Contents:

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 α-AApeptide building blocks were synthesized following previously reported procedure. [1] NMR spectra of the α-AApeptide building blocks were obtained on an Agilent DD800 instrument. Cyclic lipo-α-AApeptides were prepared on a Rink amide resin in peptide synthesis vessels on a Burrell Wrist-Action shaker. The cyclic lipo-α-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 lipo-α-AApeptides were identified on a Bruker AutoFlex MALDI-TOF mass spectrometer.

2. Synthesis and characterization of AApeptide building blocks

The AApeptide building blocks were synthesized following the previously reported procedure (Scheme 1). [1] The structures of building blocks used for the solid phase synthesis are shown below (Figure S1). Compounds m1[2] and m2[1a] were reported previously.

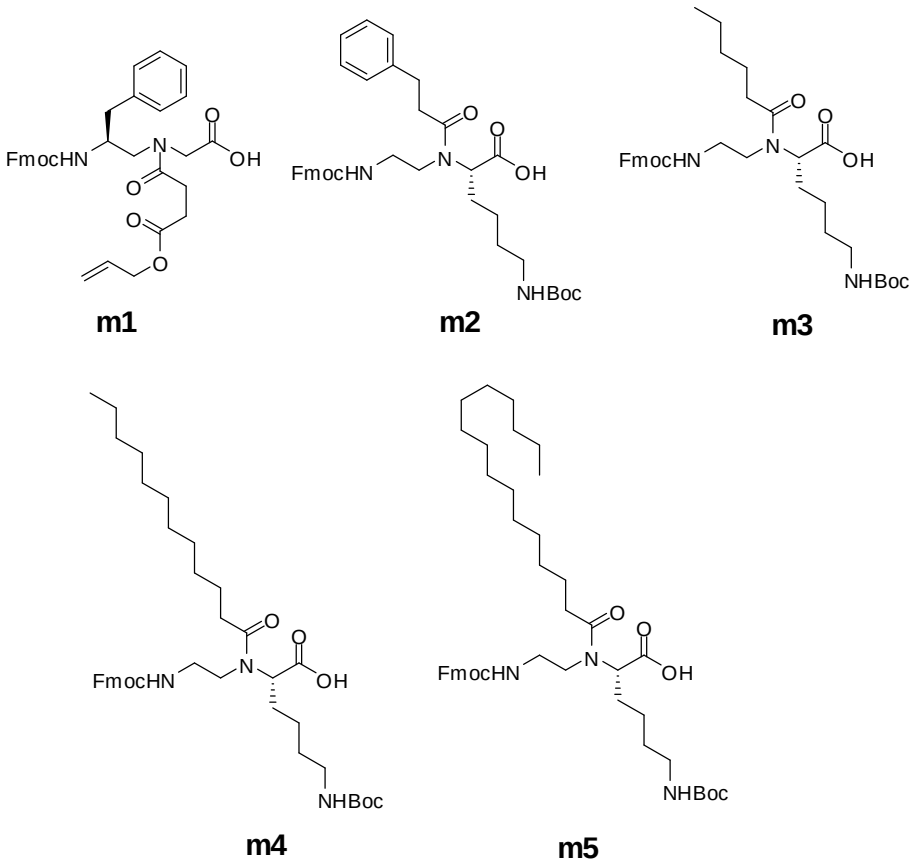
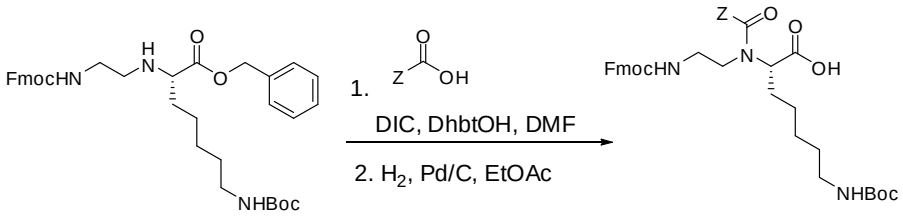


Figure S1. Building blocks used for the solid phase synthesis.



Z = C_5H_{11} **m3**
$C_{11}H_{23}$ **m4**
$C_{15}H_{31}$ **m5**

Scheme 1. Synthesis of α-AApeptide building blocks. DhbtOH = 3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine.

Compound m3. Yield 70% in two steps. ^{1}H NMR ($CDCl_3$, 800 MHz) δ (two rotamers) 7.73 (2H, t, *J* = 8), 7.56 (2H, d, *J* = 8), 7.37 (2H, t, *J* = 8), 7.29 (2H, t, *J* = 8), 5.98-5.87 (1H, m), 4.60-4.25 (4H, m), 3.79 (3H, m), 3.09 (2H, m), 2.40-2.00 (4H, m), 1.42 (9H,s), 1.14 (10H, d), 0.9-0.8 (3H, m). ^{13}C NMR ($CDCl_3$, 200 MHz) δ 175.0, 174.3, 157.2, 156.6, 143.8, 141.2 127.7, 127.1, 125.1, 120.0, 79.1, 67.1, 60.4, 48.1, 47.1, 40.2, 39.6, 33.8, 33.2, 31.4, 29.9, 28.4, 24.8, 24.4, 24.0, 23.9, 23.4, 22.9, 22.4 13.9. HR-ESI: $[M+H]^+$ cacl: 610.3487, found:610.3498.

Compound m4. Yield 52% in two steps. ^{1}H NMR ($CDCl_3$, 800 MHz) δ (two rotamers) 7.72 (2H, t, *J* = 8), 7.55 (2H, d, *J* = 8), 7.36 (2H, t, *J* = 8), 7.27 (2H, t, *J* = 8), 6.01 (1H, m), 4.70-4.05 (4H, m), 3.70-3.00 (5H, m), 2.40-2.00 (4H, m), 1.80-1.00(31H, m), 0.95 (3H, t, *J* = 8). ^{13}C NMR ($CDCl_3$, 200 MHz) δ 174.0, 172.1, 156.6, 156.1, 143.8, 141.2, 127.7, 127.1, 125.1, 119.9, 79.2, 67.1, 63.1, 48.4, 47.1, 41.0, 39.6, 34.2, 33.3, 31.9, 30.0, 29.6, 29.3, 29.1, 29.4, 25.2, 23.8, 22.7, 14.1. HR-ESI: $[M+H]^+$cacl: 694.4426, found:694.4446.

Compound m5. Yield 60% in two steps. ^{1}H NMR ($CDCl_3$, 800 MHz) δ (two rotamers) δ = 7.72 (2H, t, *J* = 8), 7.55 (2H, d, *J* = 8), 7.36 (2H, t, *J* = 8), 7.27 (2H, t, *J* = 8), 5.95 (1H, m), 4.80-3.80 (5H, m), 3.70-3.20 (5H, m), 3.06 (2H, m), 2.31 (2H, m), 1.80-1.50(2H, m), 1.41(9H, s), 1.30-1.10 (28H, m), 0.86 (3H, t, *J* = 8). ^{13}C NMR ($CDCl_3$, 200 MHz) δ 175.2, 174.5, 156.7, 156.1, 148.0, 141.2, 127.7, 127.1, 125.1, 119.9, 79.1, 67.1, 61.1, 48.4, 47.1, 40.2, 39.6, 33.8, 33.4, 31.9, 30.6, 29.7, 29.4, 29.3, 29.1, 28.4, 25.2, 23.8, 22.7, 14.1. HR-ESI: $[M+H]^+$cacl: 750.5052 , found: 750.5036.

