7.12 (7H), 7.13-7.05(4H, m), 5.80 (1H, m), 4.56-4.14 (4H, m), 3.94-3.21 (6H, m), 3.07-2.67 (6H, m), 1.44-1.26 (13H, m). ^{13}C NMR ($CDCl_3$, 100MHz) δ 171.2, 158.0, 156.3, 156.1, 143.8141.2, 140.7, 128.4, 127.7, 127.1, 126.1, 125.1, 119.9, 67.0, 60.4, 48.2, 47.1, 42.4, 40.2, 39.7, 35.5, 34.7, 31.2, 29.6, 28.4, 23.9, 23.4. HR-ESI: $[M+H]^+$cacl: 644.3330, found: 644.3330.



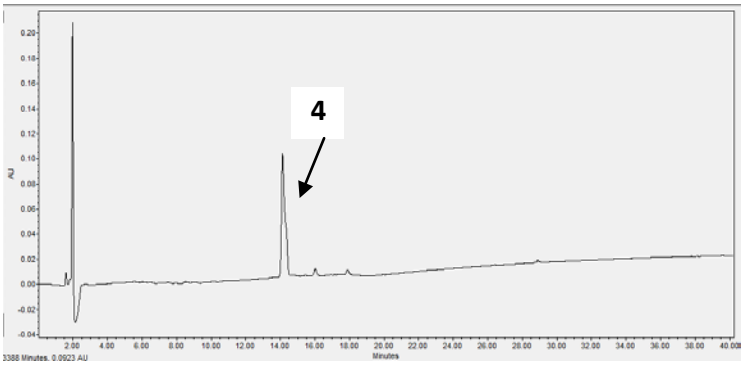
Compound **m2**. Yield 66% (two steps). ^{1}H NMR (DMSO-d_6, 400MHz) δ = 7.88 (2H, d, J=8), 7.67 (2H), 7.41 (2H, t, J=8), 7.32 (2H, t, J=8), 7.13-7.05(4H, m), 6.75 (1H, m), 4.35-4.21 (4H, m), 3.15-2.89 (6H, m), 2.30 (2H, m), 1.87-1.20 (18H, m), 0.85 (6H, t, J=8). ^{13}C NMR ($CDCl_3$, 100MHz) δ 156.6, 143.8, 141.2, 127.7, 127.1, 125.1, 119.9, 67.1, 47.1, 40.2, 39.6, 33.9, 33.8, 31.7, 31.2, 29.8, 28.4, 27.8, 27.7, 23.8, 23.7, 22.4, 22.3. HR-ESI: $[M+H]^+$cacl: 610.3487, found: 610.3469.

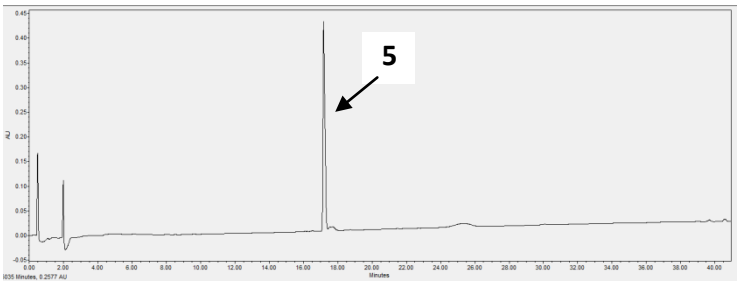
3. Solid phase synthesis, purification and characterization of AApeptides.

