Electronic Supplementary Material (ESI) for Chemical Communications

Identification of γ-AApeptides with potent and broad-spectrum antimicrobial activity

Youhong Niu,[a,1] Shruti Padhee,[a,1] Haifan Wu,[a] Ge Bai,[a] Lacey Harrington,[b] Whitney N. Burda,[b] Lindsey N. Shaw,[b] Chuanhai Cao,[c] and Jianfeng Cai*[a]

[1]These authors contributed to the work equally.

[a]Department of Chemistry, CHE 205, University of South Florida, 4202 E. Fowler Ave., Tampa, FL 33620; [b]Department of Cell Biology, Microbiology and Molecular Biology, University South Florida, 4202 E. Fowler Ave., Tampa, FL 33620; and [c] Dept. of Molecular Pharmacology and Physiology, USF-Health Byrd Alzheimer's Institute, 4001 E. Fletcher Ave., Tampa, FL 33613, USA

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. α-peptides **m1** and **p1** were purchased from USF peptide facility and used without further purification. γ-AApeptide building blocks were synthesized following previously reported procedure. [1] NMR spectra of γ-AApeptide building blocks were obtained on a Varian Inova 400 instrument. γ-AApeptides were prepared on Knorr resin in peptide synthesis vessels on a Burrell Wrist-Action shaker. The 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 γ-AApeptides were identified on a Bruker AutoFlex MALDI-TOF mass spectrometer.

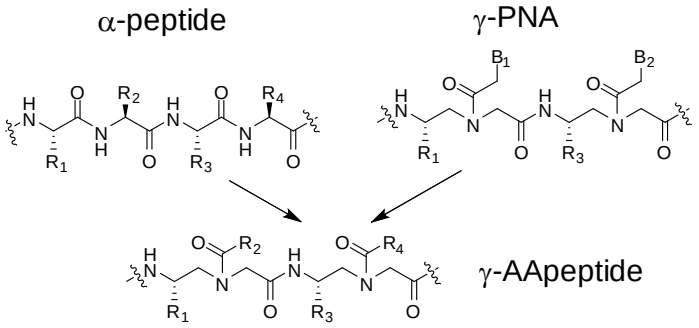


Figure S1. The structure of an α-peptide and the corresponding γ-AApeptide and γ-PNA.

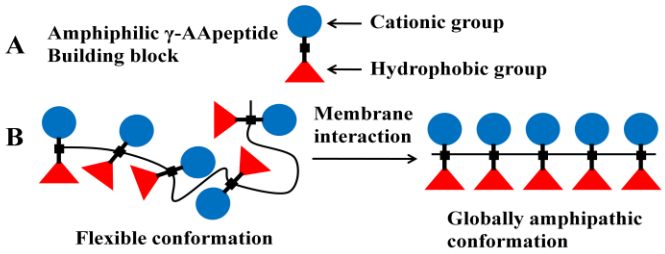
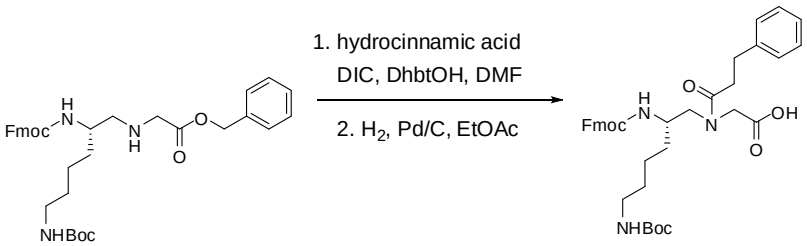


Figure S2. Illustration of antimicrobial γ-AApeptide design. **A**, Basic representation of amphiphilic γ-AApeptide structure; **B**, conformational change of γ-AApeptide upon interaction with bacterial cell membranes.

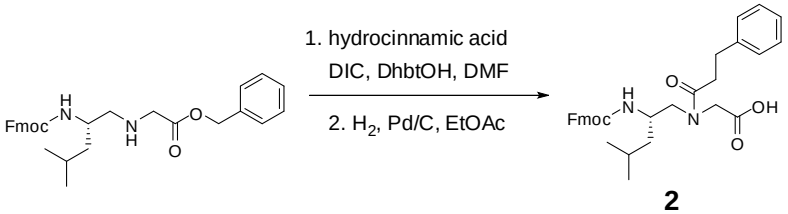
2. Synthesis[1] and characterization of γ-AApeptide building blocks.

