Supplementary Information for

Continuous N-to-C Peptide Synthesis

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Experimental Section

General information. All experiments described were carried out under a nitrogen atmosphere for general moisture control. IR spectra were measured using a Thermo Fisher Nicolet iS5 FT-IR. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AVANCE III HD 400 MHz, 500 MHz, 600 MHz, and 800 MHz spectrometers. Chemical shifts were reported in parts per million (ppm) relative to the residual deuterated solvent. Reactions were monitored by reverse-phase HPLC on an Agilent 1260 instrument. High-resolution mass spectra were recorded on an Agilent 1290 UPLC coupled to a G6230 TOF mass spectrometer with electrospray ionization. Chiral amino acid analysis was performed via extracted-ion chromatograms (EICs) following deuterium chloride (DCl) hydrolysis. Data were acquired using an Agilent 1290 UPLC system coupled to a G6475A mass spectrometer. Reaction assay yield (AY) is the yield of the desired product in the reaction mixture before purification, quantified by HPLC or another suitable analytical method against a pure product reference standard. Reaction isolated yield (IY) is the yield of purified product recovered after isolation and purification, quantified against a pure product reference standard. We thank Y.L. Zhong and H. Zi (Apeloa) for the synthesis (route B) of 2-ethyl-3-methylbutanoyl chloride (**R1**).

General HPLC methods for figures 2, 3, and 4a:

For reactions with unstable intermediates such as mixed anhydrides and oxazolones, HPLC samples were prepared using an in situ benzylamine ($BnNH_2$) quench: 30 μL of the reaction mixture was added at -20 °C to 1 mL of a precooled (-20 °C) 8.6 mM solution of $BnNH_2$ in DMAc and stirred for 10 min before being warmed to ambient temperature for analysis.

Method 1: SB-Aq 4.6 × 100 mm, particle size 1.8 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 55-95% (solvent B) in 11 min, hold 1 min; flow rate: 1.2 mL/min; detection wavelength: 210 nm.

Method 2: CSH C18 4.6 × 150 mm, particle size 2.5 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 55-85% (solvent B) in 10 min, 85-95% (solvent B) in 15 min, hold 1 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

Method 3: SB-Aq 4.6 × 100 mm, particle size 1.8 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 55-75% (solvent B) in 10 min, 75-95% (solvent B) in 11 min, hold 1 min; flow rate: 1.2 mL/min; detection wavelength: 210 nm.

Method 4: Express C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% Trifluoroacetic acid TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 10-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 5: SB-Aq 4.6 × 100 mm, particle size 1.8 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 10-95% (solvent B) in 8 min, hold 2 min; flow rate: 1.2 mL/min; detection wavelength: 210 nm.

Method 6: Express C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 50-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 7: Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5-95% (solvent B) in 5.5 min, hold 2.5 min; flow rate = 0.5 mL/min; detection wavelength = 210 nm.

Method 8: CSH C18 4.6 × 150 mm, particle size 2.5 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 55-95% (solvent B) in 12 min, hold 3 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

Method 9: HALO C8 4.6 × 100 mm, particle size 2.7 µm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN (solvent B); gradient 60-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

Method 10: HALO C18 4.6 × 100 mm, particle size 2.7 μm column; (0.1% H_3PO_4+10mM NH_4PF_6) in H_2O (solvent A), MeCN (solvent B); gradient 60-85% (solvent B) in 8 min, hold 2 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

Method 11: PCS-C18 4.6 × 150 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), MeOH (solvent B); gradient 70-85% (solvent B) in 10 min, 85-95% (solvent B) in 12 min, hold 2 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

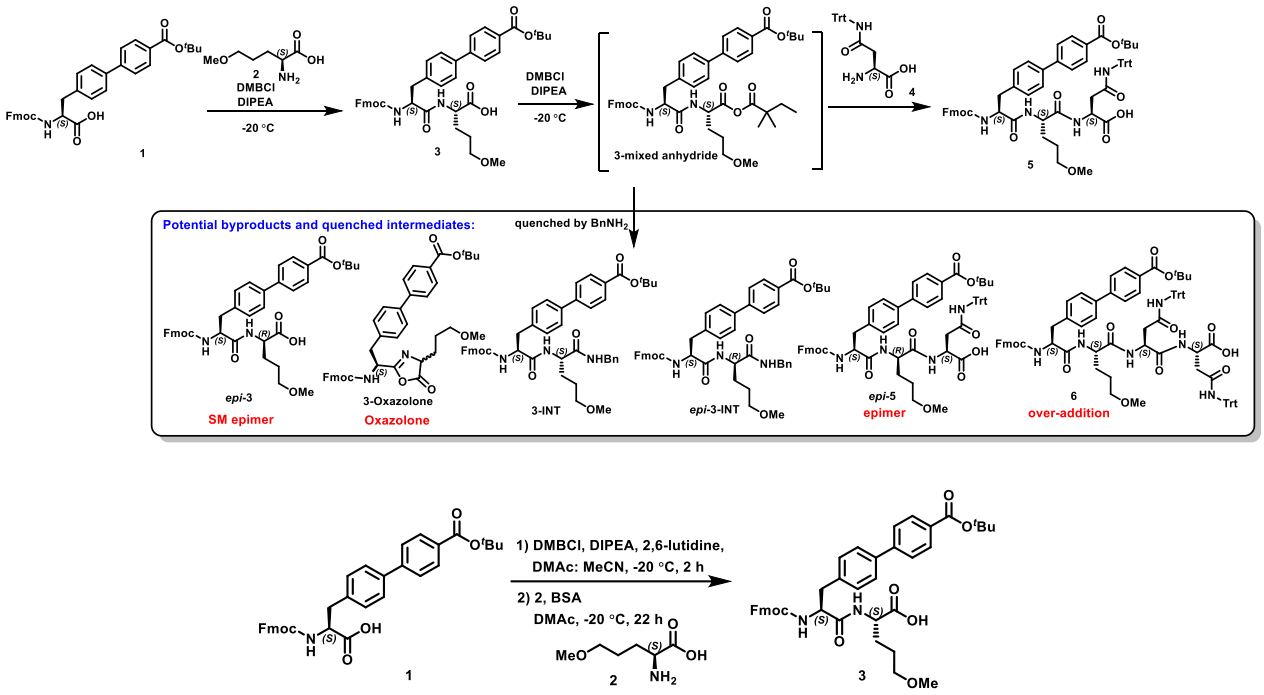
Method 12: PCS-C18 4.6 × 150 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), MeOH (solvent B); gradient 50-80% (solvent B) in 10 min, 80-95% (solvent B) in 15 min, hold 2 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

Method 13: Express C18 3.0 × 100 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 30-95% (solvent B) in 8 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 14: HALO C8 4.6 × 150 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 70-95% (solvent B) in 12 min, hold 3 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

Experimental procedures and characterizations for Figure 2

1) Experimental Procedures for Fig. 2a

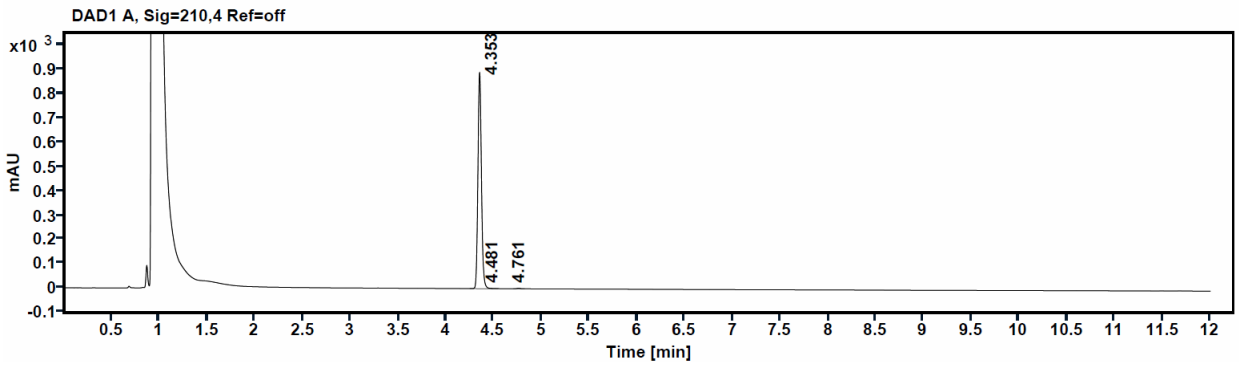


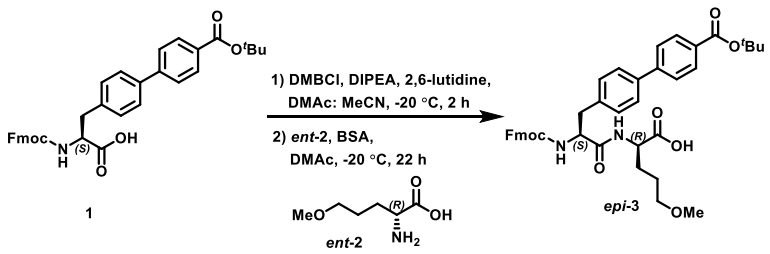
Synthesis of Fmoc-Bip-4-CO_2*t*Bu-5-OMe-NVA-OH (3). Into a 250-mL three-neck jacketed vessel, Fmoc-Bip-4-CO_2*t*Bu-OH **(1)** (10.00 g, 17.74 mmol) was dissolved in *N, N*-dimethylacetamide (DMAc)/ MeCN (1:1, 60 mL) at 25 °C. After the solution was cooled to -20 °C, 2,6-lutidine (1.90 g, 17.74 mmol) and *N*, *N*-diisopropylethylamine (DIPEA) (2.29 g, 17.72 mmol) were added. Then 2,2-dimethylbutanoyl chloride (DMBCl) (2.63 g, 19.54 mmol) was added, and the reaction mixture was stirred at -20 °C for 2 h. A solution of H-5-OMe-NVA-OH **(2)** and bis(trimethylsilyl)acetamide (BSA) in DMAc, which was prepared by stirring **2** (2.87 g, 19.50 mmol) and BSA (5.41 g, 26.59 mmol) in DMAc (40 mL) at 50 °C for 1 h, was added to the reaction at -20 °C over 10 min. The reaction mixture was stirred at -20 °C for 22 h. Upon completion, the reaction mixture was added slowly to H_2O (200 mL) over 1 h and stirred at 20 °C for 3 h and the product precipitated during the addition. The solid was collected by filtration and then slurried with MeCN (100 mL) at 75 °C for 3 h. After cooling to 25 °C, the solid was collected by filtration and dried

under vacuum to provide 11.6 g of **3** as a white solid (99.6% LCAP, 99.6 wt%) in 94% isolated yield (**Method 1**).

mp 202-203 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 12.67 (s, 1H), 8.33 (d, J = 7.6 Hz, 1H), 7.94 (d, J = 8.4 Hz, 2H), 7.87 (d, J = 7.6 Hz, 2H), 7.73 (d, J = 8.4 Hz, 2H), 7.69 - 7.62 (m, 5H), 7.47 (d, J = 8.4 Hz, 2H), 7.39 (q, J = 6.8 Hz, 2H), 7.32 - 7.24 (m, 2H), 4.40 - 4.34 (m, 1H), 4.27 - 4.22 (m, 1H), 4.14 (s, 3H), 3.31 (t, J = 6.4 Hz, 2H), 3.21 (s, 3H), 3.08 (dd, J = 14.0, 3.6 Hz, 1H), 2.83 (dd, J = 13.6, 11.2 Hz, 1H), 1.86 - 1.76 (m, 1H), 1.72 - 1.62 (m, 1H), 1.62 - 1.52 (m, 11H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 173.95, 172.13, 165.26, 156.31, 144.64, 144.25, 144.19, 141.12, 139.03, 137.28, 130.51, 130.37, 130.08, 128.07, 127.49, 127.07, 126.98, 126.51, 125.79, 120.53, 81.14, 71.73, 66.12, 58.27, 56.32, 52.22, 47.00, 37.56, 28.36, 28.28, 25.99. HRMS (ESI): m/z calcd. for $C_{41}H_{45}N_2O_8$ $[M+H]^+$: 693.3170, found: 693.3184. FTIR (neat): 3265, 2978, 1750, 1734, 1709, 1686, 1625, 1537, 1369, 1292, 1259, 1238, 1162, 1119, 1111, 1019, 982, 941, 824, 788, 771, 760, 741, 700, 649, 540 cm^{-1}.

HPLC of isolated **3**:

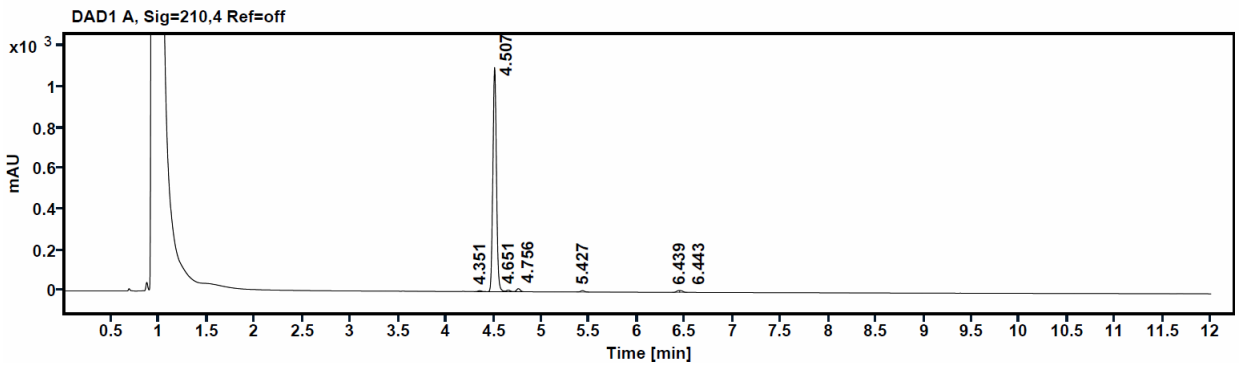


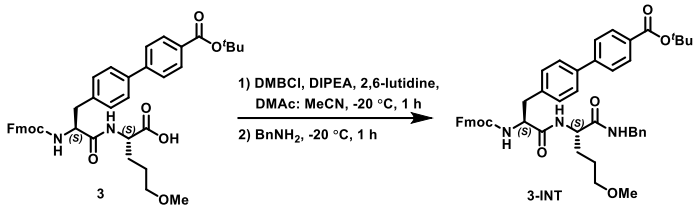


Synthesis of Fmoc-Bip-4- CO_2*t*Bu-D-5-OMe-NVA-OH (*epi*-3). In a 250-mL three-neck jacketed vessel, **1** (10.00 g, 17.74 mmol) was dissolved in DMAc/MeCN (1:1, 60 mL) at 25 °C. After the solution was cooled to -20 °C, 2,6-lutidine (1.90 g, 17.74 mmol) and DIPEA (2.29 g, 17.72 mmol) were added. Then DMBCl (2.63 g, 19.50 mmol) was added, and the reaction mixture was stirred at -20 °C for 2 h. A solution of **H-D-5-OMe-NVA-OH (*ent*-2)** and BSA in DMAc, which was prepared by stirring ***ent*-2** (2.87 g, 19.50 mmol) and BSA (5.41 g, 26.59 mmol) in DMAc (40 mL) at 50 °C for 1 h, was added to the reaction at -20 °C over 10 min. The reaction mixture was stirred at -20 °C for 22 h. Upon completion, the reaction mixture was added slowly to H_2O (200 mL) over 1 h, and the mixture was stirred at 20 °C for 3 h. The solid was collected by filtration and then slurried with MeCN (100 mL) at 75 °C for 3 h. Then, the mixture was cooled to 25 °C. The solid was collected by filtration and dried under vacuum to provide 10.3 g of ***epi*-3** as a white solid (95.6% LCAP) in 88% isolated yield (**Method 1**).

mp 182-183 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 12.74 (s, 1H), 8.41 (d, J = 8.0 Hz, 1H), 7.94 (d, J = 8.4 Hz, 2H), 7.87 (d, J = 7.6 Hz, 2H), 7.74 - 7.61 (m, 15H), 7.46 - 7.37 (m, 4H), 7.33 - 7.25 (m, 2H), 4.45 - 4.39 (m, 1H), 4.27 - 4.21 (m, 1H), 4.14 (s, 3H), 3.23 (t, J = 6.0 Hz, 2H), 3.16 (s, 3H), 3.03 (dd, J = 13.2, 4.0 Hz, 1H), 2.86 (dd, J = 13.2, 10.0 Hz, 1H), 1.77 - 1.70 (m, 1H), 1.63 - 1.57 (m, 10H), 1.47 - 1.41 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 173.88, 171.80, 165.25, 156.22, 144.61, 144.27, 144.19, 141.11, 138.82, 137.27, 130.56, 130.36, 130.06, 128.08, 127.50, 127.04, 126.95, 126.95, 125.85, 120.52, 81.13, 71.66, 66.20, 58.18, 56.48, 52.07, 46.99, 38.20, 28.60, 28.28, 25.77. HRMS (ESI): m/z calcd. for $C_{41}H_{45}N_2O_8$ $[M+H]^+$: 693.3170, found: 693.3195. FTIR (neat): 3404, 3320, 2972, 2921, 2870, 1710, 1640, 1608, 1540, 1521, 1449, 1391, 1368, 1301, 1244, 1605, 1119, 1068, 1033, 864, 849, 820, 786, 775, 757, 741, 621, 515 cm^{-1}.

HPLC of isolated ***epi*-3**



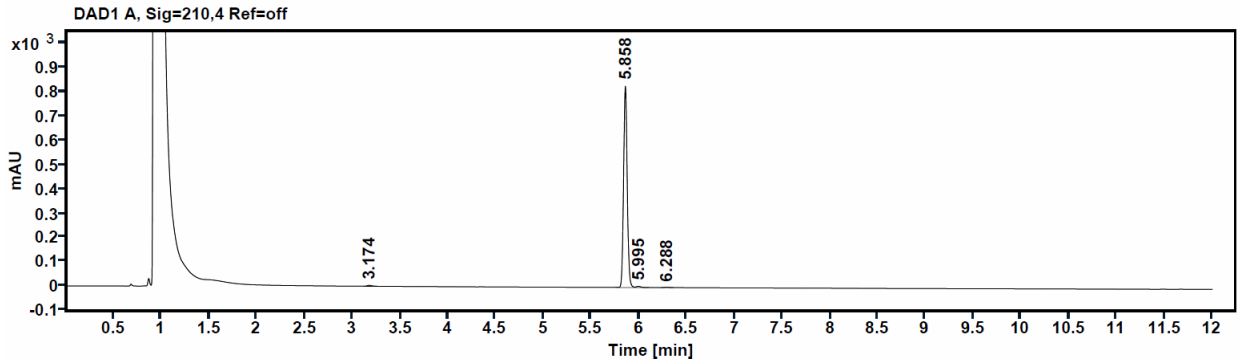


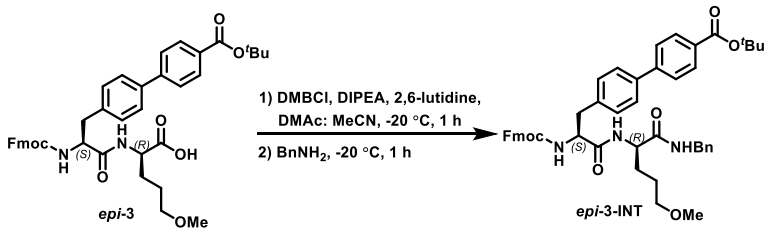
Synthesis of Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-O-BnNH$_2$ (3-INT). In a 40-mL vial, **3** (1.00 g, 1.43 mmol) was dissolved in DMAc/MeCN (1:1, 10 mL). After the solution was cooled to -20 °C, 2,6-lutidine (0.18 g, 1.73 mmol) and DIPEA (0.19 g, 1.44 mmol) were added. Then DMBCl (0.21 g, 1.59 mmol) was added, and the reaction mixture was stirred at -20 °C for 1 h. BnNH$_2$ (0.23 g, 2.17 mmol) was added to the reaction. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (Method 1). Upon completion, the reaction mixture was purified by Prep-HPLC under the conditions: Column: C18, 20-40 μm, 330 g; Mobile phase A: H_2O, Mobile phase B: MeCN; Flow rate:100 mL/min; Gradient: 70-100% B in 30 min; Wavelength: 210 nm. The collected fractions were concentrated to dryness under vacuum at a temperature below 40 °C, yielding 0.96 g of **3-INT** as a white solid (98.6% LCAP) in 85% isolated yield (**Method 1**).

mp 174-183 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.45 (t, J = 6.0 Hz, 1H), 8.19 (t, J = 8.0 Hz, 1H), 7.94 (d, J = 8.4 Hz, 2H), 7.88 (d, J = 7.6 Hz, 2H), 7.73 - 7.59 (m, 15H), 7.45 - 7.37 (m, 4H), 7.32 - 7.22 (m, 15H), 4.40 - 4.29 (m, 4H), 4.15 (s, 3H), 3.28 (t, J = 6.4 Hz, 2H), 3.18 (s, 3H), 3.11 - 3.06 (m, 1H), 2.84 (dd, J = 13.6, 10.8 Hz, 1H), 1.79 - 1.70 (m, 1H), 1.66 - 1.49 (m, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 171.84, 165.26, 156.33, 144.62, 144.24, 144.19, 141.13, 139.77, 139.00, 137.26, 130.50, 130.37, 130.08,

128.71, 128.07, 127.53, 127.50, 127.22, 127.06, 126.96, 125.77, 120.54, 81.14, 71.88, 66.13, 58.21, 56.42, 53.05, 47.01, 42.47, 37.51, 29.51, 28.29, 25.90. HRMS (ESI): m/z calcd. for $C_{48}H_{52}N_3O_7$ $[M+H]^+$: 782.3800, found: 782.3820. FTIR (neat): 3281, 2927, 1708, 1637, 1533, 1449, 1391, 1367, 1290, 1254, 1165, 1115, 1031, 772, 695 cm^{-1}.

HPLC of isolated **3-INT**

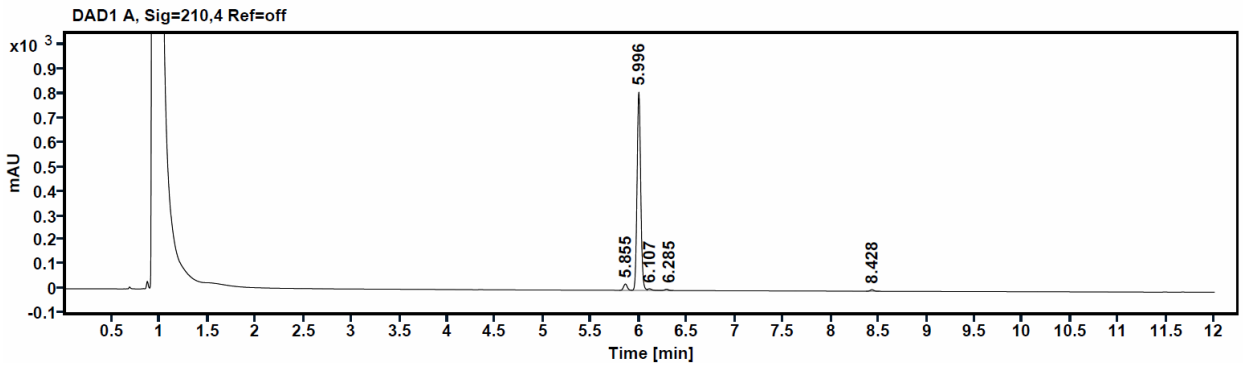


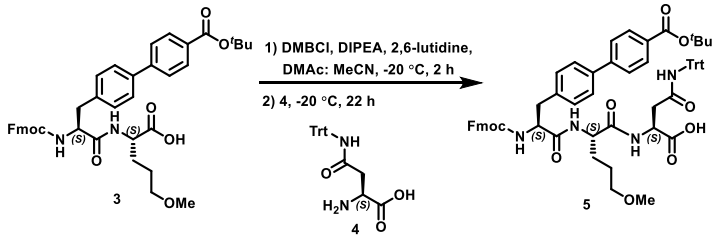


Synthesis of Fmoc-Bip-4-CO_2*t*Bu-D-5-OMe-NVA-O-$BnNH_2$ (*epi*-3-INT). In a 40-mL vial, ***epi*-3** (1.00 g, 1.43 mmol) was dissolved in DMAc/MeCN (1:1, 10 mL) at 25 °C. After the solution was cooled to -20 °C, 2,6-lutidine (185 mg, 1.72 mmol) and DIPEA (222 mg, 1.72 mmol) were added. Then DMBCl (0.21 g, 1.59 mmol) was added, and the reaction mixture was stirred at -20 °C for 1 h. $BnNH_2$ (0.23 g, 2.17 mmol) was added to the reaction. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC. Upon completion, the reaction mixture was purified by Prep-HPLC under the conditions: Column: C18, 20-40 μm, 330 g; Mobile phase A: H_2O, Mobile phase B: MeCN; Flow rate: 100 mL/min; Gradient: 70-100% B in 30 min; Wavelength: 210 nm. The collected fractions were concentrated to dryness under vacuum at a temperature below 40 °C, yielding 0.90 g of ***epi*-3-INT** as a white solid (94.7% LCAP) in 80% isolated yield (**Method 1**).

mp 202-206 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.38 - 8.34 (m, 2H), 7.95 (d, J = 8.4 Hz, 2H), 7.88 (d, J = 7.6 Hz, 2H), 7.81 (d, J = 7.6 Hz, 1H), 7.73 (d, J = 8.4 Hz, 2H), 7.64 - 7.61 (m, 4H), 7.43 - 7.38 (m, 4H), 7.33 - 7.15 (m, 15H), 4.38 - 4.33 (m, 1H), 4.28 - 4.22 (m, 3H), 4.15 - 4.09 (m, 2H), 4.06 - 4.03 (m, 1H), 3.18 - 3.14 (m, 2H), 3.12 (s, 3H), 3.00 (dd, J = 13.6, 6.0 Hz, 1H), 2.89 (dd, J = 13.2, 10.2 Hz, 1H), 1.75 - 1.67 (m, 1H), 1.57 (s, 9H), 1.53 - 1.43 (m, 1H), 1.33 - 1.26 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 171.90, 171.77, 165.26, 156.51, 144.60, 144.21, 144.18, 141.14, 139.64, 138.52, 137.36, 130.54, 130.39, 130.07, 128.65, 128.09, 127.51, 127.45, 127.14, 127.09, 126.97, 125.74, 120.54, 81.15, 71.76, 66.19, 58.14, 56.76, 52.90, 46.95, 42.44, 37.62, 29.15, 28.28, 25.78. HRMS (ESI): m/z calcd. for $C_{48}H_{52}N_3O_7$ $[M+H]^+$: 782.3800, found: 782.3828. FTIR (neat): 3291, 3063, 2975, 2927, 2869, 1706, 1689, 1644, 1536, 1449, 1288, 1256, 1169, 1115, 1032, 736, 669, 527 cm^{-1}.

