Lipidated cyclic γ-AApeptides display both antimicrobial and anti-inflammatory activity

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Contents:

1. General experimental methods.

Rink amide MBHA resins (200-400 mesh, 0.7 mmol/g) were purchased from Chem-Impex Int'l Inc. Other chemicals were ordered from either Sigma-Aldrich or Fisher Scientific, and used without further purification. ^{1}H NMR spectra of the building blocks were obtained on an Agilent DD800 instrument. The solid phase syntheses of the lipidated cyclic γ-AApeptides were carried out in a peptide reaction vessel on a Burrell Wrist-Action shaker, were analyzed and purified using an analytical and preparative Waters HPLC system, respectively. The final products were dried in a Labcono lyophilizer. Molar masses were identified using a Bruker AutoFlex MALDI-TOF mass spectrometer.

2. Synthesis and characterization of the lipidated cyclic γ-AApeptides building blocks.

The building blocks (Figure S1) used for the preparation of lipidated γ-AApeptides were synthesized following previous reported protocols.(*1-7*) Compounds **2**, **3** and **4** have been previously reported.(*1-7*) The ^{1}H and ^{13}C NMR spectra of structures **1**, **5** and **6** are obtained and shown below.

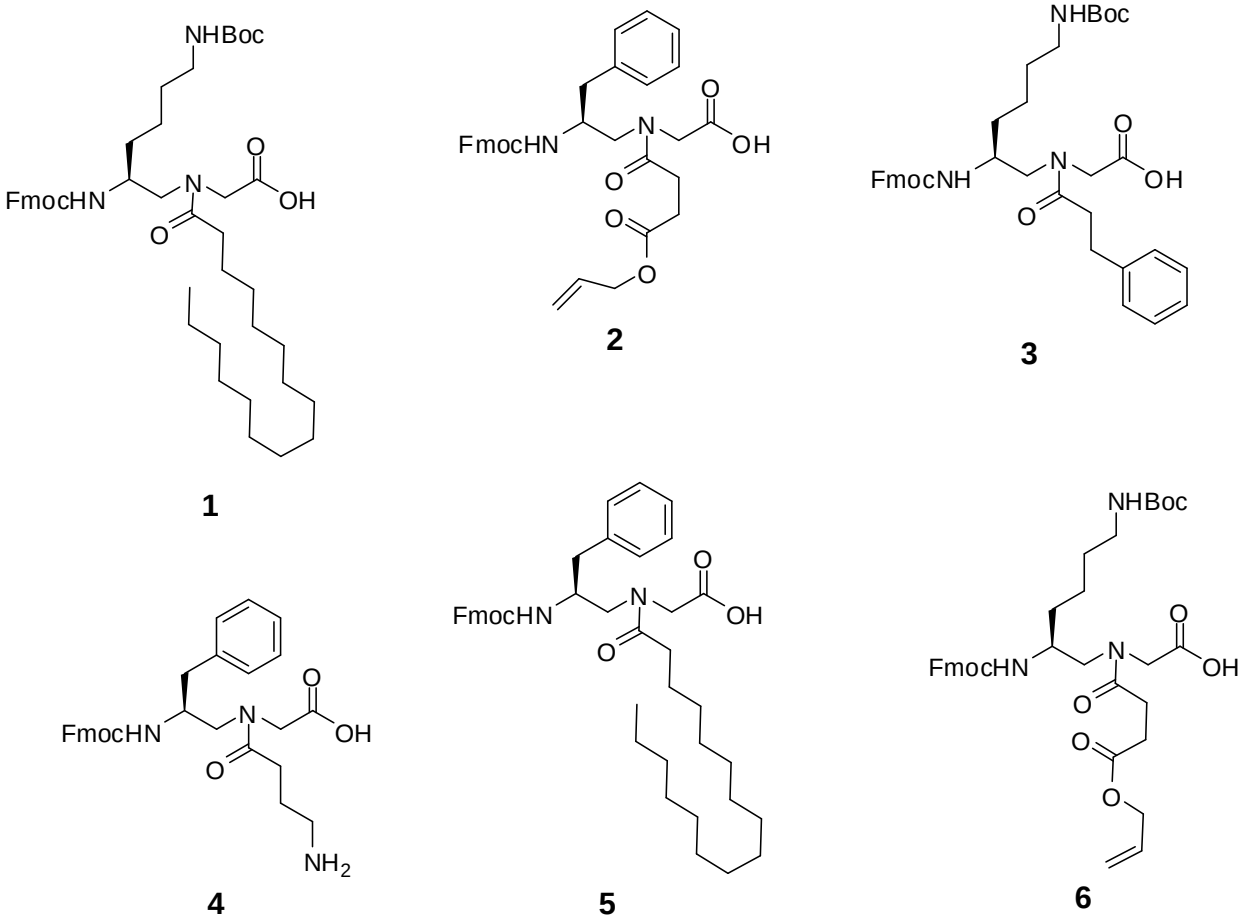


Figure S1. Lipidated cyclic γ-AApeptide building blocks

Compound 1. Yield 60%. ^{1}H NMR ($CDCl_3$, 800 MHz) δ (two rotamers) 7.71 (d, *J* = 8 Hz, 2H), 7.55 (d, *J* = 8 Hz, 2H), 7.35 (t, *J* = 8 Hz, 2H), 7.27 (t, *J* = 8 Hz, 2H), 4.70-4.26 (m, 3H), 4.14-4.02 (m, 2H), 3.85-3.33 (m, 3H), 3.05 -2.99 (m, 2H), 2.33-2.17 (m, 2H), 1.52-1.40 (m, 15H), 1.24-1.15 (m, 24H) ppm. ^{13}C NMR ($CDCl_3$, 200 MHz) δ (two rotamers) 175.3, 156.9, 156.2, 143.9, 143.8, 141.2 127.6, 127.0, 125.2,

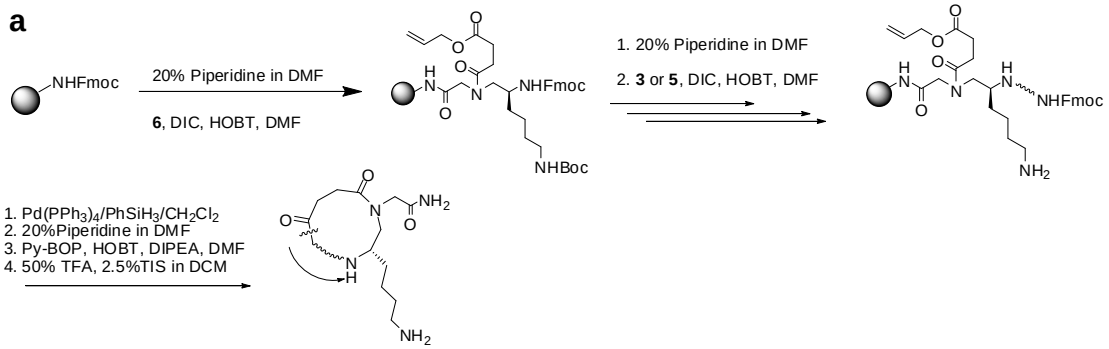
119.9, 79.2, 66.9, 66.7, 50.5, 50.2, 47.2, 47.1, 40.2, 33.2, 32.5, 31.9, 29.7, 29.6, 29.4, 28.4, 25.1, 22.7, 14.2, 14.1 ppm. HR-ESI: $[M+H]^+$ calc: 750.5052, found: 750.5069.