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

Standard Fmoc-chemistry protocol of solid phase synthesis was used to synthesize lipidated cyclic α-AApeptides on a Burell Wrist-Action shaker on Rink amide resin using peptide vessels (Figure S2). [1-2] Every coupling cycle consisted of Fmoc deprotection using 20% piperidine/DMF, 6 h coupling of 2 equiv. of building blocks in the presence of 4 equiv. of DIC (Diisopropylcarbodiimide) and DhbtOH (3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine) in DMF. Firstly, the lipidated building block **m3, m4** or **m5** was attached to the solid phase support, followed by coupling with building block **m1**, and then desired number of building blocks using standard Fmoc-chemistry. After the desired sequence was assembled, the allyl group was removed by usage of $Pd(PPh_3)_4/PhSiH_3$ (0.2 equiv./10 equiv.) in CH_2Cl_2 for 2 hours (repeated twice). The Fmoc group was then removed and the intramolecular cyclization was achieved by PyBop/HOBt/DIEA/DMF. Lastly, the resin was transferred into a 4 mL vial and the cyclic-lipo-α-AApeptide was cleaved from the solid support in 50:48:2(v/v) TFA/ CH_2Cl_2/Triisopropylsilane in 5 hours. The solvent was evaporated and the residues were analyzed and purified by Waters HPLC system on analytical (1 mL/min) and preparative (20 mL/min) modules, 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 (for sequences 2,

4, and 6) or 60 min (for sequences 1, 3, and 6). The desired fractions were lyophilized to yield cyclic-lipo-α-AApeptides (Figure S3).

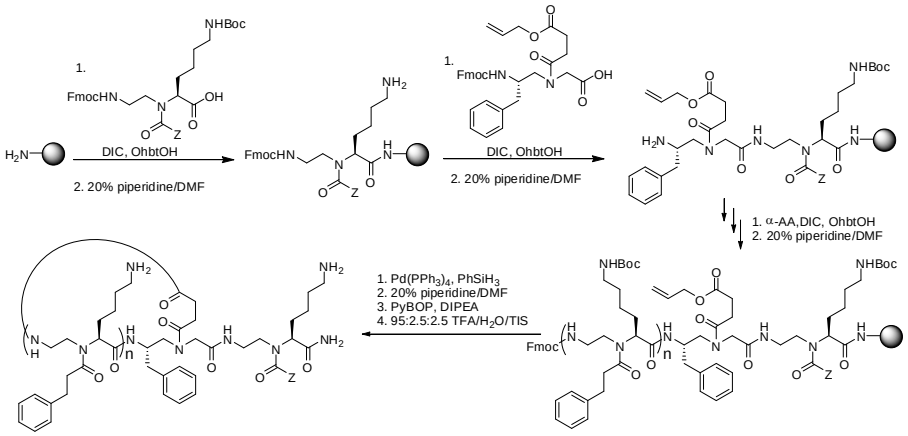


Figure S2. Solid phase synthesis of cyclic lipo-α-AApeptides.

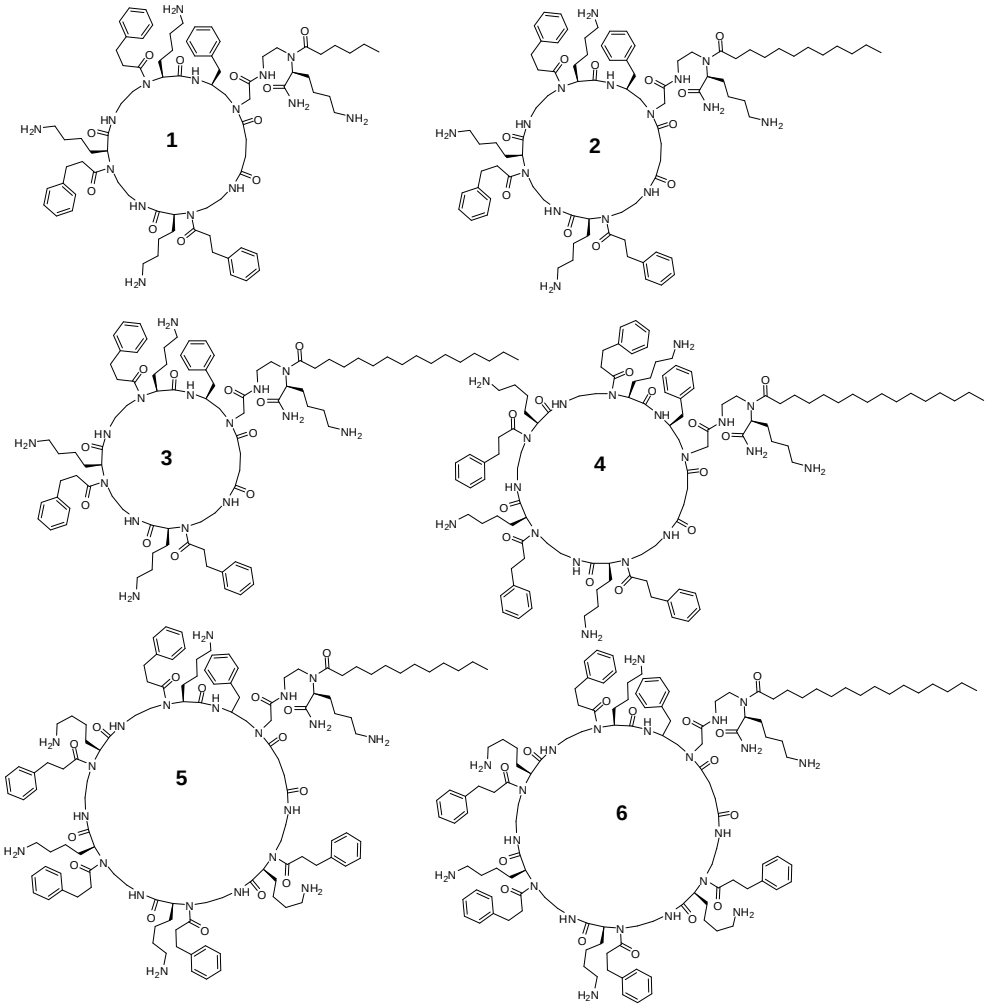
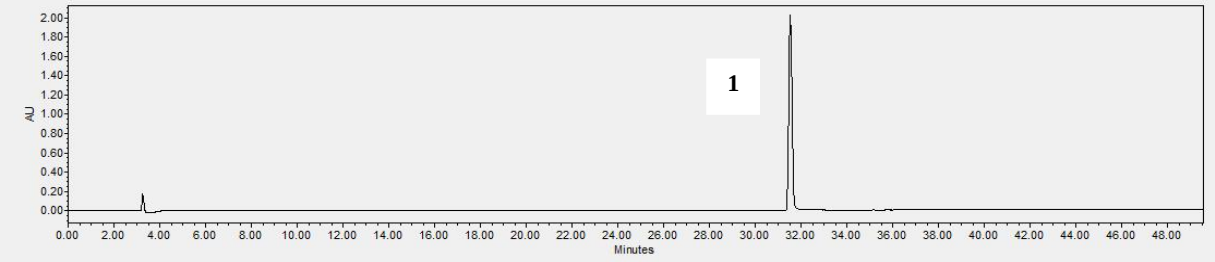