HPLC of isolated ***epi*-3-INT**



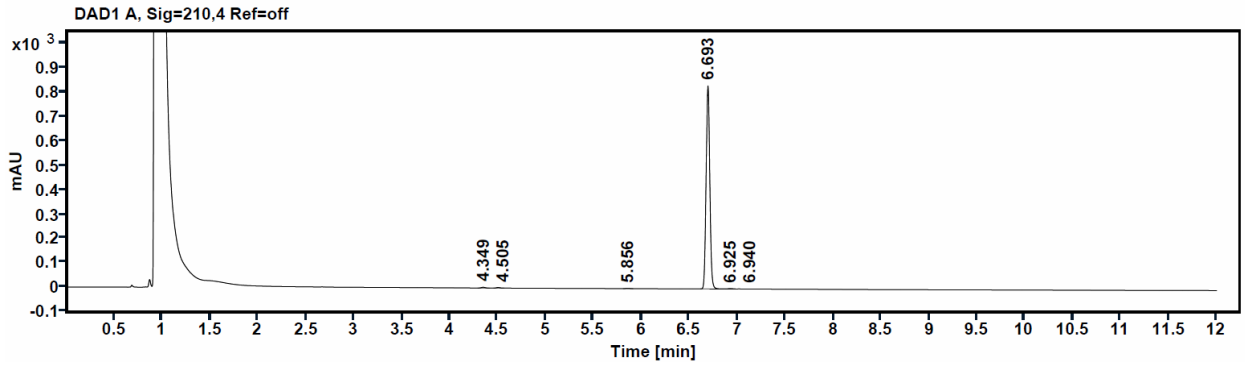


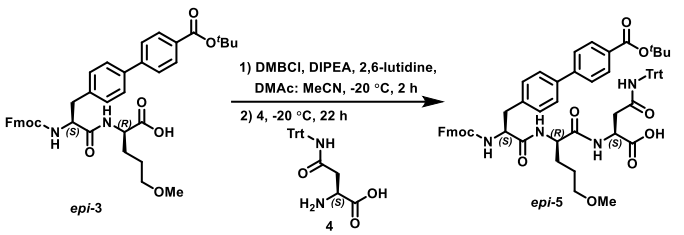
Synthesis of Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-L-Gln(Trt)-OH (5). In a 250-mL three-neck jacketed vessel, **3** (10.00 g, 14.43 mmol) was dissolved in DMAc/MeCN (1:1, 100 mL) at 25 °C. After the solution was cooled to -20 °C, 2,6-lutidine (1.85 g, 17.27 mmol) and DIPEA (1.86 g, 14.39 mmol) were added. Then DMBCl (2.14 g, 15.90 mmol) was added, and the reaction mixture was stirred at -20 °C for 2 h and monitored by HPLC* (HPLC sample was prepared by quenching with BnNH$_2$), which showed the ratio of

3:3-INT：*epi*-3-INT was 0.6:98.2:0.7. Compound **4** (5.95 g, 15.89 mmol) was then added to the reaction. The reaction mixture was stirred at -20 °C for 22 h and monitored by HPLC (Method 1). The HPLC showed the reaction assay yield was 97% and the ratio of **3:*epi*-3-INT:5:*epi*-5:6** was 0.7:0.5:97.9:0.4:0.2. Upon completion, the reaction mixture was added slowly to H_2O (300 mL) over 1 h, stirred at 0 °C for 3 h, and the solid was collected by filtration. The solid was stirred as a slurry in MeCN (100 mL, 10 V) at 25 °C for 3 h, collected by filtration, and dried under vacuum to provide 13.80 g of **5** as a white solid (98.9% LCAP, 98.5 wt%) in 91% isolated yield (**Method 1**).

mp 224-228 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 12.66 (s, 1H), 8.74 (br s, 1H), 8.31 (d, J = 7.2 Hz, 1H), 8.14 (d, J = 8.4 Hz, 1H), 7.93 (d, J = 8.4 Hz, 2H), 7.87 (d, J = 7.6 Hz, 2H), 7.71 - 7.69 (m, 3H), 7.64 - 7.59 (m, 4H), 7.43 (d, J = 8.4 Hz, 2H), 7.40 - 7.36 (m, 2H), 7.31 - 7.24 (m, 8H), 7.20 - 7.16 (m, 9H), 4.50 (q, J = 7.2 Hz, 1H), 4.43 - 4.35 (m, 2H), 4.14 - 4.04 (m, 3H), 3.29 - 3.26 (m, 2H), 3.17 (s, 3H), 3.09 - 3.05 (m, 1H), 2.86 - 2.72 (m, 2H), 2.67 - 2.61 (m, 1H), 1.80 - 1.70 (m, 1H), 1.60 - 1.54 (m, 12H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 173.30, 171.72, 171.57, 169.23, 165.27, 156.30, 145.21, 144.67, 144.26, 144.17, 141.14, 141.11, 139.09, 137.23, 130.51, 130.35, 130.07, 129.06, 128.08, 128.06, 127.94, 127.52, 127.06, 126.97, 126.82, 125.78, 125.77, 120.53, 81.14, 72.10, 69.89, 66.13, 58.17, 56.47, 52.57, 49.61, 47.01, 38.36, 37.61, 30.13, 28.30, 25.57. HRMS (ESI): m/z calcd. for $C_{64}H_{64}N_4NaO_{10}$ $[M+Na]^+$: 1071.4515, found: 1071.4542. FTIR (neat): 3293, 2930, 1712, 1693, 1643, 1528, 1448, 1392, 1367, 1290, 1255, 1167, 1115, 1035, 757, 737, 698, 564 cm^{-1}.

HPLC of isolated **5**

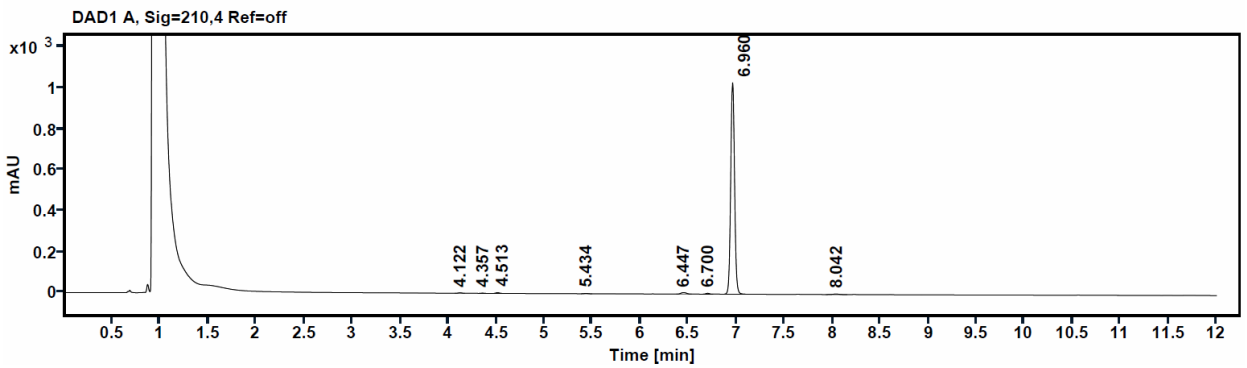


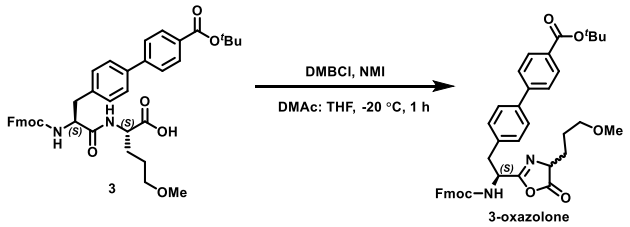


Synthesis of Fmoc-Bip-4-CO$_2$*t*Bu-D-5-OMe-NVA-L-Gln(Trt)-OH (*epi*-5). In a 40-mL vial, ***epi-3*** (1.00 g, 1.44 mmol) was dissolved in DMAc/MeCN (1:1, 10 mL) at 25 °C. After the solution was cooled to -20 °C, 2,6-lutidine (0.18 g, 1.73 mmol) and DIPEA (0.19 g, 1.44 mmol) were added. Then DMBCl (0.21 g, 1.59 mmol) was added, and the reaction mixture was stirred at -20 °C for 2 h. **4** (0.60 g, 1.59 mmol) was added to the reaction. The reaction mixture was stirred at -20 °C for 22 h and monitored by HPLC (Method 1). Upon completion, the reaction mixture was purified by Prep-HPLC under the conditions: Column: C18, 20-40 μm, 330 g; Mobile phase A: H_2O, Mobile phase B: MeCN; Flow rate: 100 mL/min; Gradient: 70-100% B in 30 min; Wavelength: 210 nm. The collected fractions were concentrated to dryness under vacuum at a temperature below 40 °C to provide 1.30 g of ***epi*-5** as a white solid (97.2% LCAP) in 91% isolated yield (**Method 1**).

mp 152-159 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 12.66 (s, 1H), 8.75 (br s, 1H), 8.35 (d, J = 7.6 Hz, 1H), 8.27 (d, J = 8.0 Hz, 1H), 7.94 (d, J = 8.4 Hz, 2H), 7.88 (d, J = 7.6 Hz, 2H), 7.74 - 7.72 (m, 3H), 7.66 (dd, J = 7.6, 3.6 Hz, 2H), 7.61 (d, J = 8.0 Hz, 2H), 7.45 (d, J = 8.0 Hz, 2H), 7.41 - 7.37 (m, 2H), 7.30 - 7.23 (m, 8H), 7.21 - 7.15 (m, 9H), 4.49 (q, J = 6.0 Hz, 1H), 4.44 - 4.38 (m, 2H), 4.17 - 4.12 (m, 3H), 3.16 (t, J = 6.4 Hz, 2H), 3.11 (s, 3H), 3.04 (dd, J = 13.2, 4.4 Hz, 1H), 2.89 - 2.77 (m, 2H), 2.60 (dd, J = 15.6, 6.8 Hz, 1H), 1.71 - 1.64 (m, 1H), 1.57 (s, 9H), 1.51 - 1.47 (m, 1H), 1.40 - 1.33 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 173.28, 171.60, 171.59, 169.29, 165.27, 156.32, 145.28, 144.63, 144.33, 144.17, 141.13, 138.75, 137.28, 130.58, 130.37, 130.07, 129.08, 128.08, 128.06, 127.89, 127.52, 127.05, 126.96, 126.80, 125.85, 125.81, 120.52, 81.14, 71.93, 69.90, 66.22, 58.15, 56.68, 52.32, 49.51, 47.01, 38.61, 38.00, 30.00, 28.29, 25.70. HRMS (ESI): m/z calcd. for $C_{64}H_{64}N_4NaO_{10}$ $[M+Na]^+$: 1071.4515, found: 1071.4543. FTIR (neat): 3289, 2928, 1709, 1643, 1525, 1491, 1447, 1293, 1254, 1165, 1114, 1035, 848, 738, 697, 638, 567 cm^{-1}.

HPLC of isolated ***epi*-5**





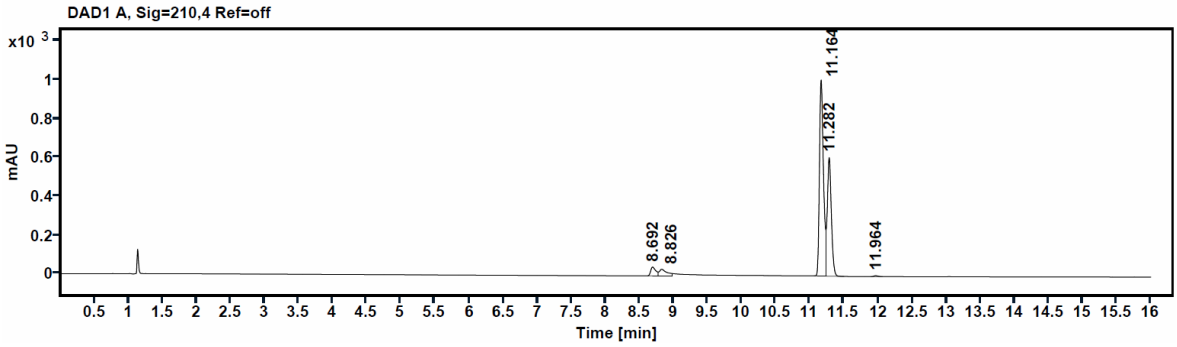
Synthesis of Fmoc-Bip-4-CO_2*t*Bu-5-OMe-NVA-oxazolone (3-oxazolone*).

* Most oxazolones are not stable under HPLC and purification conditions and are quenched by $BnNH_2$ in situ for HPLC analysis. **3-Oxazolone** has reasonable stability and can be isolated with a low level of decomposition.

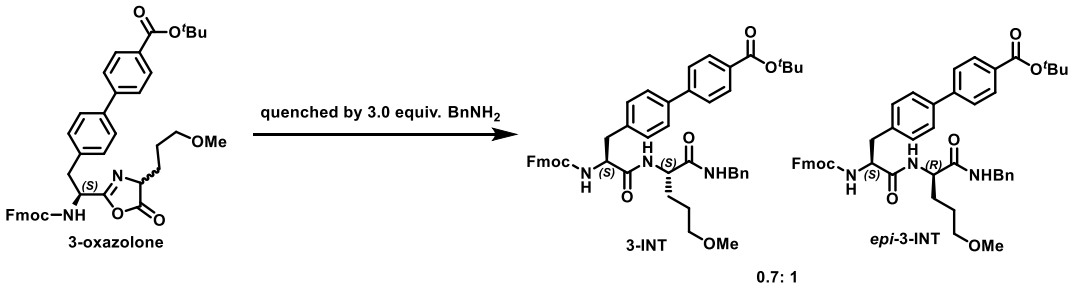
Into a 40-mL vial, **3** (2.00 g, 2.89 mmol) was dissolved in DMAc/THF (1:1, 20 mL) at 25 °C. After the solution was cooled to -20 °C, *N*-methylimidazole (NMI) (284 mg, 1.03 g/mL, 0.276 mL, 3.46 mmol) and DMBCl (0.43 g, 3.17 mmol) were added. The reaction mixture was stirred at -20 °C for 1 h and then purified by Prep-HPLC under the following conditions: Column: C18, 20-40 μm, 330 g; Mobile phase A: H_2O, Mobile phase B: MeCN; Flow rate: 100 mL/min; Gradient: 70-100% B in 30 min; Wavelength: 210 nm. The collected fractions were concentrated under vacuum at a temperature below 10 °C, then freeze-dried to provide 1.12 g of **3-oxazolone** (92.5% (57.1% + 35.4%) LCAP) as a white solid in a 57% isolated yield (**Method 2**), consisting of two isomers. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.10 (t, J = 8.8 Hz, 1H), 7.95 (d, J = 8.4 Hz, 2H), 7.88 (d, J = 7.6 Hz, 2H), 7.72 (d, J = 8.4 Hz, 2H), 7.66 - 7.62 (m, 4H), 7.44 - 7.38 (m, 4H), 7.33 - 7.26 (m, 2H), 4.69 - 4.64 (m, 1H), 4.56 - 4.51 (m, 1H), 4.32 - 4.15 (m, 3H), 3.31 (t, J = 10.0

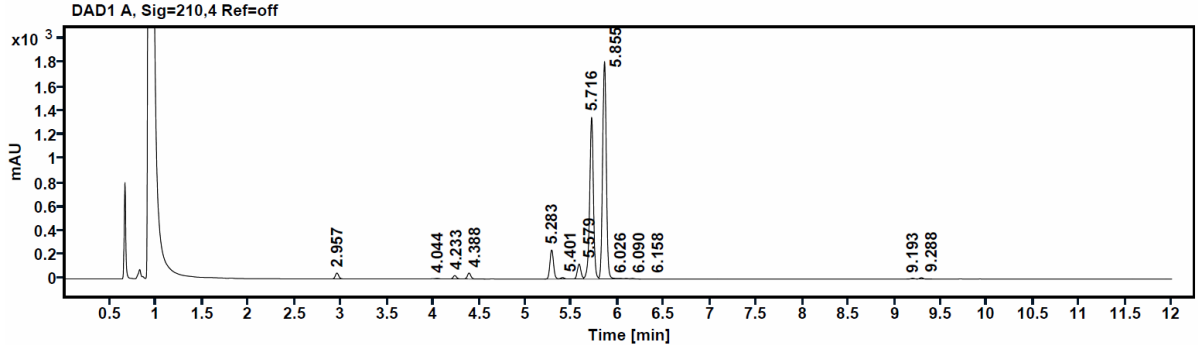
Hz, 1H), 3.24 - 3.17 (m, 5H), 3.11 - 3.02 (m, 1H), 1.91 - 1.76 (m, 1H), 1.72 - 1.63 (m, 1H), 1.57 (s, 9H), 1.53 - 1.36 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 176.99, 176.95, 163.12, 162.18, 161.92, 154.02, 142.35, 142.04, 142.00, 139.06, 135.78, 135.66, 135.53, 135.49, 128.40, 128.34, 127.97, 125.96, 125.37, 125.14, 124.88, 123.55, 118.44, 79.04, 69.41, 69.35, 64.06, 62.18, 56.11, 56.02, 49.37, 49.31, 44.91, 34.27, 26.15, 25.70, 25.60, 23.05, 22.91. HRMS (ESI): m/z calcd. for $C_{41}H_{43}N_2O_7$ $[M+H]^+$: 675.3065, found: 675.3077. FTIR (neat): 3358, 2969, 2932, 2870, 1816, 1703, 1516, 1447, 1298, 1239, 1163, 1115, 1018, 892, 761, 737, 557 cm^{-1}

HPLC (Method 2) of isolated **3-oxazolone** (no $BnNH_2$ quench)

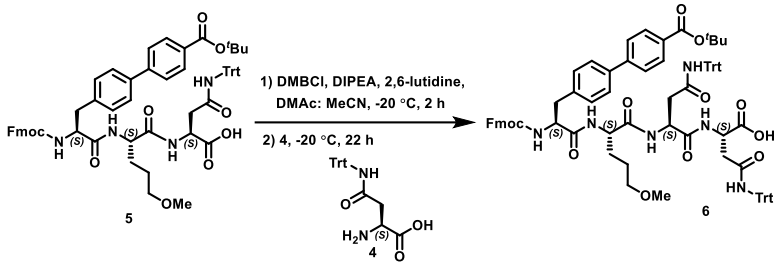


HPLC (Method 1) profile of **3-oxazolone** quenched by $BnNH_2$:





Compound ID	Time	Area	Height	Area%
3	4.233	73.7049	28.5	0.7
***epi*-3**	4.388	131.0645	49.9	1.3
3-INT	5.716	3882.8792	1351.8	35.4
***epi*-3-INT**	5.855	5021.3833	1818.3	50.4
Total	-	-	-	87.8

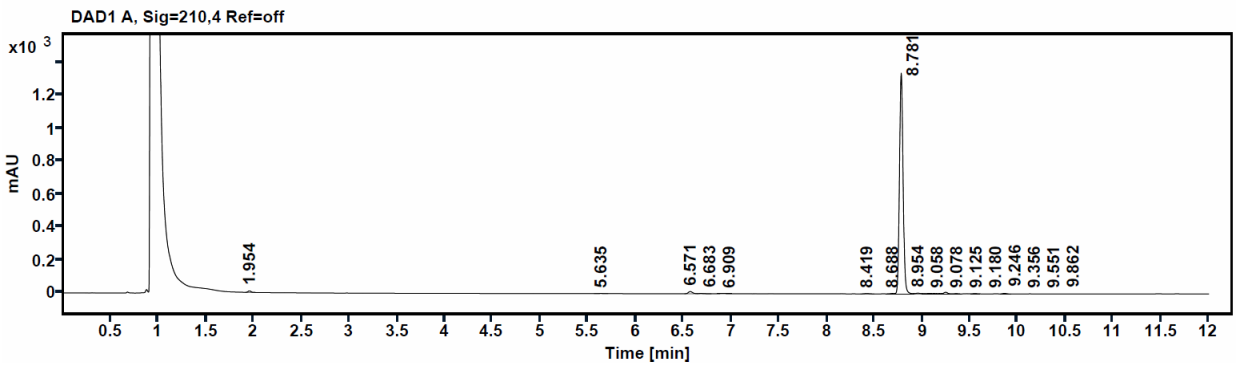


Synthesis of Fmoc-Bip-4-CO$_2$*t*Bu -5-OMe-NVA-L-Gln(Trt)-L-Gln(Trt)-OH (6). In a 40-mL vial, **5** (2.00 g, 1.91 mmol) was dissolved in DMAc/MeCN (1:1, 20 mL) at 25 °C. After the solution was cooled to -20 °C, 2,6-lutidine (0.25 g, 2.29 mmol) and DIPEA (0.25 g, 1.90 mmol) were added. Then DMBCl (0.28 g, 2.10 mmol) was added, and the reaction mixture was stirred at -20 °C for 2 h. Subsequently, **4** (0.79 g, 2.10 mmol) was added to the reaction mixture, which was then stirred at -20 °C for 22 h and monitored by HPLC (Method 1). Upon completion, the reaction mixture was purified by Prep-HPLC under the conditions: Column: C18, 20-40 μm, 330 g; Mobile phase A: H_2O, Mobile phase B: MeCN; Flow rate: 100 mL/min; Gradient: 70-100% B in 30 min; Wavelength: 210 nm. The collected fractions were concentrated to dryness under vacuum at a temperature below 40 °C to yield 2.14 g of **6** as a yellow solid (95.0% LCAP) in 80% isolated yield (**Method 1**).

mp 208-211 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 12.65 (br s, 1H), 8.64 (s, 2H), 8.27 (d, J = 8.0 Hz, 1H), 8.09 - 8.04 (m, 2H), 7.93 (d, J = 8.4 Hz, 2H), 7.87 (d, J = 7.6 Hz, 2H), 7.69 (d, J = 8.4 Hz, 2H), 7.62 (d, J = 7.6 Hz, 2H), 7.58 (d, J = 8.4 Hz, 2H), 7.43 (d, J = 8.0 Hz, 2H), 7.40 - 7.36 (m, 2H), 7.27 - 7.24 (m, 14H), 7.21 - 7.14 (m, 18H), 4.68 (q, J = 7.2 Hz, 1H), 4.49 - 4.38 (m, 3H), 4.16 - 4.11 (m, 4H), 3.24 (t,

J = 6.0 Hz, 2H), 3.15 - 3.05 (m, 4H), 2.89 - 2.73 (m, 2H), 2.65 - 2.56 (m, 3H), 1.83 - 1.76 (m, 1H), 1.62 - 1.57 (m, 12H). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 173.26, 171.78, 171.56, 171.39, 169.34, 169.15, 165.28, 156.37, 145.20, 144.68, 144.29, 144.14, 141.15, 141.12, 139.13, 137.24, 130.54, 130.36, 130.08, 129.07, 128.09, 127.96, 127.52, 127.07, 126.96, 126.82, 126.78, 125.77, 120.52, 81.12, 72.17, 69.93, 69.88, 66.18, 58.17, 56.58, 52.71, 50.37, 49.75, 47.02, 38.26, 37.63, 30.41, 28.29, 25.68. HRMS (ESI): m/z calcd. for $C_{87}H_{85}N_6O_{12}$ $[M+H]^+$: 1405.6220, found: 1405.6251. FTIR (neat): 3289, 2928, 1709, 1643, 1525, 1491, 1447, 1293, 1254, 1165, 1114, 1035, 848, 738, 697, 638, 567 cm^{-1}.

HPLC of isolated **6**



Into a 20-mL vial, **Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-L-Gln(Trt)-OH (3)** (0.50 g, 0.72 mmol) was dissolved in DMAc (5 mL). After the solution was cooled to -20 °C, the coupling reagent (0.79 mmol) was added, followed by DIPEA (0.11 g, 0.87 mmol). The reaction mixture was stirred at -20 °C for 5 h. **H-L-Gln(Trt)-OH (4)** (0.30 g, 0.79 mmol) was added to the reaction, and the reaction mixture was stirred at -20 °C for 16 h.

For EDCI: Follow the general procedure. The coupling reagents were EDCI (0.15 g, 0.79 mmol) and HOBt (0.11 g, 0.79 mmol), DIPEA (0.21 g, 1.60 mmol).

For DIC: Follow the general procedure. The coupling reagents were DIC (0.10 g, 0.79 mmol) and Oxyma (0.87 mmol).

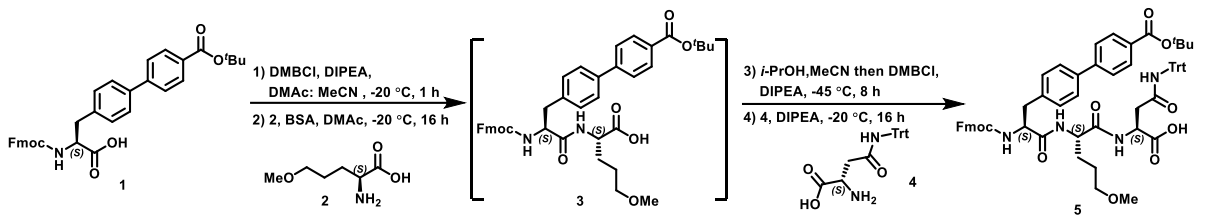
For DCC: Follow the general procedure. The coupling reagents were DCC (0.16 g, 0.79 mmol) and HOSu (0.09 g, 0.79 mmol).

HATU, Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium. EDCI, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide Hydrochloride. HOBt, 1-Hydroxybenzotriazole. COMU, (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate. DIC, *N,N′*-Diisopropylcarbodiimide. Oxyma, Ethyl Cyano(hydroxyimino)acetate. DCC, *N, N′*-Dicyclohexylcarbodiimide. HOSu, *N*-Hydroxysuccinimide. PivCl, Pivaloyl chloride. DMBCl, 2,2-Dimethylbutanoyl chloride. EHCl, 2-Ethylhexanoyl chloride. IBCF, Isobutyl chloroformate. DMAP, 4-Dimethylaminopyridine.

Supplementary Table 1. Study of activating reagents

	Condition			**HPLC result (16 h, LCAP, 210 nm)**					
Entry	**Coupling Reagent**	**Base (1.2 equiv.)**	**Temp. (°C)**	**3**	**3-mixed anhydride**	**5**	***epi*-5**	**3-Oxazolone**	**6**
1	HATU (1.1 equiv.)	DIPEA	-20	0.2	3	45.6	33.8	3.6	6.5
2	EDCI (1.1 equiv.) HOBt (1.1 equiv.)	DIPEA (2.2 equiv.)	-20	11	3.5	19.2	13.3	32.1	2.7
3	COMU (1.1 equiv.)	DIPEA	-20	9.4	0.0	51.0	8.2	16.1	1.1
4	DIC (1.1 equiv.) + oxyma (1.2 equiv.)	NA	-20	31.3	0	40.2	1.5	1.5	14.6
5	DCC (1.1 equiv.) + HOSu (1.1 equiv.)	DIPEA	0	30.4	3.2	45.2	2.6	1.6	3.8
6	IBCF (1.1 equiv.)	DIPEA	-20	0.8	0	39.9	10.0	45.5	0.6
7	PivCl (1.1 equiv.)	DIPEA	-20	5.6	0	89.3	0.4	2.6	0.3
8	DMBCl (1.1 equiv.)	DIPEA	-20	1.2	0.1	93.8	0.6	3.5	0.4

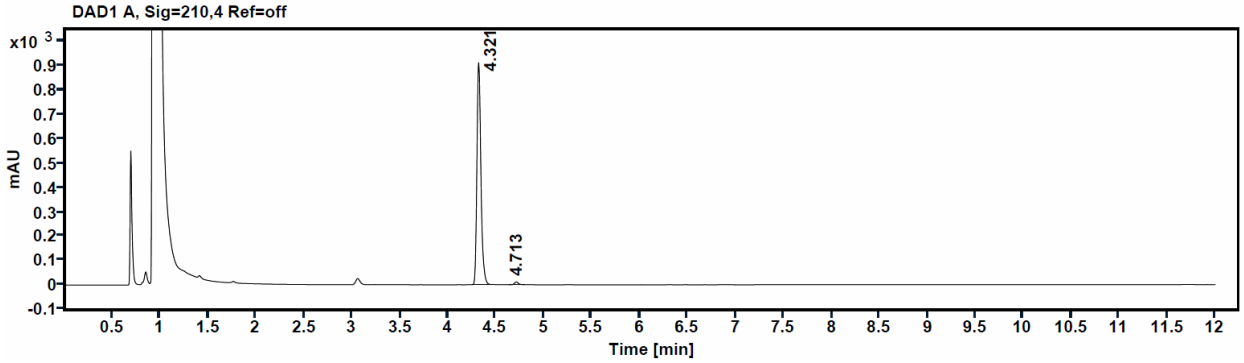
3-Oxazolone* level is reported based on the calculation: **3-oxazolone*** = ***epi*-3-INT** × 1.7. Upon quenching with $BnNH_2$, **3-oxazolone** gave **3-INT** and ***epi*-3-INT** in a 0.7:1 ratio.



Continuous Elongation to Synthesize Fmoc-Bip-4-CO_2*t*Bu -5-OMe-NVA-L-Gln(Trt)-OH (5).