Compound 5. Yield 60%. ^{1}H NMR ($CDCl_3$, 800 MHz) δ (two rotamers) 7.74 (t, *J* = 8 Hz, 2H), 7.52-7.45 (m, 2H), 7.38 (t, *J* = 8 Hz, 2H), 7.29-7.15 (m, 7H), 4.33 (t, *J* = 8 Hz, 1H), 4.22-4.12 (m, 2H), 4.06-3.99 (m, 3H), 3.75-3.24 (m, 2H), 2.97-2.76 (m, 2H), 2.21-2.17 (m, 2H), 1.54-1.52 (m, 2H), 1.24-1.16 (m, 24H), 0.88 (t, *J* = 8 Hz, 3H) ppm. ^{13}C NMR ($CDCl_3$, 200 MHz) δ (two rotamers) 175.7, 175.6, 172.6, 171.9, 156.8, 155.9, 143.8, 143.7, 143.5, 141.2, 141.1, 137.1, 136.9, 131.2, 128.6, 127.0, 125.3, 125.2, 125.0, 124.9, 120.0, 119.9, 52.6, 52.3, 51.8, 50.4, 49.0, 46.9, 38.7, 38.0, 33.1, 32.6, 31.9, 29.7, 29.6, 29.4, 25.0, 22.7, 14.2 ppm. HR-ESI: $[M+H]^+$ calc: 669.4262, found: 669.4269.

Compound 6. Yield 60%. ^{1}H NMR ($CDCl_3$, 800 MHz) δ (two rotamers) 8.65 (s, 2H), 7.75 (d, *J* = 8 Hz, 2H), 7.57 (d, *J* = 8 Hz, 2H), 7.38 (t, *J* = 8 Hz, 2H), 7.30 (t, *J* = 8Hz, 2H), 5.89-5.81 (m, 1H), 5.29-5.25 (m, 1H), 5.21-5.18 (m, 1H), 4.52 (d, J = 8 Hz, 2H), 4.35-4.12 (m, 3H), 3.76 (s, 2H), 3.64-3.39 (m, 1H), 3.28-2.97 (m, 4H), 2.73-2.44 (m, 4H), 1.57-1.42 (m, 15H) ppm. ^{13}C NMR ($CDCl_3$, 200 MHz) δ (two rotamers) 173.7, 173.2, 172.9, 172.8, 172.7, 159.9, 159.6, 158.6, 157.0, 156.7, 156.5, 143.8, 143.7, 143.5, 143.4, 141.2, 131.9, 131.8, 127.8, 127.1, 127.0, 125.2, 125.0, 124.9, 120.0, 119.9, 118.6, 118.5, 118.1, 67.6, 67.3, 65.5, 52.0, 51.4, 51.1, 50.6, 50.2, 47.0, 40.7, 39.9, 32.1, 31.3, 29.4, 29.1, 28.3, 27.8, 27.5, 22.6 ppm. HR-ESI: $[M+H]^+$ calc: 652.3229, found: 652.3255.

3. Solid phase synthesis, purification and characterization of lipidated cyclic γ-AA peptides.

The syntheses of our lipidated cyclic γ-AA peptides are based on building blocks strategy as reported previously.(*8*, *9*) For each coupling cycle, 20% Piperidine in DMF was used to remove the Fmoc protecting group, followed by the coupling of 1.5 equiv of building blocks with 4 equiv of HOBT (1-hydroxybenzotriazole monohydrate)/DIC (diisopropylcarbodiimide) in DMF for 6 h. The allyl group was removed by 0.2 equiv of $Pd(PPh_3)_4$ in the presence of 10 equiv of $PhSiH_3/CH_2Cl_2$ (2 h for each, repeated twice). The exposed carboxyl group reacted with the N-terminus of the sequence to complete the cyclization using PyBOP/HOBT/DIPEA/DMF. The lipidated γ-peptides was cleaved from solid support in 50:48:2 TFA/CH_2Cl_2/TIS (triisopropylsilane) for 2 h. The solvent was evaporated and the sequences were analyzed and purified using a Waters HPLC system monitored at 215 nm. The desired fractions were collected and lyophilized, and their molecular weights were confirmed by the Bruker AutoFlex MALDI-TOF mass spectrometer (Table S1).