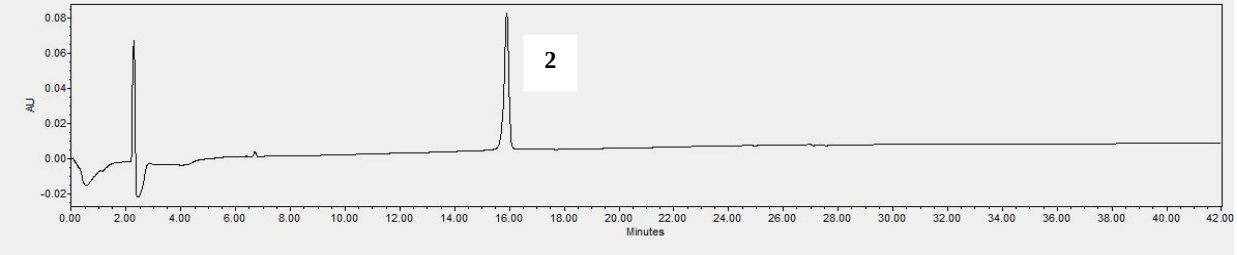
Figure S3. The structures of synthesized cyclic lipo-α-AApeptides.

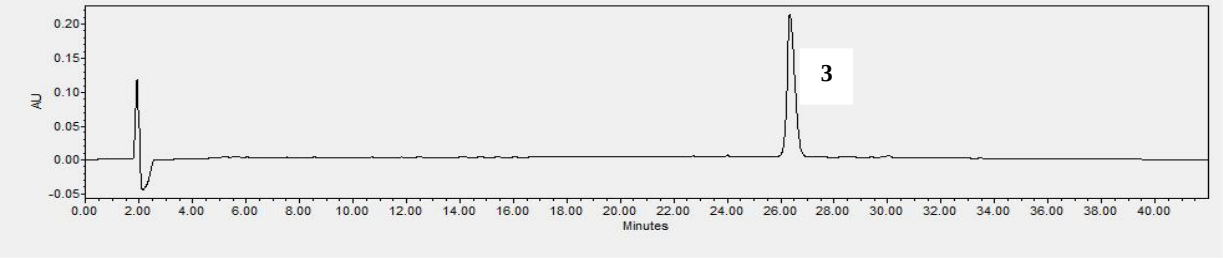
Table S1. MALDI analysis of cyclic lipo-α-AApeptides.

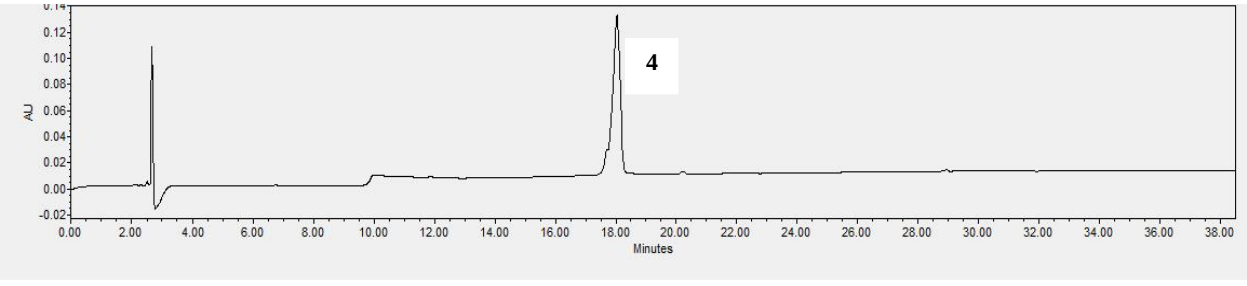
Oligomers	Molecular formula	Molecular weight (actual)	Molecular weight (Found-MALDI)
1	$C_{80}H_{121}N_{15}O_{11}$	1468.911	1470.56$(M+H)^+$
2	$C_{89}H_{121}N_{21}O_{11}$	1553.07	1554.59$(M+H)^+$
3	$C_{90}H_{141}N_{15}O_{11}$	1609.176	1610.47$((M+H)^+$
4	$C_{107}H_{166}N_{18}O_{13}$	1912.58	1913.861$(M+H)^+$
5	$C_{120}H_{183}N_{21}O_{15}$	2159.869	2162.15$(M+H)^+$
6	$C_{124}H_{191}N_{21}O_{15}$	2215.98	2217.485$(M+H)^+$

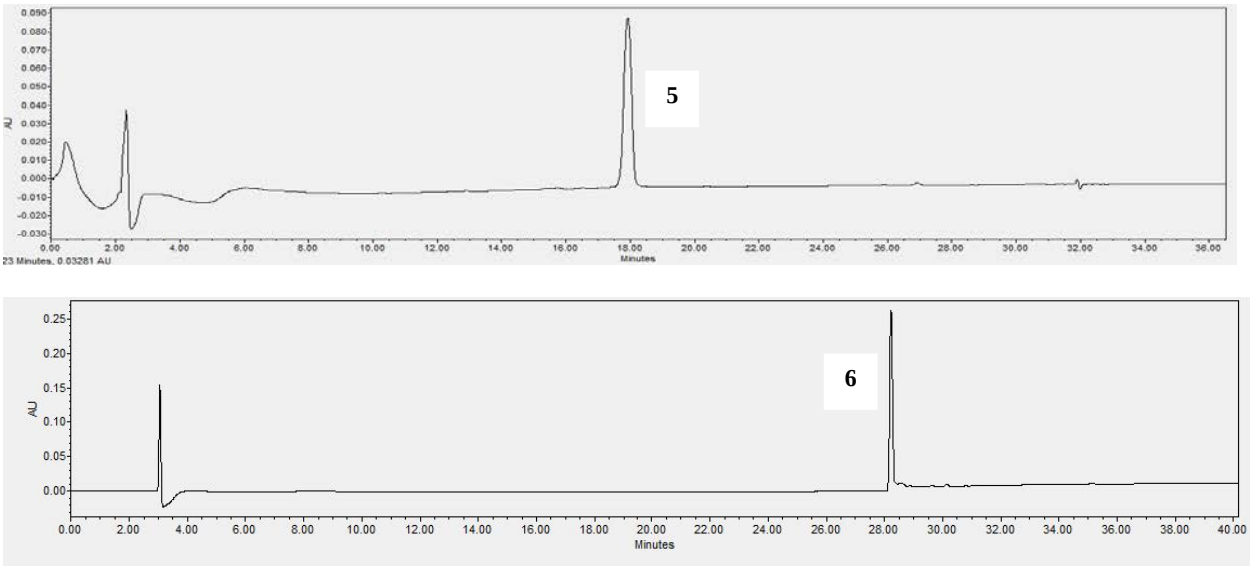
Purified HPLC traces of the cyclic lipo-α-AApeptides-detected at 215nm











4. Antimicrobial assay for MIC determination[1-3]

The bacterial strains used for testing the efficacy of cyclic lipo-α-AApeptides were multi-drug resistant *S. epidermidis* (RP62A), Vancomycin-resistant *E. faecalis* (ATCC 700802), methicillin resistant *S.aureus* (ATCC 33591), *K. pnuemoniae* (ATCC 13383) and multi-drug resistant *P. aeruginosa* (ATCC 27853). The antimicrobial activities of the cyclic lipo-α-AApeptides developed were determined in sterile 96-well plates by serial dilution method. Bacterial cells were grown overnight at 37 ˚C in 5 mL medium after which a bacterial suspension of approximately 10^6 CFU/mL in Luria broth or trypticase soy was prepared ensuring that the bacterial cells were in the mid-logarithmic phase. Aliquots of 50 µL bacterial suspension were added to 50µL of medium containing the cyclic lipo-α-AApeptides for a total volume of 100µL in each well. The α-AApeptides were dissolved in PBS buffer in two–fold serial dilutions. The concentration range used for peptides was 25 to 0.5 µg/mL. The 96-well plates were incubated at 37 ˚C for about 20 h. The Biotek microplate reader was used to measure the optical density (OD) at a wavelength of 600 nm after about 20 h. The experiments were carried out as three independent biological replicates, each in duplicate. The lowest concentration at which complete inhibition of bacterial growth is observed is defined as the minimum inhibitory concentration (MIC). The experiments were repeated for three times and each time in duplicate.