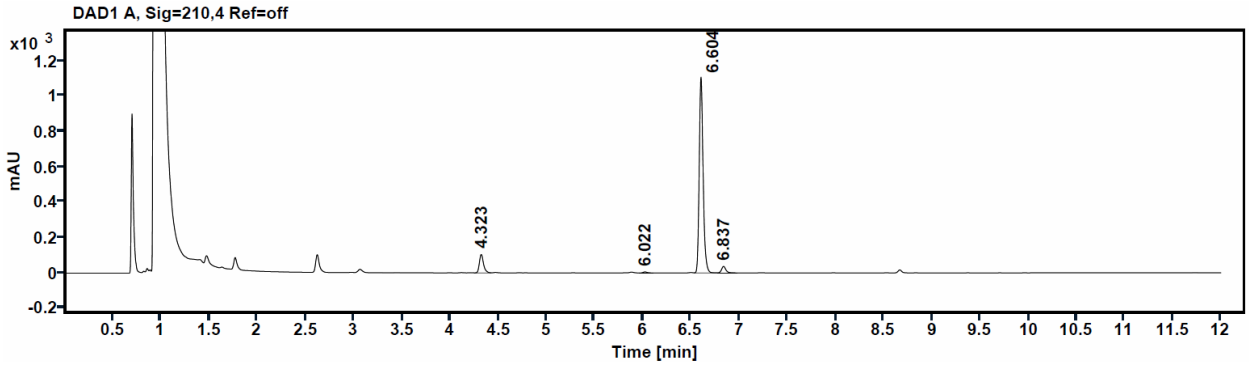
In a 100-mL three-neck jacketed vessel, **1** (5.00 g, 8.87 mmol) was dissolved in DMAc/MeCN (1:1, 30 mL) at 25 °C. Then DIPEA (1.26 g, 9.76 mmol) and DMBCl (1.31 g, 9.76 mmol) were added at -20 °C. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC **(Method 1)**, which showed the ratio of **1:1-INT** was 1.7:98.3. A BSA solution of **2**, which was prepared by stirring **2** (1.57 g, 10.64 mmol) and BSA (3.61 g, 17.74 mmol) in DMAc (15 mL) at 50 °C for 1 h then cooling down to ambient temperature, was added to the reaction at -20 °C over 10 min. Then DIPEA (0.23 g, 1.77 mmol) was added at -20 °C. The reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC **(Method 1)**, which showed the ratio of **1:3:3-over-addition** was 1.3:98.7:0.0. Then isopropyl alcohol (*i*-PrOH) (2.13 g, 35.48 mmol) was added to the solution at 0 °C and stirred for 1 h. MeCN (15 mL) was added at 0 °C. Then the mixture was cooled to -45 °C. DMBCl (2.03 g, 15.08 mmol) and DIPEA (1.61 g, 12.42 mmol) were added and stirred at -45 °C for 8 h and monitored by HPLC **(Method 1)**, which showed the ratio of **3:3-INT:*epi*-3-INT** was 6.5:90.9:2.6. Compound **4** (4.64 g, 12.42 mmol) and DIPEA (0.23 g, 1.77 mmol) were added to the reaction at -45 °C. The reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC **(Method 1)**, which showed the ratio of **3:*epi*-3-INT:5:*epi*-5:6** was 8.7:0.5:87.5:3.3:0.0. The reaction assay yield was 82% over two steps. (coupling 1 assay yield was 96%, coupling 2 assay yield was 85%)

HPLC of **3** formation (reaction mixture)



Compound ID	Time	Area	Height	Area%
3	4.321	2616.8992	915.2	98.7
1	4.713	34.4782	11.9	1.3
3-over-addition	4.124	-	-	0.0
Total	-	-	-	100.0

HPLC of **5** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
3	4.323	307.9737	104.2	8.7
***epi*-3-INT**	6.022	17.4107	5.8	0.5
5	6.604	3104.0186	1107.6	87.5
***epi*-5**	6.837	116.7635	37.0	3.3
6	8.672	-	-	0.0
Total	-	-	-	100.0

2) General Procedure for Fig. 2b

Additive screening procedure:

In a 20-mL vial, **3** (0.50 g, 0.72 mmol) was dissolved in the solvent(s) (5 mL). After the solution was cooled to -20 °C, DIPEA (0.10 g, 0.79 mmol) and additives were added. Then DMBCl (0.11 g, 0.79 mmol) was added, and the reaction mixture was stirred at -20 °C for 5 h. Subsequently, **4** (0.30 g, 0.79 mmol) was added to the reaction mixture. The reaction mixture was stirred at -20 °C for 16 h.

3-Oxazolone* level is reported based on the calculation: **3-Oxazolone***= ***epi*-3-INT**×1.7. **3-Oxazolone** was converted to 0.7:1 of **3-INT:*epi*-3-INT** by quenching with $BnNH_2$.

Total Oxazolone Formed** level is reported based on the calculation: ***epi*-3-INT**×1.7+ ***epi*-5**×2.

For entries 47***and 48***: **3-Oxazolone*** level is reported based on the calculation: **3-Oxazolone***= ***epi*-3-INT**×1.7+ ***epi*-3**×**2**.

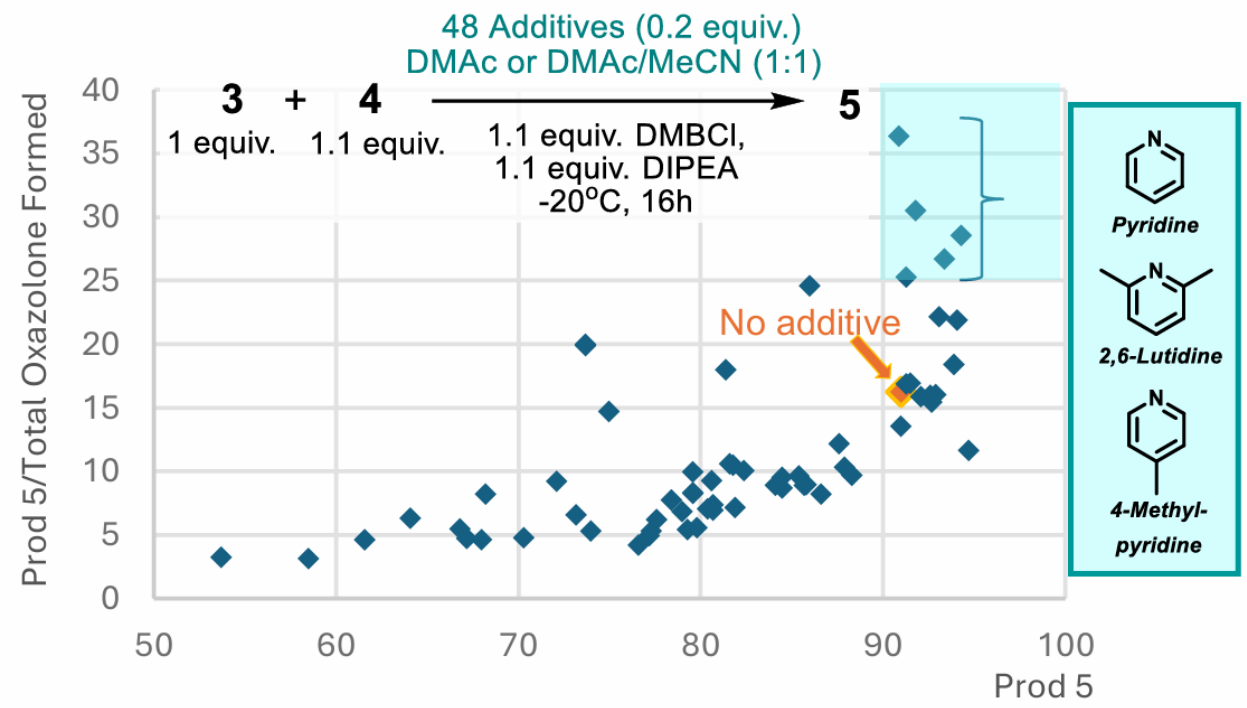
Supplementary Table 2. Additive screening for model reaction

	Condition		HPLC (Method 1) result (LCAP, 210 nm)					
Entry	**Base, solvent and temperature**	**Additive (20 mol%)**	**3**	**5**	***epi*-5**	**3-oxazolone***	**6**	**Total oxazolone formed****
1		-	1.5	90.2	1.2	5.3	0.1	7.7
2		$ZnCl_2$	2.9	86.6	1.4	7.7	0.0	10.5
3		$AlCl_3$	64.1	0.9	0.0	6.0	0.0	6.0
4		$MnCl_2$	26.5	38.3	0.5	7.8	0.1	8.8
5		$FeCl_3$	23.6	31.0	0.3	6.3	0.0	6.9
6		$FeCl_2$	1.8	87.9	0.7	7.1	0.1	8.5
7		$MgCl_2$	2.1	84.5	0.7	8.3	8.9	9.7
8		$ZrCl_4$	52.3	0.9	0.2	6.5	0.0	6.9
9		$BiCl_3$	0.9	85.8	0.7	8.2	0.0	9.6
10		$CeCl_3$	19.4	47.0	0.6	7.5	0.9	8.7
11		$FeBr_3$	26.3	29.4	0.4	10.0	0.0	10.8
12		$InCl_3$	4.4	68.2	0.5	7.3	0.0	8.3
13		LiCl	4.4	86.0	0.4	2.7	2.5	3.5

14	1.1 equiv. DIPEA, 1.1 equiv. DMBCl, 10 vol. DMAc, -20 °C	$CoCl_2$	0.8	82.4	0.7	6.8	0.0	8.2
15		$Cu(OTf)_2$	11.6	30.9	0.7	12.1	0.0	13.5
16		$Mg(OTf)_2$	12.1	79.6	1.0	6.0	0.5	8.0
17		$Al(OTf)_3$	46.6	9.5	0.3	8.5	0.0	9.1
18		$Ca(OTf)_2$	5.3	80.6	0.7	7.3	1.8	8.7
19		$Sc(OTf)_3$	23.2	45.5	0.9	14.8	0.0	16.6
20		$Fe(OTf)_2$	3.6	81.9	0.7	10.0	0.1	11.4
21		$Fe(OTf)_3$	22.5	36.0	0.5	11.2	0.0	12.2
22		$Ni(OTf)_2$	0.9	91.0	0.7	5.3	0.0	6.7
23		$Zn(OTf)_2$	7.5	77.3	2.1	10.4	0.0	14.6
24		$Y(OTf)_3$	31.4	27.5	0.8	11.7	0.2	13.3
25		AgOTf	1.5	88.3	0.9	7.3	0.0	9.1
26		$In(OTf)_3$	8.1	68.0	0.7	13.3	0.0	14.7
27		$Sn(OTf)_2$	3.3	76.6	0.9	16.5	0.0	18.3
28		$Ba(OTf)_2$	7.1	75.0	0.6	3.9	3.5	5.1
29		$La(OTf)_3$	28.2	38.5	0.9	12.1	1.6	13.9
30		$Pr(OTf)_3$	26.4	38.4	0.7	12.4	0.9	13.8
31		$Nd(OTf)_3$	25.0	41.7	0.8	11.2	1.1	12.8
32		$Eu(OTf)_3$	27.1	34.0	0.6	12.1	0.4	13.1
33		$Er(OTf)_3$	28.2	34.4	0.7	11.7	0.5	11.7
34		$Yb(OTf)_3$	28.1	30.0	0.6	10.5	0.0	13.3
35		$Bi(OTf)_3$	10.6	70.3	0.7	13.3	0.0	14.7
36		$Pd(OAc)_2$	4.5	81.8	1.7	4.4	0.0	7.8
37		TBAB	1.0	91.3	0.5	4.4	0.1	5.4
38		TBACl	1.1	92.9	0.8	3.6	0.0	5.8
39		TBAI	0.9	92.6	0.6	4.6	0.1	5.8
40		Et_3N	1.1	92.7	0.6	4.8	0.2	6.0
41		NMM	0.8	93.9	0.6	3.9	0.0	5.1
42		pyridine	0.9	93.4	1.0	1.5	0.2	3.5
43		2,6-lutidine	0.8	94.3	0.3	2.7	0	3.3
44		4-Methyl pyridine	0.8	94.3	0.3	2.7	0.0	2.5
45		2-Methyl pyridine	1.3	90.9	0.3	1.9	0.8	5.8
46		2,4,6-Collidine	1.0	92.1	0.5	4.8	0.1	5.4
47***		DMAP***	0.9	91.5	0.9	3.6	0.3	55.8
48***		NMI***	9.7	30.1	27.2	1.4	8.1	74.8

	Condition		HPLC result (LCAP, 210 nm)					
entry	**Base, solvent and temperature**	**Additive (20 mol%)**	**3**	**5**	***epi*-5**	**3-oxazolone***	**Total oxazolone formed****	**6**
1	1.1 equiv. DIPEA, 1.1 equiv. DMBCl, 10 Vol 1/1 DMAc:MeCN	-	5.9	79.8	2.2	9.9	14.3	0.7
2		$ZnCl_2$	8.4	80.4	2.1	7.2	11.4	0.4
3		$AlCl_3$	36.5	9.3	0.5	7.0	8.0	0.0
4		$MnCl_2$	10.3	74.0	1.3	11.3	13.9	0.9
5		$FeCl_3$	29.0	45.1	0.4	5.3	6.1	0.1
6		$FeCl_2$	23.5	47.4	0.2	6.4	6.8	0.2
7		$MgCl_2$	28.7	34.9	0.6	16.7	17.9	9.4
8		$ZrCl_4$	83.8	0.4	0.0	1.8	1.8	0.1
9		$BiCl_3$	4.1	84.1	0.6	8.2	9.4	0.2
10		$CeCl_3$	36.8	34.3	0.1	10.3	10.5	1.8
11		$FeBr_3$	25.2	36.8	0.1	9.3	9.5	0.1
12		$InCl_3$	6.7	73.2	0.7	9.7	11.1	0.1
13		LiCl	18.3	42.7	6.1	24.8	37.0	4.0
14		$CoCl_2$	7.1	64.1	0.5	9.1	10.1	0.2
15		$Cu(OTf)_2$	8.7	73.7	0.3	3.1	3.7	0.3
16		$Mg(OTf)_2$	9.1	78.4	1.0	8.2	10.2	1.8
17		$Al(OTf)_3$	27.6	15.1	0.6	10.0	11.2	0.1
18		$Ca(OTf)_2$	13.9	67.2	1.0	12.2	14.2	2.2
19		$Sc(OTf)_3$	18.1	47.8	0.5	9.0	10.0	0.1
20		$Fe(OTf)_2$	4.1	81.6	0.3	7.1	7.7	0.2
21		$Fe(OTf)_3$	7.4	79.6	0.5	8.6	9.6	0.9
22		$Ni(OTf)_2$	4.3	87.6	0.7	5.8	7.2	0.3
23		$Zn(OTf)_2$	6.2	79.3	2.3	10.1	14.7	0.2
24		$Y(OTf)_3$	4.1	85.7	1.2	7.2	9.6	0.4
25		AgOTf	5.1	85.4	0.9	7.0	8.8	0.3
26		$In(OTf)_3$	9.2	58.5	0.8	17.1	18.7	0.2
27		$Sn(OTf)_2$	6.1	79.0	0.5	10.5	11.5	0.2
28		$Ba(OTf)_2$	13.0	66.8	0.9	10.4	12.2	2.3
29		$La(OTf)_3$	36.3	32.9	0.1	15.3	15.5	1.3
30		$Pr(OTf)_3$	34.9	33.6	0.1	15.1	15.3	1.2
31		$Nd(OTf)_3$	34.4	34.2	0.1	14.1	14.3	0.9
32		$Eu(OTf)_3$	34.7	33.2	0.1	13.6	13.8	0.7
33		$Er(OTf)_3$	32.0	36.4	0.1	12.6	12.8	0.3
34		$Yb(OTf)_3$	31.4	33.3	0.4	12.0	12.8	0.2
35		$Bi(OTf)_3$	5.5	77.6	0.6	11.3	12.5	0.2
36		$Pd(OAc)_2$	25.1	61.6	3.9	5.6	13.4	0.2
37		TBAB	10.4	53.7	1.5	13.4	16.4	0.5

38		TBACl	8.8	49.8	2.0	17.5	21.5	0.5
39		TBAI	4.9	81.4	0.9	2.7	4.5	0.4
40		Et_3N	7.3	77.2	3.1	9.4	15.6	0.8
41		NMM	7.1	81.7	1.4	8.2	11.0	0.7
42		pyridine	3.7	91.8	0.4	2.2	3.0	0.6
43		2,6-lutidine	1.1	94.1	0.6	3.1	4.3	0.3
44		4-methylpyridine	4.3	91.3	0.7	2.2	3.6	0.3
45		2-methylpyridine	5.4	84.5	1.2	6.5	8.9	0.5
46		2,4,6-Collidine	6.0	94.7	1.0	6.1	8.1	0.5
47***		DMAP	14.7+ ***epi-3***:12.1	25.9	22.6	29.9	75.1	4.5
48***		NMI	9.7+ ***epi-3***: 6.7	22.1	14.0	38.9	66.9	3.6



2,6-Lutidine, pyridine, and 4-methylpyridine were further evaluated through loading and kinetic studies.

Loading and kinetic studies identified 1.0 equiv. 2,6-lutidine as the optimal base, maximizing SM conversion while minimizing ***epi*-5** and **3-oxazolone** formation. Pyridine and 4-methylpyridine showed similar benefits at lower loadings and shorter reaction times; however, prolonged aging increased epimer and oxazolone levels.

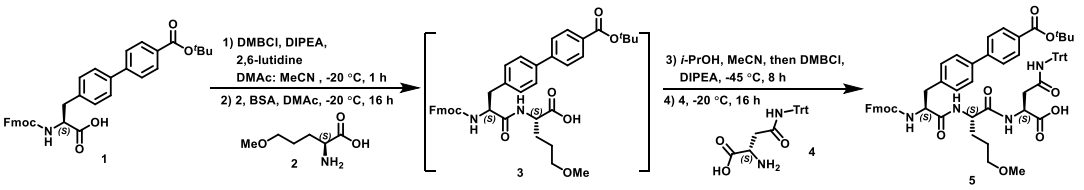
Experimental:

In a 20-mL vial, **3** (0.50 g, 0.72 mmol) was dissolved in DMAc/MeCN (1:1, 5 mL). After the solution was cooled to -20 °C, DIPEA (0.09 g, 0.72 mmol) was added. Then DMBCl (0.11 g, 0.79 mmol) was added, and the reaction mixture was stirred for 30 min at -20 °C. 2,6-Lutidine, pyridine or 4-methyl pyridine was added, and the reaction mixture was stirred at -20 °C for 20 h. **4** (0.30 g, 0.79 mmol) was added to the reaction. The reaction mixture was stirred at -20 °C for 20 h.

3-mixed anhydride ** level is reported based on the calculation: **3-mixed anhydride** **= **3-INT** - ***epi*-3-INT**× 0.7. **3-Oxazolone** was converted to 0.7:1 of **3-INT**:***epi*-3-INT** by quenching with $BnNH_2$, so the LCAP of **3-INT** is the sum of **3-mixed anhydride** and ***epi*-3-INT**× 0.7.

Supplementary Table 3. Loading study of 2,6-lutidine, pyridine, and 4-methylpyridine

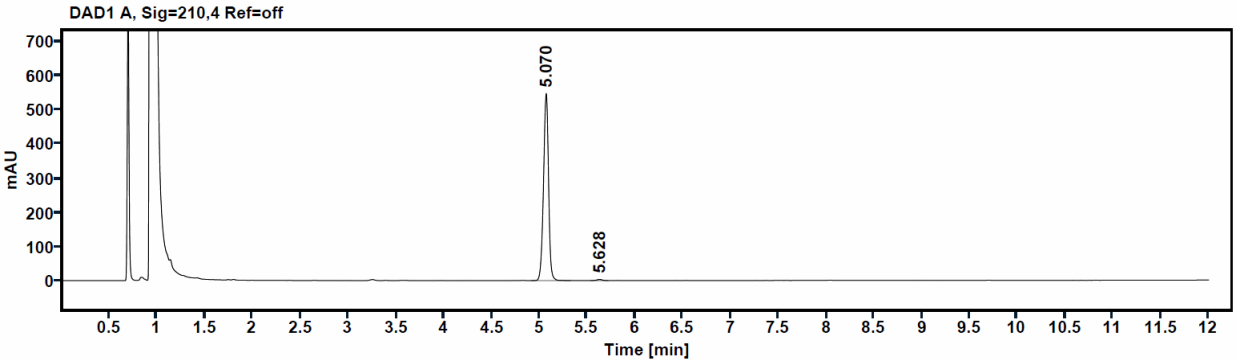
Entry	HPLC result (LCAP, 210 nm)			Additive	HPLC result (LCAP, 210 nm)									HPLC result (LCAP, 210 nm)					
	30 min				1 h			4 h			20 h			20 h					
	1	3-mixed anhydride	3-oxazolone		3	3-mixed anhydride	3-oxazolone	3	3-mixed anhydride	3-oxazolone	3	3-mixed anhydride	3-oxazolone	3	3-mixed anhydride	5	*epi*-5	3-oxazolone	6
1	10.1	85.8	4.1	/	1.8	92.1	6.1	1.7	91.5	6.8	5.8	77.7	16.5	5.6	0.5	86.5	2.6	4.8	0.0
2	10.6	84.7	4.6	2,6-lutidine (0.1 equiv.)	4.9	90.0	5.1	4.3	90.4	5.3	3.7	90.4	6.0	3.0	0.8	91.9	0.6	3.7	0.1
3	9.7	85.9	4.4	2,6-lutidine (0.2 equiv.)	1.4	93.5	5.1	1.7	93.0	5.3	2.9	90.8	6.3	2.6	0.3	91.6	0.8	4.5	0.1
4	9.7	86.1	4.3	2,6-lutidine (0.5 equiv.)	0.8	94.6	4.6	0.9	94.4	4.8	0.8	93.9	5.3	0.7	0.3	95	0.5	3.4	0.1
5	10.2	85.6	4.3	2,6-lutidine (1.0 equiv.)	0.7	94.7	4.6	0.8	94.5	4.6	1.1	94.0	4.9	1.0	0.2	95	0.6	3.3	0.1
6	9.6	86.1	4.3	pyridine (0.1 equiv.)	0.7	94.7	4.6	0.7	94.5	4.8	0.8	93.9	5.4	1.1	0.2	94.2	0.6	3.9	0.1
7	10.5	85.3	4.3	pyridine (0.2 equiv.)	1.1	94.4	4.4	1.0	94.2	4.8	0.9	93.6	5.6	1.0	0.3	94.2	0.7	3.8	0.1
8	9.8	86.0	4.3	pyridine (0.5 equiv.)	0.6	94.8	4.6	0.6	94.6	4.8	0.6	93.3	6.1	0.6	0.2	94.4	1	3.7	0.1
9	9.2	86.4	4.4	pyridine (1.0 equiv.)	0.5	94.9	4.6	0.5	94.2	5.3	0.5	91.9	7.7	0.5	0.2	93.8	1.1	3.9	0.1
10	10.0	85.9	4.3	4-methyl pyridine (0.1 equiv.)	0.7	94.7	4.6	0.6	94.5	4.9	0.5	93.0	6.5	0.5	0.3	94.2	1.0	4.1	0.1
11	10.0	85.7	4.4	4-methyl pyridine (0.2 equiv.)	0.5	94.7	4.8	0.5	94.2	5.3	0.5	91.0	8.5	0.5	0.3	93.3	1.7	4.2	0.1
12	13.8	81.6	4.6	4-methyl pyridine (0.5 equiv.)	0.6	94.0	5.4	0.4	91.9	7.7	0.5	79.5	20.1	0.6	0.3	88.9	5.8	4.2	0.1
13	11.7	84.0	4.4	4-methyl pyridine (1.0 equiv.)	0.5	93.3	6.1	0.5	88.1	11.4	1.4	60.7	37.9	1.7	0.2	82.2	10.6	5.4	0.1



Synthesis of Fmoc-Bip-4-CO_2*t*Bu-5-OMe-NVA-L-Gln(Trt)-OH (5).

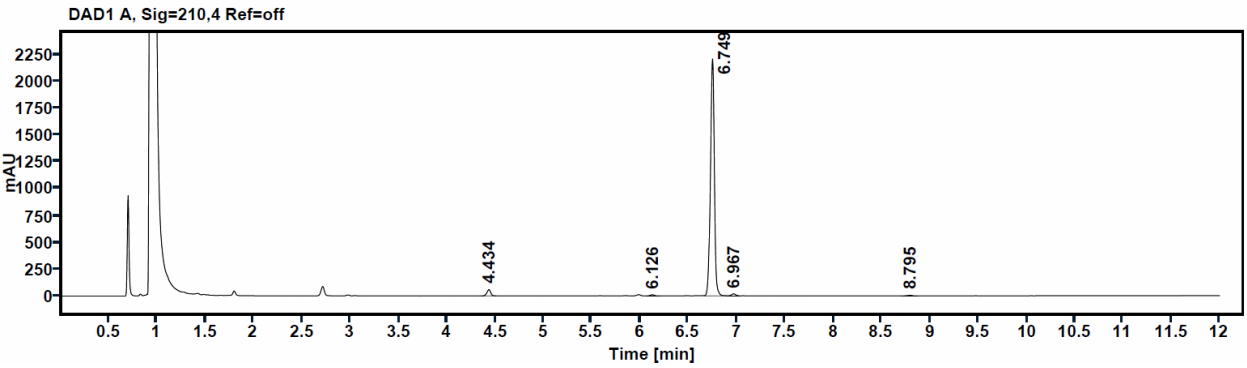
With 2,6-lutidine: In a 100-mL three-neck jacketed vessel, **1** (5.00 g, 8.87 mmol) was dissolved in DMAc/ MeCN (1:1, 30 mL) at 25 °C. Then 2,6-lutidine (1.05 g, 9.76 mmol) and DIPEA (1.31 g, 9.76 mmol) were added at -20 °C. Then DMBCl (1.31 g, 9.76 mmol) was added, and the reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC **(Method 3)**, which showed the ratio of **1:1-INT** was 0.6:99.4. A BSA solution of **2**, which was prepared by stirring **2** (1.43 g, 9.76 mmol) and BSA (3.61 g, 17.74 mmol) in DMAc (15 mL) at 50 °C for 1 h, was added to the reaction at -20 °C over 10 min. Then DIPEA (0.23 g, 1.77 mmol) was added. The reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC **(Method 3)**, which showed the ratio of **1**:**3**:**3-over-addition** was 0.7:99.3:0.0. Then *i*-PrOH (2.13 g, 35.48 mmol) was added to the solution at 0 °C and stirred for 1 h. MeCN (15 mL) was added at 0 °C. Then the mixture was cooled to -45 °C, DMBCl (1.67 g, 12.42 mmol) and DIPEA (0.34 g, 2.66 mmol) were added and stirred at -45 °C for 8 h and monitored by HPLC **(Method 3)**, which showed the ratio of **3:3-INT:*epi*-3-INT** was 3.0:96.9:0.1. **4** (3.98 g, 10.64 mmol) and DIPEA (0.23 g, 1.77 mmol) were added to the reaction at -45 °C. The reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC **(Method 1)**, which showed the ratio of **3:*epi*-3-INT:5:*epi*-5:6** was 2.4:0.5:95.9:0.9:0.3. The reaction assay yield was 91% over two steps. (coupling1 assay yield was 98%, and coupling 2 assay yield was 94%)

HPLC of **3** formation (reaction mixture)

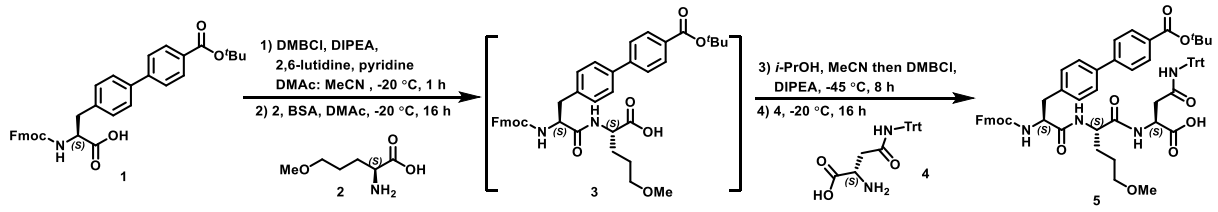


Compound ID	Time	Area	Height	Area%
3	5.070	1992.1921	550.7	99.3
1	5.628	13.1334	3.5	0.7
3-over-addition	4.928	-	-	0.0
Total	-	-	-	100.0

HPLC of **5** formation (reaction mixture)



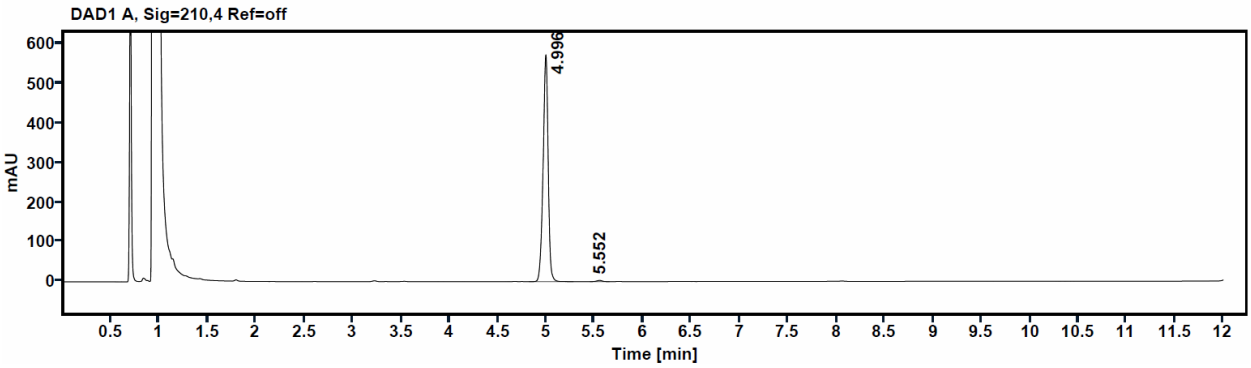
Compound ID	Time	Area	Height	Area% ratio
3	4.434	158.8523	59.0	2.4
***epi*-3-INT**	6.126	32.2052	11.2	0.5
5	6.749	6284.6719	2214.3	95.9
***epi*-5**	6.967	58.2257	18.8	0.9
6	8.795	17.7890	5.7	0.3
Total	-	-	-	100.0