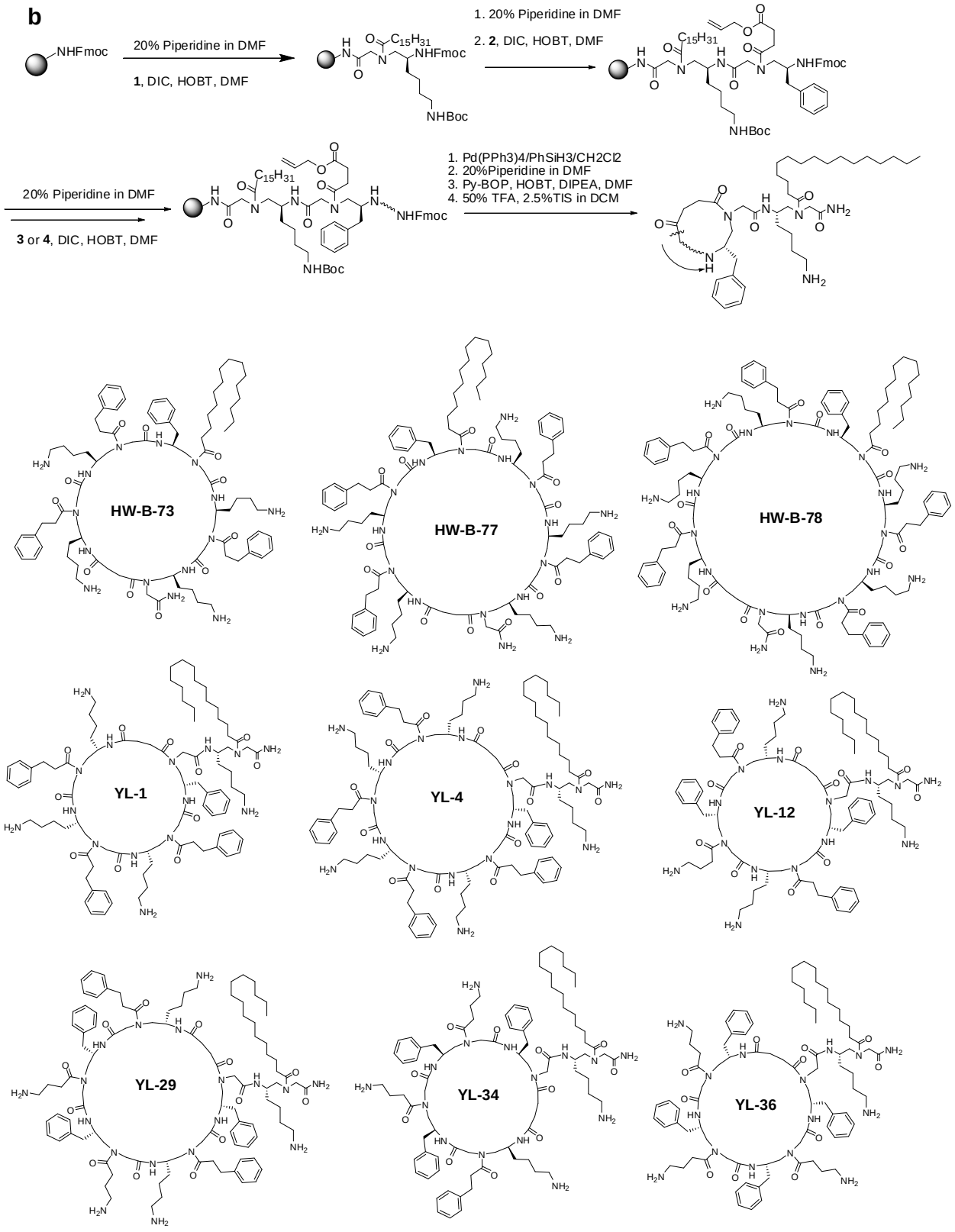
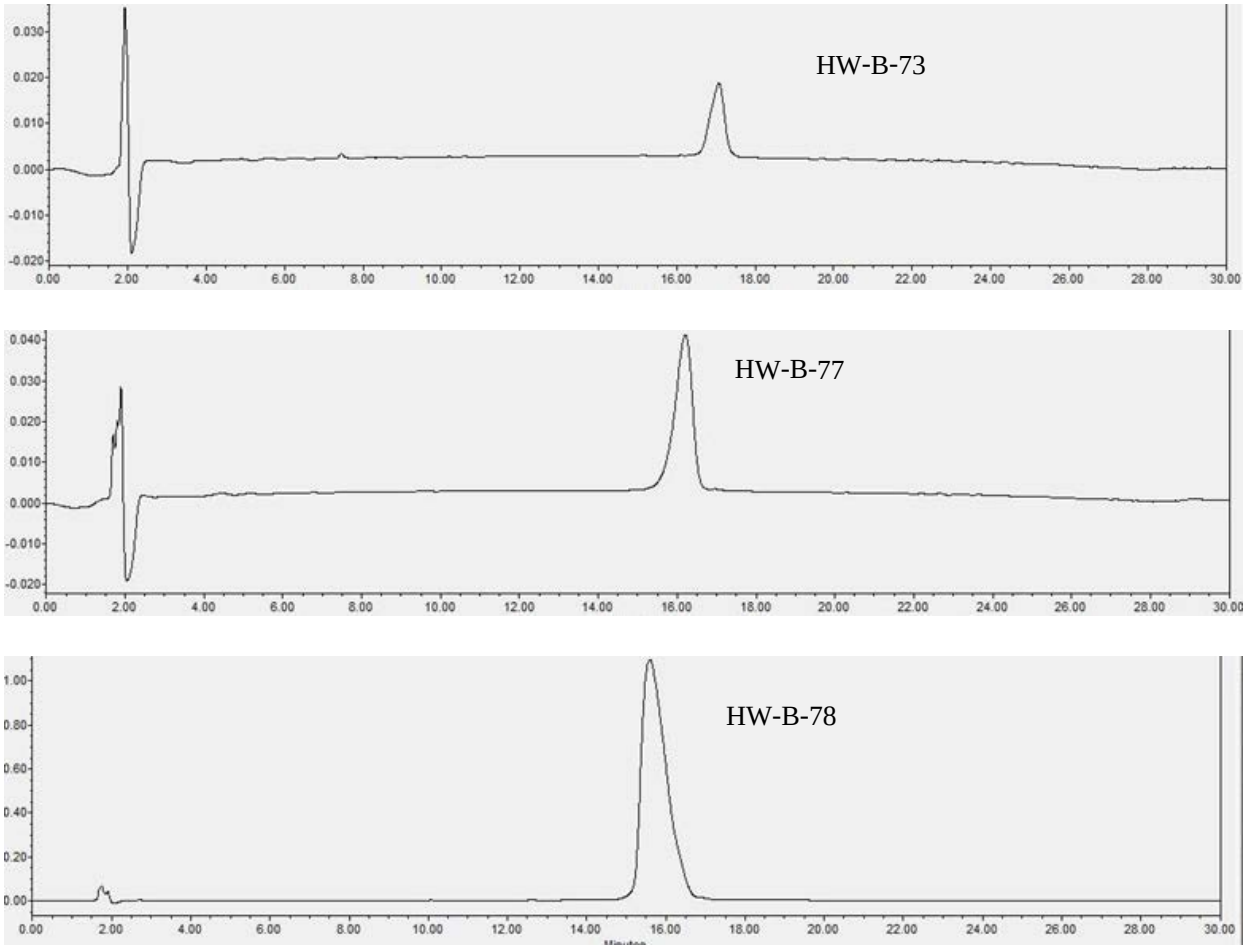