5. Hemolysis assay[1-3]

Freshly drawn human red blood cells (hRBC's) were used for the assay. The blood sample was washed with PBS buffer several times and centrifuged at 700 g for 10 min until a clear supernatant was observed. The hRBC's were re-suspended in 1 × PBS to get a 5% v/v suspension which was used to perform the assay. Two-fold serial dilutions of cyclic lipo-α-AApeptides were prepared in PBS buffer. Concentrations ranging from 250 µg/ml through 1.56 µg/mL were tested by adding the cyclic lipo-α-AApeptides solutions to sterile 96-well plates 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. The 96 well plate was incubated at

37 ˚C for 1h and centrifuged at 3500 rpm for 10 min. The supernatant (30 µL) was then diluted with 100 µL of 1 × PBS and hemoglobin was detected by measuring the optical density at 360nm by Biotek microtiter plate reader (Type: Synergy HT).

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

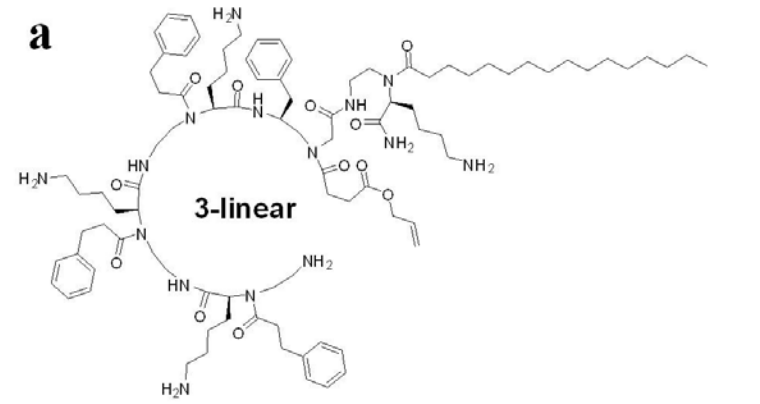
Peptide concentrations corresponding to 50% hemolysis were determined from the dose-response curves. The experiments were repeated for three times and each time in duplicate.

6. Fluorescence microscopy[1-3]

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 *S. aureus* (ATCC 33591) cells. DAPI being a double stranded DNA binding dye, stains all bacterial cells irrespective of their viability. 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. The bacterial cells were first stained with PI and then with DAPI. The bacterial cells were grown until they reached mid-logarithmic phase and then ~ 2×10^3 cells were incubated with the cyclic lipo-α-AApeptide **3** at 2 X MIC for 4 h. Then the cells were pelleted by centrifugation at 3000 g for 15 min in an Eppendorf microcentrifuge. The supernatant was decanted and the cells were washed with 1X PBS several times and then incubated with PI (5 µg/mL) in the dark for 15 min at 0 ºC .The excessive PI was removed by washing the cells several times with 1 × PBS several times. Lastly, the cells were incubated with DAPI (10 µg/mL in water) for 15min in the dark at 0 ºC. Then finally the excessive DAPI solution was removed by washing it with 1 × PBS. The controls were performed following the exact same procedure for bacteria without **3**. The bacteria were then examined by using the Zeiss Axio Imager Z1optical microscope with an oil-immersion objective (100 ×). [1-3]

7. Fluorescent Detection of Nitric Oxide

Raw 264.7 (Mouse leukaemic monocyte macrophage cell line) cells were grown in RPMI 1640 medium with 10 % fetal bovine serum (FBS), 1% Penicillin/streptomycin, and 1% L-glutamine. On day one, cells were plated in a 96-well plate at 75,000 cells/well in complete RPMI 1640 medium. Cells were grown overnight at 37°C and 5% CO_2 in a humidified incubator. On day 2, media was removed, and cells were placed in unsupplemented RPMI 1640 medium. 20 ng/mL LPS and the appropriate concentration of AApeptides were added to a final volume of 200 µL. All stock solutions of AApeptides as 20 mM stocks were prepared in PBS, and then diluted to the desired concentration with PBS. There are PBS controls in each experiment. Plates were then incubated for 24 hours after treatment. Following incubation, 100 µL of media was removed from each well and added to a flat black 96-well microfluor plate (Thermo Scientific, MA, USA). 10 µL of 0.05 mg/mL 2,3-diaminonamthalene in 0.62 M HCl was added to the media and incubated for 20 min in the dark. The reaction was quenched with 5 µL of 3.0 M NaOH, and the plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with excitation at 360 nm and emission at 430 nm. Data was normalized with the ligand only control as 100% activation, and the untreated cells as 0% activation. Fold inhibition = [(Sample $_{430\ nm}$ − Untreated cells $_{430\ nm}$)/(Ligand Control $_{430\ nm}$ − Untreated cells $_{430\ nm}$)]. The IC_{50} values were calculated graphically using OriginPro v8.6 software.



Oligomers	MIC (μg/mL)					Hemolysis
	Gram-negative		Gram-positive			
	K. pneumoniae	*P. aeruginosa*	*S. epidermidis*	*E. faecalis*	*S. aureus*	HC_{50} (μg/mL)
3-linear	>25	20	>25	25	>25	>250

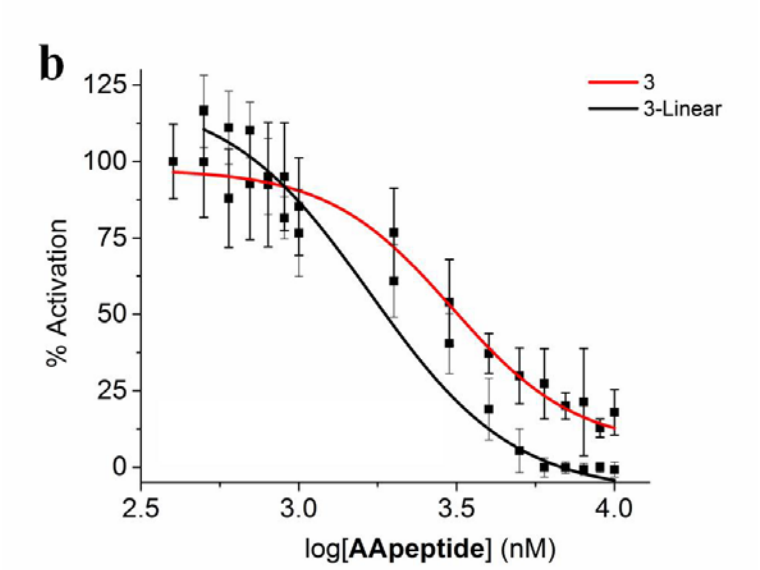


Figure S4. a, the structural of **3-Linear** (non-cyclic analog of **3**) and its antimicrobial and hemolytic activity. **b.** TLR4-induced nitric oxide production in the presence of **3** or **3-Linear.** RAW 264.7 were treated with 20 ng/mL LPS and varying concentrations of **3** or **3-Linear**. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation. Our results indicate that **3** and **3-Linear** reduce nitric oxide production in a comparable fashion**.**