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



1

Compound **1**. Yield 65% (two steps). ^{1}H NMR (DMSO-d_6, 400MHz) δ = 7.84 (d, J = 7.2 Hz, 2H), 7.65-7.60 (m, 2H), 7.37 (t, J = 7.6 Hz,2H), 7.27(m, 2H),7.22-7.08 (m, 5H), 6.71 (b, 1H), 4.28-4.02 (m, 3H), 3.98-3.57 (m, 2H), 3.47-3.44(m, 1H), 3.45 (dd, J = 5.2, 13.2 Hz, 1H), 3.04-2.99 (m, 1H), 2.85-2.84 (m, 2H), 2.73-2.53(m, 3H), 2.47-2.31 (m, 1H), 1.33-1.11 (m, 15H). ^{13}C NMR (DMSO-d_6, 100MHz) δ 172.7, 172.3, 156.5, 156.4, 156.0, 144.4, 144.3, 144.28, 144.2, 141.9, 141.8, 141.2, 128.7, 128.61, 128.59, 128.0, 127.5, 127.4, 126.2, 126.1, 125.62, 125.58, 125.5, 120.5, 77.7, 65.7, 65.6, 52.5, 51.1, 50.2, 51.1, 50.11, 50.09, 48.3, 47.3, 41.1, 34.5, 34.0, 32.1, 31.7, 31.2, 31.0, 29.8, 29.7, 28.7, 23.7, 23.3, 23.27. HR-ESI: $[M+H]^+$cacl: 644.3330, found: 644.3338.



2

Compound **2**. Yield 53% (two steps). ^{1}H NMR (DMSO-d_6, 400MHz) δ = 7.83 (d, J = 8.0 Hz, 2H), 7.65-7.59 (m, 2H), 7.38-7.07 (m, 10H), 4.29-4.21 (m,2H), 4.17-4.10 (m, 1H),3.96-3.67 (m, 3H), 3.47-3.2.99 (m,2H), 2.75-2.64(m,2H), 2.63-2.55 (m,1H), 2.42-2.35 (m, 1H),1.53-1.51 (m, 1H), 1.30-1.19 (m, 1H), 1.10-1.07 (m, 1H), 0.83-0.76 (m, 6H). ^{13}C NMR (DMSO-d_6, 100MHz) δ 172.7, 156.4, 56.3, 144.4, 144.3, 144.25, 144.17, 129.3, 128.7, 128.6, 128.57, 128.00, 127.7, 127.5, 127.4, 126.2, 126.1, 125.6, 125.5, 11.8, 120.5, 65.6, 52.7, 51.5, 48.2, 47.3, 41.6, 41.6, 34.7, 34.6, 34.1, 31.3, 31.0, 24.7, 24.9, 23.8, 22.2, 21.9. HR-ESI: $[M+H]^+$cacl: 529.2697, found: 529.2695.

