Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Radiolabeled γ-AApeptides: A New Class of Tracers for Positron Emission Tomography

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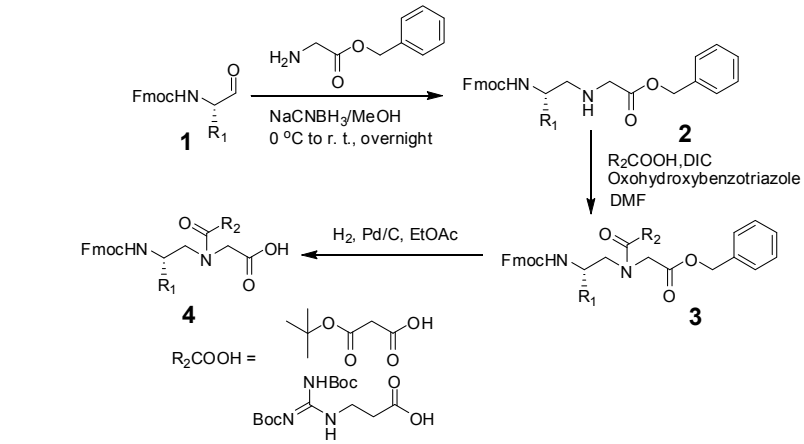
1. General experimental methods.

Fmoc protected α-amino acids and Knorr resin were obtained from Chem-Impex International, Inc. DOTA-NHS ester was purchased from Macrocyclics, Inc. (Dallas, TX). Pronase and Chelex 100 resin (50-100 mesh) were from Sigma-Aldrich (St. Louis, MO). Water and all buffers were of Millipore grade and pre-treated with Chelex 100 resin to ensure that the aqueous solution was heavy metal-free. All other reaction buffers and chemicals were from Thermo Fisher Scientific (Fair Lawn, NJ). NMR spectra of γ-AApeptide building blocks were obtained on a Varian Inova 400. γ-AApeptide sequences were prepared on a Knorr resin in peptide synthesis vessels on a Burrell Wrist-Action shaker. The γ-AApeptides were analyzed and purified on a Waters HPLC with analytical and preparative modules, and the desired fractions were then lyophilized using a Labconco lyophilizer. Molecular weight of γ-AApeptides were identified on a Bruker AutoFlex MALDI-TOF mass spectrometer.

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

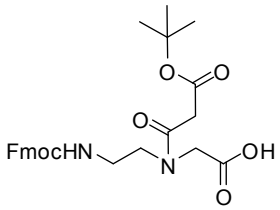


General procedure

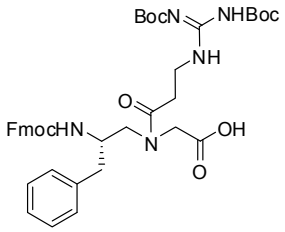
Synthesis of 2.[1-3] To a glycine benzyl ester hydrochloride in 15 ml methanol in a 100 ml round bottom flask was added 1.1 equiv. of triethylamine and the reaction mixture was stirred at 0 °C for 15 min. Stoichiometric amount of a Fmoc protected amino acid aldehyde was added and the mixture was stirred for another 30 min. Catalytic amount of acetic acid was then added, followed by 2 equivalence of $NaBH_3CN$. The solution was stirred at 0 °C for 1 h and continued at room temperature overnight. The solvent was evaporated and 100 ml ethyl acetate and 100 ml saturated sodium bicarbonate solution were added to the residue. The organic layer was separated and washed with 100 ml brine, dried over anhydrous sodium sulfate, and removed *in vacuo*. Flash chromatography using ethyl acetate/hexane 1:1 gave **2** (R_f = 0.2) as a colorless oil.

Synthesis of 3. Compound **2,** 1.2 equiv. of DIC, Oxohydroxybenzotriazole, and R_2COOH were stirred in 20 ml DMF overnight. The solution was then partitioned in 100 ml ethyl acetate and 100 ml water. The organic layer was separated and washed with water (3 ×100 ml) and Brine (2 ×100 ml), dried over anhydrous sodium sulfate, and then concentrated *in vacuo*. Flash chromatography using ethyl acetate/hexane 1:3 gave **3** (R_f = 0.1) as a colorless oil.