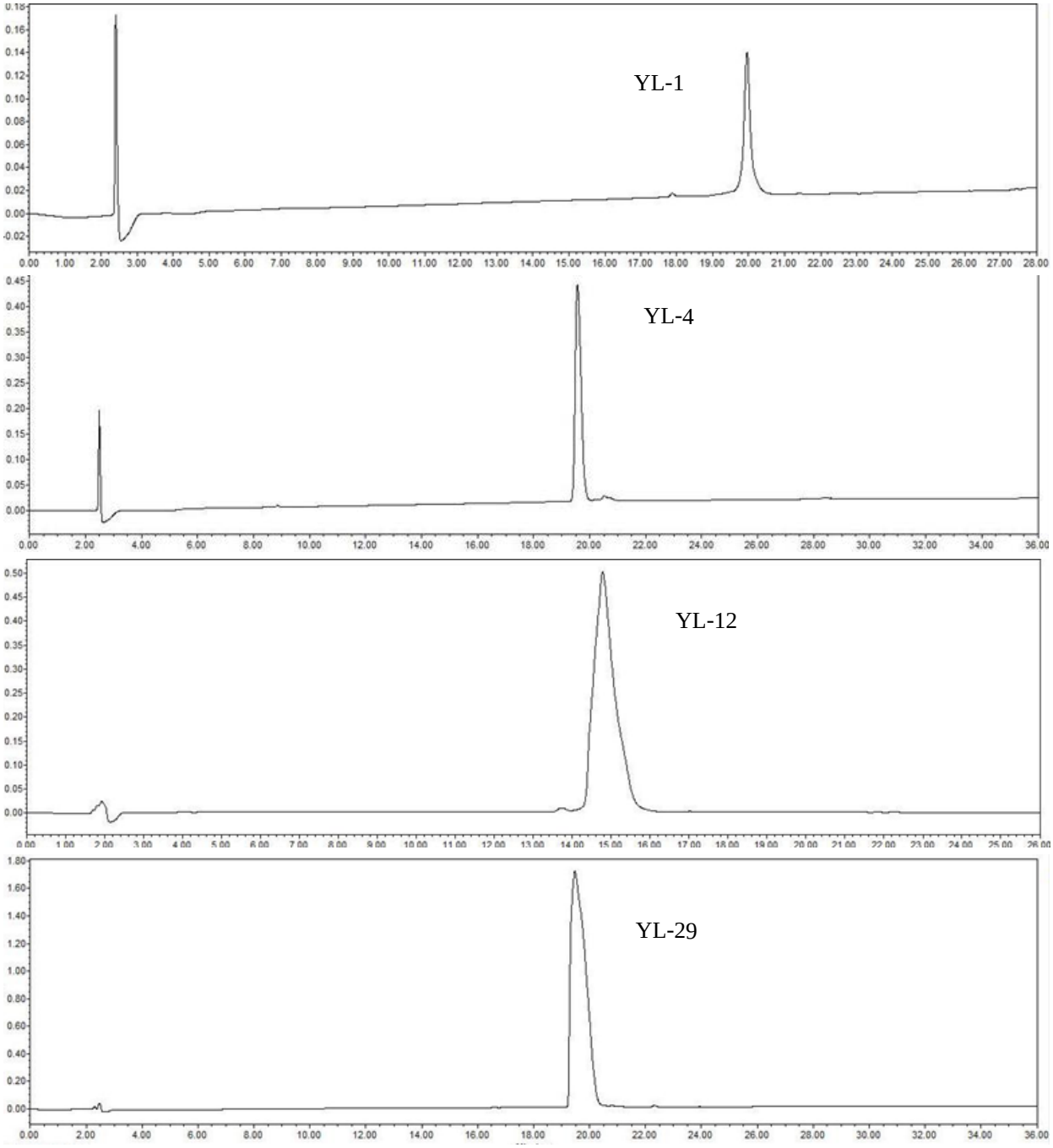
Figure S2. The synthesis and structures of lipidated cyclic γ-AApeptides. **a**, synthesis of HW-B-73, HW-B-77, and HW-B-78; **b**, synthesis of YL-1, YL-4, YL-12, YL-29, YL-34 and YL-36.

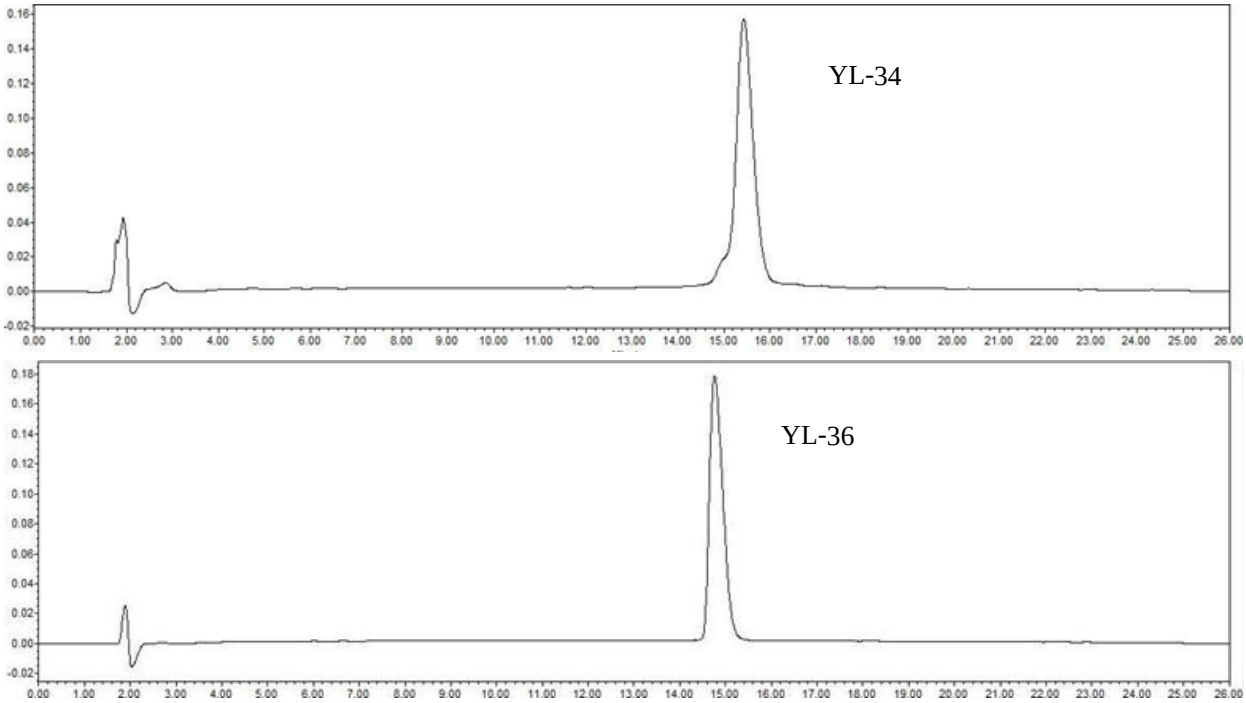
Table S1. MALDI analysis of lipidated cyclic γ-AA peptides.

Lipidated Cyclic γ-AA peptides	Yield (based on resin loading)	Molecular Weight (Actual)	Molecular Weight (found)
HW-B-73	8.5%	1608.11	1609.388 ($M+H^+$)
HW-B-77	8.0%	1911.29	1912.755 ($M+H^+$)
HW-B-78	8.3%	2214.48	2216.346 ($M+H^+$)
YL-1	12.0%	1608.09	1661.941 ($M+3NH_4^+$)
YL-4	9.8%	1911.29	1965.442 ($M+3NH_4^+$)
YL-12	10.1%	1580.06	1634.593 ($M+3NH_4^+$)
YL-29	9.7%	1827.19	1828.014 ($M+H^+$)
YL-34	11.3%	1552.03	1605.503 ($M+3NH_4^+$)
YL-36	9.7%	1524.00	1525.010 ($M+H^+$)

HPLC traces at 215 nm




YL-1
YL-4
YL-12
YL-29



4. Antimicrobial assays

The lipidated cyclic γ-AApeptides were tested for their antimicrobial activity against various microbial organisms including *E. coli* (ATCC 25922), *K. pneumonia* (ATCC 13383), multi-drug resistant *P. aeruginosa* (ATCC 27853), Methicillin-resistant *S. epidermidis* (MRSE, RP62A), Vancomycin-resistant *E. faecalis* (ATCC 700802), Methicillin-resistant *S. aureus* (ATCC 33592). The highest concentration of the tested AA-peptides was 25 μg/mL. The bacteria in 5 mL of medium were grown at 37 ºC overnight and then diluted to make a suspension of approximate 1×10^6 CFU/mL. Aliquots of 50 μL of bacterial suspension were mixed with 50 μL of medium containing different concentrations of lipidated cyclic γ-AA-peptides. The plate was incubated at 37 ºC overnight with cell growth monitored by a Biotek Synergy HT microtiter plate reader under the 600 nm wavelength. MIC was determined when the lowest concentration of the compounds inhibit the cell growth completely in 24 h. The results were repeated at least three times with duplicates for each time.