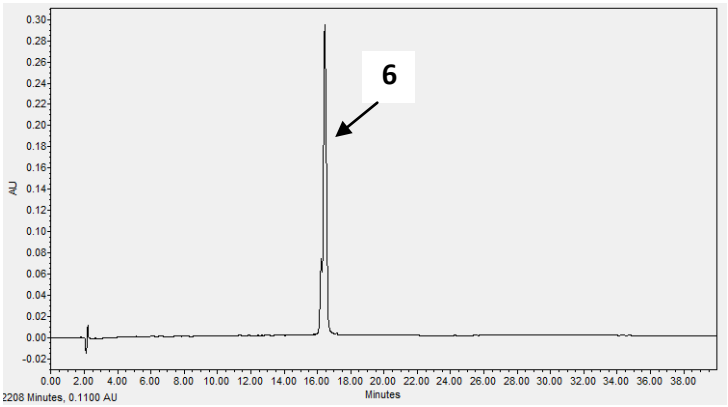
α-AApeptides were prepared on Knorr resin in peptide synthesis vessels on a Burrell Wrist-Action shaker following standard Fmoc chemistry protocol of solid phase peptide synthesis using synthesized α-AApeptides building blocks. Each coupling cycle included an Fmoc deprotection using 20% Piperidine in DMF, and 8 h coupling of 1.5 equiv of AApeptide building blocks onto resin in the presence 4 equiv of DIC (diisopropylcarbodiimide) /DhbtOH (3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine) in DMF. After desired sequences were assembled, they were transferred into 4 ml vials and cleaved from solid support in 74:24:2 TFA/CH_2Cl_2/triisopropylsilane for 2 h. 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 desired fractions were eluted as single peaks at > 95% purity. They were collected and lyophilized. The molecular weights of α-AApeptides were obtained on Bruker AutoFlex MALDI-TOF mass spectrometer using α–cyano-4-hydroxy-cinnamic acid, or on an Agilent LC-MS.

Table S1. MS analysis of α-AApeptides.

α-AApeptides	molecular weight (Actual)	molecular weight (found)
1	307.4	154.1(LC-MS)
2	927.3	984.3 $(M+K+NH_4)^+$ (LC-MS)
3	1230.6	1254.2 $(M+Na)^+$ (MALDI)
4	1534.0	1535.18 $(M+H)^+$ (MALDI)
5	1837.4	1839.2 $(M+H)^+$ (MALDI)
6	2141.0	2142.4 $(M+H)^+$ (MALDI)
7. Magainin	2466.9	2467.2 (MALDI)
8	1944.5	1944.5 (MALDI)
9	1944.5	1967.3 $(M+Na)^+$ (MALDI)







4. CD measurements

Circular Dichroism (CD) spectra were measured on an Aviv 215 circular dichroism spectrometer using a 1 mm path length quartz. 10 scans were averaged for each sample, and 3 times of independent experiments were carried out and the spectra were averaged. The final spectra were normalized by subtracting the average blank spectra. Molar ellipticity $[\theta]$ (deg.cm^2.dmol^{-1}) was calculated using the equation:

$$[\theta] = \theta_{obs}/(n \times l \times c \times 10)$$

Where is the measured ellipticity in millidegrees, while n is the number of side groups, l is path length in centimeter, and c is the concentration of AApeptide in molar units.

5. Preparation of Lipid vesicles

The phospholipids L-α-phosphatidyl-choline (EYPC) and egg yolk L-α-phosphatidyl-DL- glycerol (EYPG) were purchased from Sigma-Aldrich. A mixture of lipids (EYPC: EYPG=70:30 molar ratio) in chloroform was evaporated and dried under nitrogen flow in a small diameter glass test tube. [2] The solvent (chloroform) was completely evaporated by flowing nitrogen gas over the mixture until a thin film of lipids was formed on the inner walls of the test tube. Then 1 mL of 1× PBS (pH = 7.4) was added to the thin film and it was vigorously vortexed for about 2 minutes until a turbid homogenous suspension was formed. Small unilamellar liposomes 10 mM are obtained by sonicating the solution for 30 to 40 minutes. [3]

6. Antimicrobial assays

The bacterial strains used for the assays were a gram negative strain *Escherichia coli* (JM109), two gram positive strains *Bacillus subtilis* (BR151), *Staphylococcus epidermidis* (RP62A, multi drug- resistant). The fungal strain used was *Candida albicans* (ATCC 10231). The antimicrobial activities of the α-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 ^{o}C in 5 ml medium, after which a bacterial suspension (approximately 10^6 CFU/ml) or fungal suspension (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 α-AApeptides for a total volume of 100 µL in each well. The α-AApeptides were prepared in PBS buffer in 2 –fold serial dilutions, with the final concentration range of 0.5 to 100 µg/ml. Plates were then incubated at 37 ^{0}C for 24 h, with the cell growth monitored at time intervals of 6 h by turbidities, which were determined by visualization. The experiments were carried out independently three times in duplicates. The lowest concentration at which complete inhibition of bacterial growth is observed throughout 24 h is defined as the minimum inhibitory concentration (MIC).