Synthesis of 4. 3 in 20 ml of ethyl acetate was added to 10% Pd/C and hydrogenated at atmospheric pressure and room temperature overnight. The solution was evaporated and the residue was purified by flash chromatography 5-7% MeOH/CH_2Cl_2 to give **4** (R_f = 0.2 in 7% MeOH/CH_2Cl_2) as a white foam solid.



4a. 81% overall yield from **2**. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.89 (d, *J* = 6.0 Hz, 2H), 7.68 (d, *J* = 6.0 Hz, 2H), 7.66 (d, *J* = 6.0 Hz, 2H), 7.43-7.38 (m, 2H), 7.35-7.31 (m, 2H), 4.32 (d, J = 12 Hz, 1H), 4.28-4.19 (m, 2H), 3.97-3.93 (m, 2H), 3.40-3.26 (m, 4H), 3.19-3.09 (m, 2H), 1.38 (s, 9H), ^{13}C NMR (150 MHz, DMSO-d_6) δ 171.9, 171.2, 167.6, 167.1, 167.0 166.98, 156.7, 156.6, 144.3, 144.2, 141.2, 141.1, 128.0, 127.5, 125.6, 125.5, 120.54, 120.51, 81.0, 80.9, 65.9, 48.4, 47.9, 47.1, 46.9, 42.4, 41.8, 39.1, 38.4, 28.1, 28.04. HRMS: $[M+Na]^+$ cacl: 505.1945, found: 505.1955.



4b. 62% overall yield from **2**. ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.4-10.8(s, 1H), 8.87-8.76 (m, 1H), 7.88-7.86 (m, 2H), 7.60-7.55 (m, 2H), 7.41-7.14 (m, 10H), 4.21-4.16 (m, 2H), 4.15-4.10 (m, 2H), 4.09-4.06 (m, 1H), 4.03-4.01 (m, 1H), 3.71-3.31 (m, 4H), 3.06-2.46 (m, 4H).1.42 (s, 9H), 1.39 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.3, 171.8, 171.5, 171.0, 156.2, 156.1, 154.93, 154.91, 152.0, 151.96, 144.2, 144.17, 144.1, 141.1, 141.06, 139.2, 138.99, 129.5, 128.53, 128.5, 128.0, 127.4, 126.5, 126.3, 125.5, 125.4, 120.5, 84.0, 83.6, 65.8, 65.6, 52.4, 51.9, 51.4, 50.9, 50.3, 48.0, 47.1, 47.0, 38.1,37.9, 37.5, 32.4, 32.2, 32.0, 31.7, 28.3, 27.9 HRMS: $[M+Na]^+$ cacl: 766.3422, found: 766.3426.

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

γ-AA1 and γ-AA2 were prepared on a Knorr resin in peptide synthesis vessels on a Burrell Wrist-Action shaker following the standard Fmoc chemistry of solid phase peptide synthesis protocol. Each coupling cycle included an Fmoc deprotection using 20% Piperidine in DMF, and 4 h coupling of 1.5 equiv of γ-AApeptide building blocks onto resin in the presence 2 equiv of DIC (diisopropylcarbodiimide)/Oxohydroxybenzotriazole in DMF. After the desired sequences were assembled, they were transferred into a 4 ml vial and cleaved from solid support in 48:50:2 TFA/CH_2Cl_2/triisopropylsilane overnight. Then solvent was evaporated and the residue was analyzed and

purified on an analytical (1 ml/min) and a preparative Waters (20 ml/min) HPLC systems, respectively. The same methods were used by running 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 collected with > 95% purity and lyophilized. The molecular weights of γ-AA1 and γ-AA2 were obtained on Bruker AutoFlex MALDI-TOF mass spectrometer using α–cyano-4-hydroxy-cinnamic acid as the matrix.

Table S1. MALDI-TOF MS analysis of γ-AApeptides.

Sequence	Formula	Mass calcd.	Mass found
γ-AA1	$C_{22}H_{34}N_8O_6$	506.6	507.2 ($M+H^+$)
γ-AA2	$C_{44}H_{71}N_{13}O_{14}$	1006.0	1038.8 ($M+2NH_4^+ - 4H^+$)

4. Cell lines and animal model.

U87MG human glioblastoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum and incubated at 37 ºC with 5% CO_2. Cells were used for in vitro and in vivo experiments when they reached ~75% confluence.