8. Secreted Embryonic Alkaline Phosphatase (SEAP) Reporter of NF-κB Transcription

HEK293 (Human Embryonic Kidney 293) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% Penicillin/streptomycin, and 1% L-glutamine. HEK293 cells have been stably transfected with human TLR4, as well as the required accessory proteins MD-2 and

CD14. Additionally, the cells possess an optimized alkaline phosphatase reporter gene under the control of a NF-κB inducible promoter. [4] On day one, cells were plated in a 96-well plate at 40,000 cells/well in complete DMEM medium. Cells were grown overnight at 37°C and 5% CO_2 in a humidified incubator. On day 2, media was removed, and cells were placed in Optimem + 0.5% FBS medium. 20 ng/mL LPS and the appropriate concentration of AApeptides were added to a final volume of 200 µL. All stock solutions of AApeptides were prepared as 20 mM stocks in PBS, and then diluted to the desired concentration with PBS. There were PBS controls in each experiment. Plates were then incubated for 24 hours after treatment. Following incubation, the medium was assayed per the instructions of the Phospha-Light™ SEAP Reporter Gene Assay System (Applied Biosystems, NY, USA). The plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with luminescence at 430 nm. Data was normalized with the ligand only control as 100% activation, and the untreated cells as 0% activation. Fold inhibition = [(Sample $_{430\ nm}$ – Untreated cells $_{430\ nm}$)/(Ligand Control $_{430\ nm}$ – Untreated cells $_{430\ nm}$)]. The IC_{50} values were calculated graphically using OriginPro v8.6 software.

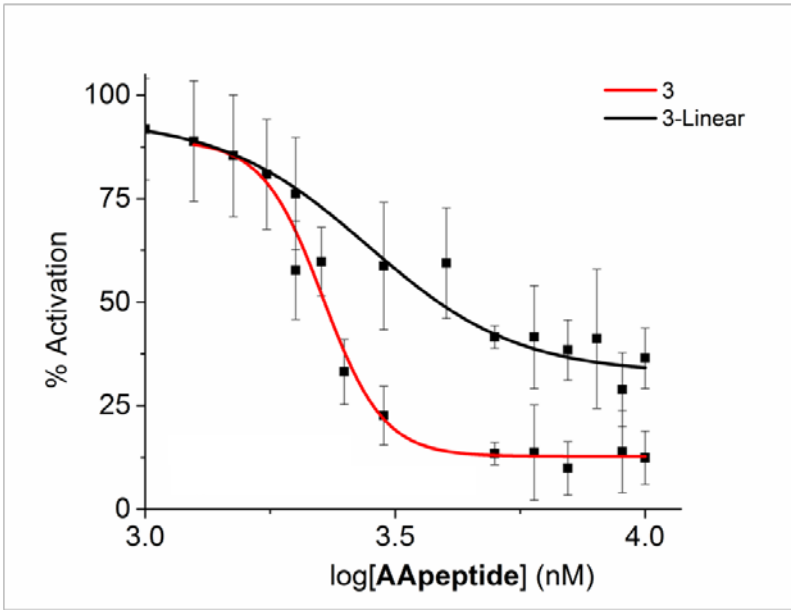


Figure S5. NF-κB activation is inhibited in HEK 293 cells by **3** and **3-Linear**. Cells were plated in 96-well plate and treated with 20 ng/mL LPS and various concentrations of **3** and **3-Linear**. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation**.**

9. Enzyme-Linked Immunosorbent Assay (ELISA) Detection of TNF-α

Raw 264.7 (Mouse leukaemic monocyte macrophage cell line) cells were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1% Penicillin/streptomycin, and 1% L-glutamine. On day one, cells were plated in a 96-well plate at 75,000 cells/well in complete RPMI 1640 medium. Cells were grown overnight at 37°C and 5% CO_2 in a humidified incubator. On day 2, media was removed, and cells were placed in unsupplemented RPMI 1640 medium. 20 ng/mL LPS and the appropriate concentration of AApeptides were added to a final volume of 200 µL. We prepared all stock solutions of AApeptides as 20 mM stocks in PBS, and then diluted to the desired concentration with PBS. There are PBS controls in each experiment. Plates were then incubated for 24 hours after treatment. Following incubation, samples were assayed for TNF-α per the method outlined in the BD Biosciences Mouse TNF (Mono/Mono) ELISA Set (BD Biosciences, CA, USA). The plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with absorbance at 450 nm. Data was normalized with the ligand only control as 100% activation, and the untreated cells as 0% activation. Fold inhibition = [(Sample $_{450\ nm}$

– Untreated cells $_{450\ nm}$)/(Ligand Control $_{450\ nm}$ – Untreated cells $_{450\ nm}$)]. The IC_{50} values were calculated graphically using OriginPro v8.6 software.

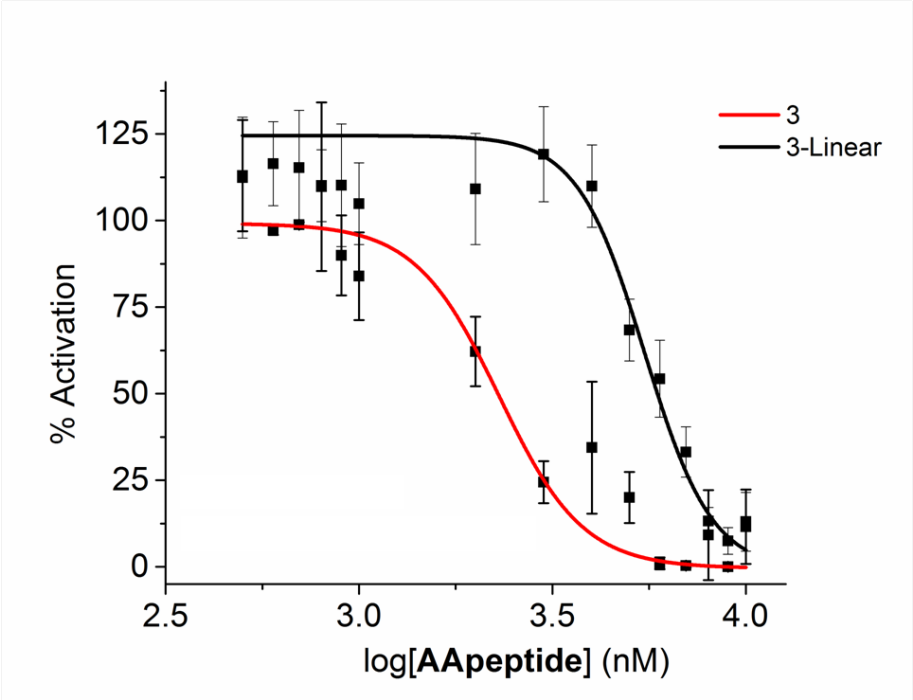


Figure S6. Inhibition of TLR4-induced TNF-α production by **3** and **3-Linear.** Mouse TNF (Mono/Mono) enzyme-linked immunosorbent assay (ELISA) in RAW 264.7 cells demonstrates that TNF-α production is decreased with treatment of **3** and **3-Linear**. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation**.**

10. Crystal Violet Toxicity Assay

Cells which were treated with compound for nitric oxide experimentation were also tested for compound toxicity using crystal violet stain. Media was decanted, and cells were fixed for 20 min in 4% paraformaldehyde. After fixing, formaldehyde was removed and cells were incubated for one hour with 0.05% crystal violet stain. After incubation, cells were rinsed with deionized water to remove excess stain, and reconstituted in 100% methanol for 10 min. The plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with absorbance at 535 nm. Data was normalized with the untreated cells control as 100% survival, and the blank wells as 0% survival. Fold inhibition = [(Sample $_{535\ nm}$ – Blank $_{535\ nm}$)/(Untreated cells $_{535\ nm}$ – Blank $_{535\ nm}$)].