5. Hemolysis assay

Freshly drawn, K_2 EDTA treated human red blood cells (hRBCs) were washed with PBS buffer twice and centrifuged at 1000g for 10 min. After the clear supernatant was removed, the cell pellets were mixed with serial diluted lipo-cyclic γ-AApeptides in a 96-well plate. The plate was incubated at 37 ºC for 1 h and centrifuged at 3500 rpm for 10 min. The supernatant was separated and diluted in PBS, and the absorbance was detected at 360 nm using a Biotek Synergy TH plate reader. % hemolysis = $(Abs_{sample} - Abs_{PBS})/(Abs_{Triton} - Abs_{PBS}) \times 100\%$. 0% hemolysis (negative control) was determined by mixing blood with PBS and 100% hemolysis (positive control) was determined by mixing blood with Triton X-100 (final concentration 0.1%). The results were repeated at least three times with duplicates for each time.

6. Fluorescence microscopy

DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride, Sigma, >98%) and PI (Propidium iodide, Sigma) were used to stain the bacteria cells of *E. coli* or *S. aureus*. DAPI is a DNA binding dye staining all bacterial cells regardless of their viabilities, and PI is an ethidium derivative which only can pass through damaged bacterial membranes and intercalates with their nucleic acids. Briefly, bacteria in mid-logarithmic phase were incubated with lipidated cyclic γ-AA peptides (2 × MIC) for 2 h, and then were centrifuged at 3000g for 15 min. The bacteria cell pellets were separated then incubated with PI, followed by washing and incubation with DAPI (each dye incubation was performed at 0 ºC for 15 minutes in dark). Controls were bacteria culture without peptides following the same procedure described above. The stained bacteria cells were observed under Zeiss Axio Imager Zloptical microscope using the 100X oil-immersion objective.

7. Fluorescent Detection of Nitric Oxide

Raw 264.7 (Mouse leukaemic monocyte macrophage cell line) cells were grown in RPMI 1640 medium containing 1% L-glutamine, 1% Penicillin/streptomycin and 10% fetal bovine serum (FBS). Cells were plated in a 96-well plate at 75,000 cells/well in complete RPMI 1640 medium, and allowed to grow overnight at 37°C and 5% CO_2 in a humidified incubator. The media was removed, and cells were placed in unsupplemented RPMI 1640 medium. 20 ng/mL LPS and the appropriate concentration of lipidated cyclic γ-AApeptides (20 mM stock solutions in PBS) were added to a final volume of 200 µL. PBS controls were included in each experiment. Plates were then incubated for 24 h, and then 100 µL of media was transferred to a flat black 96-well microfluor plate (Thermo Scientific, MA, USA). Following that, 10 µL of 0.05 mg/mL 2,3-diaminonamthalene in 0.62 M HCl was added to the media and incubated for 20 minutes 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 EC_{50} values were calculated graphically using OriginPro v8.6 software.

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) in the presence of 1% Penicillin/streptomycin, 10% fetal bovine serum (FBS), and 1% L-glutamine. HEK293 cells are stably transfected with human TLR4, as well as the required accessory proteins MD-2 and CD14. Moreover, the cells also possess an optimized alkaline phosphatase reporter gene under the control of a NF-κB inducible promoter. (*10*) Cells were first plated in a 96-well plate at 40,000 cells/well in complete DMEM medium and allow to grow overnight at 37°C and 5% CO_2 in a humidified incubator. Then the media was removed, and cells were placed in Optimem + 0.5% FBS medium. 20 ng/mL LPS and the appropriate concentration of lipidated cyclic γ-AApeptides were added to a final volume of 200 µL. PBS buffer was included as control. Plates were then incubated for 24 h, and then 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 EC_{50} values were calculated graphically using OriginPro v8.6 software.

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. Cells were plated in a 96-well plate at 75,000 cells/well in complete RPMI 1640 medium and allowed to grow overnight at 37°C and 5% CO_2 in a humidified incubator. Then media was removed, and cells were placed in unsupplemented RPMI 1640 medium. 20 ng/mL LPS and the appropriate concentration of lipidated cyclic γ-AApeptides (20 mM stock solutions) were added to a final volume of 200 μL. PBS was included as the control. Plates were then incubated for 24 hours, and then 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 EC_{50} values were calculated graphically using OriginPro v8.6 software.

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. Cells were fixed for 20 minutes in 4% paraformaldehyde after the media was removed. After fixing, formaldehyde was removed and cells were incubated for 1h with 0.05% crystal violet stain. After incubation, cells were rinsed with deionized water and reconstituted in 100% methanol for 10 minutes. 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 $_{535\ nm}$)].

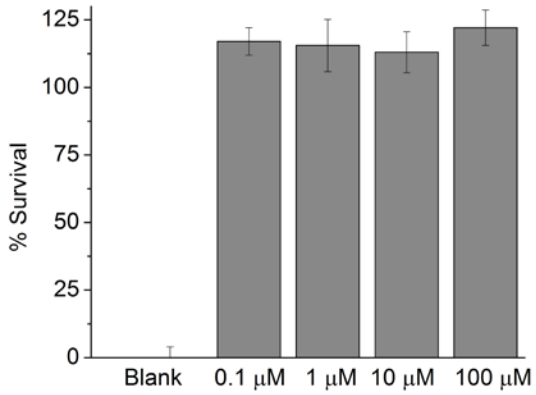


Figure S3. YL-36 is non-toxic with treatments up to 100 μM as demonstrated by a Crystal Violet cell viability assay. Data is normalized with untreated cells as 100% survival, and blank wells as 0% survival.