With 2,6-lutidine and pyridine: In a 250-mL round-bottom flask, **1** (10.00 g, 17.74 mmol) was dissolved in DMAc/MeCN (1:1, 60 mL). After the solution was cooled to -20 °C, 2,6-lutidine (2.09 g, 19.51 mmol), pyridine (0.28 g, 3.54 mmol) and DIPEA (2.52 g, 19.50 mmol) were added. Then DMBCl (2.63 g, 19.54 mmol) was added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC **(Method 3)**, which showed the ratio of **1:1-INT** was 0.5:99.5. A BSA solution of **2**, which was prepared by stirring **2** (2.87 g, 19.50 mmol) and BSA (7.22 g, 35.50mmol) in DMAc (30 mL) at 50 °C for 1 h, was added to the reaction at -20 °C over 10 min. Then DIPEA (0.46 g, 3.56 mmol) was added. The reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC **(Method 3)**, which showed the ratio of **1:3:3-over-addition** was 0.6:99.4:0.0. Then *i*-PrOH (4.26 g, 70.8 mmol) was added to the solution at 0 °C and stirred for 1 h. MeCN (30 mL) was added. Then the mixture was cooled to -45 °C, DMBCl (3.34 g, 24.81 mmol) and DIPEA (0.69 g, 5.34 mmol) were added and stirred at -45 °C for 8 h and monitored by HPLC **(Method**

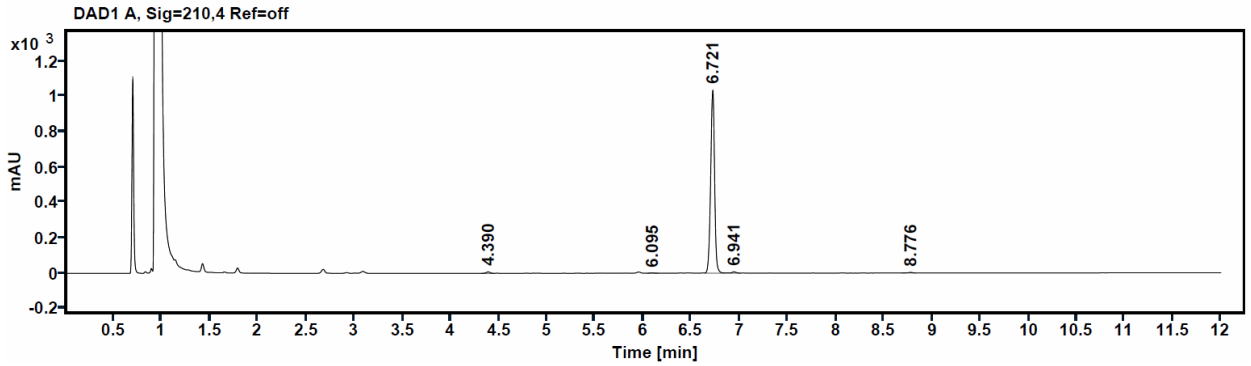
3), which showed the ratio of **3:3-INT:*epi*-3-INT** was 0.7:99.2:0.1. **4** (7.95 g, 21.23 mmol) and DIPEA (0.46 g, 3.56 mmol) were added to the reaction at -45 °C. The reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC **(Method 1)**, which showed the ratio of **3:*epi*-3-INT:5:*epi*-5:6** was 0.7:0.2:98.0:0.7:0.4. The reaction assay yield was 96% over two steps. (coupling 1 assay yield was 99%, and coupling 2 assay yield was 97%) The reaction mixture was added slowly to H_2O (300 mL) at 0 °C over 1 h, and the mixture was stirred at 0 °C for 3 h. The product was collected by filtration and dried under vacuum at 40 °C, yielding 18.4 g of **5** as a white solid (96.1% LCAP, 95.2 wt%) in 94% isolated yield. **(Method 1)**

HPLC of **3** formation (reaction mixture)



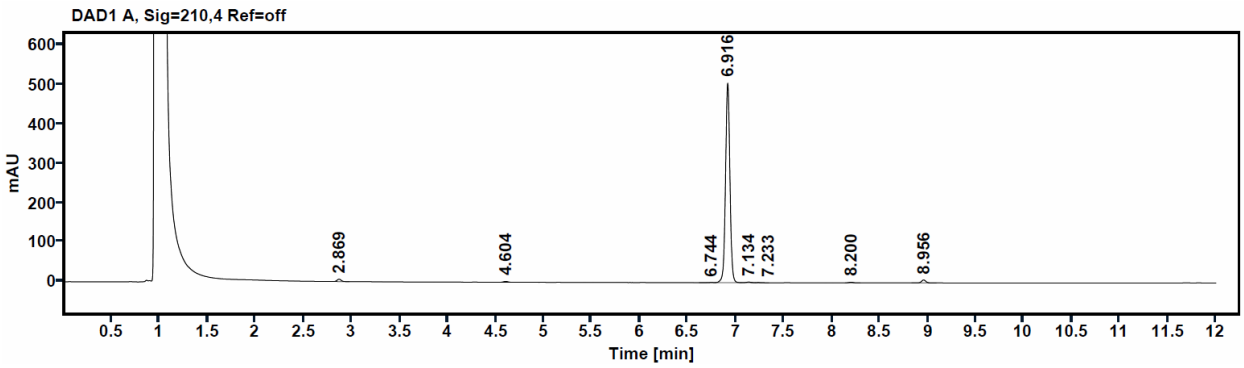
Compound ID	Time	Area	Height	Area% ratio
3	4.996	2086.5112	572.8	99.4
1	5.552	11.7503	3.1	0.6
3-over-addition	4.841	-	-	0.0
Total	-	-	-	100.0

HPLC of **5** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
3	4.39	21.8382	8.2	0.7
***epi*-3-INT**	6.095	5.6387	1.9	0.2
5	6.721	2883.302	1036.3	98.0
***epi*-5**	6.941	19.9443	7	0.7
6	8.776	11.2398	3.6	0.4
Total	-	-	-	100.0

HPLC of isolated **5**



Experimental procedures and characterizations for Figure 3

Experimental Procedures for Fig. 3a -1 and -2

Entry 1: In a 20-mL vial, **3** (0.50 g, 0.72 mmol) was dissolved in DMAc/MeCN (1:1, 5 mL). After the solution was cooled to -20 °C, DIPEA (0.09 g, 0.72 mmol) was added. Then DMBCl (0.11 g, 0.79 mmol) was added, and the reaction mixture was stirred at -20 °C for 17.5 h. Subsequently, **4** (0.30 g, 0.79 mmol) was added to the reaction mixture. The reaction mixture was stirred at -20 °C for 16 h.

Entry 2: In a 20-mL vial, **3** (0.50 g, 0.72 mmol) was dissolved in DMAc/MeCN (1:1, 5 mL). After the solution was cooled to -20 °C, DIPEA (0.09 g, 0.72 mmol) was added. Then DMBCl (0.11 g, 0.79 mmol) was added, and the reaction mixture was stirred at -20 °C for 30 min. Then 2,6-lutidine (0.08 g, 0.72 mmol) was added and the reaction mixture was stirred at -20 °C for 17 h. Subsequently, **4** (0.30 g, 0.79 mmol) was added to the reaction mixture. The reaction mixture was stirred at -20 °C for 16 h.

Entry 3: In a 20-mL vial, **3** (0.50 g, 0.72 mmol) was dissolved in DMAc/MeCN (1:1, 5 mL). After the solution was cooled to -20 °C, DIPEA (0.09 g, 0.72 mmol) and 2,6-lutidine (0.08 g, 0.72 mmol) were added. Then DMBCl (0.11 g, 0.79 mmol) was added, and the reaction mixture was stirred at -20 °C for 17.5 h. Subsequently, **4** (0.30 g, 0.79 mmol) was added to the reaction mixture. The reaction mixture was stirred at -20 °C for 16 h.

Supplementary Table 4. Effect of 2,6-lutidine in accelerating mixed anhydride formation and suppressing oxazolone

	Mixed anhydride formation HPLC (Method 1) result (LCAP, 210 nm)								
	Entry 1: no 2,6-lutidine			**Entry 2: added 2,6-lutidine after 30 min**			**Entry 3: added 2,6-lutidine at the beginning**		
Time	**3**	**3-mixed anhydride**	**3-oxazolone**	**3**	**3-mixed anhydride**	**3-oxazolone**	**3**	**3-mixed anhydride**	**3-oxazolone**
30 min	12.0	84.7	3.3	11.5	85.1	3.3	0.9	98.3	0.9
	/			added 2,6-lutidine at 30 min			/		
40 min	9.0	87.0	3.9	1.2	95.1	3.6	0.8	98.3	1.0
1 h	6.0	89.4	4.6	0.9	95.3	3.7	0.7	98.5	0.9
1.5 h	1.6	92.8	5.6	0.8	95.5	3.7	0.6	98.4	1.0
2.5 h	1.4	91.8	6.8	0.7	95.4	3.9	0.6	98.4	1.0
4.5 h	2.0	90.6	7.5	0.7	95.4	3.9	0.6	98.5	1.0
6.5 h	2.0	90.6	7.5	0.7	95.4	3.9	0.6	98.5	1.0
8.5 h	2.1	90.3	7.7	0.7	95.4	3.9	0.6	98.5	1.0
17.5 h	4.9	71.8	23.3	0.8	94.8	4.4	0.6	97.9	1.5

Entry	Amide coupling, 20 h HPLC result (LCAP, 210 nm)					
	3	**3-mixed anhydride**	**5**	***epi*-5**	**3-oxazolone**	**6**
Entry 1: no 2,6-lutidine	4.4	0.7	75.7	2.5	15.6	1.1
Entry 2: added 2,6-lutidine after 30 min	0.7	0.2	95.5	0.4	3.1	0.0
Entry 3: added 2,6-lutidine at the beginning	0.5	0.5	97.7	0.2	1.2	0.0

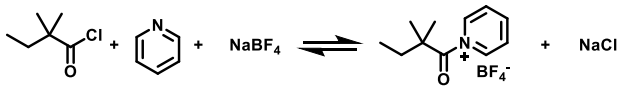
Oxazolone formation vs. 2,6-lutidine equiv.

In a 20-mL vial, **3** (0.50 g, 0.72 mmol) was dissolved in DMAc/MeCN (1:1, 5 mL). After the solution was cooled to -20 °C, DIPEA (0.09 g, 0.72 mmol) and 2,6-lutidine (0.0-2.0 equiv.) were added. Then DMBCl (0.11 g, 0.79 mmol) was added, and the reaction mixture was stirred at -20 °C for 5 h. **4** (0.30 g, 0.79 mmol) was added to the reaction. The reaction mixture was stirred at -20 °C for 16 h.

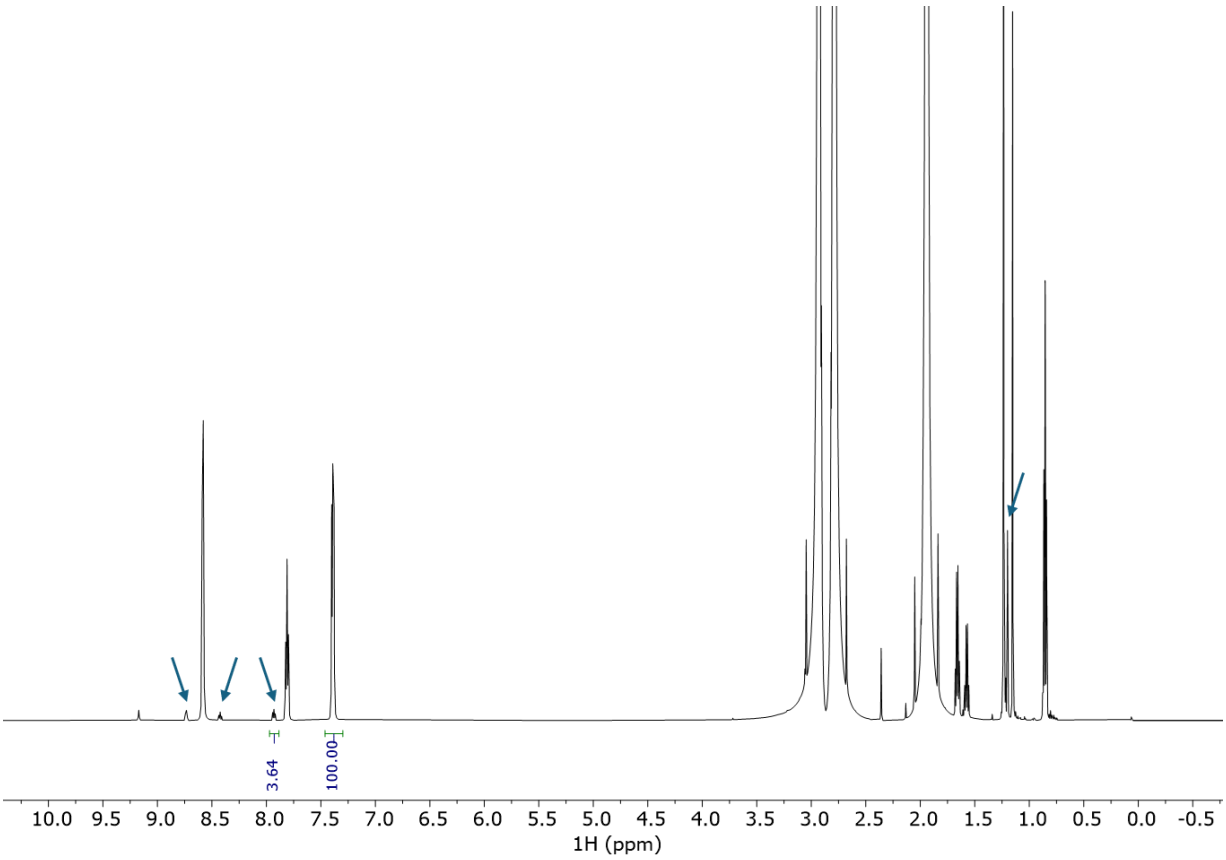
Supplementary Table 5. Oxazolone formation vs. 2,6-lutidine equivalent.

	Condition	**HPLC result (5 h, LCAP, 210 nm)**		
Entry	2,6-lutidine (equiv.)	**3**	**3-mixed-anhydride**	**3-oxazolone**
1	0.0	0.8	94.1	4.1
2	0.2	1.4	95.2	3.3
3	0.4	1.4	95.8	2.6
4	0.8	0.7	96.7	2.2
5	1.0	0.6	97.6	1.4
6	1.2	0.6	97.8	1.2
7	1.6	0.5	97.9	1.2
8	2.0	0.5	97.8	1.2

NMR study of DMB-acylpyridinium intermediate formation:



To a solution of 0.2 M 2,2-dimethylbutanoyl chloride in 600 µL 1:1 CD_3CN/d_9-DMAc in a 5 mm NMR tube was added 2 equiv. pyridine and 1 equiv. $NaBF_4$. The white precipitation was then centrifuged prior to NMR analysis. ^1H NMR showed the formation of ~3% DMB-acylpyridinium salt, in consistent with literature reported chemical shifts of similar analogs.[55]



^1H NMR showing formation of acylpyridinium salt intermediate in the presence of $NaBF_4$. (600 MHz, 1:1 CD_3CN/d_9-DMAc, 298 K)

[55] Pabel, J. *et al.* Generation of N-acylpyridinium ions from pivaloyl chloride and pyridine derivatives by means of silyl triflates. *J. Org. Chem.* **65**, 9272–9275 (2000).

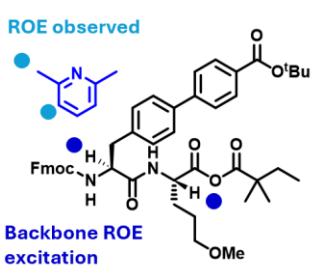
NMR study of the interaction between 2,6-lutidine and 3-mixed anhydride

60 mg dipeptide **3** was dissolved in 600 µL 1:1 CD_3CN/d_9-DMAc that had been dried over 4 Å molecular sieves. To this solution were added 15.1 µL (1.0 equiv.) DIPEA and 12.1 µL (1.2 equiv.) 2,6-lutidine before being transferred to a 5 mm NMR tube. 13.1 µL (1.1 equiv.) DMBCl was then added at 20 °C, and the NMR tube was immediately transferred into the NMR spectrometer that was pre-cooled to 253 K. At this time, the formation of the mixed anhydride was already completed.

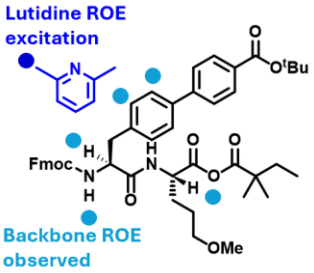
The sample was then analyzed by NMR using a Bruker 800 MHz Neo spectrometer equipped with a 5 mm TCI cryoprobe and a Bruker 600 MHz Avance III HD spectrometer equipped with a 5 mm TCI cryoprobe. The peak assignment of the crude mixture was achieved through a combination of COSY, HSQC, HMBC and TOCSY analysis. The selective 1D ROE with 250 ms mixing time was then performed to probe the interaction between 2,6-lutidine and the mixed anhydride. With selective irradiation at 4.457 ppm (α-proton region of the mixed anhydride) with a 14.36 ms Rsnob selective pulse, ROE of the 2,6-lutidine Me at C2/C6 position and the H3/H5 proton were observed.

1D selective ROE of 2,6-lutidine and mixed anhydride. (600 MHz, 253 K, 1:1 CD_3CN/d_9-DMAc)

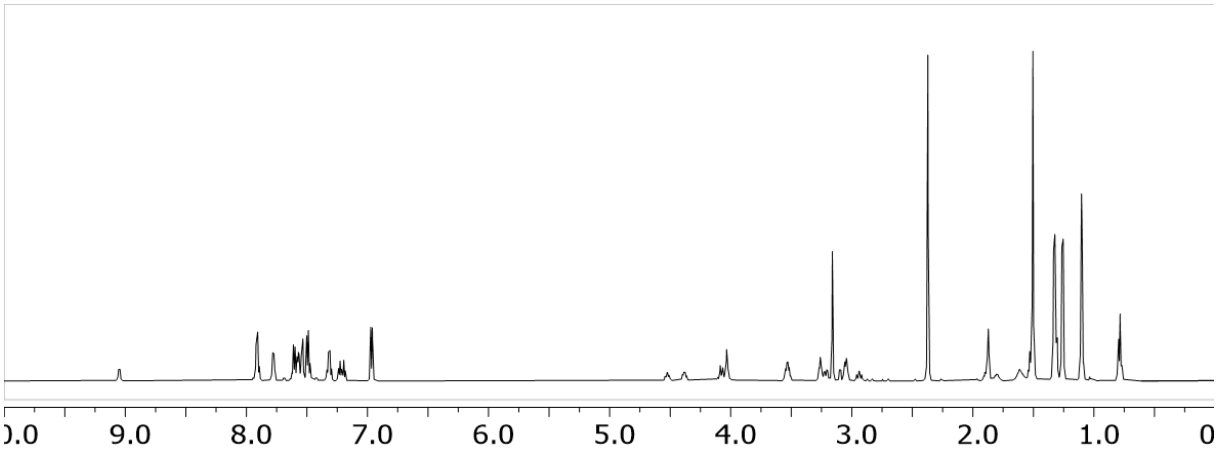
Top: ^{1}H NMR spectrum; middle: 1D selective ROE with selective excitation at 4.457 ppm, highlighting the observed NOE signals from 2,6-lutidine; bottom: 1D selective ROE with selective excitation at 2.359 ppm, highlighting the observed NOE signals from backbone α-protons and NHs and aromatic sidechains of the mixed anhydride.

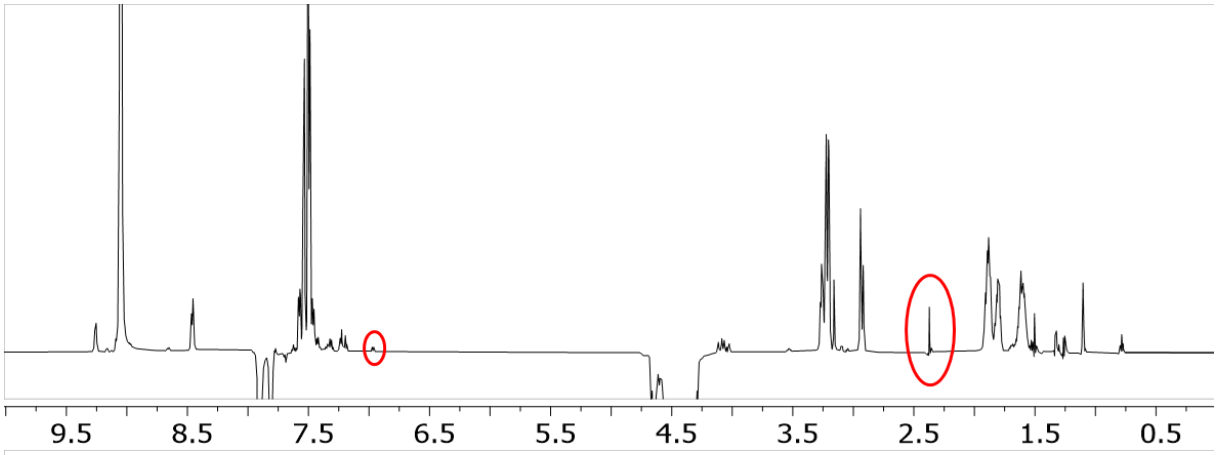


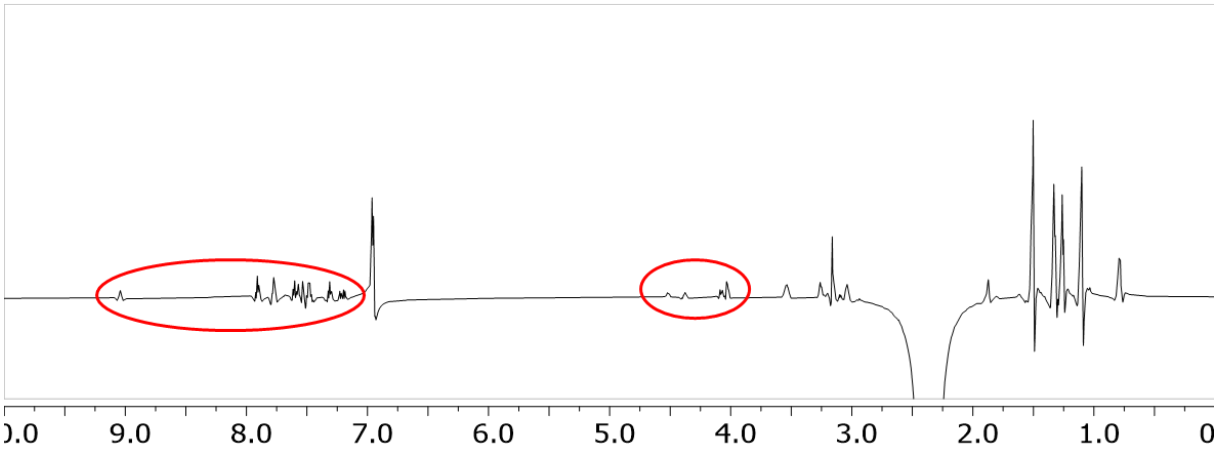
(middle)



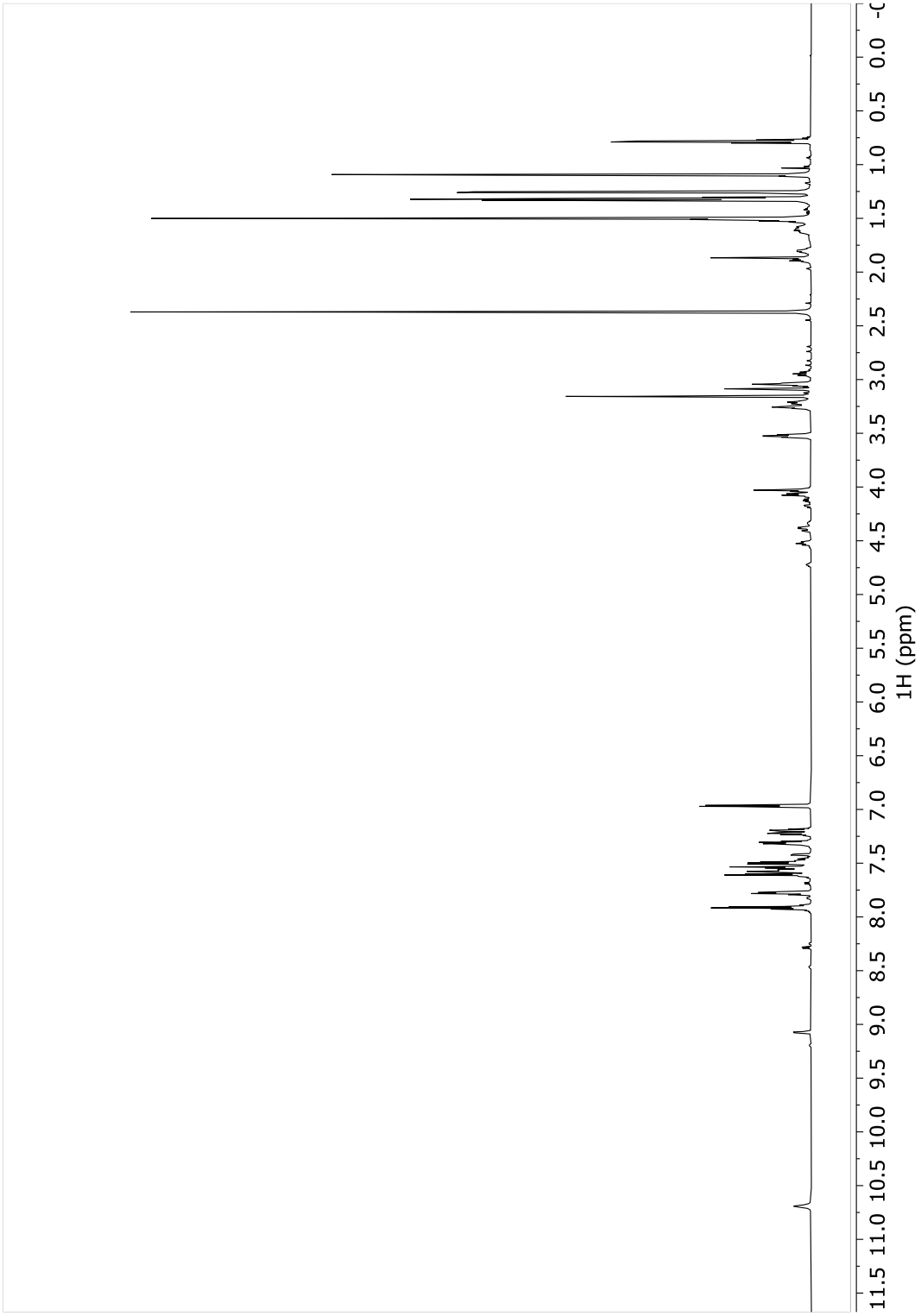
(bottom)