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Bai, H. Wu, F. Costanza, L. West, L. Harrington, L. Shaw, C. Cao, J. Cai, *Chemical Communications* **2011**, *47*, 9729-9731.

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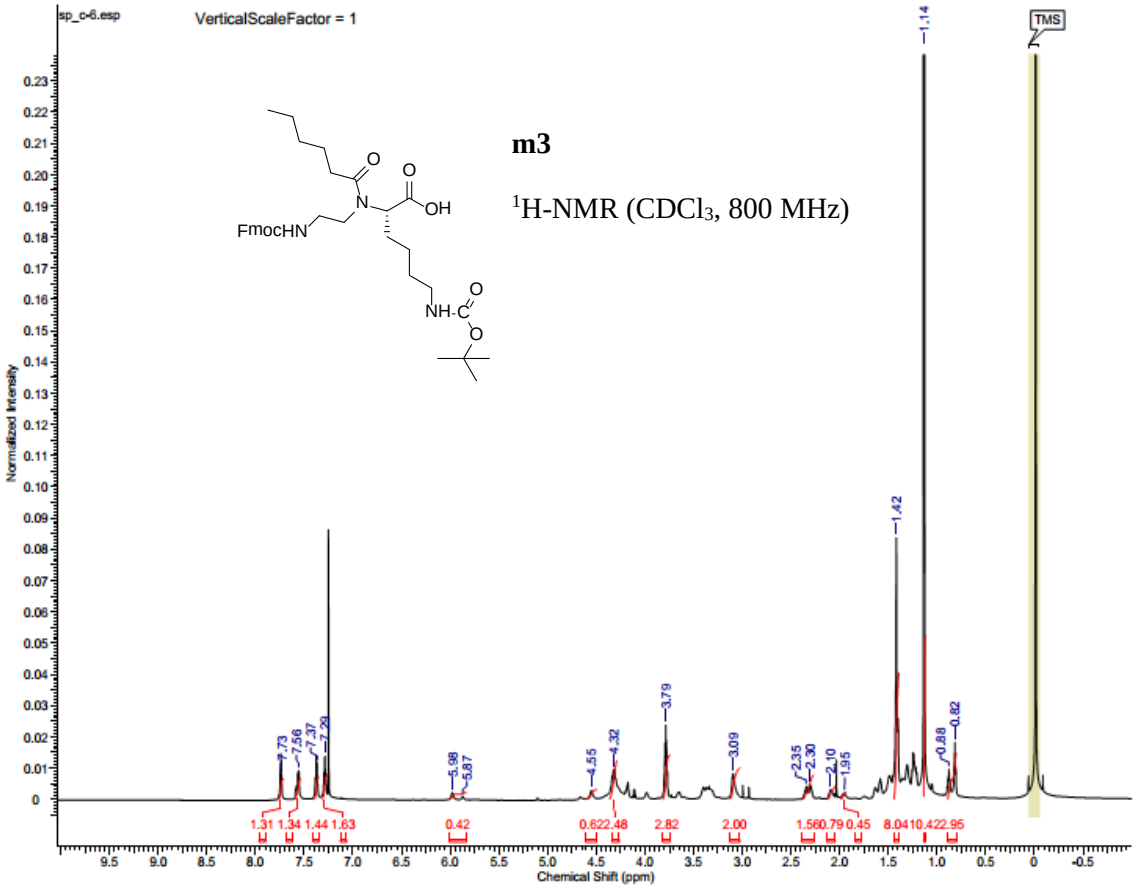
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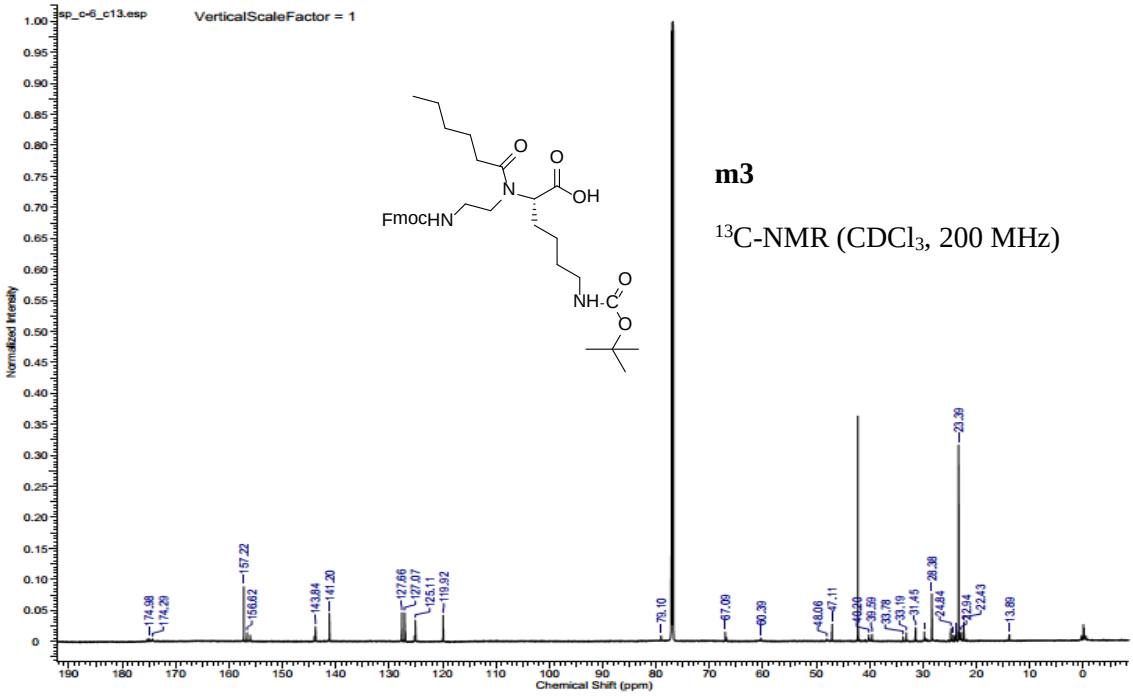