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

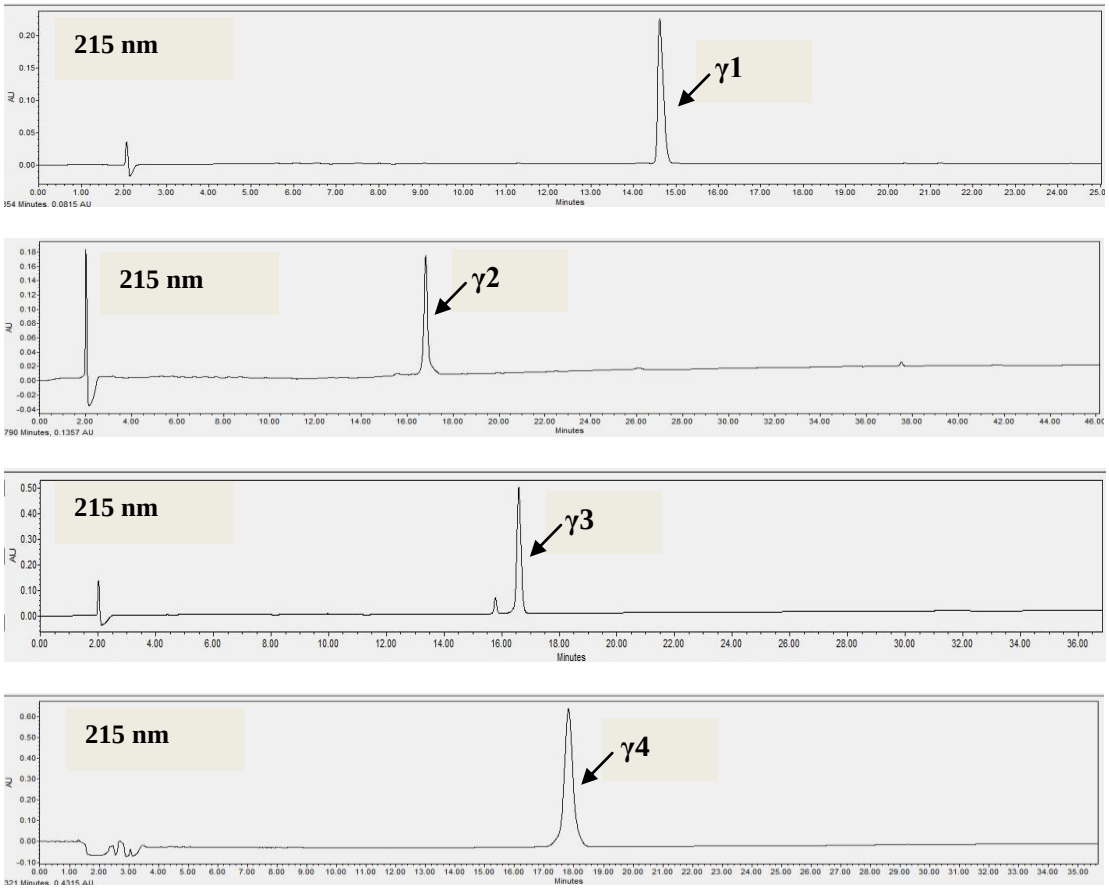
γ-AApeptides were prepared on Knorr resin in peptide synthesis vessels on a Burrell Wrist-Action shaker following standard Fmoc chemistry protocol of solid phase peptide synthesis using synthesized γ-AApeptide building blocks. Each coupling cycle included an Fmoc deprotection using 20% Piperidine in DMF, and 8 h coupling of 1.5 equiv of γ-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 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)

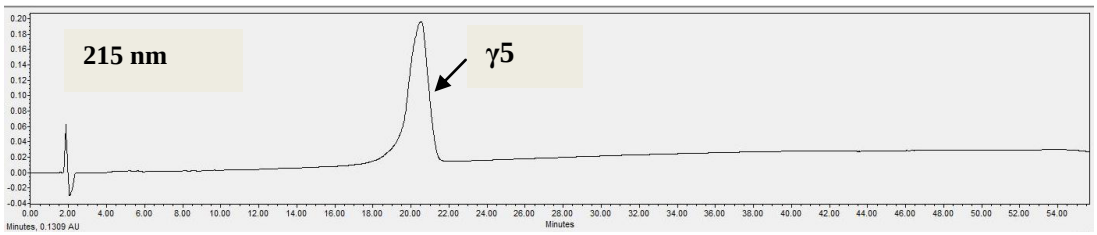
HPLC systems, respectively, using 5% to 100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 40 min, followed by 100% solvent B over 10 min. The HPLC traces were detected at 215 nm. The desired fractions were eluted as single peaks at > 95% purity. They were collected and lyophilized. The molecular weights of γ-AApeptides were obtained on Bruker AutoFlex MALDI-TOF mass spectrometer using α–cyano-4-hydroxy-cinnamic acid.

Table S1. MS analysis of γ-AApeptides (γ1-γ5) and α-peptides (p1 and m1).

γ-AApeptides and α-peptides	molecular weight (Actual)	molecular weight (found)
γ1	927.2	927.4 (MALDI)
γ2	1534.0	1535.0 $(M+H)^+$ (LC-MS)
γ3	2140.8	2141.3 (LC-MS)
γ4	2125.8	2125.8 (MALDI)
γ5	2110.8	2111.7 $(M+H)^+$ (MALDI)

HPLC traces





4. Antimicrobial assays

The microbial organisms used were E. coli (JM109), B. subtilis (BR151), S. epidermidis (RP62A), C. albicans (ATCC 10231), E. faecalis (ATCC 700802), S. aureus (ATCC 33592), K. pneumoniae (ATCC 13383), Methicillin-Resistant S. aureus (MRSA, USA100 lineage), and B. anthracis. 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 100 µg/ml. The antimicrobial activities of the γ-AApeptides were determined in a sterile 96 -well plates by broth micro-dilution method. Bacterial cells 2 and fungi 3 were grown overnight at 37 oC in 5 ml medium, after which a bacterial suspension (approximately 106 CFU/ml) or fungal suspension Candida albicans (ATCC 10231) (approximately 103 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 γ-AApeptides for a total volume of 100 µL in each well. The γ-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 0C 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.