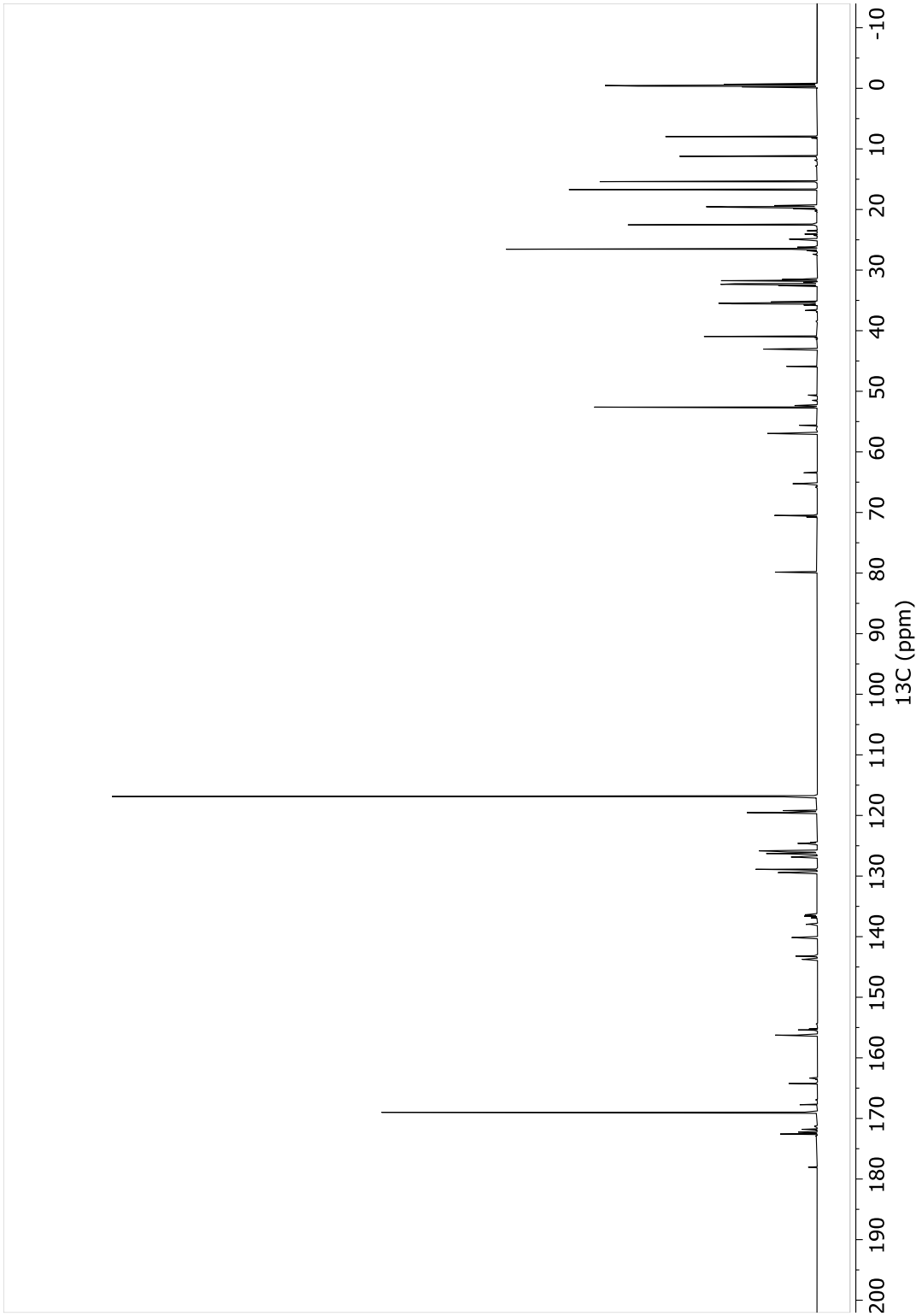




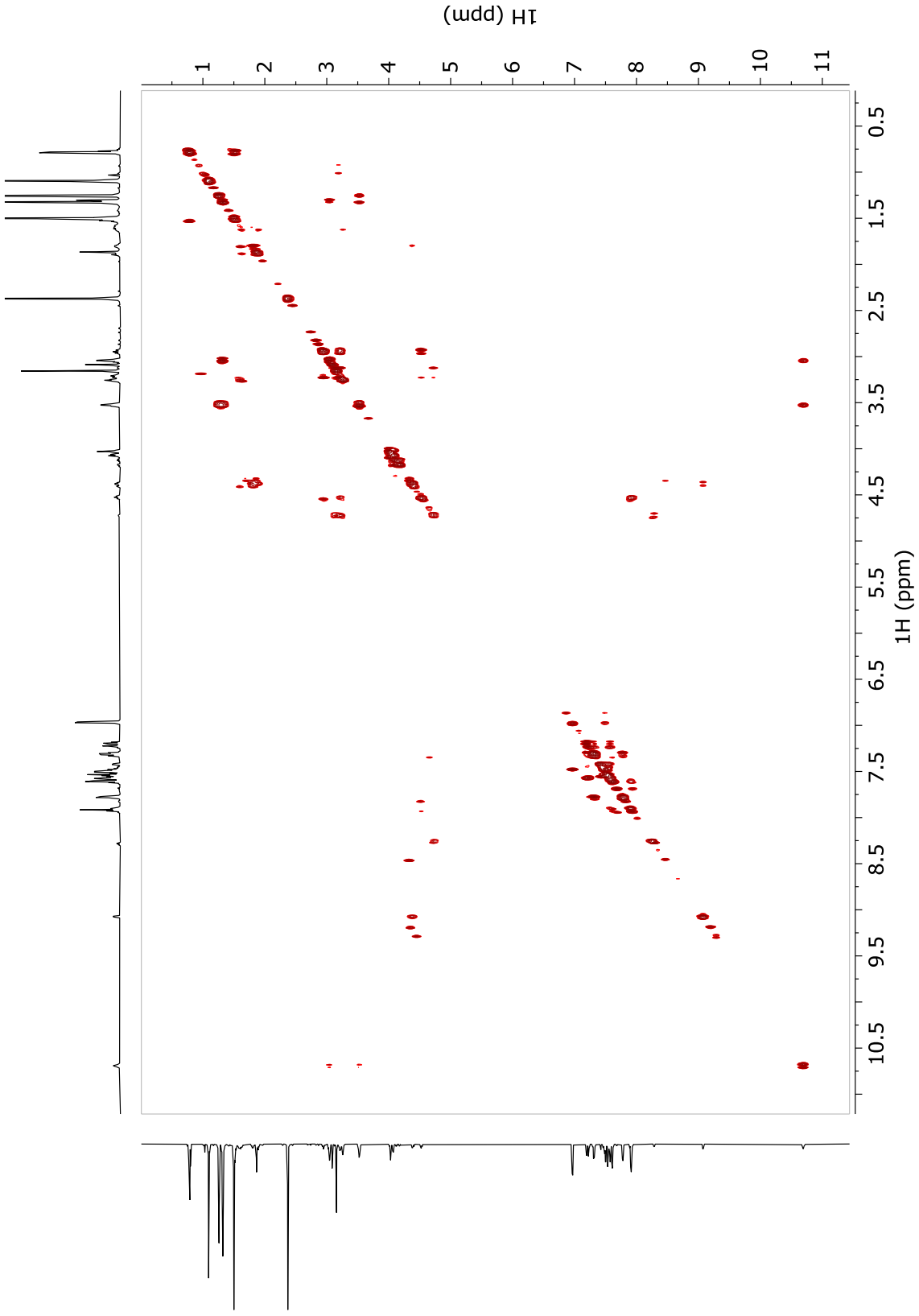
^{1}H spectrum of the mixed anhydride reaction mixture with 2,6-lutidine as additive (800 MHz, 256 K, 1:1 CD_3CN/d_9-DMAc)



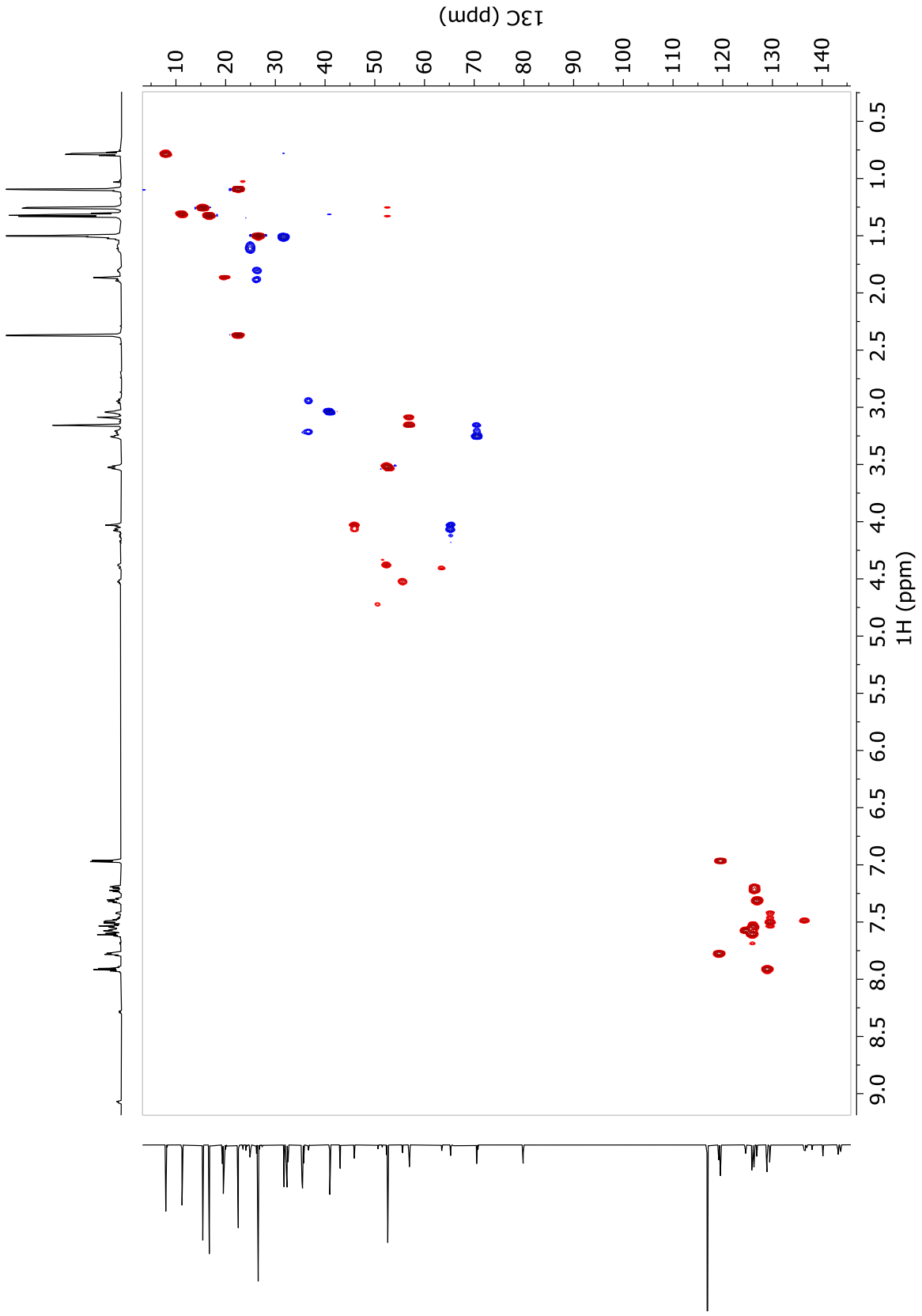
$^{13}C\{1H\}$ spectrum of the mixed anhydride reaction mixture with 2,6-lutidine as additive (201 MHz, 256 K, 1:1 CD_3CN/d_9-DMAc)



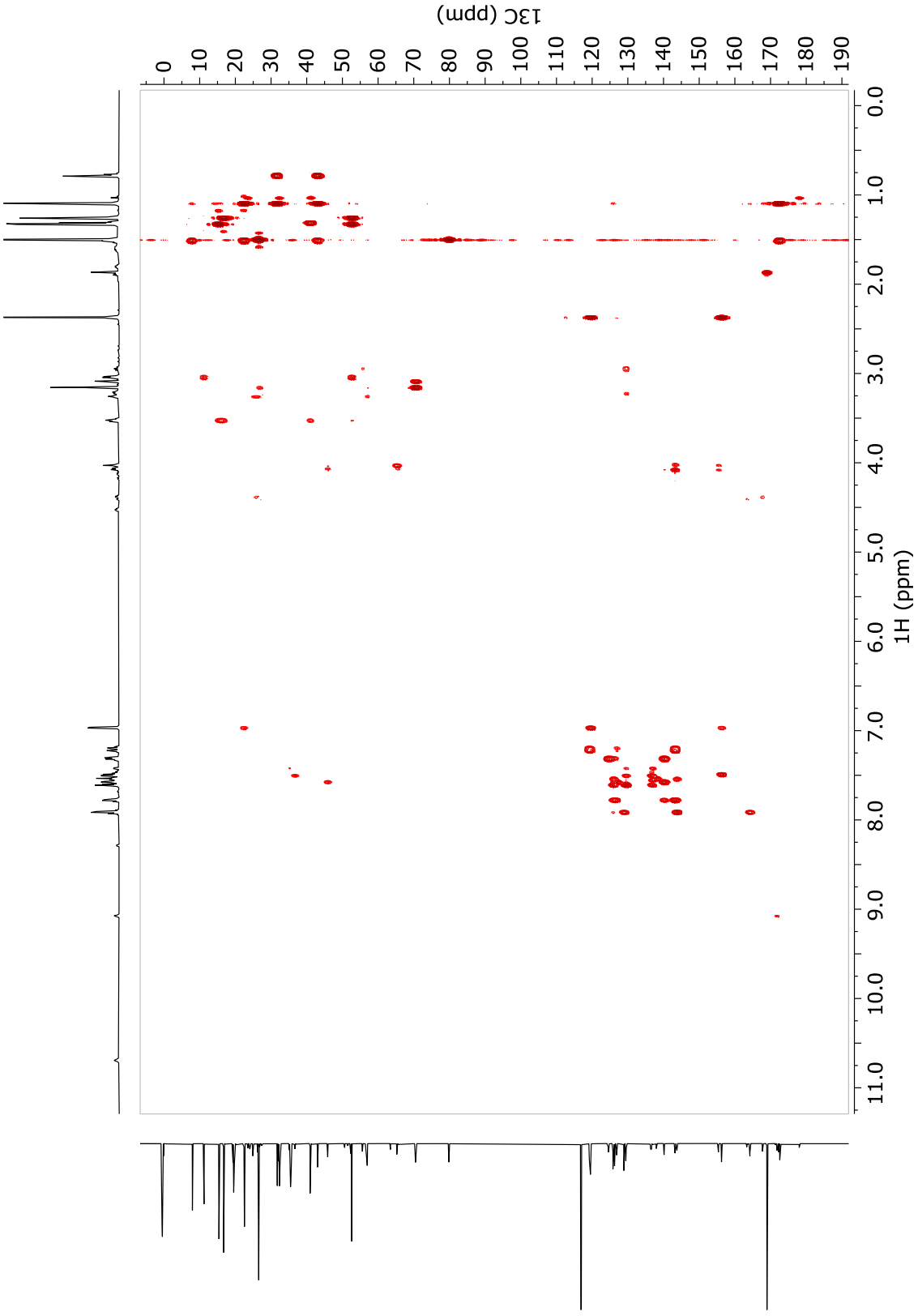
COSY spectrum of the mixed anhydride reaction mixture with 2,6-lutidine as additive (800 MHz, 256 K, 1:1 CD_3CN/d_9-DMAc)



HSQC spectrum of the mixed anhydride reaction mixture with 2,6-lutidine as additive (800 MHz, 256 K, 1:1 CD_3CN/d_9-DMAc)



HMBC spectrum of the mixed anhydride reaction mixture with 2,6-lutidine as additive (800 MHz, 256 K, 1:1 CD_3CN/d_9-DMAc)



TOCSY spectrum (80 ms mixing time) of the mixed anhydride reaction mixture with 2,6-lutidine as additive (800 MHz, 256 K, 1:1 CD_3CN/d_9-DMAc)

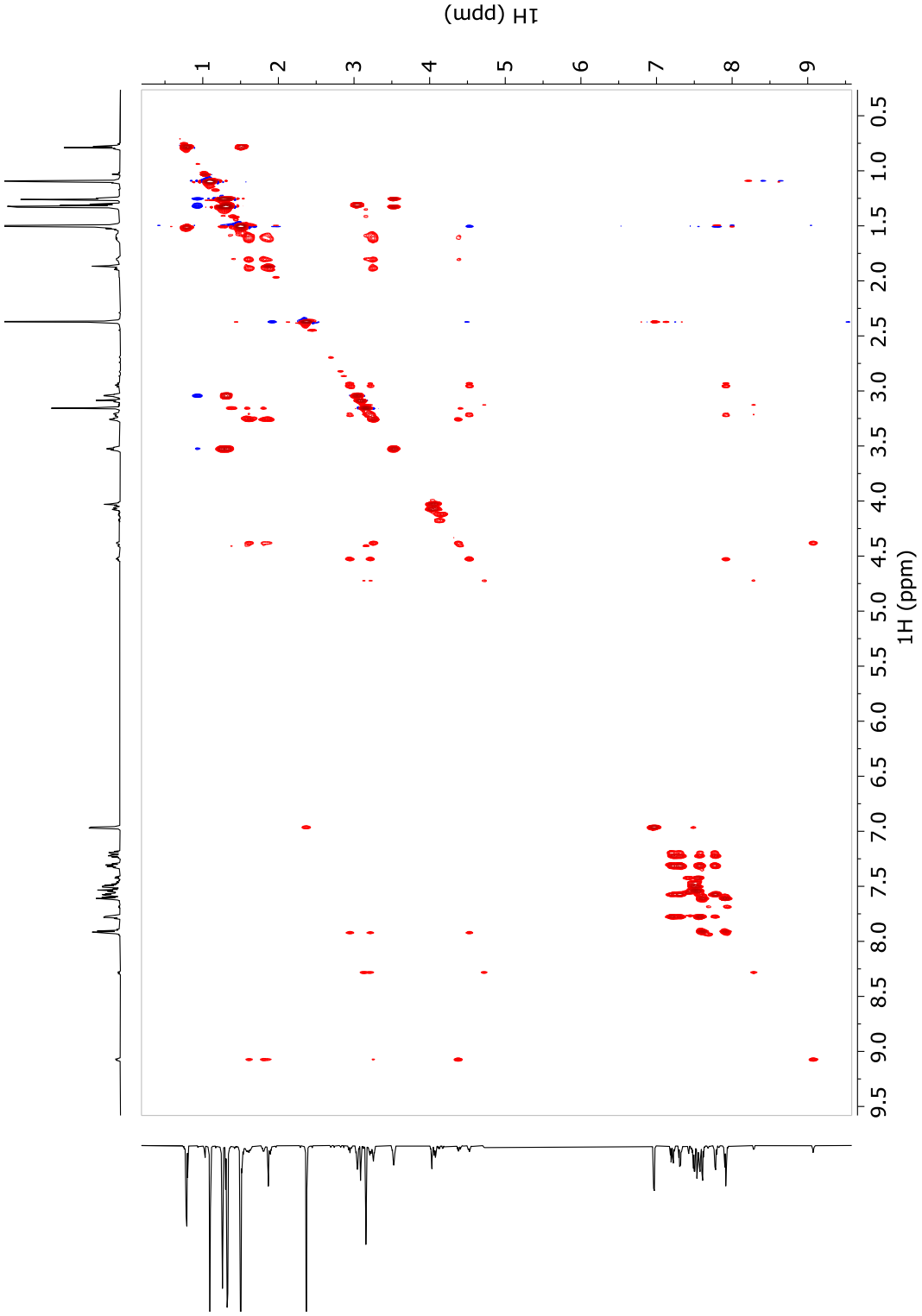
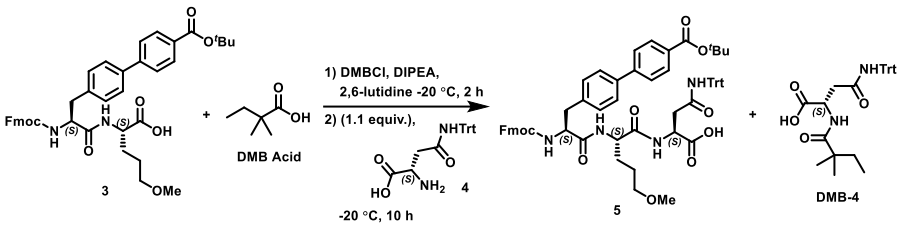


Fig. 3a-3 Additional experiments on 2,6-lutidine minimizing DMB acid interference

1) Lutidine suppresses DMB acid interference in the model reaction of **3** + **4**



Supplementary Table 6.

Entry	Conditions for Stage 1				Reaction HPLC area%	
	DMBCl	**DMB acid**	**DIPEA**	**2,6-lutidine**	**5**	**DMB-4**
1	0.95	1.0	2.0	0.0	34.4	9.0
2	0.95	1.0	1.0	1.0	85.5	0.3

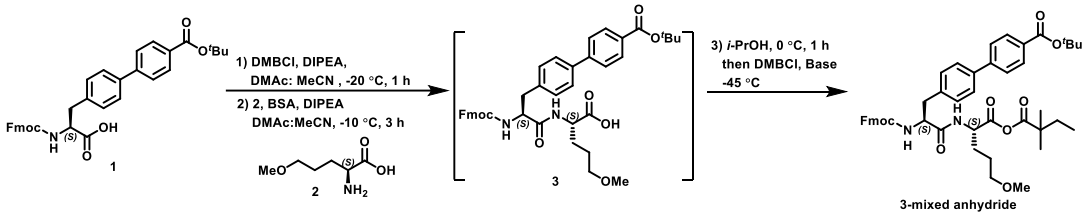
1.0 equiv. of DMB acid was added to the model reaction to test DMB acid interference. The results showed poor conversion to **5** with significant **DMB-4** formation, likely through DMB acid activation by DMBCl (entry 1). Lutidine suppressed this pathway (entry 2), likely by increasing the selectivity for activation of **3** over DMB acid.

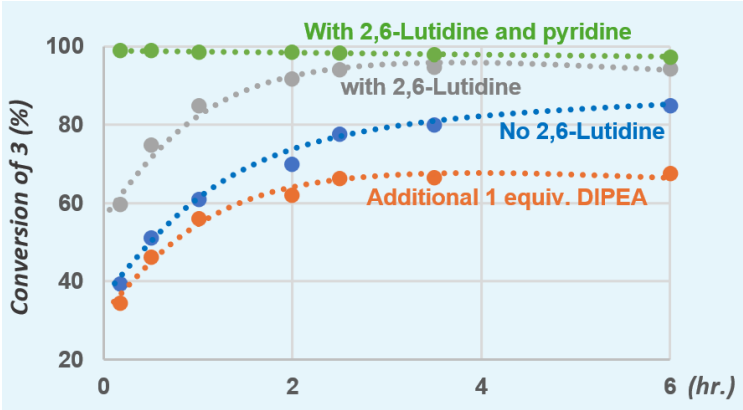
Experimental:

Entry 1: In a 20-mL vial, **3** (0.50 g, 0.72 mmol) and DMB acid (90 µL, 0.72 mmol) was dissolved in DMAc/MeCN (1:1, 5 mL). After the solution was cooled to -20 °C, DMBCl (96 µL, 0.69 mmol) was added. Then DIPEA (123 µL, 0.72 mmol) was added, and the reaction mixture was stirred at -20 °C for 2 h. Subsequently, **4** (0.30 g, 0.79 mmol) was added to the reaction mixture. The reaction mixture was stirred at -20 °C for 10 h and monitored by HPLC (**Method 1**).

Entry 2: In a 20-mL vial, **3** (0.50 g, 0.72 mmol) and DMB acid (90 µL, 0.72 mmol) was dissolved in DMAc/MeCN (1:1, 5 mL). After the solution was cooled to -20 °C, DMBCl (96 µL, 0.69 mmol) was added. Then 2,6-lutidine (84 µL, 0.72 mmol) and DIPEA (246 µL, 1.44 mmol) was added, and the reaction mixture was stirred at -20 °C for 2 h. Subsequently, **4** (0.30 g, 0.79 mmol) was added to the reaction mixture. The reaction mixture was stirred at -20 °C for 10 h and monitored by HPLC (**Method 1**).

2) Additive effects on activation of dipeptide **3** in continuous elongation





The crude reaction stream of dipeptide **3** was prepared from **1** and **2** using 1.1 equiv. of DMBCl without 2,6-lutidine or pyridine. This stream was then activated under four sets of conditions: (1) no additive; (2) 1 equiv. of 2,6-lutidine; (3) 1 equiv. of 2,6-lutidine and 0.2 equiv. of pyridine; and (4) an additional 1 equiv. of DIPEA. Without additives, conversion was incomplete relative to that obtained using isolated **3** (Fig. 2b), suggesting that DMB acid in the crude stream competed with **3** for DMBCl. Addition of 2,6-lutidine accelerated the activation and increased the conversion of **3**, consistent with selective acceleration of the activation of **3** over that of DMB acid. Pyridine further accelerated the activation. A general base effect was ruled out by the lower conversion observed upon addition of DIPEA.

Experimental:

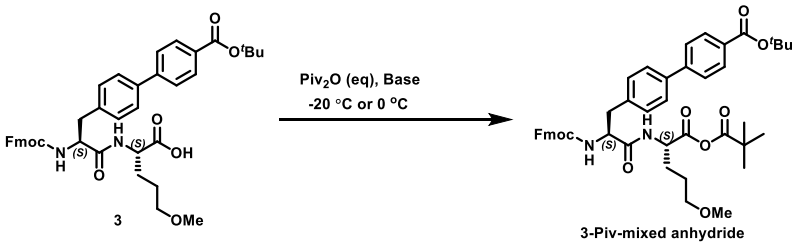
Into a 20-mL vial, **Fmoc-Bip-4-CO$_2$*t*Bu-D-5-OMe-NVA-OH (1)** (500 mg, 0.89 mmol) was dissolved in DMAc/MeCN (1:1, 2.5 mL). The solution was cooled to -20 °C, then DMBCl (137 µL, 0.98 mmol) and DIPEA (166 µL, 0.98 mmol) were added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1**, mixed anhydride intermediate quenched by BnNH$_2$). The reaction mixture was added to a DMAc and MeCN solution of **H-5-OMe-NVA-OH (2)** and BSA at -10 °C, which was prepared by mixing **2** (144 mg, 0.98 mmol) and BSA (434 µL, 1.78 mmol) in DMAc/MeCN (1:1, 2.5 mL), stirring at 40 °C for 1 h, and then cooling to -10 °C before use. The reaction mixture was stirred at -10 °C for 3 h and monitored by HPLC (**Method 4**). Then *i*-PrOH (270 µL, 3.55 mmol) was added to the solution at 0 °C and stirred for 1 h. The reaction was cooled to -45 °C. DMBCl (174 µL, 1.24 mmol) was added, followed by

DIPEA (181 μL, 1.07 mmol) or 2,6-lutidine (103 μL, 0.89 mmol) or pyridine (14 μL, 0.18 mmol). The reaction mixture was stirred at -45 °C and monitored by HPLC (**Method 1**).

Supplementary Table 7.

	Mixed anhydride formation HPLC result (LCAP, 210 nm)											
	Entry 1: DIPEA (1.2 equiv.)			Entry 2: DIPEA (1.2 equiv.), 2,6-lutidine (1.0 equiv.)			Entry 3: DIPEA (1.2 equiv.), 2,6-lutidine (1.0 equiv.), pyridine (0.2 equiv.)			Entry 4: DIPEA (2.2 equiv.)		
Times	**3**	**3-mixed anhydride**	**3-oxazolone**	**3**	**3-mixed anhydride**	**3-oxazolone**	**3**	**3-mixed anhydride**	**3-oxazolone**	**3**	**3-mixed anhydride**	**3-oxazolone**
10 min	60.6	38.7	0.7	40.3	58.8	0.9	0.9	98.6	0.5	65.5	33.8	0.7
30 min	48.8	50.0	1.2	25.1	73.7	1.2	1.0	98.5	0.5	53.7	44.9	1.4
1.0 h	39.0	59.3	1.7	14.9	83.4	1.7	1.3	97.9	0.8	43.8	53.8	2.4
2.0 h	29.3	68.5	2.2	8.2	89.6	2.2	1.4	97.6	1.0	37.8	58.3	3.9
2.5 h	22.3	74.6	3.1	5.7	91.6	2.7	1.7	96.9	1.4	33.8	60.4	5.8
3.5 h	19.8	76.8	3.4	5.2	91.9	2.9	1.9	96.2	1.9	33.3	60.2	6.5
6.0 h	14.9	80.8	4.3	5.4	90.9	3.7	2.5	95.0	2.5	32.5	57.6	9.9
20 h	9.3	84.4	6.3	6.4	85.4	8.2	5.6	88.1	6.3	36.4	39.3	24.3

3) Hypothesis of activation of dipeptide by pivalic anhydride



We studied the activation of isolated **3** by pivalic anhydride to test another possible role of lutidine in minimizing interference from DMB acid: lutidine might promote the activation of **3** by DMB anhydride formed upon reaction of DMB acid with DMBCl. Because DMB anhydride was not readily available, commercially available pivalic anhydride (Piv_2O) was used as a surrogate. However, very little activation of **3** was observed, even at 0 °C, either with lutidine additive or with extra DIPEA. These results ruled out this mechanistic possibility.