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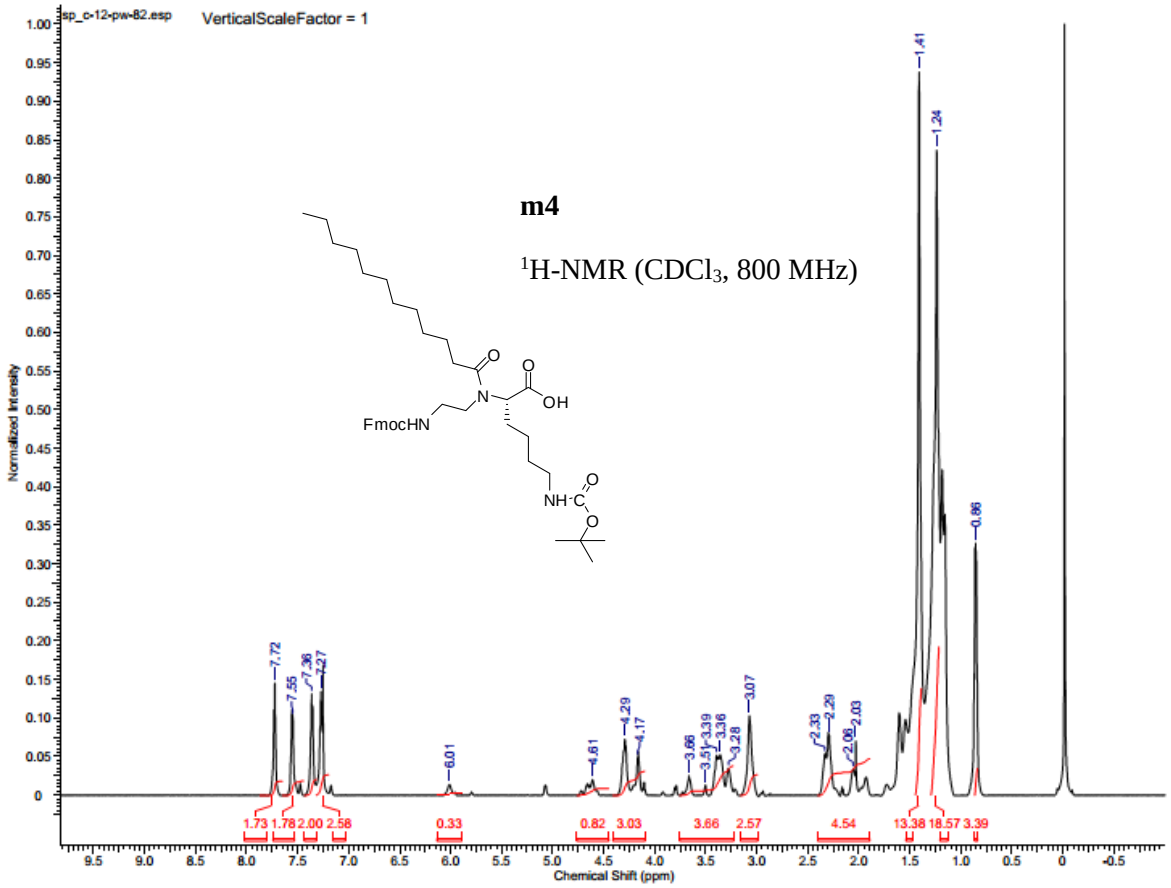
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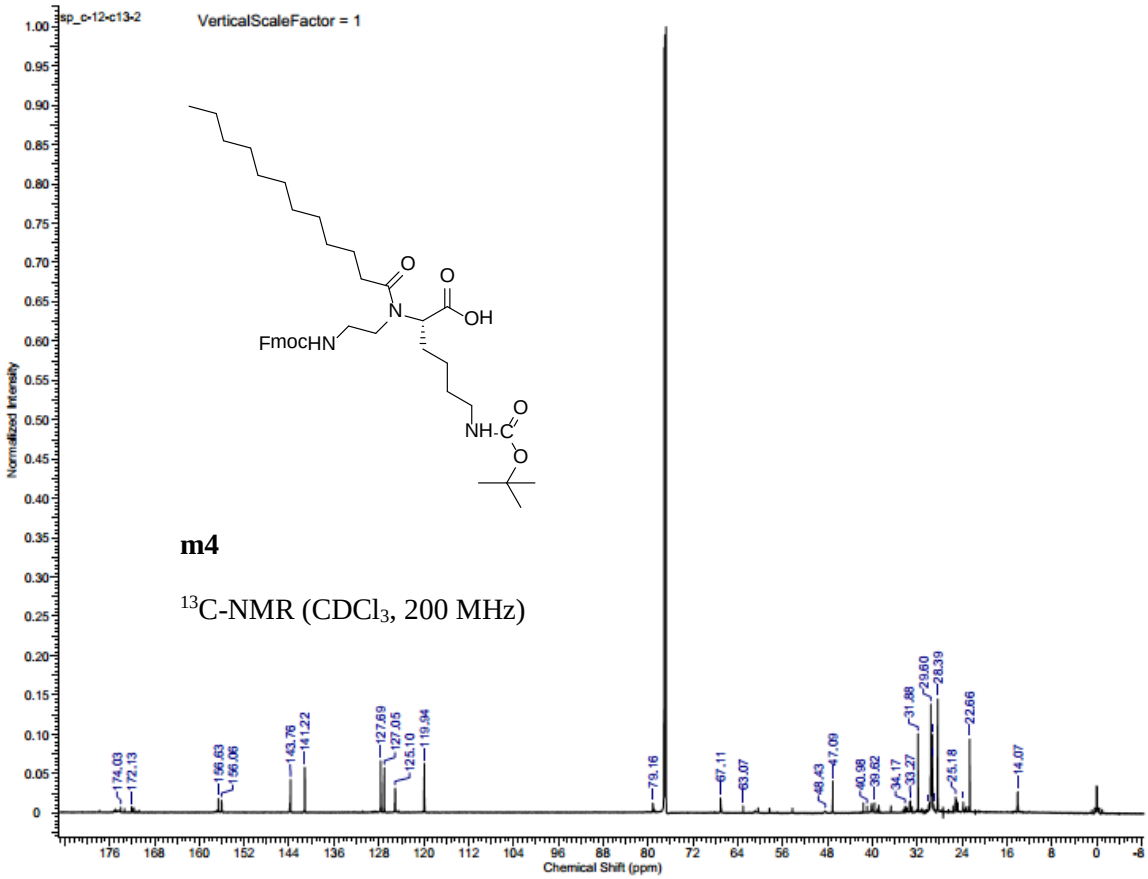
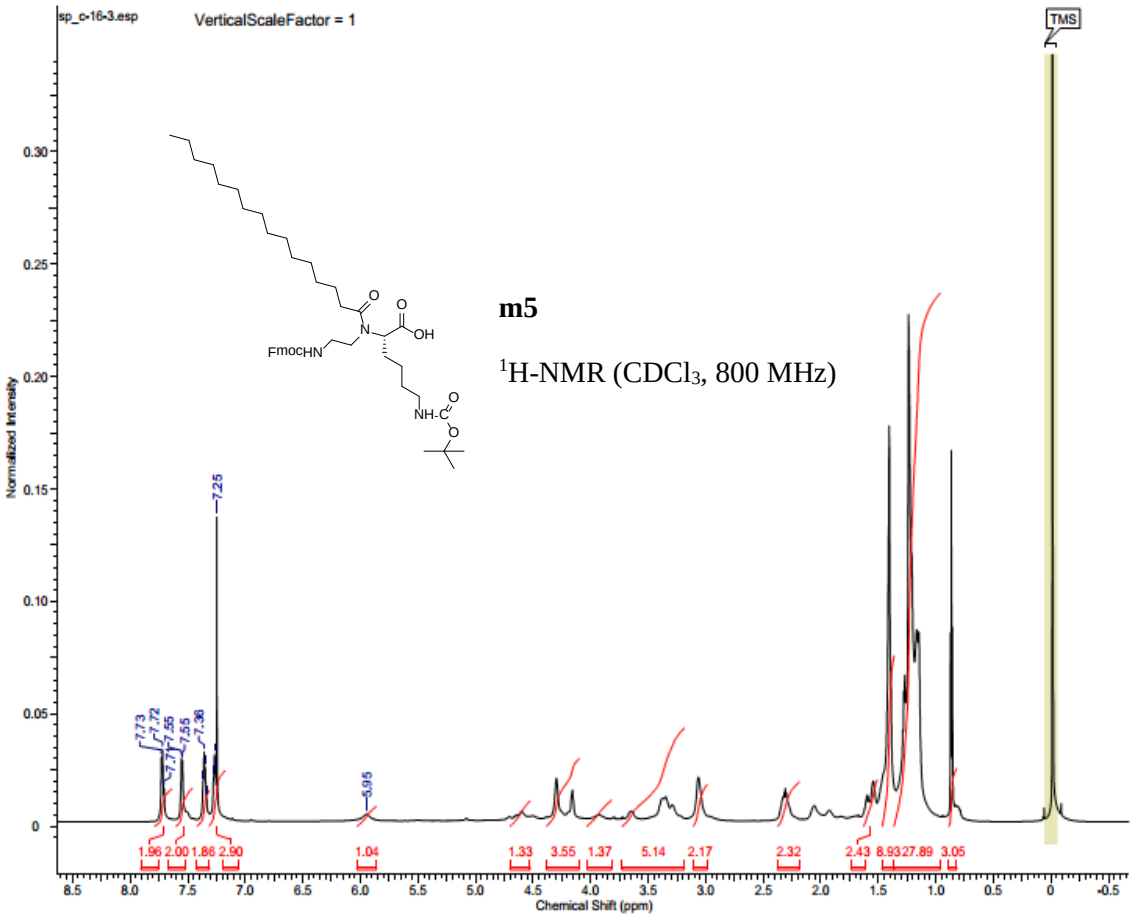
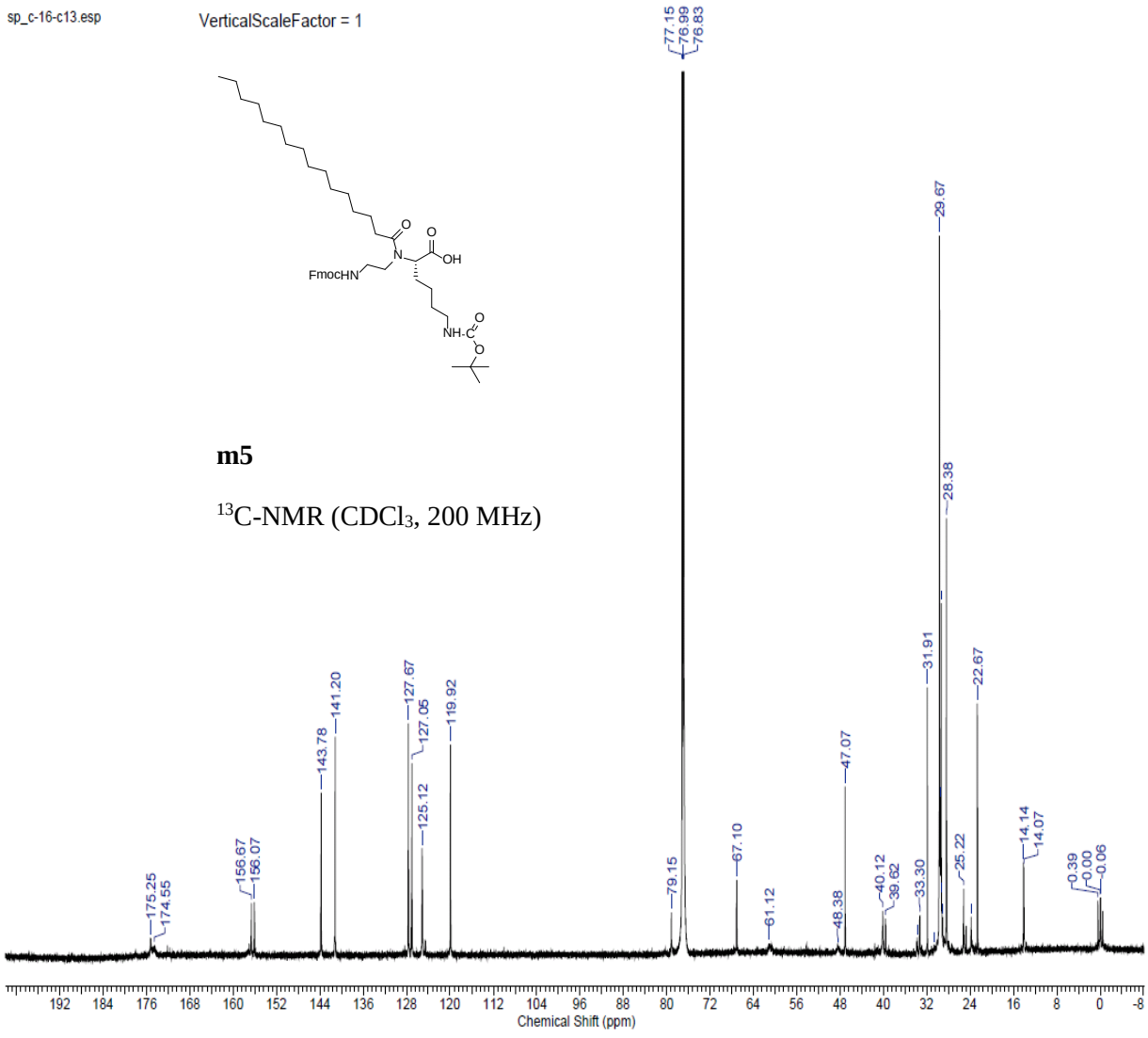
18.57
3.39