7. 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 1000g 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 AApeptides dissolved in 1× PBS from 250 µg/ml through1.6 µg/ml were added to 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 360nm by Biotek Synergy HT microtiter plate reader. Peptide concentrations corresponding to 10% hemolysis were determined from the dose-response curves.

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

8. 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 *E. coli* or *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 intercalates with the nucleic acids of injured and dead cells to form a bright red fluorescent complex. [6] 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 α-AApeptide **6** at the concentration of 2× MIC (10 μg/ml) for 2 h. Then the cells were pelleted by centrifugation at 3000g for 15 min in an eppendorf microcentrifuge. The supernatant was then decanted and the cells were washed with 1× PBS several times and then incubated with PI (5 μg/ml) in dark for 15 min at 0 ^oC. The excess PI was removed by washing the cells with 1× PBS several times. Then the cells were incubated with DAPI (10 μg/ml in water) for 15 mins in dark at 0 ^oC. The DAPI solution was removed and cells were washed with 1× PBS several times. Controls were performed following the exact same procedure for bacteria without the addition of α-AApeptide **6**. The bacterial cells were then examined by using the Zeiss Axio Imager Z1optical microscope with an oil-immersion objective (100×). [7]

9. SEM microscopy

In order to prepare the bacteria for SEM, *E. coli* or *B. subtilis* were grown to an exponential phase and approximately 2 × 10^6 cells were incubated with the α-AApeptide **6** (10 μg/ml) for about 2 h. The cells were then harvested by centrifugation (3000g) for 15 min. After pelleting the cells, the cells were washed twice with DI water. The cells were then fixed with 2.5% (w/v) glutaraldeyhyde in nanopure water for about 30 min. The cells were washed extensively to get rid of excess glutaralhedyde. The cells were dehydrated with a graded ethanol series (30%, 50%, 70%, 95% and 100%, 5 min each). [8] Hexamethyldisilazane addition followed the dehydration of cells for 2 min. Then about 10 μL of sample was added to the SEM grid followed by gold sputter coating. The samples were observed at 25 KV with a HITACHI S-800 scanning electron microscope.

Fig. S1 Oligomers for the antimicrobial assay. **1-6** are α-AApeptides; **7** is magainin II; **8** is the 14-mer regular peptide with alternative phenylalanine and lysine residues.