References

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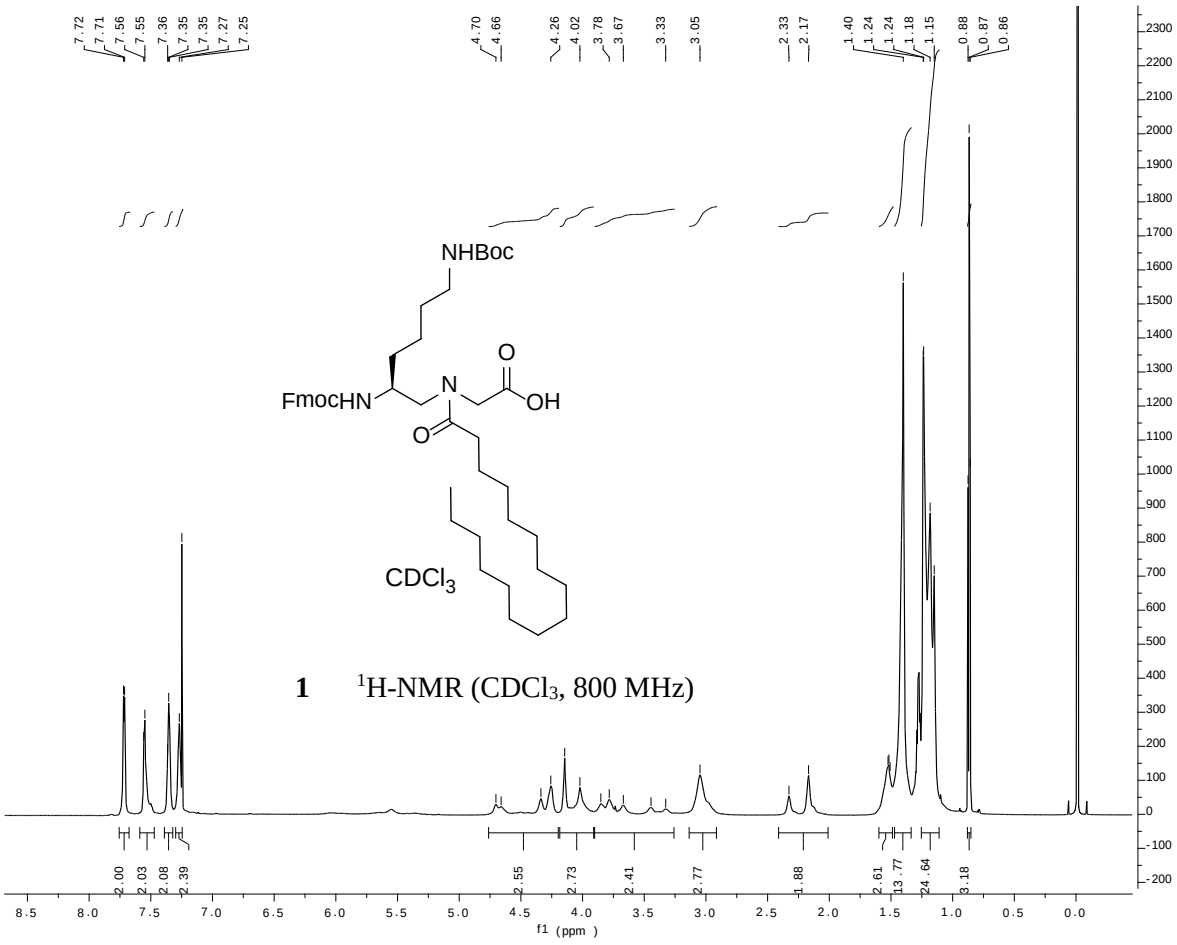

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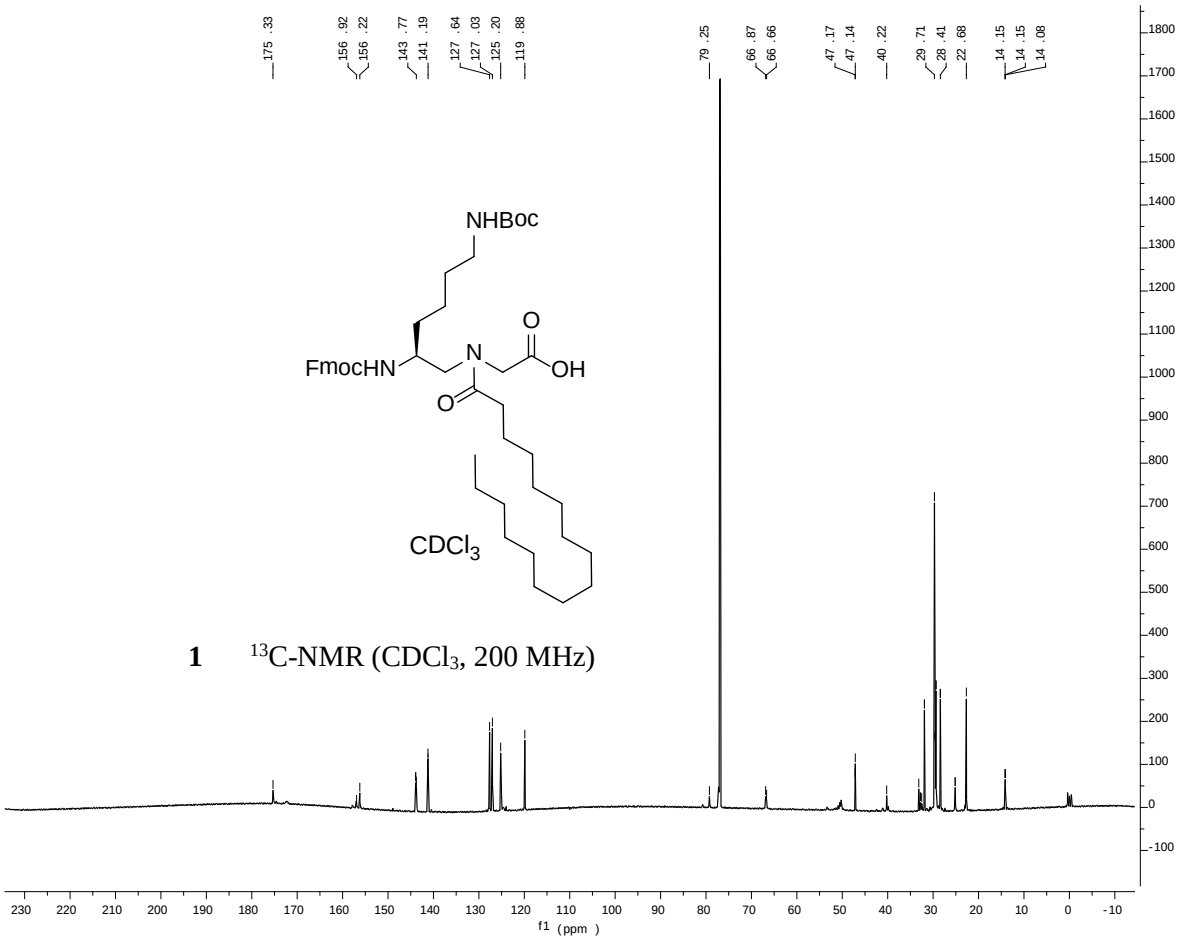