5. Drug resistance study[4]

The initial MIC of γ5 and control antibiotics norfloxacin against *S. aureus* was obtained as described above. Bacteria from duplicate wells at the concentration of one-half MIC were then used to prepare the bacterial dilution (approximately 10^6 CFU/ml) for the next experiment. These bacterial suspensions were then incubated with γ5 and norfloxacin respectively. After incubation at 37°C for 24 h, the new MIC was determined. The experiment was repeated each day for 17 passages.

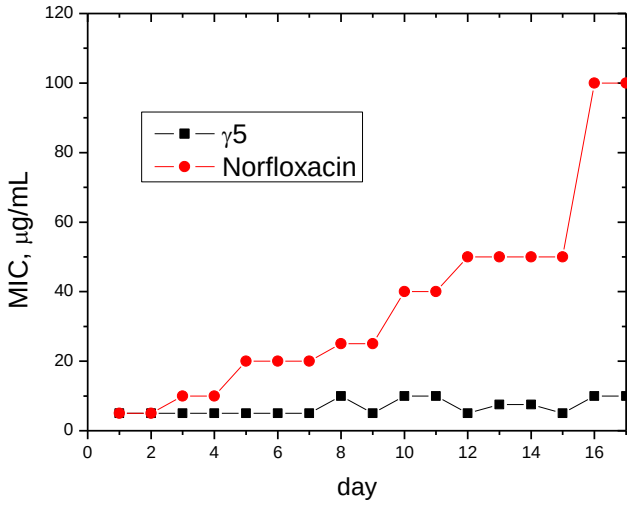


Figure S3. The development of resistance by *S. aureus* ATCC 33592 towards γ5 and norfloxacin.

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 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 γ-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. [2] 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. % hemolysis was determined by the following equation:

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

H50 is the concentration of γ-AApeptide amphiphiles 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 *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. [5] 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 γ-AApeptide γ5 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 °C. 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 °C. 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 γ5. The bacterial cells were then examined by using the Zeiss Axio Imager Z1optical microscope with an oil-immersion objective (100×). [6]