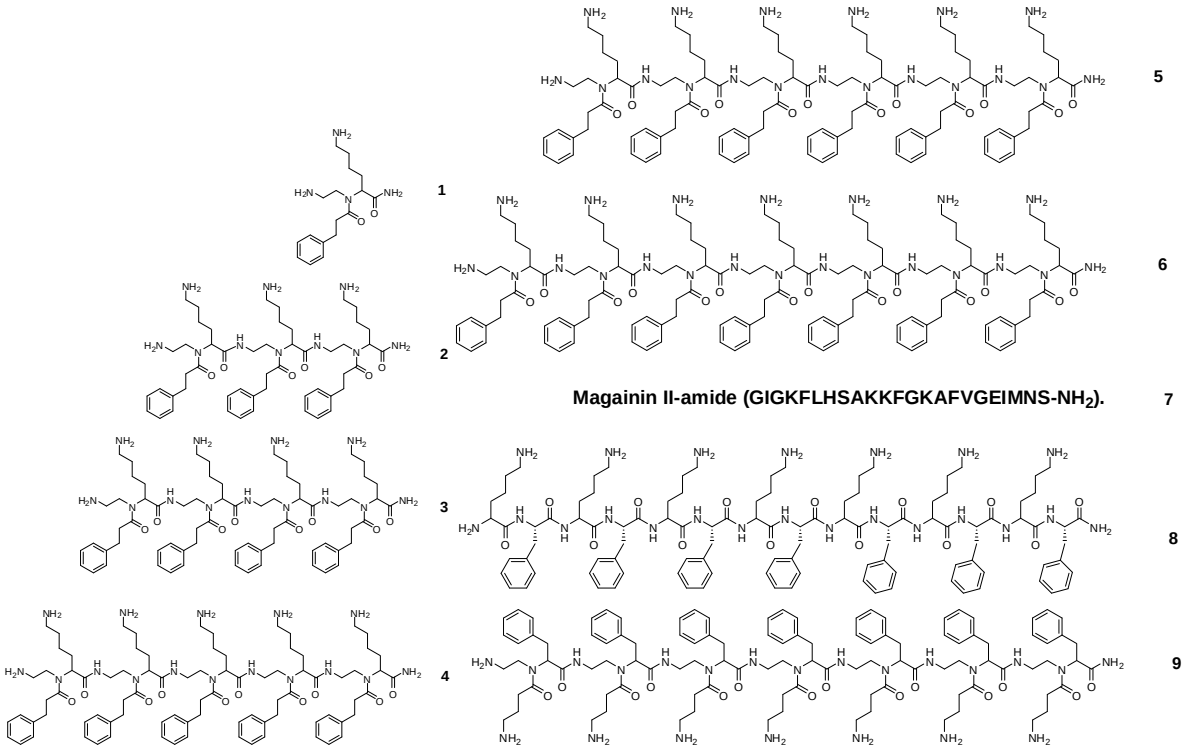


Fig. S2. MIC (based on both μg/ml and μM) of *B. subtilis* vs. α-AApeptides.

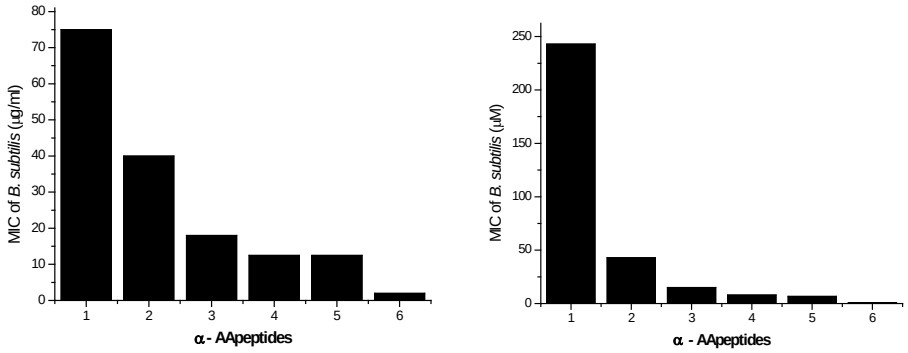


Figure S3. Hemolytic activities of **1**-**8** versus the percentage human blood cells lysed following 1 h incubation. α-AApeptides **4, 5, 6** and magainin **7** exhibit negligible hemolysis at concentrations as high as 250 μg/mL.

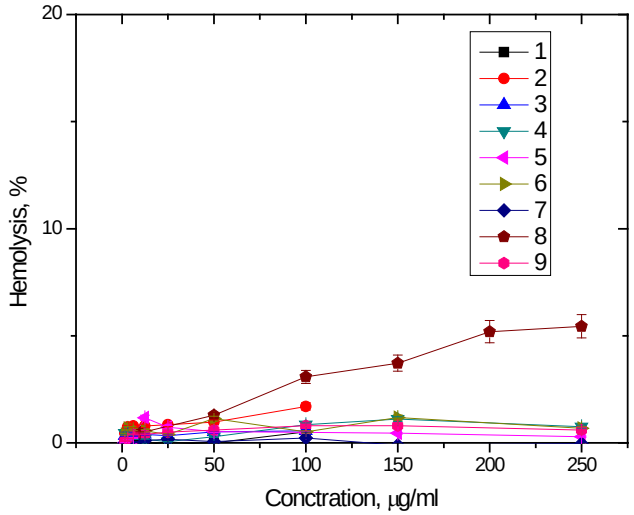


Fig. S4 The CD spectra of oligomers. 4a, CD spectra of **1-8** in PBS buffer. 4b, CD spectra of **6-8** in 5mM lipid vesicle, 10 mM PBS buffer. The concentration of oligomers are approximately 100 μM.