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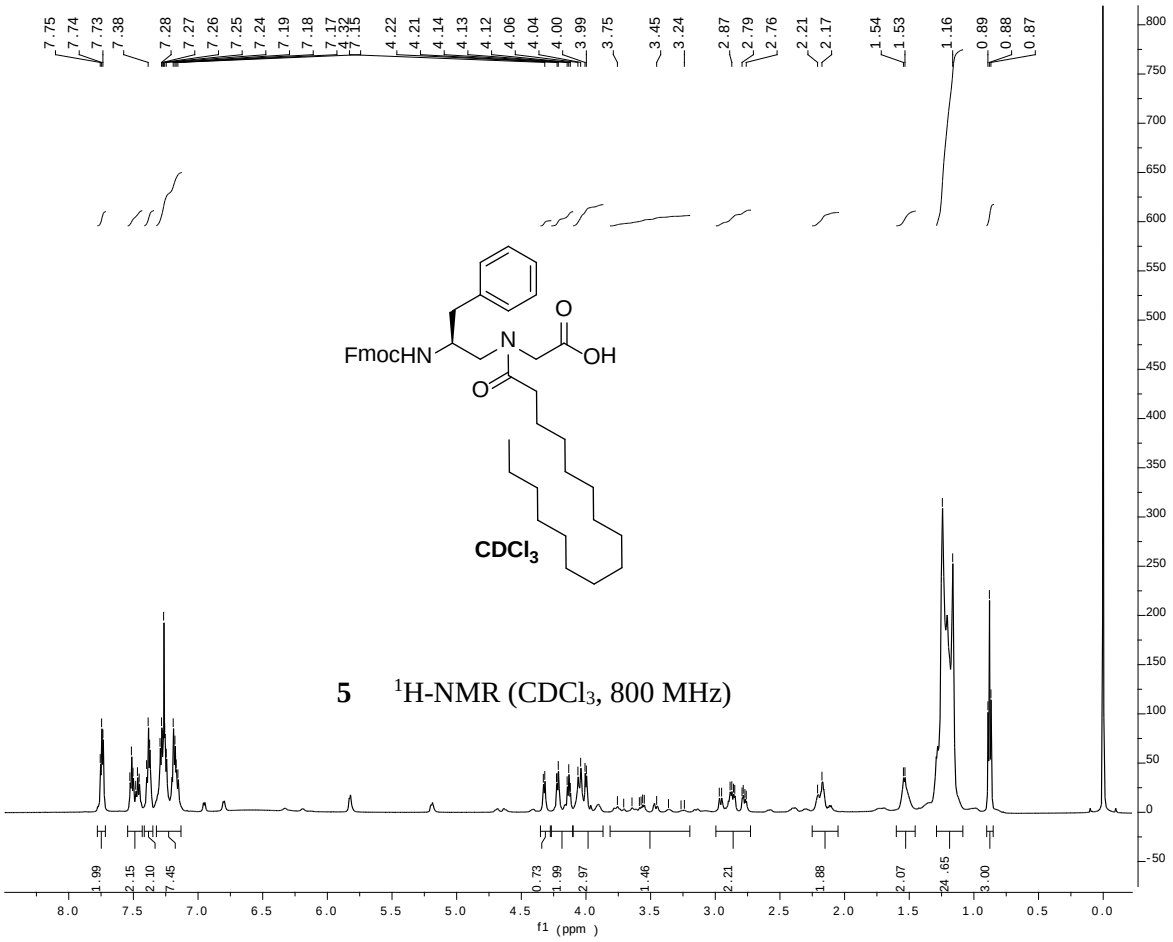

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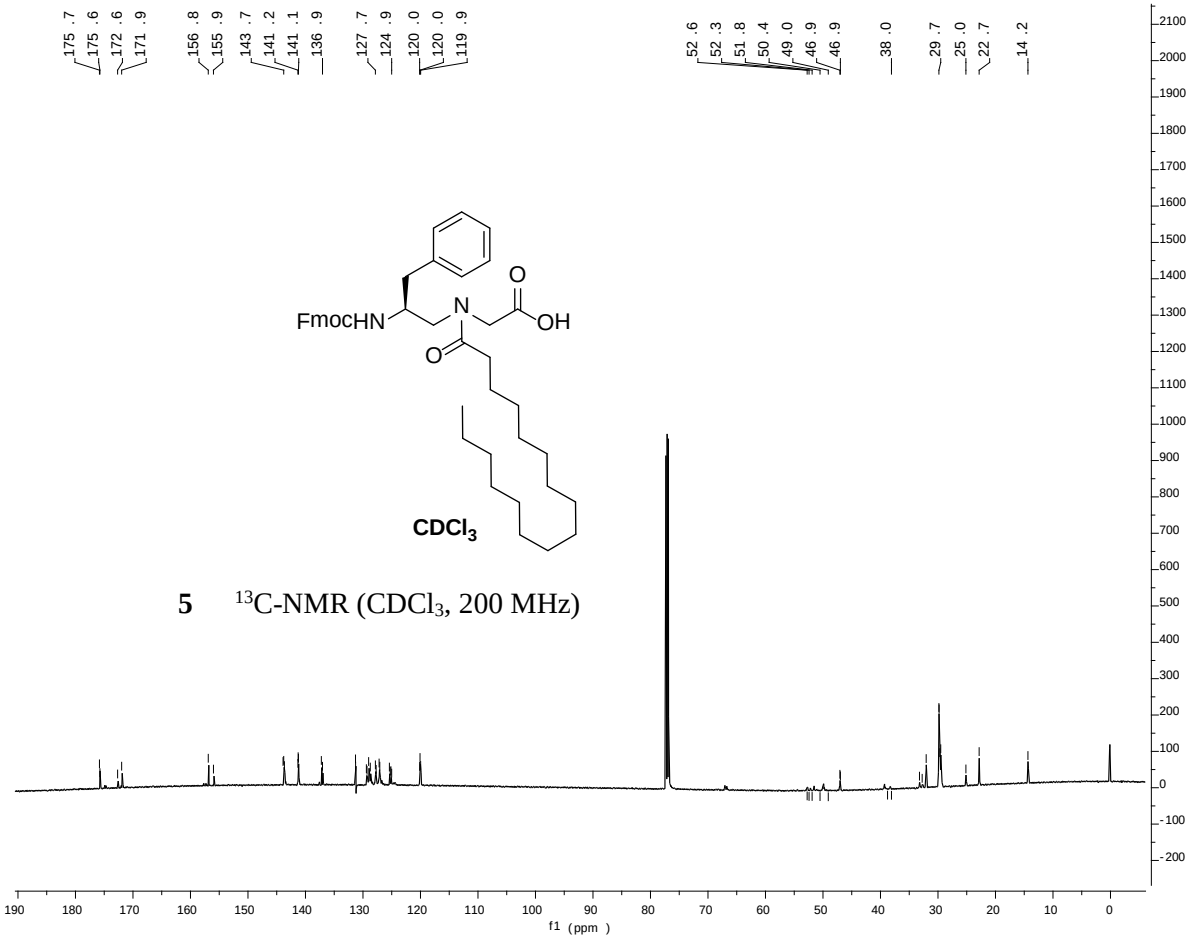