All animal studies were conducted under a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee. To generate the xenograft tumor model, four- to five-week-old female nude mice were purchased from Harlan (Indianapolis, IN) and tumors were established by subcutaneously injecting 5×10^6 U87MG cells, suspended in 100 μL of 1:1 mixture of DMEM and Matrigel (BD Biosciences, Franklin lakes, NJ), into the front flank of mice. Tumor sizes were monitored every other day and mice were used for in vivo experiments when the diameter of tumors reached 5-8 mm (typically 4 weeks after inoculation).

5. ^{64}Cu-labeling.

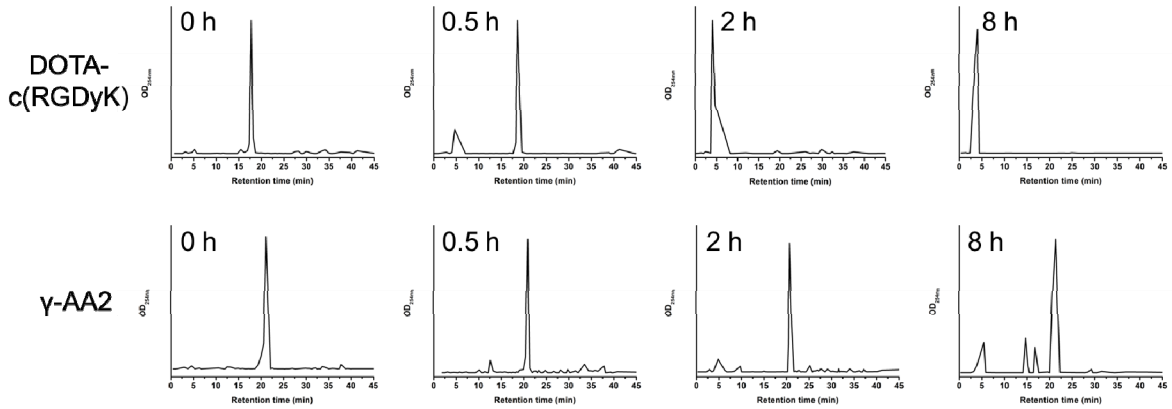
$^{64}CuCl_2$ (111 MBq) was diluted in 300 μL of 0.1 M sodium acetate buffer (pH 6.5) and added to 6 μg of γ-AA2. The reaction mixture was incubated for 30 min at 40 °C with constant shaking. ^{64}Cu-γ-AA2 was purified by a Dionex Ultimate 3000 HPLC system equipped with a radioactivity and UV detector using a C-18 column. A solvent gradient (A: water with 0.1% TFA; B: acetonitrile with 0.1% TFA) was used, where solvent B was gradually increased from 5% to 65% over a period of 30 min. After collection of the radioactive peak, acetonitrile was removed from the solution with continuous argon flow. The remaining solution was reconstituted into a final concentration of 1×PBS. The tracer was passed through a 0.2 μm syringe filter before in vivo experiments.

6. Flow cytometry studies.

The binding affinity and specificity of FITC-γ-AA1 for integrin $\alpha_v\beta_3$ was evaluated in U87MG cells in a binding buffer (20mM Tris, 150mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 1mM $MnCl_2$, 0.10% BSA pH=7.4) at 37 °C, by fluorescence-activated cell sorting (FACS) analysis. Briefly, cells were harvested and suspended in the binding buffer at a concentration of 5×10^6 cells/mL. The cells were incubated with FITC-c(RGDyK) or FITC-γ-AA1 (5 μg/mL) for 30 min at RT, washed three times with cold PBS, and centrifuged at 1,000 rpm for 5 min. Two μM of c(RGDyK) was used for "blocking" studies of the two FITC-labeled agents. Afterwards, the cells were washed and analyzed by FACS using a BD FACSCalibur 4-color analysis cytometer, which is equipped with 488nm and 633nm lasers (Becton-Dickinson, San Jose, CA) and FlowJo analysis software (Tree Star, Inc., Ashland, OR).