sp_c-12-c13-2
VerticalScaleFactor = 1
Normalized Intensity
Chemical Shift (ppm)
FmocHN
OH
NH-C
O
m4
13C-NMR (CDCl3, 200 MHz)
174.03
172.13
156.63
156.06
143.76
141.22
127.69
127.05
125.10
119.94
79.16
67.11
63.07
48.43
47.09
40.98
39.62
34.17
33.27
31.88
29.60
28.39
25.18
22.66
14.07


sp_c-16-3.esp
VerticalScaleFactor = 1
TMS
m5
^{1}H-NMR (CDCl$_3$, 800 MHz)
FmocHN
OH
NH-C
Normalized Intensity
Chemical Shift (ppm)
7.73
7.72
7.55
7.36
7.25
5.95
1.96 2.00 1.86 2.90
1.04
1.33 3.55 1.37 5.14 2.17
2.32
2.43 8.93 27.89 3.05


sp_c-16-c13.esp
VerticalScaleFactor = 1
FmocHN
OH
NH
m5
13C-NMR (CDCl3, 200 MHz)
77.15
76.99
76.83
29.67
28.38
31.91
22.67
127.67
127.05
125.12
119.92
143.78
141.20
47.07
67.10
79.15
61.12
48.38
40.12
39.62
33.30
25.22
14.14
14.07
175.25
174.55
156.67
156.07
0.39
0.00
-0.06
Chemical Shift (ppm)