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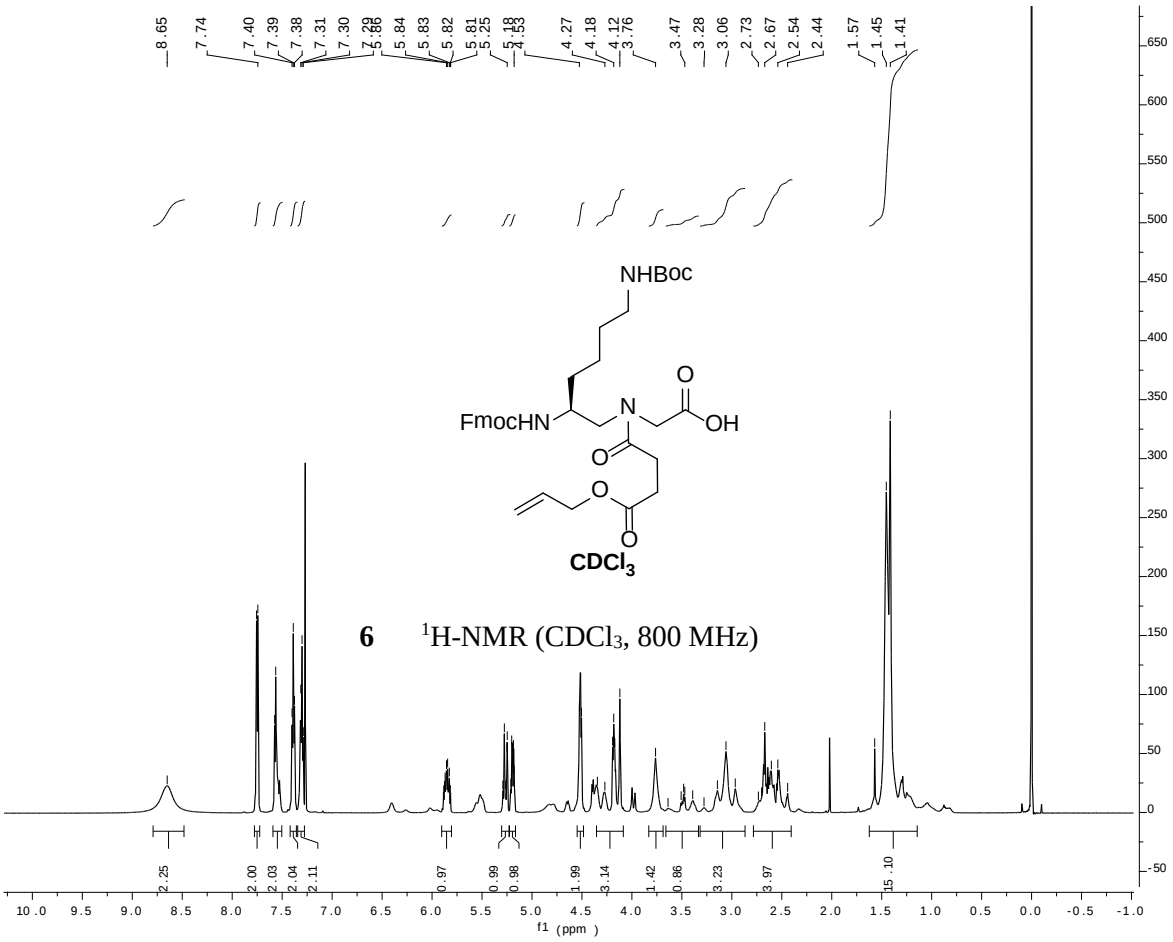
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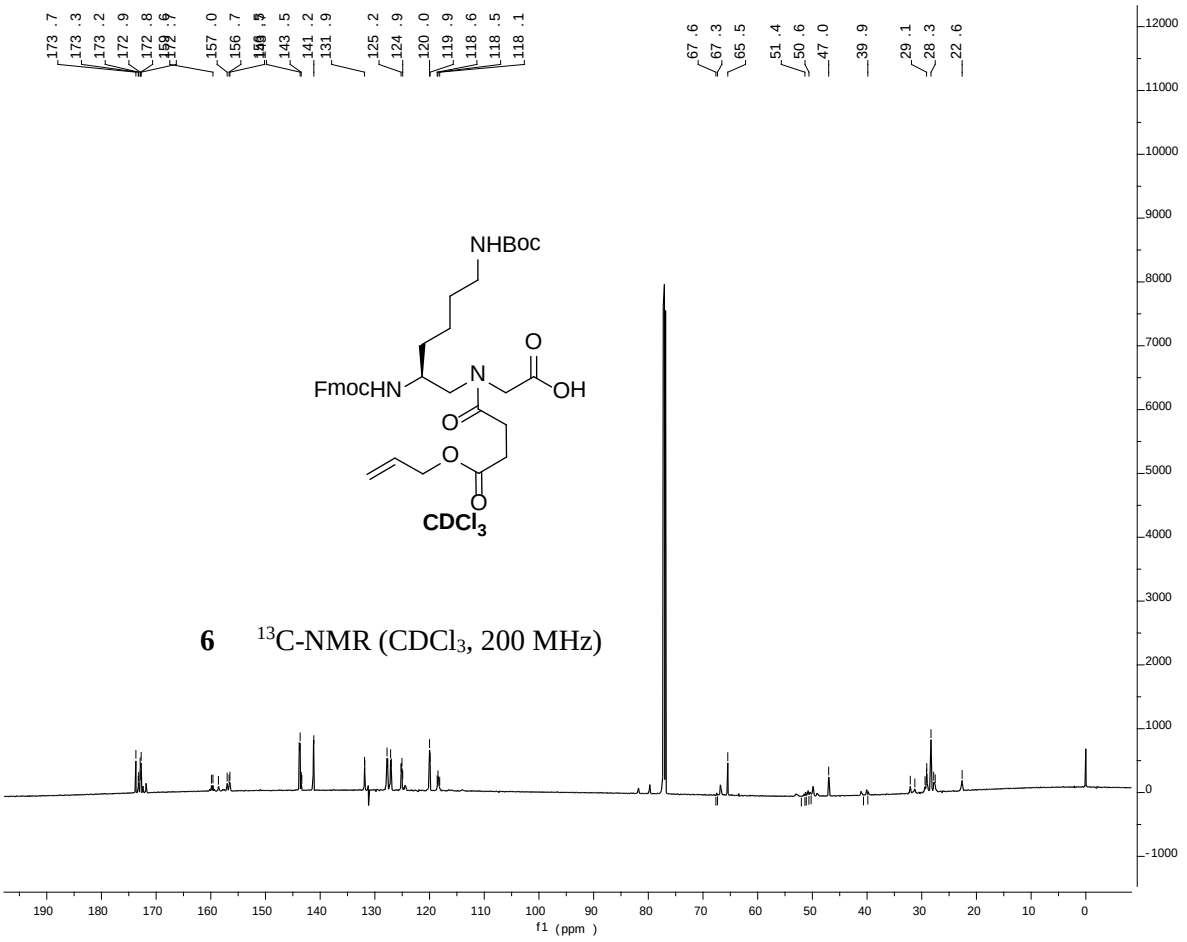

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