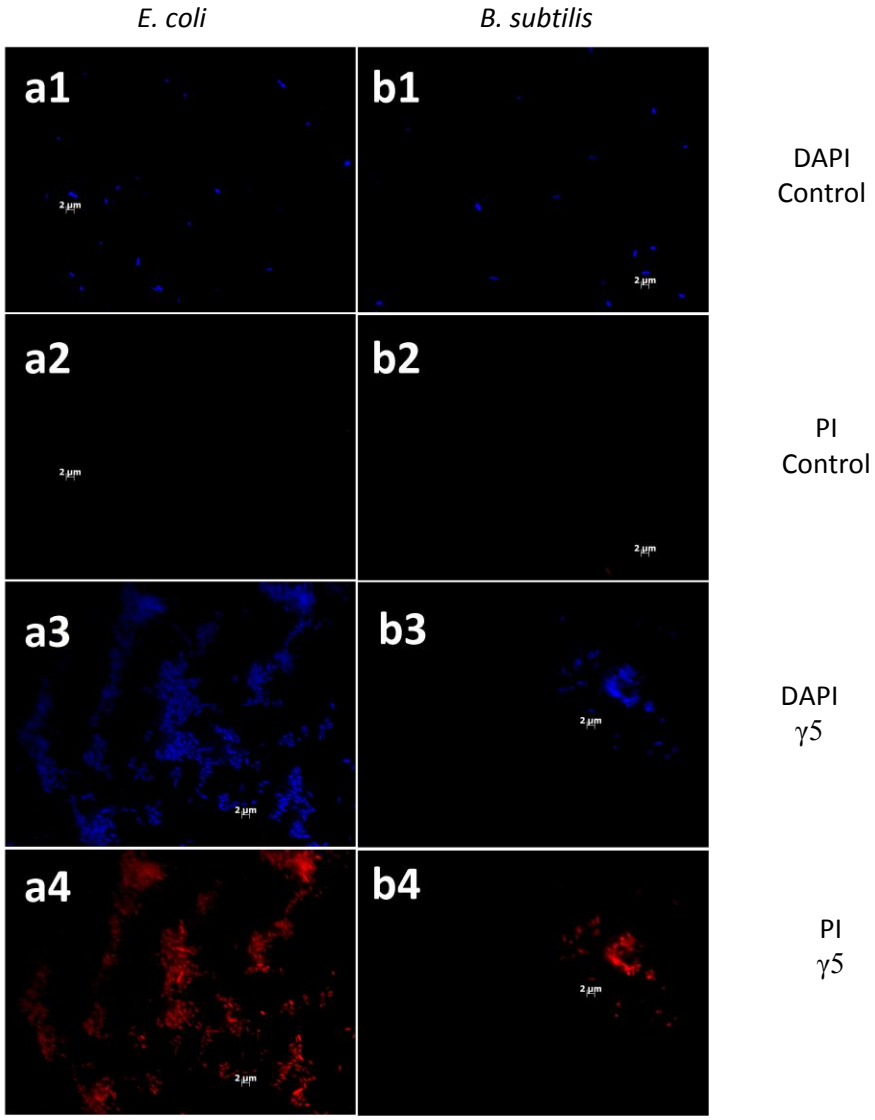


Figure S4. Fluorescence micrographs of *E. coli* and *B. subtilis* treated with 10 μg/ml γ-AApeptide γ5 for 2 h. **a1-a4**, *E. coli*. **a1**, control, no treatment, DAPI stained; **a2**, control, no treatment, PI stained; **a3**, γ5 treatment, DAPI stained; **a4**, γ5 treatment, PI stained. **b1-b4**, *B. subtilis*. **b1**, control, no treatment, DAPI stained; **b2**, control, no treatment, PI stained; **b3**, γ5 treatment, DAPI stained; **b4**, γ5 treatment, PI stained. Scale bar: 2 μm for *E. coli* and for *B. subtilis*.