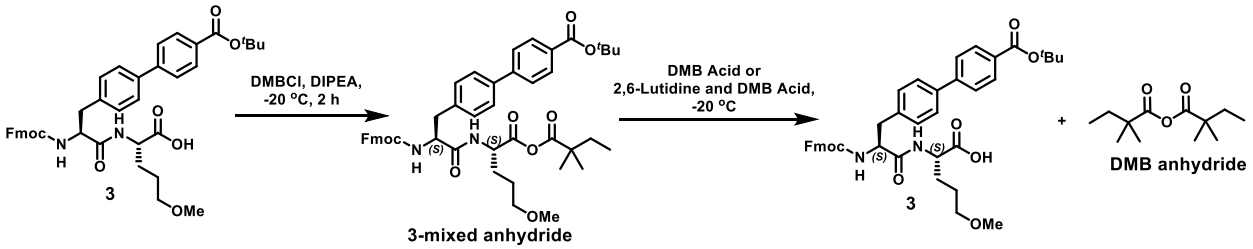
Experimental:

Into a 20-mL vial, **Fmoc-Bip-4-CO_2*t*Bu-5-OMe-NVA-OH (3)** (500 mg, 0.72 mmol) was dissolved in DMAc:MeCN (1:1, 2.5 mL). The solution was cooled to -20 °C or 0 °C, then Piv_2O (140 μL, 0.72 mmol) and DIPEA (147 μL, 0.87 mmol) or lutidine (84 μL, 0.72 mmol) were added. The reaction mixture was stirred at -20 °C or 0 °C and monitored by HPLC Method 1 (mixed anhydride intermediate quenched by $BnNH_2$).

Supplementary Table 8.

Entry	Coupling for stage-1			Result for stage-1 (%)					
	DIPEA (equiv.)	2,6-lutidine (equiv.)	Temp. (°C)	5 h			16 h		
				3	3-Piv-mixed anhydride	3-oxazolone	3	3-Piv-mixed anhydride	3-Oxazolone
1	1.2	0	-20	100.0	0.0	0.0	100.0	0.0	0.0
2	1.2	1.0	-20	100.0	0.0	0.0	100.0	0.0	0.0
3	2.2	0	-20	100.0	0.0	0.0	100.0	0.0	0.0
4	1.2	0	0	98.6	0.7	0.7	90.5	1.3	8.2
5	1.2	1.0	0	98.7	0.6	0.7	81.7	2.7	15.6
6	2.2	0	0	98.9	0.6	0.5	92.6	1.1	6.3

4) Hypothesis of DMB acid reacting with **3**-mixed anhydride



We tested an alternative hypothesis that DMB acid could react with the **3**-mixed anhydride to regenerate **3**, and that 2,6-lutidine might suppress this process. After forming the **3**-mixed anhydride in the absence of lutidine, DMB acid was added at -20 °C with or without 2,6-lutidine. In both cases, no reversion to **3** was observed, ruling out anhydride exchange under these conditions as a mechanism of DMB acid interference.

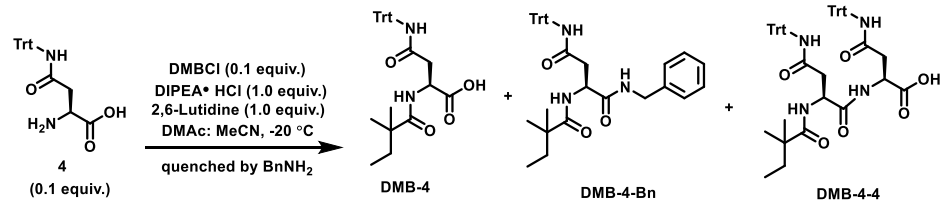
Experimental:

Into a 20-mL vial, **Fmoc-Bip-4-CO_2*t*Bu-5-OMe-NVA-OH (3)** (500 mg, 0.72 mmol) was dissolved in DMAc:MeCN (1:1, 2.5 mL). The solution was cooled to -20 °C, then DMBCl (111 µL, 0.79 mmol) and DIPEA (135 µL, 0.79 mmol) were added. The reaction mixture was stirred at -20 °C for 2 h and monitored by HPLC (mixed anhydride intermediate quenched by $BnNH_2$). DMB acid (90 µL, 0.72 mmol) was added alone or together with 2,6-lutidine (84 µL, 0.72 mmol). The reaction mixture was stirred at -20 °C and monitored by HPLC **Method 1** (mixed anhydride intermediate quenched by $BnNH_2$).

Supplementary Table 9.

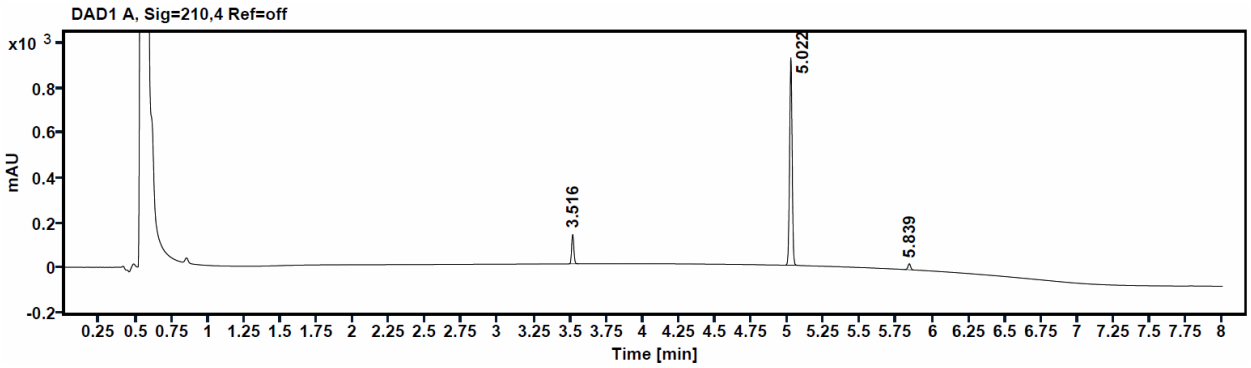
	Mixed anhydride formation HPLC result (LCAP, 210 nm)								
	Entry 1: DIPEA (1.2 equiv.), DMBCl (1.1 equiv.)			Entry 2: DIPEA (1.2 equiv.), DMBCl (1.1 equiv.)			Entry 3: DIPEA (1.2 equiv.), DMBCl (1.1 equiv.)		
Times	**3**	**3-mixed anhydride**	**3-oxazolone**	**3**	**3-mixed anhydride**	**3-oxazolone**	**3**	**3-mixed anhydride**	**3-oxazolone**
2 h	1.6	92.6	5.8	1.2	93.8	5.0	1.5	93.6	4.8
	N/A			Added DMB acid (1.0 equiv.) at 2 h			Added DMB acid(1.0 equiv.) and 2,6-lutidine (1.0 equiv.) at 2 h		
2.5 h	1.5	92.3	6.0	1.0	88.9	10.1	0.6	92.5	6.8
5.0 h	1.6	87.2	11.2	1.3	80.4	18.2	1.3	90.0	8.6
18.0 h	4.1	63.5	32.4	3.2	65.0	31.7	4.0	82.1	13.9

N-/C- selectivity with DMBCl.

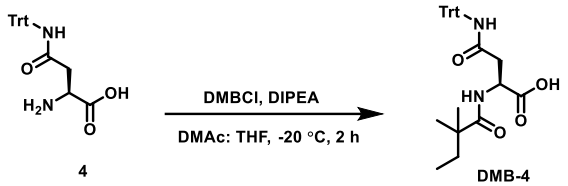


In a 40-mLvial, DMAc/MeCN (1:1, 16 mL) was charged at 25 °C followed by DIPEA HCl salt (0.43 g, 2.67 mmol). After the solution was cooled to -20 °C, 2,6-lutidine (0.29 g, 2.67 mmol) was added. Then DMBCl (0.04 g, 0.27 mmol) was added. The reaction mixture was stirred at -20 °C for 10 min. Then H-L-Gln(Trt)-OH **4** (0.10 g, 0.27 mmol) was added to the reaction. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 4**), which showed the ratio of **4**:**DMB-4**:**DMB-4-Bn**:**DMB-4-4** was 10.6:87.0:2.4:0.0. No product formation was observed from C-activation of compound **4** alone. The *N*-/C- selectivity should be higher than the *N*-activation product **DMB-4**/double activation product **DMB-4-Bn**, which is ~36:1.

HPLC of **N-/C- selectivity with DMBCl** formation (reaction mixture)

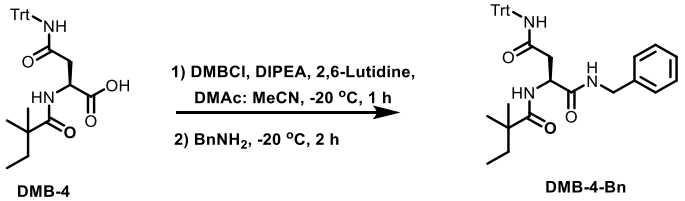


Compound ID	Time	Area	Height	Area%
4	3.516	139.3640	133.2	10.6
DMB-4	5.022	1144.8207	931.7	87.0
DMB-4-Bn	5.839	31.8391	26.5	2.4
DMB-4-4	6.420	-	-	0.0
Total	-	-	-	100.0



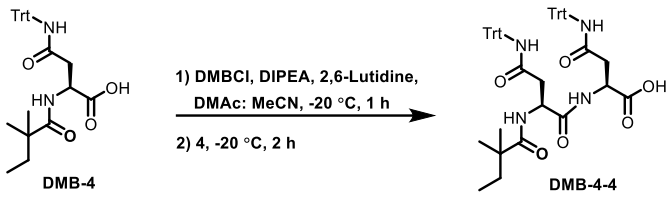
Synthesis of DMB-L-Gln(Trt)-OH (DMB-4). In a 100-mL three-neck jacketed vessel, DMAc:THF (1:1, 50 mL) was charged. After the solution was cooled to -20 °C, DIPEA (2.07 g, 16.02 mmol) was added. Then DMBCl (1.98 g, 14.69 mmol) was added. The reaction mixture was stirred at -20 °C for 1 h. **4** (5.00 g, 13.35 mmol) was added to the reaction. The reaction mixture was stirred at -20 °C for 2 h and monitored by HPLC (**Method 1**). Upon completion, the reaction mixture was purified by Prep-HPLC: Column: C18, 20-40 um, 330 g; Mobile Phase A: H_2O, Mobile Phase B: MeCN; Flow rate: 100 mL/min; Gradient: 10-50% B in 40 min; Wavelength: 210 nm. The collected fractions were concentrated to dryness under vacuum at a temperature below 40 °C to yield 4.30 g of **DMB-4** as a white solid (99.9% LCAP) in 69% isolated yield. (**Method 4**)

mp 152-159 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.49 (br s, 1H), 8.64 (s, 1H), 7.49 (d, J = 7.7 Hz, 1H), 7.28 - 7.24 (m, 6H), 7.22 - 7.19 (m, 3H), 7.18 - 7.15 (m, 6H), 4.44 - 4.38 (m, 1H), 2.79 (dd, J = 15.3, 8.1 Hz, 1H), 2.68 (dd, J = 15.3, 5.0 Hz, 1H), 1.51 - 1.41 (m, 2H), 1.04 (d, J = 5.7 Hz, 6H), 0.77 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 177.03, 173.66, 169.68, 145.22, 129.04, 127.91, 126.87, 69.93, 49.80, 41.99, 38.05, 33.57, 25.22, 25.11, 9.45. HRMS (ESI): m/z calcd. for $C_{29}H_{32}N_2NaO_4$ $[M+Na]^+$: 495.2254, found: 495.2273. FTIR (neat): 3528, 3440, 3349, 3220, 3058, 2969, 2935, 1731, 1685, 1631, 1504, 1448, 1418, 1286, 1228, 1204, 1128, 1036, 908, 768 cm^{-1}.



Synthesis of DMB-L-Gln(Trt)-BnNH$_2$ (DMB-4-Bn). In a 40-mL vial, **DMB-4** (2.00 g, 4.24 mmol) was dissolved in DMAc/MeCN (1:1, 20 mL). After the solution was cooled to -20 °C, 2,6-lutidine (0.50 g, 4.66 mmol) and DIPEA (0.60 g, 4.66 mmol) were added. Then DMBCl (0.63 g, 4.66 mmol) was added. The reaction mixture was stirred at -20 °C for 1 h. BnNH$_2$ (0.87 g, 8.08 mmol) was added to the reaction. The reaction mixture was stirred at -20 °C for 2 h and monitored by HPLC. Upon completion, the reaction mixture was purified by Prep-HPLC: Column: C18, 20-40 μm, 330 g; Mobile Phase A: H_2O, Mobile Phase B: MeCN; Flow rate: 100 mL/min; Gradient: 20-60% B in 30 min; Wavelength: 210 nm. The collected fractions were concentrated to dryness under vacuum at a temperature below 40 °C to yield 1.93 g of **DMB-4-Bn** as a white solid (99.9% LCAP) in 81% isolated yield. (**Method 4**).

mp 152-159 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.57 (s, 1H), 8.21 (s, 1H), 8.21 (t, J = 6.1 Hz, 1H), 7.50 (d, J = 7.8 Hz, 1H), 7.30 - 7.15 (m, 20H), 4.57 - 4.52 (m, 1H), 4.27 (d, J = 6.0 Hz, 1H), 2.80 (dd, J = 14.8, 9.4 Hz, 1H), 2.56 (dd, J = 14.9, 4.5 Hz, 1H), 1.52 - 1.41 (m, 2H), 1.05 (d, J = 7.0 Hz, 6H), 0.73 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 177.35, 171.85, 169.61, 145.26, 139.86, 129.04, 128.63, 127.91, 127.42, 127.10, 126.86, 69.91, 50.91, 42.55, 42.08, 38.73, 33.49, 25.15, 24.99, 9.47. HRMS (ESI): m/z calcd. for $C_{36}H_{40}N_3O_3$ $[M+H]^+$: 562.3064, found: 562.3080. FTIR (neat): 3383, 3349, 3060, 3029, 2969, 2939, 1681, 1648, 1519, 1495, 1448, 1362, 1265, 1187, 699 cm^{-1}.



Synthesis of DMB-L-Gln(Trt)-L-Gln(Trt)-OH (DMB-4-4). In a 40-mL vial, **DMB-4** (1.00 g, 2.12 mmol) was dissolved in DMAc/MeCN (1:1, 10 mL). After the solution was cooled to -20 °C, 2,6-lutidine (0.25 g, 2.33 mmol) and DIPEA (0.30 g, 2.33 mmol) were added. Then DMBCl (0.31 g, 2.33 mmol) was added. The reaction mixture was stirred at -20 °C for 1 h. H-L-Gln(Trt)-OH **4** (0.95 g, 2.54 mmol) was added to the reaction. The reaction mixture was stirred at -20 °C for 2 h and monitored by HPLC. Upon completion, the reaction mixture was purified by Prep-HPLC: Column: C18, 20-40 um, 330 g; Mobile Phase A: H_2O, Mobile Phase B: MeCN; Flow rate:100 mL/min; Gradient: 40-70% B in 30 min; Wavelength: 210 nm. The collected fractions were concentrated to dryness under vacuum below 40 °C to provide 1.06 g (99.4 wt%, yield calculated by wt%) of **DMB-4-4** as a white solid (99.5% LCAP) in 60% isolated yield (**Method 4**).

mp 170-173 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.63 (s, 1H), 8.69 (s, 1H), 8.55 (s, 1H), 7.96 (d, J = 8.0 Hz, 1H), 7.43 (d, J = 7.9 Hz, 1H), 7.28 - 7.14 (m, 30H), 4.60 - 4.54 (m, 1H), 4.47 (q, J = 6.6 Hz, 1H), 2.79 (dd, J = 14.9, 10.0 Hz, 1H), 2.72 - 2.61 (m, 2H), 2.54 (dd, J = 15.0, 3.8 Hz, 1H), 1.51 - 1.39 (m, 2H), 1.02 (d, J = 9.4 Hz, 6H), 0.73 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 177.24,

173.23, 171.67, 169.63, 169.48, 145.26, 145.20, 129.07, 129.05, 127.95, 127.92, 126.86, 126.79, 69.94, 69.90, 50.64, 49.54, 42.07, 38.91, 38.20, 33.46, 25.17, 24.99, 9.52. HRMS (ESI): m/z calcd. for $C_{52}H_{52}N_4NaO_6$ $[M+Na]^+$: 851.3887 found: 851.3749. FTIR (neat): 3409, 3088, 3058, 3027, 2967, 2932, 2879, 1735, 1672, 1597, 1493, 1448, 1366, 1321, 1284, 1206, 1036, 753, 701 cm^{-1}.

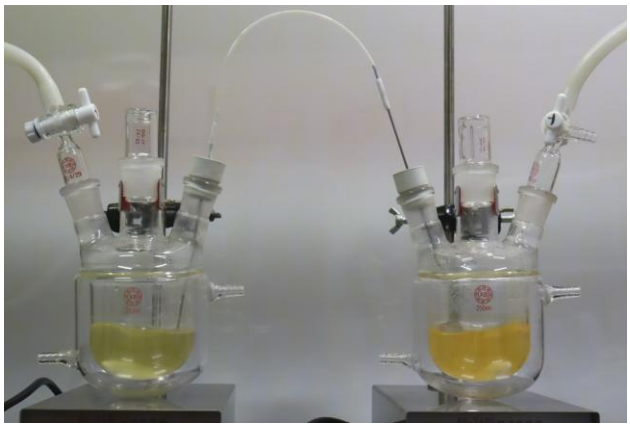
Experimental procedures and characterizations for Figure 4

Photos for lab experiment set-up

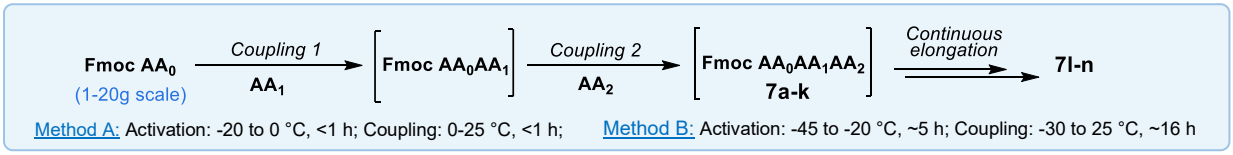
AA addition into mixed anhydride

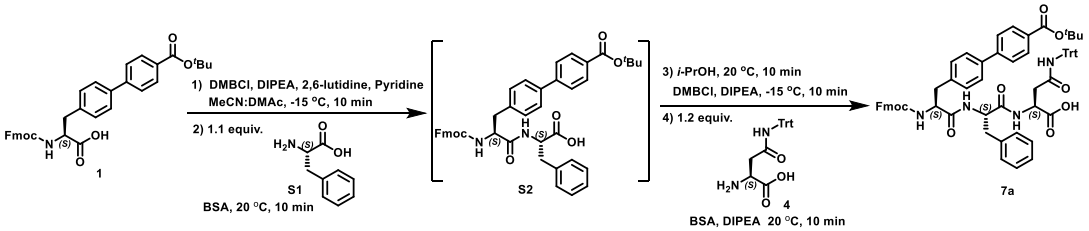


Mixed anhydride addition into AA



Experimental procedures for **7a-n**

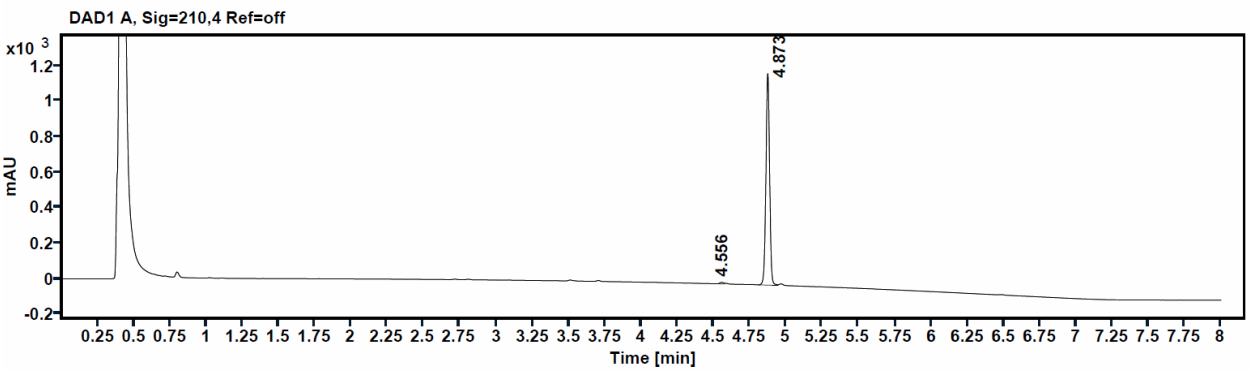




Synthesis of Fmoc-Bip-4-CO$_2$*t*Bu-L-Phe-L-Asn(Trt)-OH (7a) (**Method A**). In a 40 mL vial, **Fmoc-Bip-4-CO$_2$*t*Bu-OH (1)** (500 mg, 0.89 mmol), pyridine (15 µL, 0.18 mmol) and 2,6-lutidine (103 µL, 0.887 mmol) were dissolved in DMAc/MeCN (1:1, 2.5 mL). The solution was cooled to -15 °C, then DMBCl (131 µL, 0.93 mmol) and DIPEA (151 µL, 0.89 mmol) were added. The reaction mixture was stirred at -15 °C for 10 min and monitored by HPLC (**Method 6**) which showed the ratio of **1:1-INT** (mixed anhydride intermediate quenched by BnNH$_2$) was 0.5:99.5. The reaction mixture was added slowly to a DMAc and MeCN solution of **L-Phe-OH (S1)** and BSA at 20 °C. The solution was prepared by mixing **S1** (161 mg, 0.98 mmol) and BSA (434 µL, 1.77 mmol) in DMAc/MeCN (1:1, 2.5 mL), stirring at 40 °C for 0.5 h, and then cooling to 20 °C before use. The reaction mixture was stirred at 20 °C for 10 min and monitored by HPLC (**Method 6**), which showed the ratio of **1:S2:S2-over-addition** was 0.7:99.3:0.0. Then *i*-PrOH (270

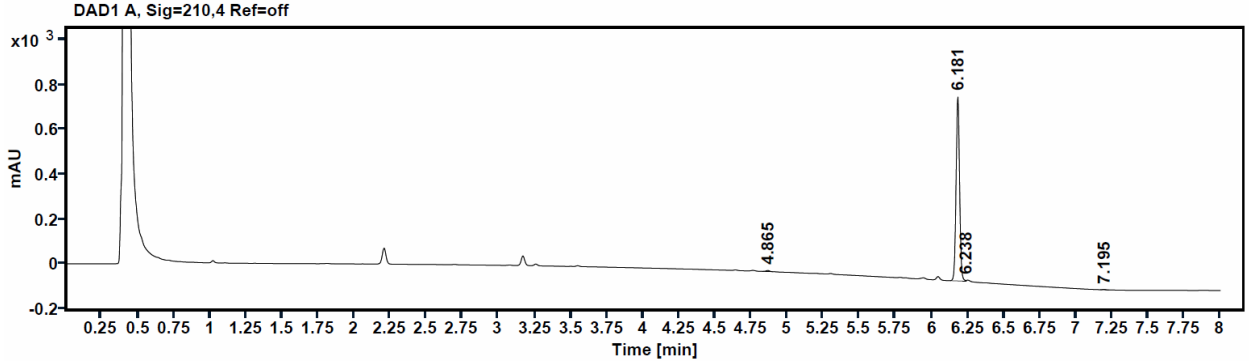
μL, 3.55 mmol) was added into the reaction mixture at 20 °C and stirred for 10 min. The reaction was cooled to -15 °C, DMBCl (162 μL, 1.15 mmol) and DIPEA (166 μL, 0.98 mmol) were added and stirred at -15 °C for 10 min, then monitored by HPLC (**Method 6**). HPLC showed the ratio of **S2:(S2-INT + *epi*-S2-INT)** (mixed anhydride quenched by $BnNH_2$) was 0.5:99.5. The reaction mixture was added to a solution of **L-Asn(Trt)-OH (4)** at 20 °C, which was prepared by mixing **4** (398 mg, 1.06 mmol) and BSA (651 μL, 2.66 mmol) in DMAc/MeCN (1:1, 2.5 mL), stirring at 40 °C for 30 min, cooling to 20 °C, and adding DIPEA (106 μL, 0.62 mmol) right before use. The reaction mixture was stirred at 20 °C for 10 min and monitored by HPLC (**Method 6**), which showed the ratio of **S2:7a:*epi*-7a:*epi*-S2-INT:7a-over-addition** was 0.5:99.1:0.2:0.0:0.2. The assay yield of the reaction was 96% over two steps.

HPLC of **S2** formation (reaction mixture)

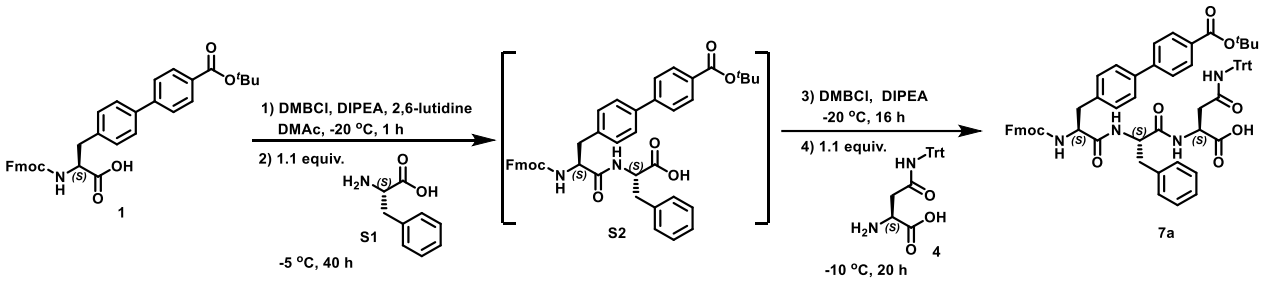


Compound ID	Time	Area	Height	Area% ratio
1	4.556	14.9015	8.4	0.7
S2	4.873	2101.7551	1196.7	99.3
S2-over-addition	5.238	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of **7a** formation (reaction mixture)



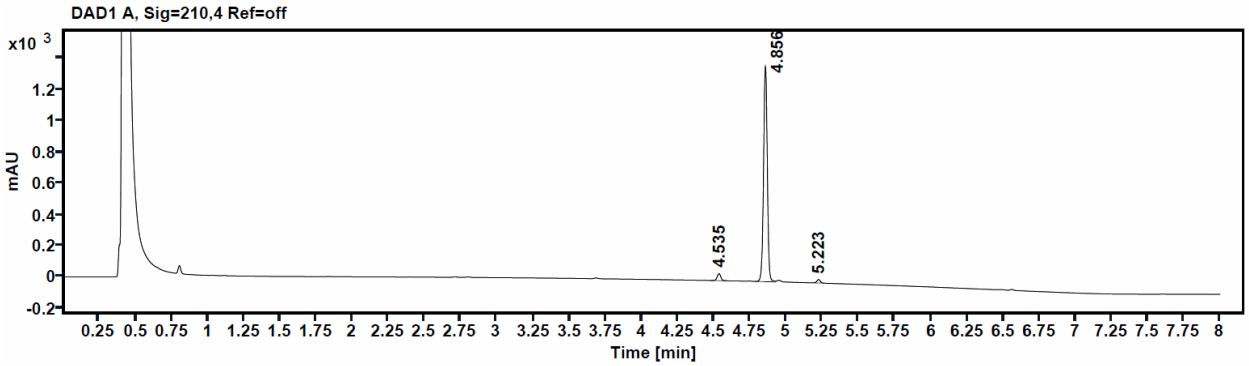
Compound ID	Time	Area	Height	Area% ratio
S2	4.865	7.2082	4.6	0.5
***epi*-S2-INT**	6.002	0.0	0.0	0.0
7a	6.181	1391.7278	827.3	99.1
***epi*-7a**	6.238	2.2344	4.1	0.2
7a-over-addition	7.195	2.7776	1.8	0.2
Total	-	-	-	100.0



Synthesis of Fmoc-Bip-4-CO_2*t*Bu-L-Phe-L-Asn(Trt)-OH (7a) (Method B). In a 100 mL 3-neck round bottom flask, **Fmoc-Bip-4-CO_2*t*Bu-OH (1)** (3.00 g, 5.32 mmol) and 2,6-lutidine (0.57 g, 5.32 mmol) were dissolved in DMAc (18 mL). Then the solution was cooled to -20 °C, DMBCl (0.75 g, 5.59 mmol) and DIPEA (0.69 g, 5.32 mmol) were added to the reaction mixture. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 6**), which showed the ratio of **1:1-INT** was 0.8:99.2. **L-Phe-OH (S1)** (0.97 g, 5.85 mmol) was added to the reaction solution at -20 °C. The reaction mixture was stirred at -5 °C for 40 h and monitored by HPLC (**Method 6**), which showed the ratio of **1:S2:S2-over-addition** was: 3.1:95.5:1.4. The reaction mixture was cooled to -20 °C, DMBCl (1.00 g, 7.45 mmol) and DIPEA (0.76 g, 5.85 mmol) were added into the reaction mixture. The reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC (**Method 6**), which showed the ratio of **S2:(S2-INT + *epi*-S2-INT)** (mixed anhydride quenched by $BnNH_2$) was 1.3:98.7. **L-Asn(Trt)-OH (4)** (2.19 g, 5.85 mmol) was added to the reaction mixture at -20 °C. The reaction mixture was stirred at -10 °C for 20 h and monitored by HPLC (**Method 6**) which showed the ratio of **S2:7a:*epi*-7a:(S2-INT + *epi*-S2-INT):7a-over-addition** was 1.3:96.9:0.5:1.3:0.0. The assay yield of the reaction was 91% over two steps. The reaction solution was added slowly into H_2O (75 mL) at 5 °C for 2 h and stirred at 5 °C for 2 h. The solid was collected by filtration, washed with H_2O (15 mL), and dried under N_2 overnight. The crude product was slurried with IPAc (90 mL) for 1 hour at 10 °C. The solid was collected by filtration and dried under N_2 for overnight to provide 5.32 g (93.7% LCAP, 91.5 wt%) of compound **7a** as a white solid in 86% isolated yield.