7. Enzymatic stability of ^{64}Cu-γ-AA2 and ^{64}Cu-DOTA-c(RGDyK).

Approximately 148 MBq of ^{64}Cu-γ-AA2 or ^{64}Cu-DOTA-c(RGDyK) was incubated with 0.1 mg/ml pronase at 37 ^{o}C in 100 mM of ammonium bicarbonate buffer (pH 7.8) for 24 h, respectively. One fourth of the reaction mixtures were analyzed by radio-HPLC at 0.5 h, 2 h, and 8 h post-treatment and compared to evaluate the stability of the two tracers. The Radio-HPLC traces are shown in the main text (Fig. 4) and the UV HPLC traces are shown below. Both confirmed the marked enhanced enzymatic stability of ^{64}Cu-γ-AA2 over ^{64}Cu-DOTA-c(RGDyK).



8. PET imaging and biodistribution studies.

PET scans were performed using an Inveon microPET/microCT rodent model scanner (Siemens Medical Solutions USA, Inc.). Each U87MG tumor-bearing mouse was injected with 5-10 MBq of the PET tracer via tail vein and 5 minute static PET scans were performed at various time points post-injection (p.i.). The images were reconstructed using a maximum a posteriori (MAP) algorithm, with no attenuation or scatter correction. For each microPET scan, three-dimensional (3D) regions-of-interest (ROIs) were drawn over the tumor and major organs by using vendor software (Inveon Research Workplace [IRW]) on decay-corrected whole-body images. Assuming a tissue density of 1 g/mL, the ROIs were converted to MBq/g using a conversion factor (pre-determined using a 20 mL centrifuge tube filled with ~37 MBq of $^{64}CuCl_2$ as a phantom), and then divided by the total administered radioactivity to obtain an image ROI-derived percentage injected dose per gram of tissue (%ID/g). Another group of three U87MG tumor-bearing mice was each injected with the similar amount of ^{64}Cu-γ-AA2 along with 10 mg/kg dose of c(RGDyK) to evaluate the integrin $\alpha_v\beta_3$ specificity of ^{64}Cu-γ-AA2 in vivo (i.e. blocking experiment).
Biodistribution studies were carried out to confirm that the quantitative tracer uptake values based on PET imaging truly represented the radioactivity distribution in tumor-bearing mice. After the last PET scans at 24 h p.i., mice were euthanized and blood, U87MG tumor, and major organs/tissues were collected and wet-weighed. The radioactivity in the tissue was measured using a gamma-counter (Perkin Elmer) and presented as %ID/g (mean ± SD).

9. Cell binding assay.

The binding affinity of γ-AA1, γ-AA2, and the c(RGDyK) peptide to integrin $\alpha_v\beta_3$ was evaluated via a displacement cell-binding assay using ^{64}Cu-DOTA-c(RGDyK) as the integrin-specific radioligand. U87MG cells were harvested, washed twice with PBS and resuspended (2×10^6 cells/mL) in binding buffer (20 mM Tris, pH = 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.1% Bovine Serum Albumin). 96-well multiscreen DV plates (filter pore size: 0.65 μm, Millipore, Billerica, MA) were seeded with 10^5 cells/well and incubated with ^{64}Cu-DOTA-c(RGDyK) (0.1 μCi/well) in the

presence of increasing concentrations of γ-AA1, γ-AA2, or the c(RGDyK) peptide. The final volume was adjusted to 200 μL per well. After the cells were incubated for 2 h at room temperature, the binding buffer was removed by vacuum. Receptor-ligand complex trapped on the filters was washed three times with 200 μL ice-cold binding buffer. After heating to complete dryness, the filters were collected and the radioactivity was measured using a gamma counter (PerkinElmer). The best-fit IC_{50} values were calculated by fitting the data by nonlinear regression using GraphPad Prism™ (GraphPad Software, Inc., San Diego, CA). Experiments were carried out twice with triplicate samples.

10. Statistical analysis

Quantitative data were expressed as mean ± SD. Means were compared using Student's t-test. P values < 0.05 were considered statistically significant.