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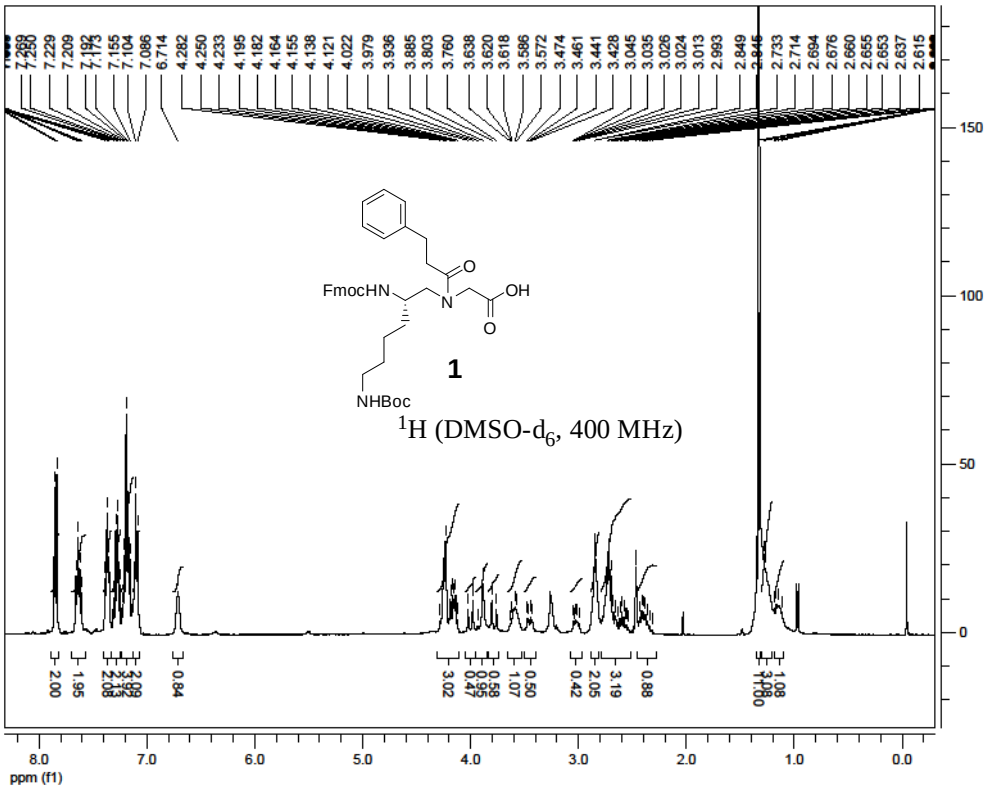
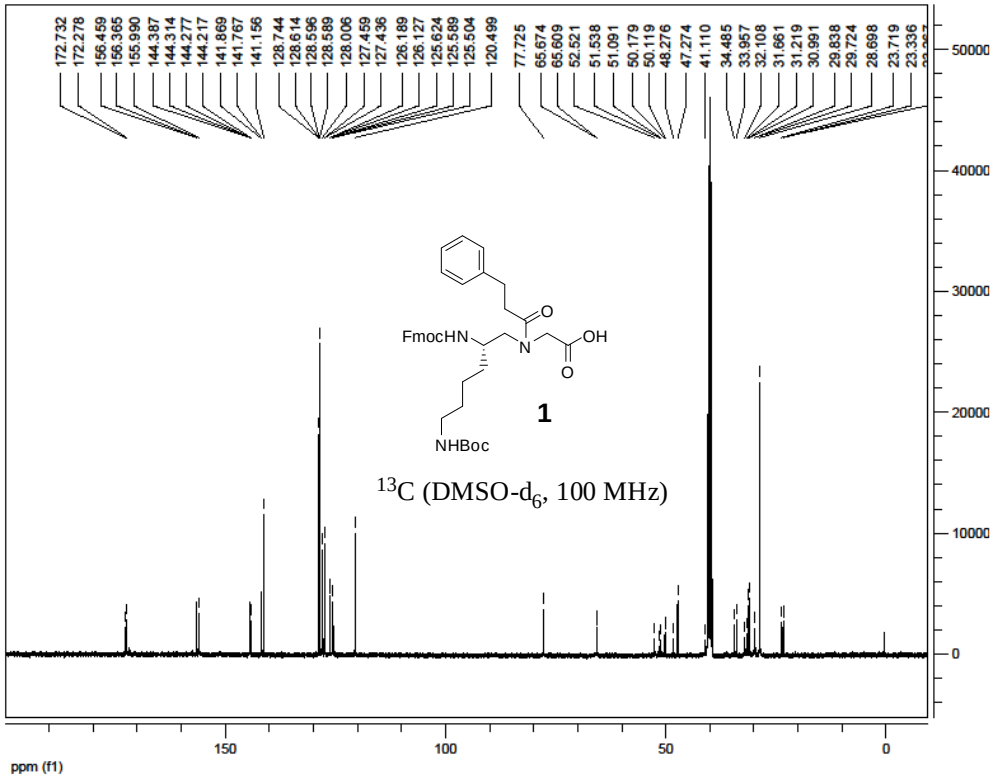
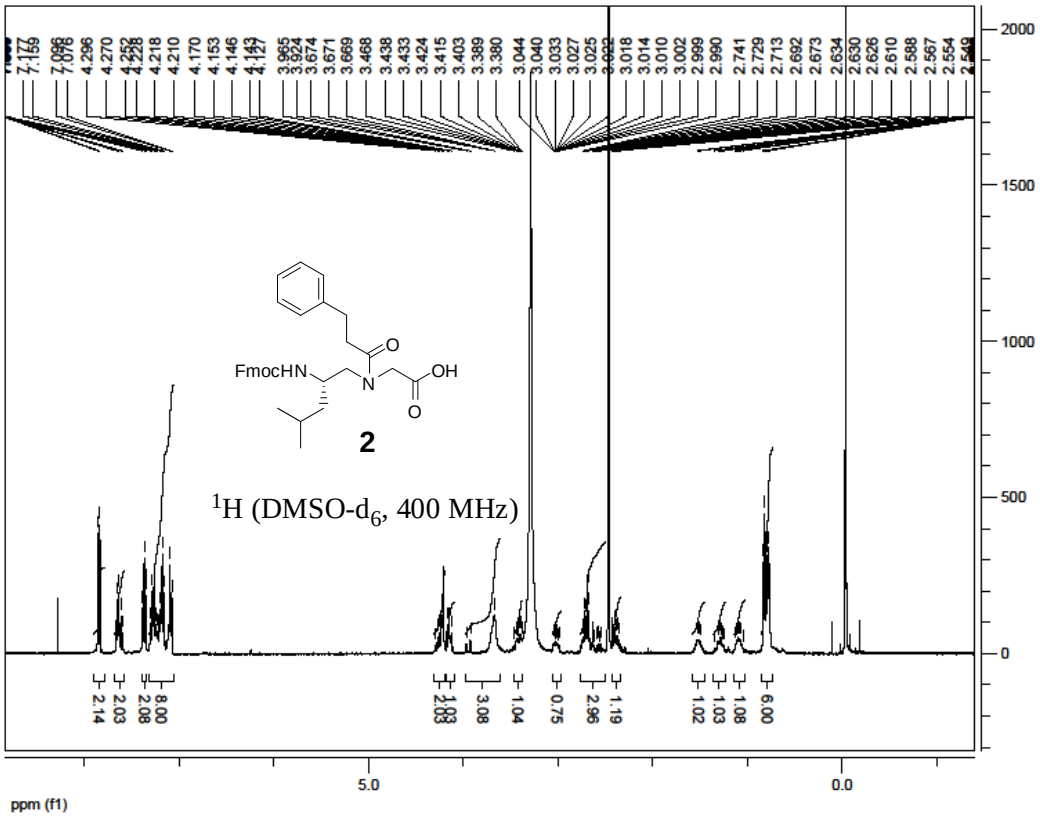
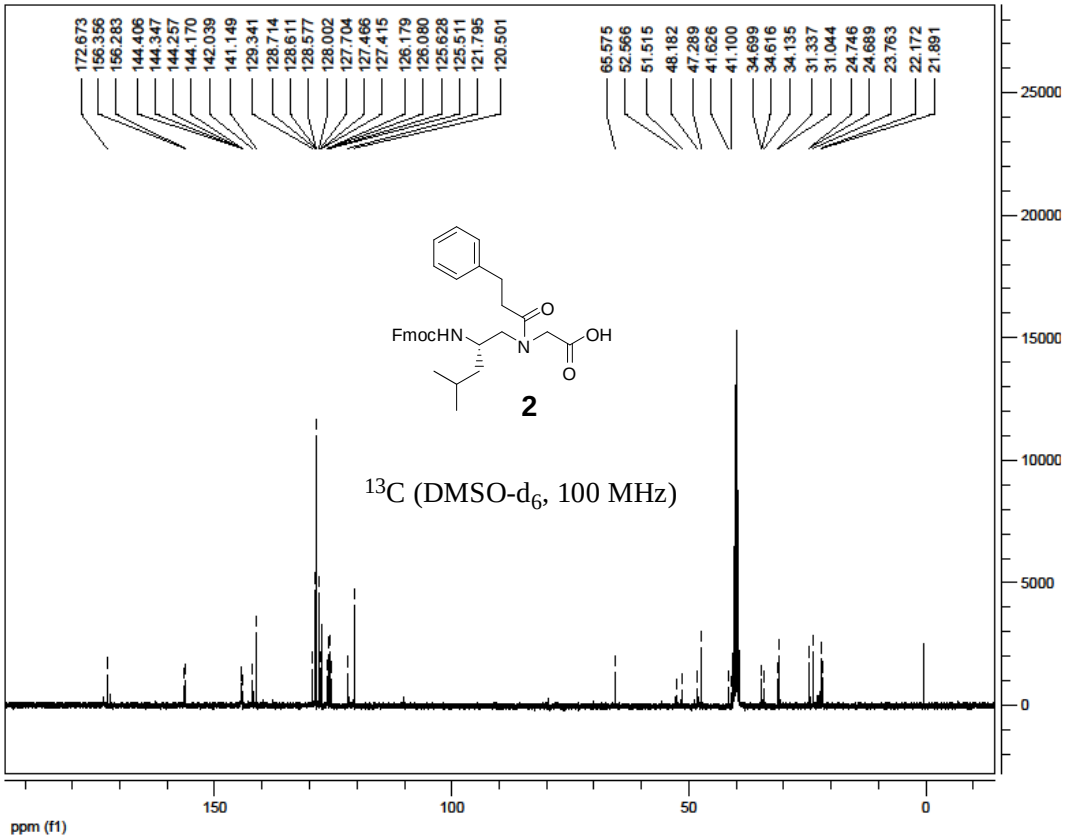


^{1}H (DMSO-d$_6$, 400 MHz)



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


FmocHN
N
OH
O
O
2
^{1}H (DMSO-d$_6$, 400 MHz)
ppm (f1)


FmocHN
N
OH
O
O
2
^{13}C (DMSO-d$_6$, 100 MHz)
ppm (f1)