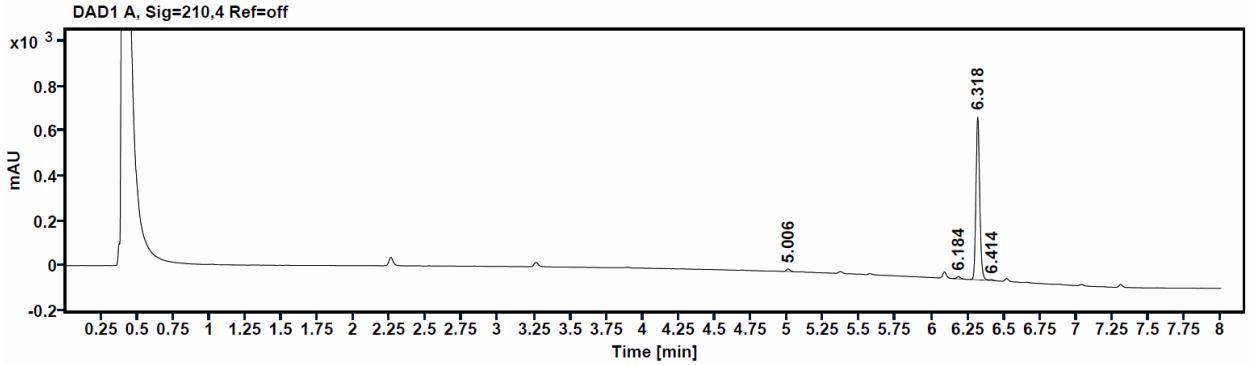
mp 205 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ(ppm) 12.74 (s, 1H), 8.30 (s, 1H), 8.54 (d, J = 8.0 Hz, 1H), 8.13 (d, J = 8.4 Hz, 1H), 7.93 (d, J = 8.0 Hz, 2H), 7.87 (d, J = 7.6 Hz, 2H), 7.70 (d, J = 7.6 Hz, 2H), 7.64 - 7.60 (m, 3H), 7.57 (d, J = 8.4 Hz, 2H), 7.40 - 7.35 (m, 4H), 7.29 - 7.17 (m, 22H), 4.68 (td, J = 8.4, 4.8 Hz, 1H), 4.58 (q, J = 7.2 Hz, 1H), 4.34 - 4.28 (m, 1H), 4.16 - 4.11 (m, 3H), 3.14 - 3.10 (m, 1H), 3.01 - 2.98 (m, 1H), 2.89 - 2.65 (m, 4H), 1.57 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6): δ(ppm) 172.28, 170.55, 170.01, 168.11, 164.18, 155.07, 144.11, 143.56, 143.17, 143.07, 140.03, 137.94, 136.70, 136.11, 129.38, 129.25, 128.98, 128.89, 127.99, 127.34, 126.99, 126.86, 126.43, 125.95, 125.87, 125.74, 125.63, 124.71, 119.44, 80.05, 68.81, 65.05, 55.44, 52.86, 48.49, 45.90, 37.28, 27.21. HRMS (ESI): m/z calcd for $C_{61}H_{62}N_4NaO_9$ $[M+Na]^+$: 1089.4409, found: 1089.4428. FTIR (neat): 3293, 3057, 2925, 2850, 1709, 1692, 1645, 1524, 1493, 1447, 1291, 1114, 1032, 1005, 847, 769, 757, 739, 697, 561, 502 cm^{-1}.

HPLC of **S2** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
1	4.535	80.2948	45.6	3.1
S2	4.856	2485.1736	1394.6	95.5
S2-over-addition	5.223	35.4677	21.8	1.4
Total	-	-	-	100.0

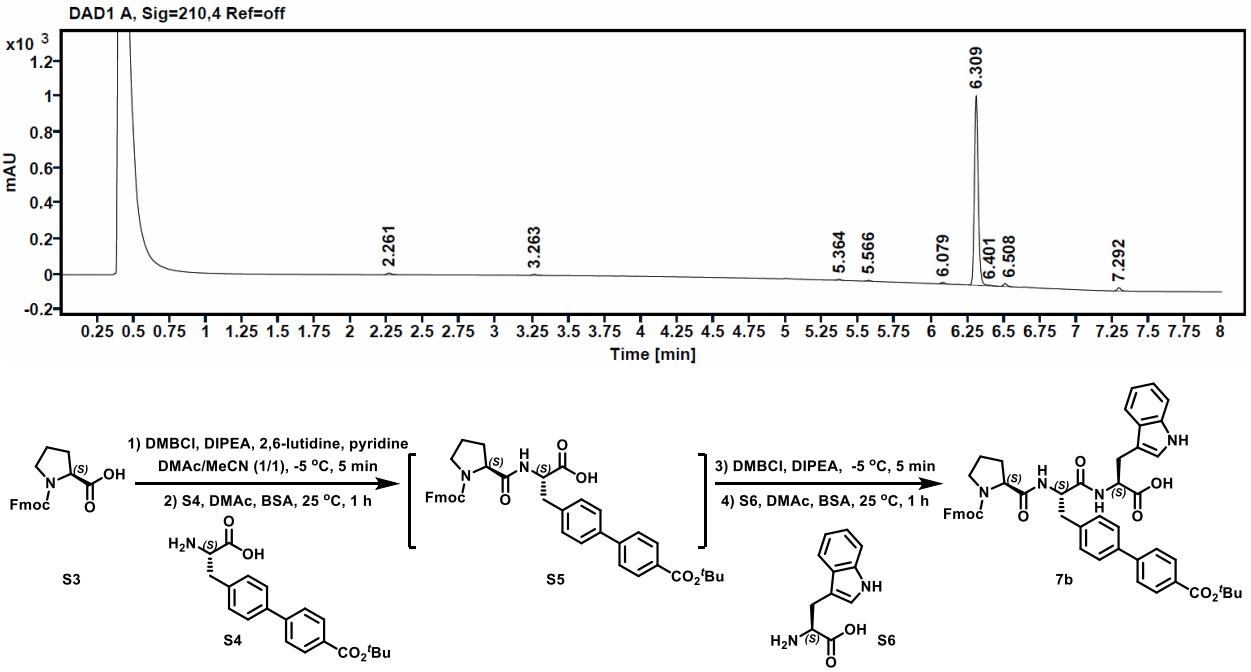
HPLC of **7a** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S2	5.006	18.3198	11.0	1.3
S2-INT + ***epi*-S2-INT**	6.184	18.3549	10.5	1.3*
7a	6.318	1325.9692	731.7	96.9
***epi* -7a**	6.414	6.2804	3.8	0.5
7a-over-addition	6.983	0.0	0.0	0.0
Total	-	-	-	100.0

*Note: Contains 0.6% ***epi*-S2-INT**

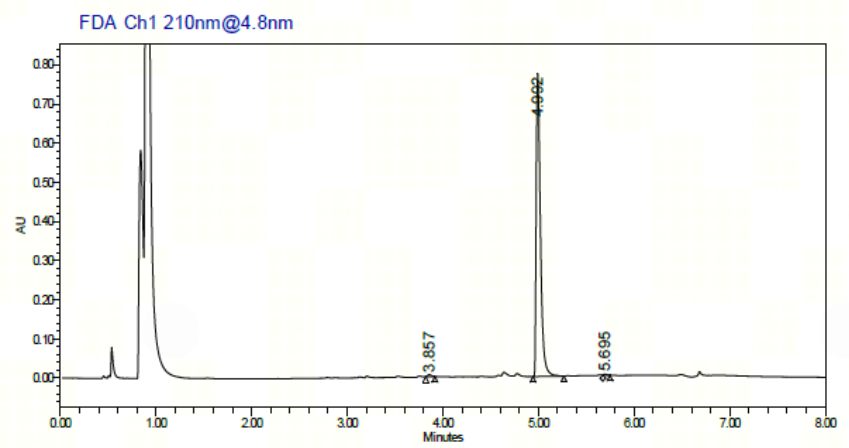
HPLC of isolated **7a**



Synthesis of Fmoc-Pro-Bip-4-CO_2*t*Bu-Trp-OH (7b) (Method A). To a 250 mL three-neck jacketed vessel, **S4** (10.63 g, 31.12 mmol), DMAc (80 mL) and BSA (12.06 g, 59.28 mmol) were added. After the mixture was stirred for 30 min at 50 °C and then cooled to 25 °C. To another 250 mL three-neck jacketed vessel, **S3** (10.00 g, 29.64 mmol) and DMAc:MeCN (1:1, 100 mL) were added. After the solution was cooled to -5 °C, 2,6-lutidine (3.18 g, 29.64 mmol), pyridine (468.93 mg, 5.93 mmol), DIPEA (3.83 g, 29.64 mmol), and DMBCl (4.19 g, 31.12 mmol) were added. The reaction mixture was stirred at -5 °C for 5 min and monitored by HPLC (**Method 7**), which showed the ratio of **S3:S3-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 1.37:98.63. Then, the reaction solution was added to above **S4** solution in one portion. The reaction mixture was stirred at 25 °C for 1 h and monitored by HPLC (**Method 1**). The HPLC showed the ratio of **S3:S5:S5-over-addition** was 0.58:99.12:0.30. *i*-PrOH (7.13 g, 118.56 mmol) was

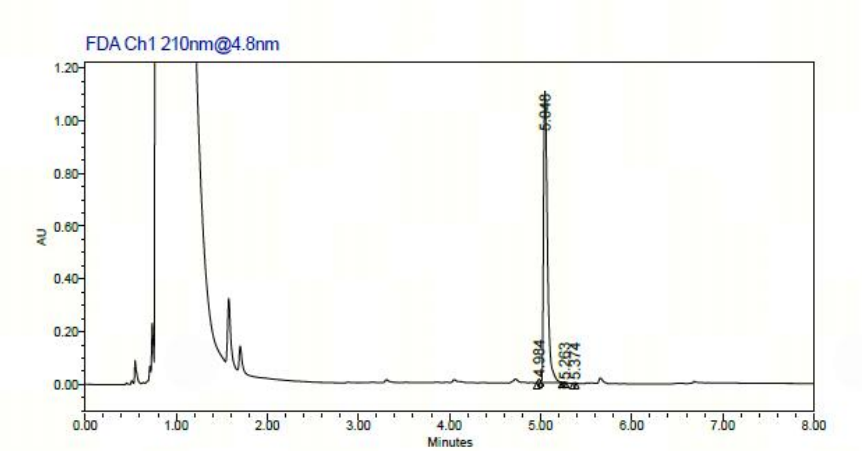
added to quench for 1 h at 25 °C. The reaction solution was directly used in the next step. To a 500 mL three-neck jacketed vessel, **S6** (7.26 g, 35.57 mmol), DMAc (100 mL) and BSA (18.09 g, 88.92 mmol) were added. The mixture was stirred for 30 min at 50 °C, and then cooled to 25 °C. The **S5** reaction solution was cooled to -5 °C. DIPEA (4.41 g, 34.09 mmol) and DMBCl (4.79 g, 35.57 mmol) were added. The reaction mixture was stirred at -5 °C for 5 min and monitored by HPLC, which showed the ratio of **S5**:**S5-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 1.16:98.84. Then the reaction solution was added to above **S6** solution in one portion. The reaction mixture was stirred at 25 °C for 1 h and monitored by HPLC. The HPLC showed the reaction assay yield was 93% over two steps, and the ratio of **S5:7b:*epi*-7b:7b-over-addition** was 0.74:99.14:0.06:0.05.

HPLC of **S5** formation (reaction mixture)

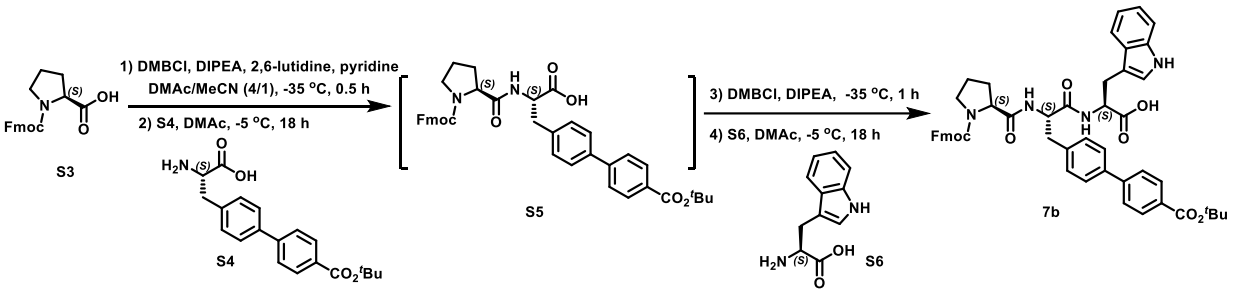


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S5-over-addition	5.695	2840	5173	0.30
S5	4.992	2387508	773034	99.12
S3	3.857	13963	2840	0.58
Total	-	-	-	100.00

HPLC of **7b** formation (reaction mixture)



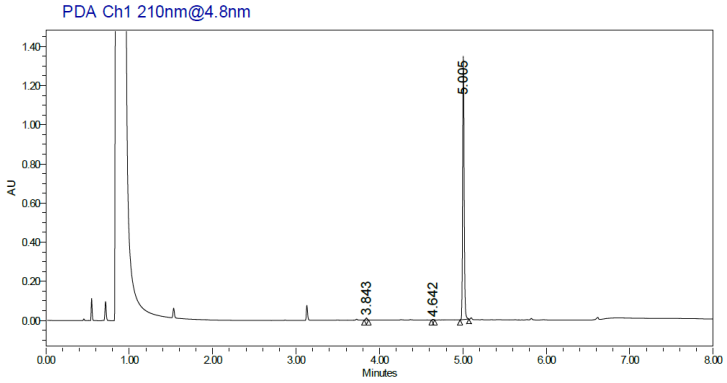
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
7b-over-addition	5.263	-	-	0.05
7b	5.048	3287670	1104333	99.14
***epi*-7b**	5.374	1415	2140	0.06
S5	4.984	24688	12203	0.74
Total	-	-	-	100.00



Synthesis of Fmoc-Pro-Bip-4-CO_2*t*Bu-Trp-OH (7b) (Method B). To a 50 mL single-neck, jacketed vessel, **S3** (1.00 g, 2.96 mmol) and DMAc/MeCN (4:1, 5 mL) were added. After the solution was cooled to -35 °C, 2,6-lutidine (318.0 mg, 2.96 mmol), pyridine (46.9 mg, 0.59 mmol), DIPEA (383.0 mg, 2.96 mmol), and DMBCl (419 mg, 3.11 mmol) were added. The reaction mixture was stirred at -35 °C for 0.5 h and monitored by HPLC (**Method 7**), which showed the ratio of **S3**:**S3-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 1.56:98.44. Then, the reaction solution was added to a pre-cooled slurry (-5 °C) of **S4** (1.1 g, 3.11 mmol) in DMAc (8 mL) over 5 min. The reaction mixture was stirred at -5 °C for 18 h and monitored by HPLC(**Method 7**). The HPLC showed the ratio of **S3**:**S5**:**S5-over-addition** was 0.79:99.03:0.18. The reaction solution was filtered, and the filtrate was directly used in the next stage. The **S5** reaction solution was cooled to -35 °C. DIPEA (440.0 mg, 3.41 mmol) and DMBCl (479.0 mg, 3.56 mmol) were added. The reaction mixture was stirred at -35 °C for 1 h and monitored by HPLC, which showed the ratio of **S5**:**S5-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.42:99.58. Then the reaction solution was added to a pre-cooled slurry (-5 °C) of **S6** (726.4 mg, 3.56 mmol) in DMAc (10 mL) solution. The reaction mixture was stirred at -5 °C for 18 h and monitored by HPLC (**Method 7**). The HPLC showed the reaction assay yield was 96% over two steps, and the ratio of **S5**:**7b**:***epi*-7b**:**7b-over-addition** was 0.54:99.25:0.21:0. And the reaction solution was quenched with 1.0 mL MeCN/H_2O (1/1) for 4 h at -5 °C. The mixture was precipitated from a 1:2 MeCN/H_2O solution (50 mL) to yield 2.36 g of **7b** (99.6% LCAP and 97.9 wt%) with an isolated yield of 92%. mp 219 °C. The compound existed as two rotamers in a ~1.1:1 ratio under NMR conditions. Rotamer 1:1H NMR (600 MHz, 3:1 CD_3CN/D_2O, 298 K): δ (ppm) 7.74 (2H), 7.67 (2H), 7.46 (1H), 7.36 (2H), 7.34 (2H), 7.34 (1H), 7.24 (1H), 7.21 (1H), 7.13 (2H), 7.12 (2H), 7.10 (1H), 7.08 (1H), 7.07 (2H), 6.99 (t, J = 7.3 Hz, 1H), 4.64 (1H), 4.58 (1H), 4.22 (1H),

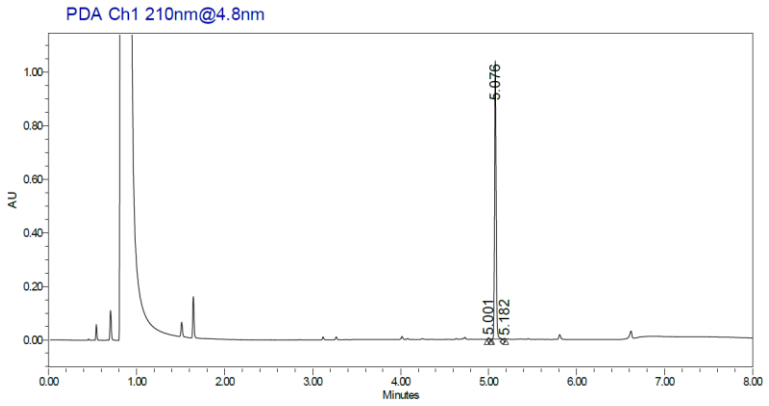
3.92 (dd, J = 10.1, 7.5 Hz, 1H), 3.80 (1H), 3.76 (1H), 3.37 (1H), 3.32 (1H), 3.27 (1H), 3.13 (1H), 3.05 (dd, J = 14.4, 4.3 Hz, 1H), 2.73 (dd, J = 14.4, 10.7 Hz, 1H), 2.06 (1H), 1.67 (1H), 1.63 (1H), 1.62 (1H), 1.55 (s, 9H); ^{13}C NMR (151 MHz, 3:1 CD_3CN/D_2O, 298 K): δ (ppm) 172.70, 172.70, 170.9, 165.0, 154.2, 143.03, 143.03, 142.98, 140.2, 136.5, 136.4, 135.4, 129.4, 128.9, 128.6, 126.9, 126.5, 126.3, 125.8, 125.3, 124.6, 124.4, 122.7, 120.6, 119.1, 118.1, 117.4, 110.6, 108.8, 80.4, 66.7, 58.9, 53.5, 52.2, 46.4, 45.8, 35.8, 30.3, 26.6, 26.2, 22.2. Rotamer 2: ^{1}H NMR (600 MHz, 3:1 CD_3CN/D_2O, 298 K): δ (ppm) 7.86 (d, *J* = 7.9 Hz, 2H), 7.64 (2H), 7.50 (3H), 7.49 (1H), 7.47 (1H), 7.41 (d, *J* = 7.6 Hz, 2H), 7.34 (1H), 7.31 (2H), 7.24 (1H), 7.22 (1H), 7.17 (2H), 7.10 (1H), 7.07 (1H), 6.95 (t, *J* = 7.3 Hz, 1H), 4.63 (1H), 4.59 (1H), 4.34 (1H), 4.09 (1H), 4.08 (1H), 4.06 (1H), 3.26 (1H), 3.25 (1H), 3.22 (1H), 3.15 (1H), 3.14 (1H), 2.79 (dd, *J* = 14.1, 9.5 Hz, 1H), 1.88 (1H), 1.64 (1H), 1.61 (1H), 1.56 (s, 9H), 1.46 (1H); ^{13}C NMR (151 MHz, 3:1 CD_3CN/D_2O, 298 K): δ (ppm) 172.64, 172.0, 170.6, 165.0, 155.2, 143.6, 142.94, 142.90, 140.08, 140.00, 137.0, 136.4, 135.4, 129.6, 129.0, 128.8, 126.8, 126.5, 126.3, 126.1, 125.7, 124.3, 124.2, 123.1, 120.6, 119.1, 118.1, 117.5, 110.6, 108.3, 80.5, 66.4, 60.0, 52.8, 52.2, 46.05, 46.00, 35.2, 28.3, 26.6, 26.1, 23.0. HRMS (ESI): m/z calcd for $C_{51}H_{51}N_4O_8$ $[M+H]^+$: 847.3701, found: 847.3707. FTIR (neat): 3307, 2977, 1676, 1523, 1421, 1298, 1160, 1118, 1006, 771, 758, 738, 701 cm^{-1}.

HPLC of **S5** formation (reaction mixture)



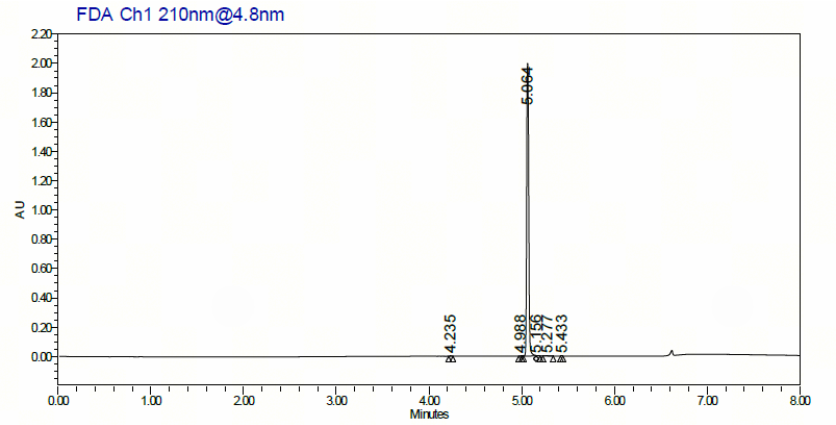
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
BnNH-DMB	3.171	-	-	-
S5-over-addition	4.642	3088	2721	0.18
S5	5.005	1731027	1346251	99.03
S3	3.843	13879	9829	0.79
Total	-	-	-	100.00

HPLC of **7b** formation (reaction mixture)

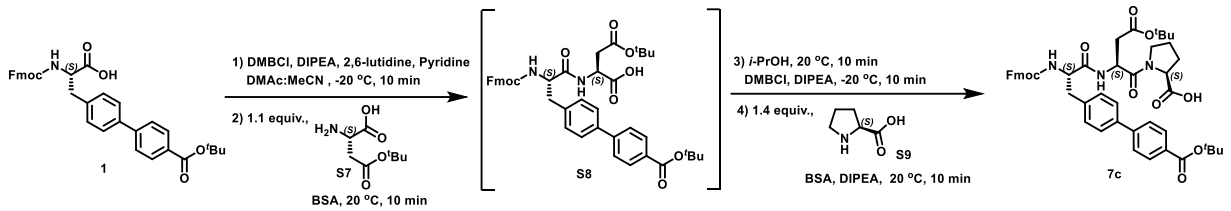


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
7b-over-addition	5.122	-	-	-
7b	5.076	1271919	1039940	99.25
***epi*-7b**	5.182	2750	2467	0.21
S5	5.001	6947	5985	0.54
Total	-	-	-	100.00

HPLC of isolated **7b**

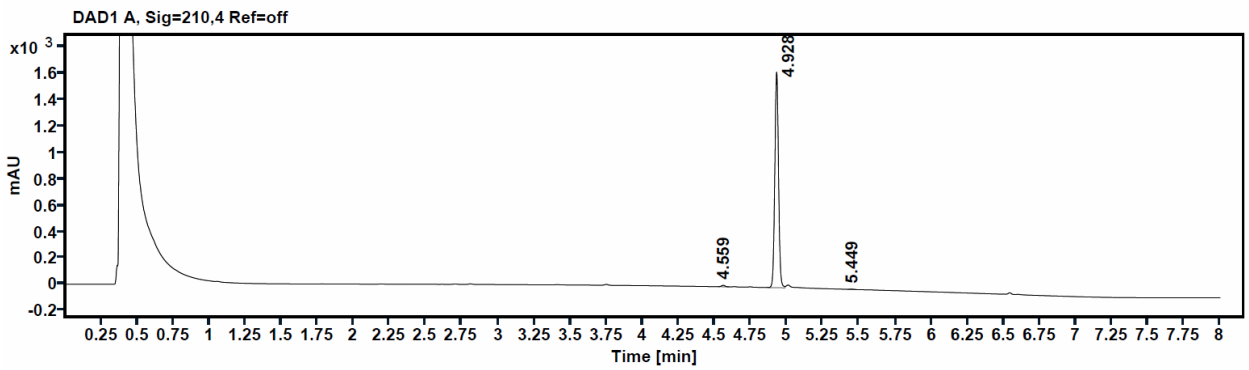


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
7b-over-addition	5.110	-	-	-
7b	5.064	3072972	1997190	99.59
***epi*-7b**	5.156	3843	3059	0.12
S5	4.988	615	662	0.02
Total	-	-	-	99.93



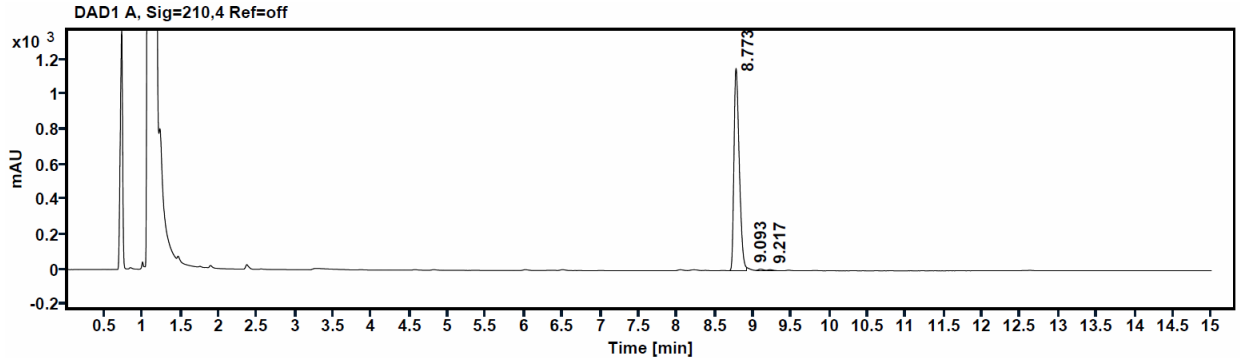
Synthesis of Fmoc-Bip-4-CO_2*t*Bu-L-Asp (O*t*Bu)-L-Pro-OH (7c) (Method A). In a 100 mL 3-neck round bottom flask, **Fmoc-Bip-4-CO_2*t*Bu (1)** (10.00 g, 17.74 mmol), pyridine (0.28 g, 3.55 mmol) and 2,6-lutidine (1.90 g, 17.74 mmol) were dissolved in DMAc/MeCN (1:1, 50 mL). The solution was cooled to -20 °C, then DMBCl (2.51 g, 18.63 mmol) and DIPEA (2.29 g, 17.74 mmol) were added into the reaction mixture. The reaction mixture was stirred at -20 °C for 10 min and monitored by HPLC (**Method 6**), which showed the ratio of **1:1-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.5:99.5. The reaction mixture was added slowly over 10 min to a DMAc and MeCN solution of **L-Asp (O*t*Bu)-OH (S7)** and BSA at 20 °C. The solution was prepared by mixing **S7** (3.69 g, 19.51 mmol) and BSA (7.22 g, 35.48 mmol) in DMAc/MeCN (1:1, 50 mL), stirring at 40 °C for 0.5 h, and then cooling to 20 °C before use. The reaction mixture was stirred at 20 °C for 10 min and monitored by HPLC (**Method 6**), which showed the ratio of **1:S8:S8-over-addition** was 0.8:99.1:0.1. Then *i*-PrOH (4.26 g, 70.96 mmol) was added into the reaction mixture. The reaction mixture was stirred at 20 °C for 10 min. The reaction was cooled to -20 ºC, DMBCl (3.10 g, 23.06 mmol) and DIPEA (2.52 g, 19.51 mmol) were added into the reaction mixture. The reaction mixture was stirred at -20 °C for 10 min and monitored by HPLC (**Method 8**). HPLC showed the ratio of **S8:S8-INT:*epi*-S8-INT** (mixed anhydride quenched by $BnNH_2$) was 0.5:99.2:0.3 The reaction mixture was added slowly over 10 min to a DMAc and MeCN solution of **L-Pro-OH (S9)**, BSA and DIPEA at 20 °C. The solution was prepared by mixing **S9** (2.86 g, 24.84 mmol) and BSA (10.83 g, 53.22 mmol) in DMAc/MeCN (1:1, 50 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C, then adding DIPEA (1.61 g, 12.42 mmol) before use. The reaction mixture was stirred at 20 °C for 10 min and monitored by HPLC (**Method 8**), which showed the ratio of **S8:7c:*epi*-7c:*epi*-S8-INT:7c-over-addition** was 0.5:98.8:0.7:0.0:0.0. The assay yield of the reaction was 94% over two steps. The reaction solution was added dropwise into 2.5% KH_2PO_4 solution (300 mL) at 20 ºC. Then extracted with MTBE (200 mL). Upon phase separation, the organic phase was washed with 5% NaCl solution (200 mL), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was dissolved in THF (50 mL) and added dropwise into n-heptane (250 mL) at 0 ºC over 0.5 h and stirred at 0 ºC for 2 h. The solid was collected by filtration and dried under N_2 for overnight to provide 13.73 g of **7c** as a white solid (96.6% LCAP, 94.8 wt%) in 88% isolated yield (**Method 8**).