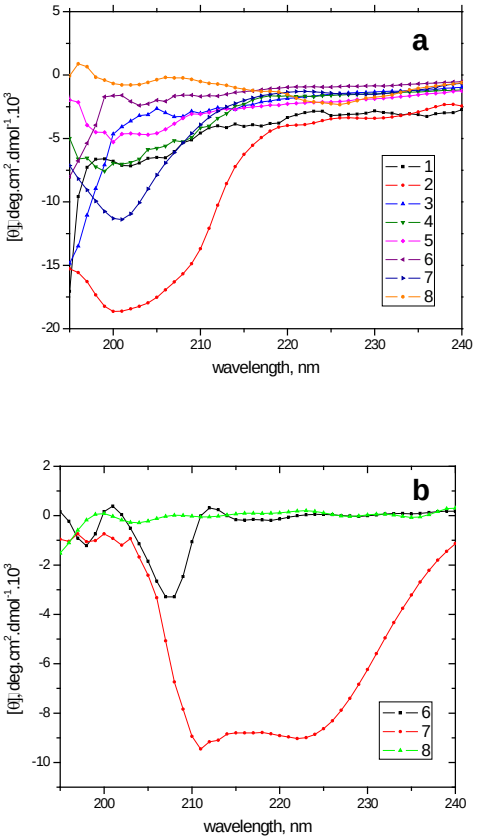


Fig. S5 Schematic representation of α-AApeptide adopting amphipathic structure upon binding to bacterial membrane.

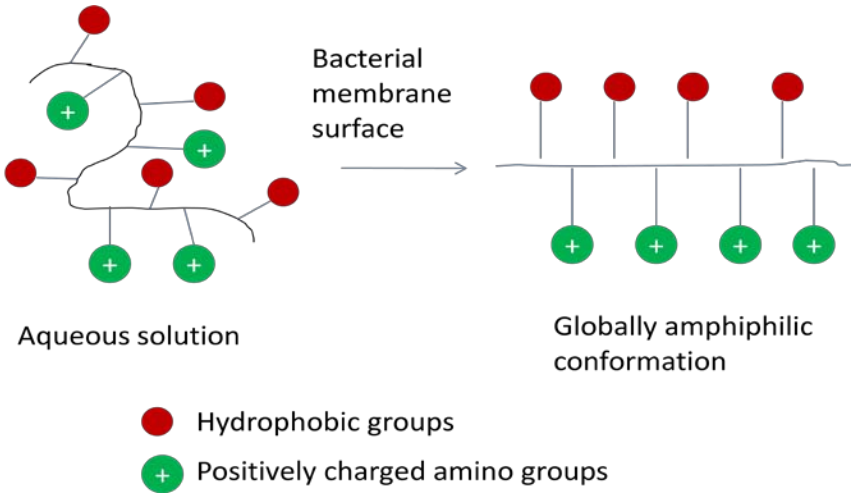


Fig. S6 Fluorescence micrographs of *E. coli* and *B. subtilis* treated with 10 μg/ml α-AApeptide **6** for 2 h. **a1-a4**, *E. coli*. **a1**, control, no treatment, DAPI stained; **a2**, control, no treatment, PI stained; **m1**, merged

pictures of **a1** and **a2**. **a3**, α-AApeptide **6** treatment, DAPI stained; **a4**, α-AApeptide **6** treatment, PI stained. **m2**, merged pictures of **a3** and **a4**. **b1-b4**, *B. subtilis*. **b1**, control, no treatment, DAPI stained; **b2**, control, no treatment, PI stained; **m3**, merged pictures of **b1** and **b2**. **b3**, α-AApeptide **6** treatment, DAPI stained; **b4**, α-AApeptide **6** treatment, PI stained. **m4**, merged pictures of **b3** and **b4**. Scale bar: 2 μm for *E. coli* and 5 μm for *B. subtilis*.