References

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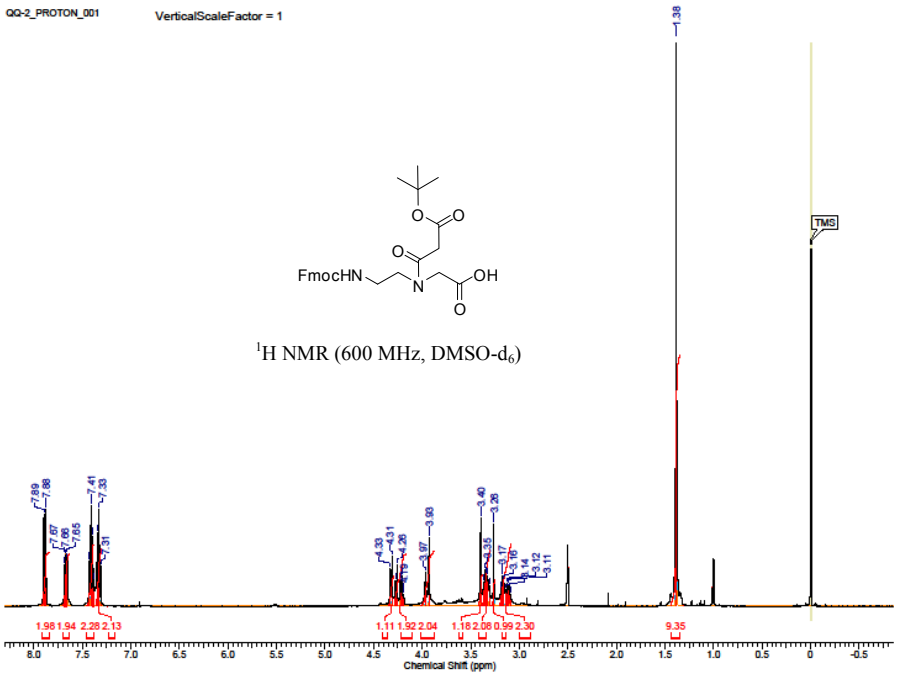
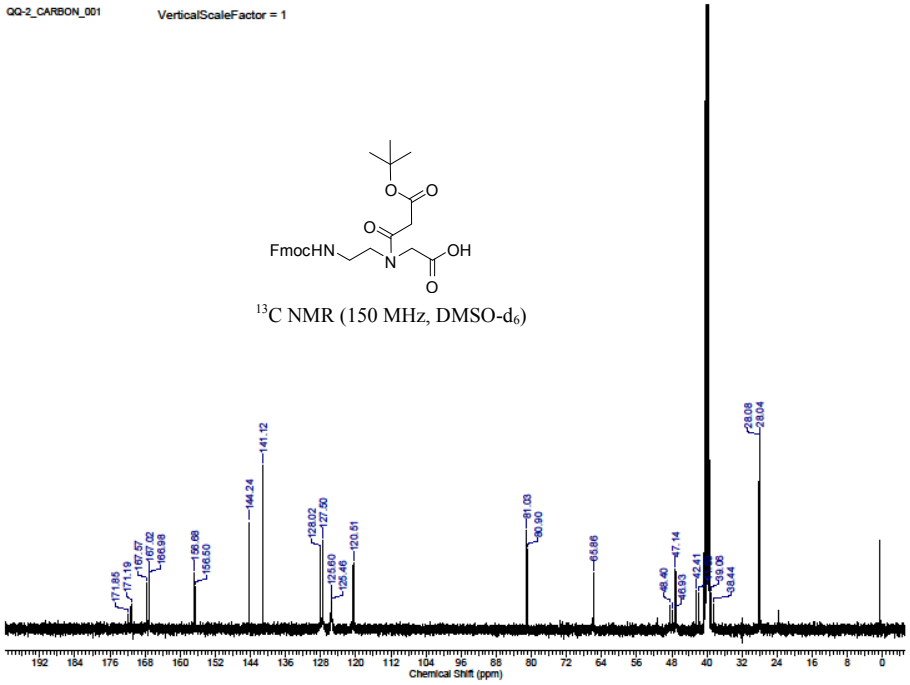
[2] Y. Niu, A. J. Jones, H. Wu, G. Varani, J. Cai, *Org Biomol Chem* **2011**, *9*, 6604.
[3] Y. Niu, S. Padhee, H. Wu, G. Bai, L. Harrington, W. N. Burda, L. N. Shaw, C. Cao, J. Cai, *Chem Commun* **2011**.