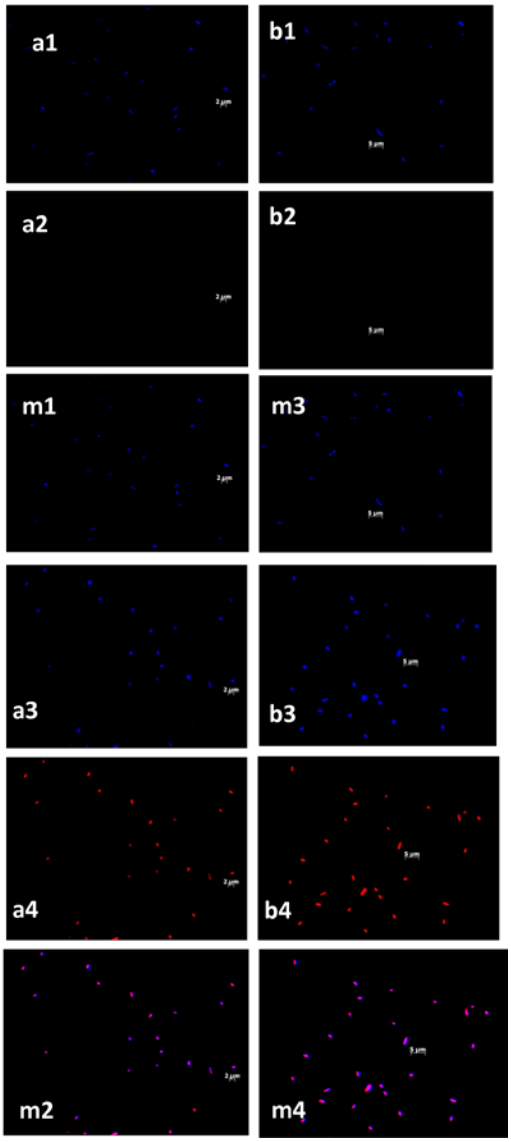
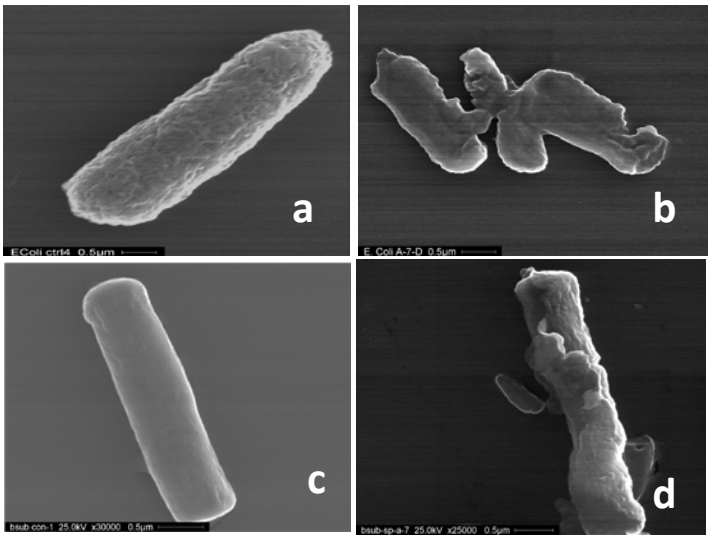


Figure S7. SEM micrographs of *E. coli* and *B. subtilis* before and after AApeptide treatment. **7a**, *E. coli*, control, no treatment; **7b**, *E. coli*, treated with 10 μg/ml AApeptide **6** for 2 h; **7c**, *B. subtilis*, control, no treatment; **7d**, *B. subtilis*, treated with 10 μg/ml AApeptide **6** for 2 h. Scale bar is 0.5 μm.



SEM analysis supports the hypothesis of bacterial membrane disruption as the mechanism of α-AApeptide action (Figure S7). Figure S7a and S7c show the characteristic *E. coli* and *B. subtilis* rod-shape with a mottled surface. Figure S7b and S7d clearly demonstrate the disruption of *E. coli* and *B. subtilis* membranes after incubation with α-AApeptide **6**.