^{1}H NMR (600 MHz, DMSO-d_6)



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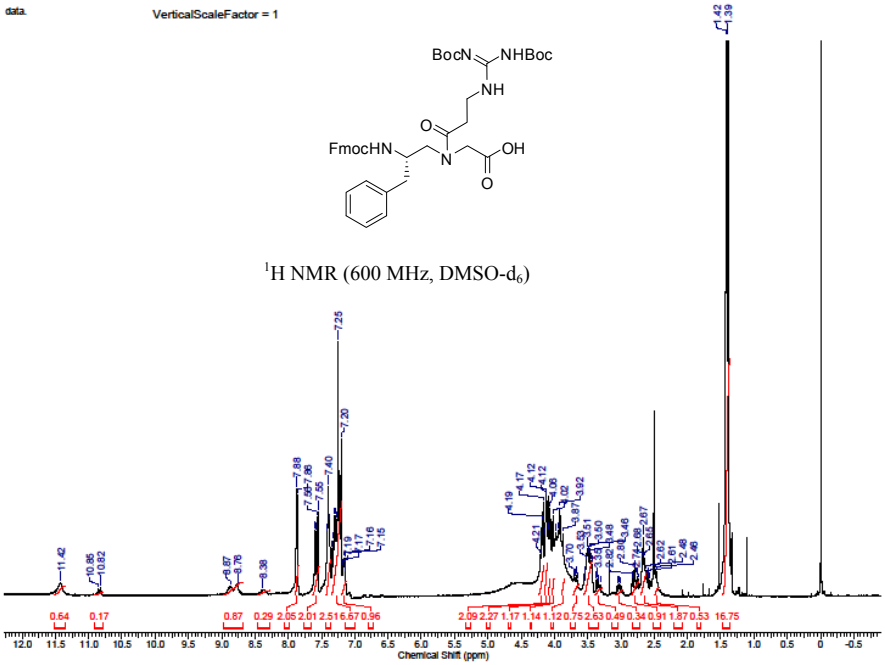
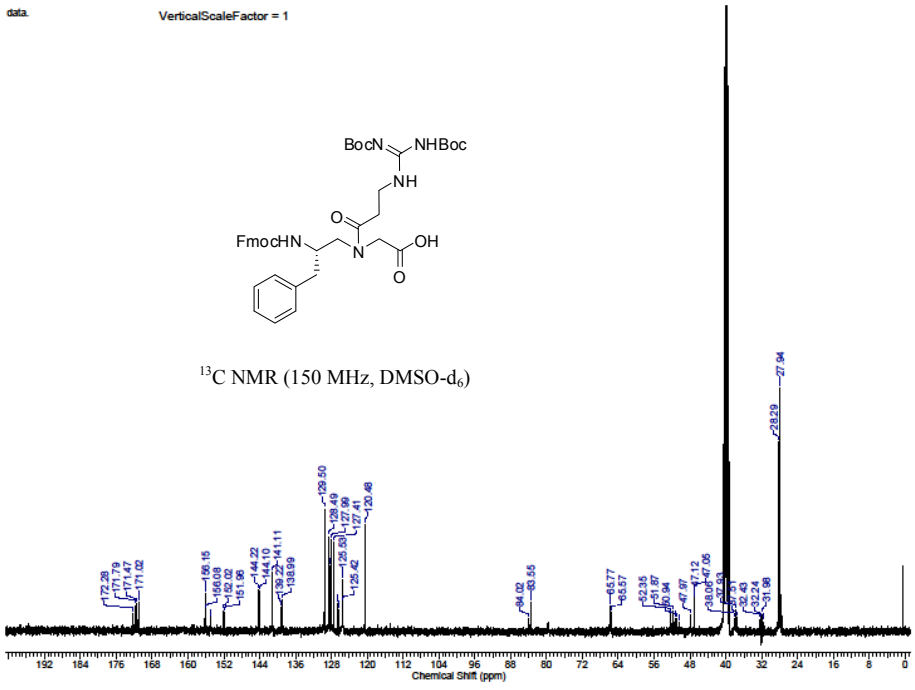


^{1}H NMR (600 MHz, DMSO-d$_6$)



^{13}C NMR (150 MHz, DMSO-d$_6$)