References

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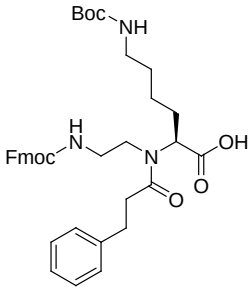
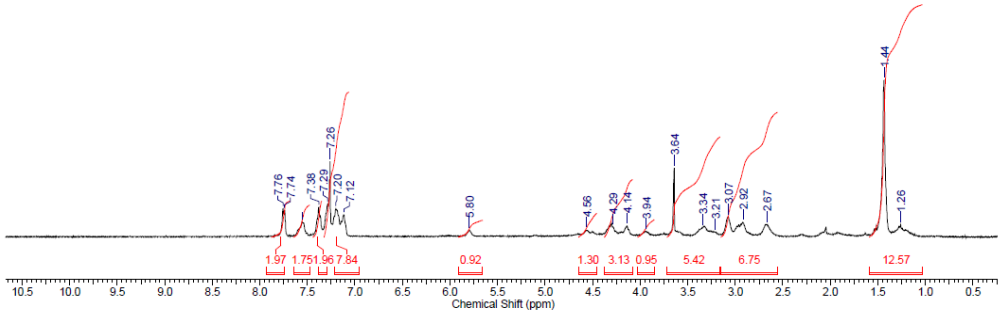
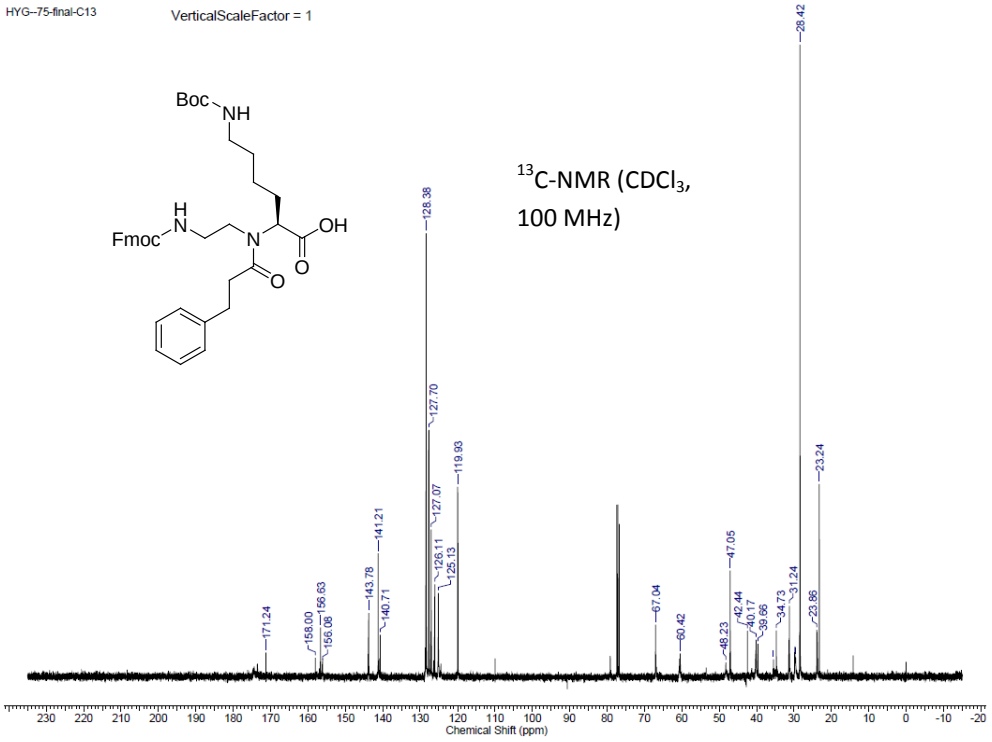
^{1}H-NMR ($CDCl_3$, 400 MHz)







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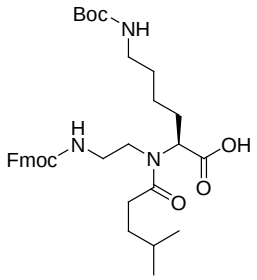

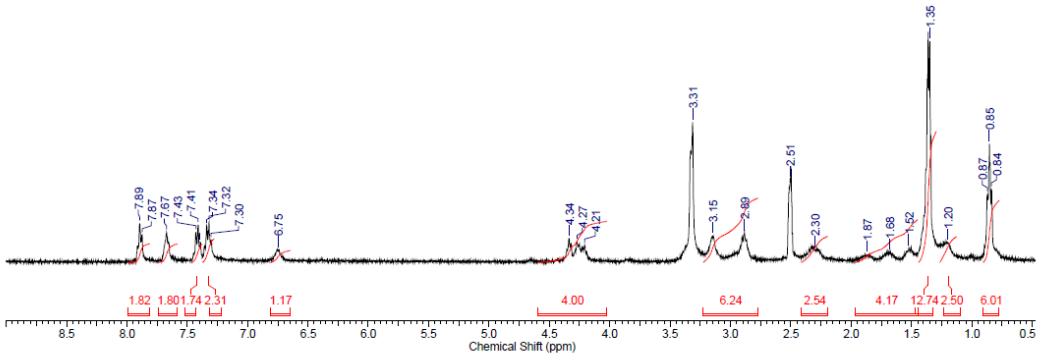

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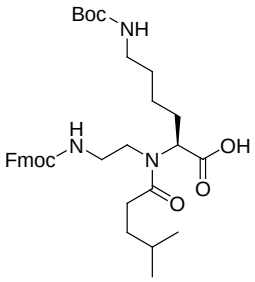

^{13}C-NMR ($CDCl_3$, 100 MHz)