HPLC of **S8** formation (reaction mixture)



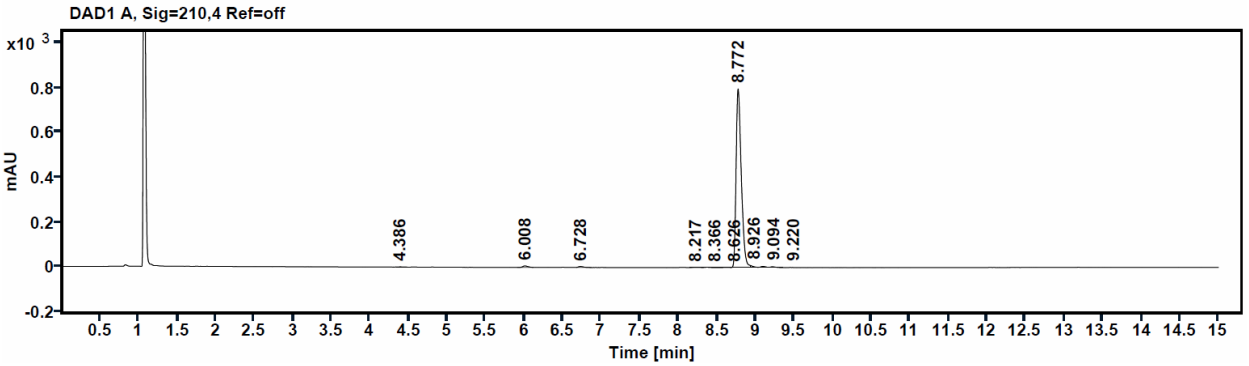
Compound ID	Time	Area	Height	Area% ratio
1	4.559	23.6654	13.2	0.8
S8	4.928	2931.5569	1644.5	99.1
S8-over-addition	5.449	3.6516	2.3	0.1
Total	-	-	-	100.0

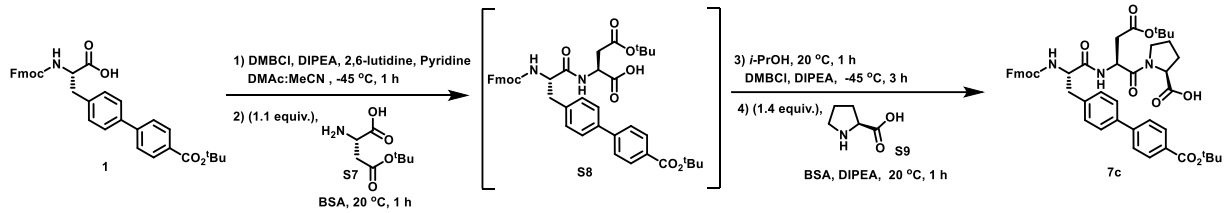
HPLC of **7c** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
7c-over-addition	8.243	0.0	0.0	0.0
7c	8.773	5596.3613	1156.2	98.8
***epi*-7c**	9.093	41.0722	8.9	0.7
S8	9.217	29.2582	6.0	0.5
***epi*-S8-INT**	10.630	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of isolated **7c**



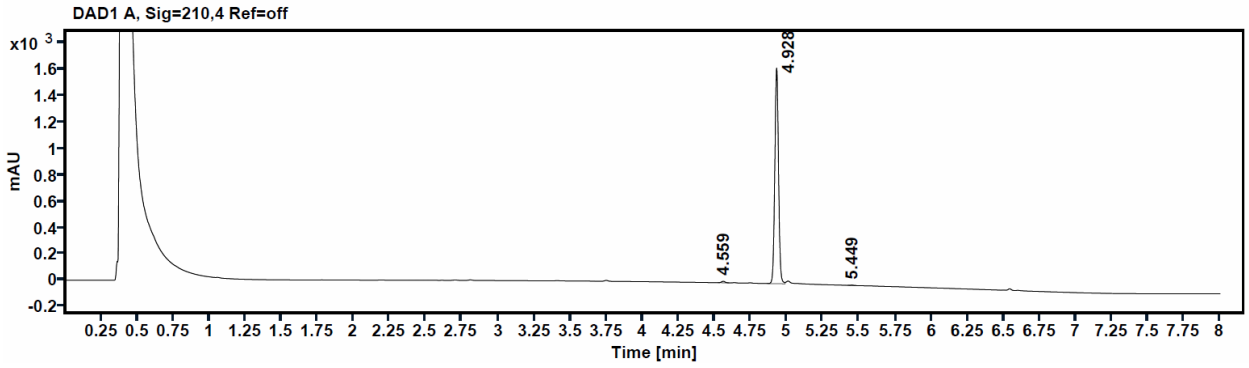


Synthesis of Fmoc-Bip-4-CO$_2$*t*Bu-L-Asp(O*t*Bu)-L-Pro-OH (7c) (Method B). In a 50 mL 3-neck round bottom flask, **Fmoc-Bip-4-CO$_2$*t*Bu (1)** (5.00 g, 8.87 mmol), pyridine (14 mg, 14.5 µL 0.18 mmol) and 2,6-lutidine (0.95 g, 8.87 mmol) were dissolved in DMAc/MeCN (1:1, 25 mL). The solution was cooled to -45 °C, then DMBCl (1.25 g, 9.31 mmol) and DIPEA (1.15 g, 8.87 mmol) were added into the reaction mixture. The reaction mixture was stirred at -45 °C for 1 h and monitored by HPLC (**Method 6**), which showed the ratio of **1:1-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.7:99.3. The reaction mixture was added slowly over 30 min to a DMAc and MeCN solution of **L-Asp (O*t*Bu)-OH (S7)** and BSA at 20 °C. The solution was prepared by mixing **S7** (1.85 g, 9.76 mmol) and BSA (3.61 g, 17.74 mmol) in DMAc/MeCN (1:1, 25 mL), stirring at 40 °C for 0.5 h, and then cooling to 20 °C before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC **(Method 6)**, which showed the ratio of **1:S8:S8-over-addition** was 0.7:99.2:0.1. Then *i*-PrOH (2.13 g, 35.48 mmol) was added into the reaction mixture. The reaction mixture was stirred at 20 ºC for 1 h. The reaction mixture was cooled to -45 °C, DMBCl (1.55 g, 11.53 mmol) and DIPEA (1.26 g, 9.76 mmol) were added into the reaction mixture. The reaction mixture was stirred at -45 °C for 3 h and monitored by HPLC **(Method 8)**, which showed a ratio of **S8**:**S8-INT:*epi*-S8-INT** was 0.6:98.3:1.1. The reaction mixture was added slowly over 30 min to a DMAc and MeCN solution of **L-Pro-OH (S9)**, BSA and DIPEA at 20 °C. The solution was prepared by mixing **S9** (1.43 g, 12.42 mmol) and BSA (5.41 g, 26.61mmol) in DMAc/MeCN (1:1, 25 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C, then adding DIPEA (0.80 g, 6.21 mmol) before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 8**), which showed the ratio of **S8:7c:*epi*-7c:*epi*-S8-INT:7c-over-addition** was**:** 0.5:98.5:0.8:0.0:0.2, the assay yield of the reaction was

94.0% over two steps. The reaction solution was added dropwise into 2.5% KH_2PO_4 solution (150 mL) at 20 °C, then extracted with MTBE (100 mL). Upon phase separation, the organic phase was washed with 5% NaCl solution (100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was dissolved in THF (25 mL) and added dropwise into n-heptane (125 mL) at 0 °C over 0.5 h and stirred at 0 °C for 2 h. The solid was collected by filtration and dried under N_2 for overnight to provide 6.82 g of compound **7c** (96.2% LCAP) was obtained as a white solid in 89% isolated yield **(Method 8).**

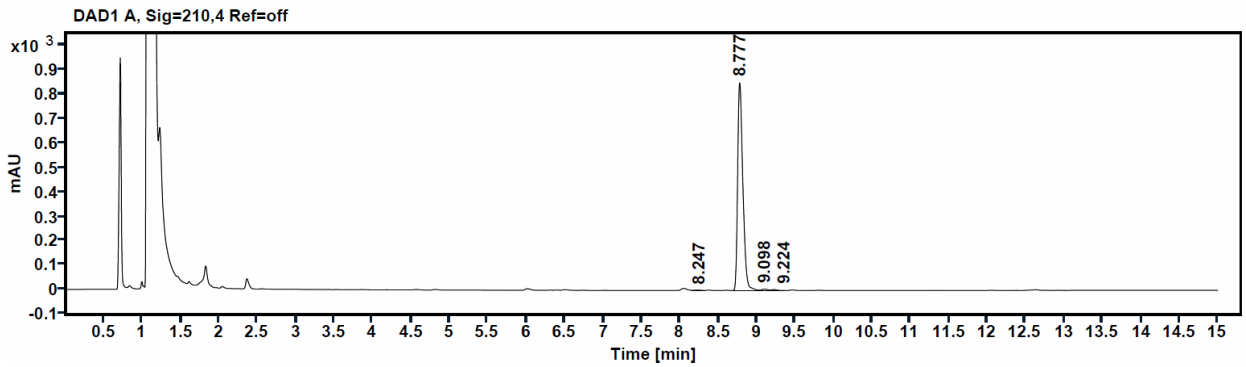
mp 120-123 °C. 1H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.55 (overlap, 2H), 7.98 - 7.92 (m, 2H), 7.90 - 7.84 (m, 2H), 7.73 (d, J = 8.4 Hz, 2H), 7.70 - 7.58 (m, 4H), 7.49 - 7.35 (m, 4H), 7.33 - 7.24 (m, 2H), 4.91 - 4.74 (m, 1H), 4.35 - 4.30 (m, 1H), 4.18 - 4.12 (m, 4H), 3.52 - 3.35 (m, 2H), 3.12 - 3.01 (m, 1H), 2.85 - 2.78 (m, 1H), 2.70 - 2.65 (m, 1H), 2.44 - 2.38 (m, 1H), 2.06 - 1.93 (m, 1H), 1.91 - 1.73 (m, 3H), 1.57 (s, 9H), 1.38 - 1.36 (m, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 174.05, 171.94, 169.50, 168.57, 165.25, 157.17, 144.60, 143.04, 139.89, 137.89, 137.22, 127.00, 121.85, 120.50, 81.13, 80.76, 66.11, 59.84, 56.15, 47.00, 46.67, 43.48, 38.12, 37.90, 29.26, 28.28, 28.06, 24.72. HRMS (ESI): m/z calcd for $C_{48}H_{54}N_3O_{10}$ $[M+H]^+$: 832.3804, found: 832.3815. FTIR (neat): 2974, 1708, 1650, 1525, 1448, 1366, 1291, 1251, 1114, 1033, 846, 772, 758, 738, 701, 620 cm^{-1}

HPLC of **S8** formation (reaction mixture)



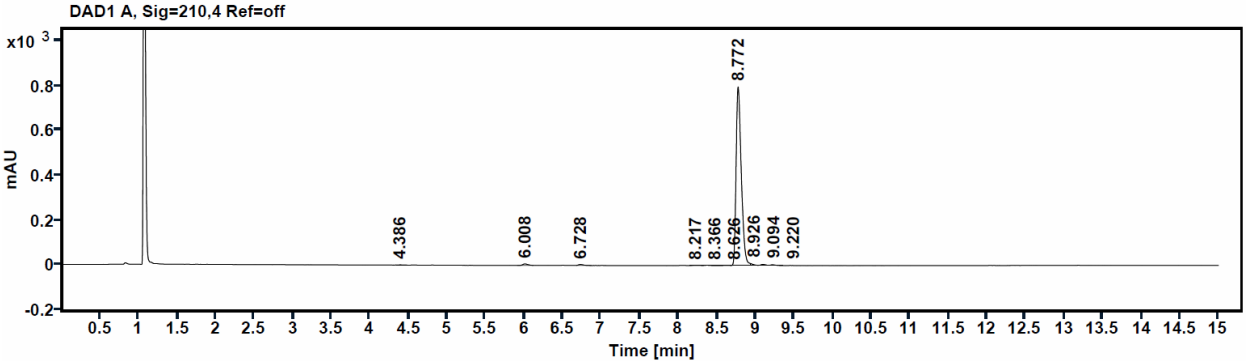
Compound ID	Time	Area	Height	Area% ratio
1	4.559	19.2594	11.2	0.7
S8	4.928	2931.5569	1644.5	99.2
S8-over-addition	5.449	3.6516	2.3	0.1
Total	-	-	-	100.0

HPLC of **7c** formation (reaction mixture)

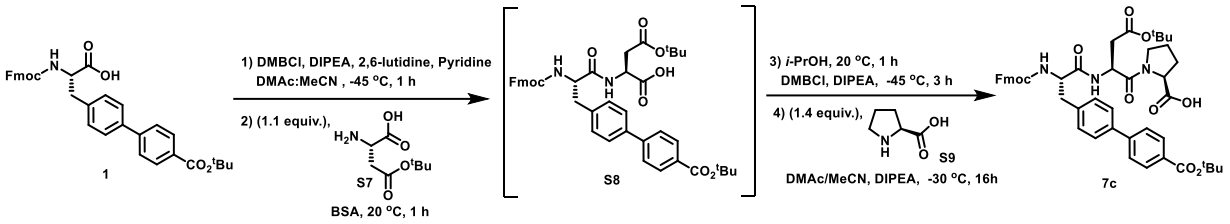


Compound ID	Time	Area	Height	Area% ratio
7c-over-addition	8.247	8.9727	1.4	0.2
7c	8.777	4025.0688	853.2	98.5
***epi*-7c**	9.098	32.5889	6.4	0.8
S8	9.224	21.3062	4.2	0.5
***epi*-S8-INT**	10.635	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of isolated **7c**

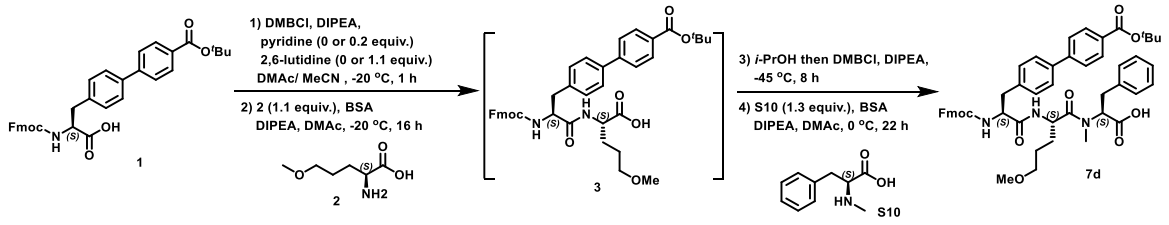


In addition to BSA, Trimethylsilyl chloride (TMSCl), Hydrochloric acid (HCl) in isopropanol, methanesulfonic acid (MSA), and 1-butanesulfonic acid were demonstrated to effectively solubilize free amino acids, enabling successful coupling reactions:



Five parallel reactions of **1** (1.00 g, 1.77 mmol) were performed to form **S8-mixed anhydride** under Method B conditions. The solutions of **L-Pro-OH (S9)**, which were prepared in 20-mL vials by stirring **S9** (0.29 g, 2.48 mmol) and different solubilizing reagents in DMAc/MeCN (1:1, 5 mL) at 50 °C for 1 h and cooled to 20 °C. The **S9** solutions were added to the reaction at -45 °C over 10 min. DIPEA was added at -45 °C. Then the reaction mixture was stirred at -30 °C for 16 h and monitored by HPLC, the results were shown in the table below:

Entry	Conditions		Result (%)				
	Solubilizing reagents	**DIPEA**	**S8**	***epi*-S8-INT**	**7c**	***epi*-7c**	**7c-over-addition**
1	1-butanesulfonic acid (2.48 mmol)	3.36 mmol	1.9	0.2	95.6	2.0	0.3
2	MSA (2.48 mmol)	3.36 mmol	1.3	0.4	96.1	1.7	0.5
3	6 M HCl in *i*-PrOH (2.48 mmol)	3.36 mmol	1.2	0.2	96.4	1.6	0.6
4	TMSCl (2.48 mmol)	3.36 mmol	1.2	0.6	93.1	1.4	0.5
5	BSA (5.31 mmol)	0.88 mmol	0.9	0.2	97.5	0.9	0.5

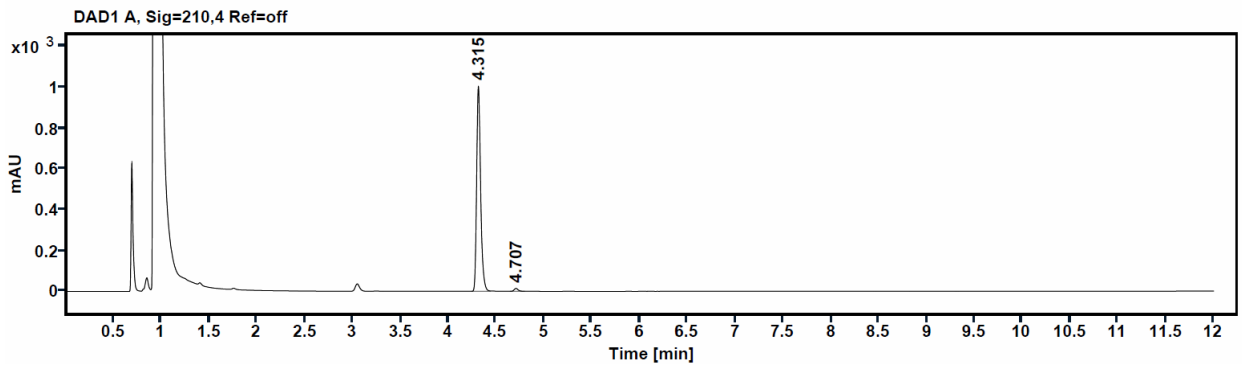


Synthesis of Fmoc-Bip-4-CO_2*t*Bu-5-OMe-NVA-MePhe-OH (7d) (Method B).

No 2,6-lutidine: In a 250-mL 3-neck round bottom flask, **1** (5.00 g, 8.87 mmol) was dissolved in DMAc/ MeCN (1:1, 60 mL). After the solution was cooled to -20 °C, DIPEA (1.26 g, 9.76 mmol) was added. Then DMBCl (1.31 g, 9.76 mmol) was added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC **(Method 1)**, which showed the ratio of **1:1-INT** was 1.9:98.1. A BSA solution of **2**, which was prepared by stirring **2** (1.44 g, 9.76 mmol) and BSA (3.61 g, 17.74 mmol) in DMAc (15 mL) at 50 °C for 1 h, and then cooling to 20 °C, was added to the reaction mixture at -20 °C over 10 min. DIPEA (0.23 g, 1.77 mmol) was added. Then the reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC (**Method 1**), which showed the ratio of **1:3:3-over-addition** was 1.4:98.6:0.0. Then *i*-PrOH (2.13 g, 35.48

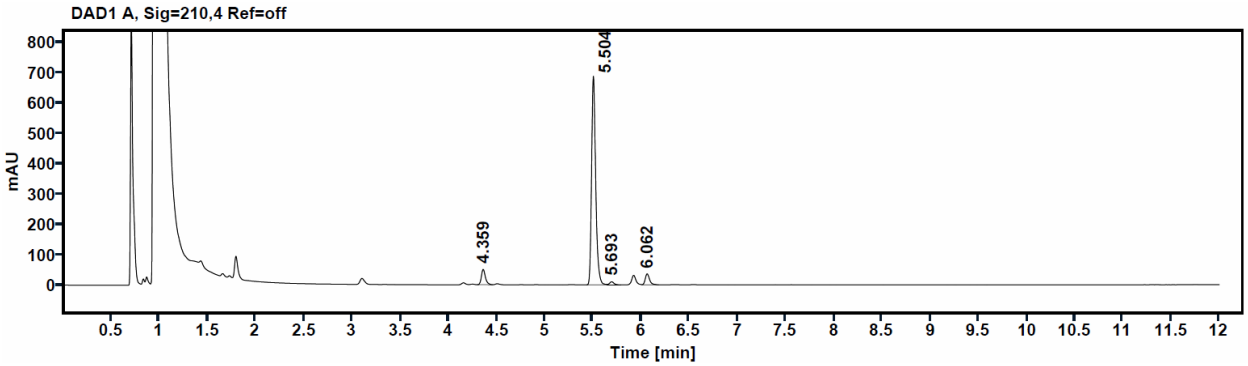
mmol) was added to the solution at 0 °C, and the mixture was stirred for 1 h. Then the mixture was cooled to -45 °C, DMBCl (2.03 g, 15.08 mmol) and DIPEA (1.61 g, 12.42 mmol) were added and stirred for 8 h and monitored by HPLC (**Method 1**), which showed the ratio of **3:3-INT:*epi*-3-INT** was 6.5:90.9:2.6. A BSA solution of **H-MePhe-OH (S10)**, which was prepared by stirring **S10** (2.07 g, 11.53 mmol) and BSA (5.41 g, 26.61 mmol) in DMAc (15 mL) at 50 °C for 1 h, and then cooling to 20 °C, was added to the reaction at -45 °C over 10 min. DIPEA (1.03 g, 7.98 mmol) was added. Then the reaction mixture was stirred at 0 °C for 22 h and monitored by HPLC (**Method 1**), which showed the ratio of **3:*epi*-3-INT:7d:*epi*-7d:7d-over-addition** was 6.4:4.8:87.4:1.4:0.0. The reaction assay yield was 77%. (coupling 1 assay yield was 96%, coupling 2 assay yield was 81%.)

HPLC of **3** formation (reaction mixture)



Compound ID	Time	Area	Height	Area%
3	4.315	2888.0476	1007.6	98.6
1	4.707	39.8325	14.2	1.4
3 over-addition	4.120	-	-	0.0
Total	-	-	-	100.0

HPLC of **7d** formation (reaction mixture)



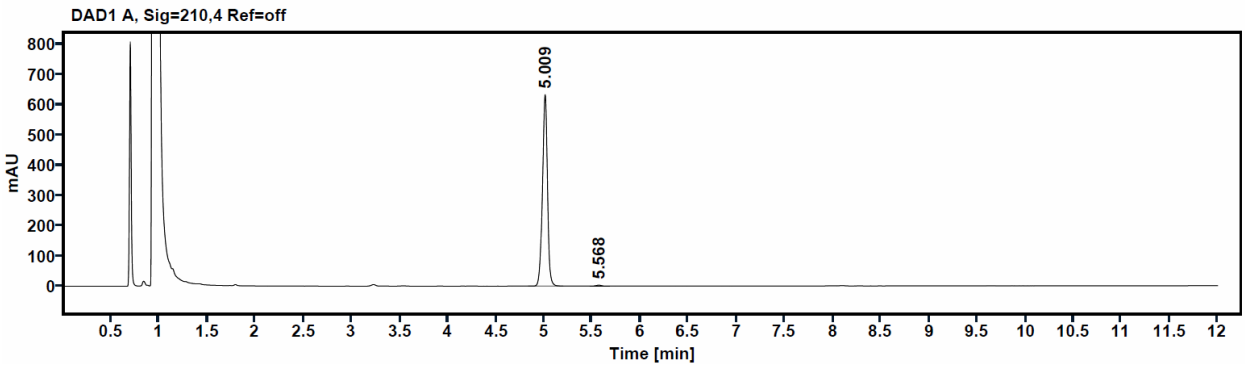
Compound ID	Time	Area	Height	Area% ratio
3	4.359	148.5128	51.0	6.4
7d	5.504	2036.1794	691.2	87.4
***epi*-7d**	5.693	32.7621	10.3	1.4
***epi*-3-INT**	6.062	112.2256	36.5	4.8
7d-over-addition	8.696	-	-	0.0
Total	-	-	-	100.0

With 2,6-lutidine and pyridine: In a 250-mL 3-neck round bottom flask, **1** (10.00 g, 17.7 mmol) dissolved in DMAc/MeCN (1:1, 60 mL). After the solution was cooled to -20 °C, 2,6-lutidine (2.09 g, 19.5 mmol), pyridine (0.28 g, 3.5 mmol), and DIPEA (2.52 g, 19.5 mmol) were added. Then DMBCl (2.63 g, 19.5 mmol) was added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 3**), which showed the ratio of **1:1-INT** was 0.6:99.4. A BSA solution of **2**, which was prepared by stirring **2** (2.87 g, 19.5 mmol) and BSA (7.22 g, 35.4 mmol) in DMAc (30 mL) at 50 °C for 1 h, and then cooling to 20 °C, was added to the reaction at -20 °C over 10 min. DIPEA (0.46 g, 3.5 mmol) was added. Then the reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC (**Method 3**), which showed the ratio of **1:3:3-over-addition** was 0.5:99.5:0.0. Then *i*-PrOH (4.26 g, 70.8 mmol) was added to the solution at 0 °C, and the mixture was stirred for 1 h. MeCN (30 mL) was added. Then the mixture was cooled to -45 °C, DMBCl (3.34 g, 24.8 mmol) and DIPEA (0.69 g, 5.3 mmol) were added and stirred for 8 h and monitored by HPLC (**Method 3**), which showed the ratio of **3:3-INT:*epi*-3-INT** was 0.6:99.3:0.2. A BSA solution of **H-MePhe-OH (S10)**, which was prepared by stirring **S10** (4.13 g, 23.0 mmol) and BSA (10.8 g, 53.1 mmol) in DMAc (30 mL) at 50 °C for 1 h, and then cooling to 20 °C, was added to the reaction at -45 °C over 10 min. DIPEA (2.06 g, 15.9 mmol) was added. Then the reaction mixture was stirred at 0 °C for 22 h and monitored by HPLC (**Method 1**), which showed the ratio of **3:*epi*-3-INT:7d:*epi*-7d:7d-over-addition** was 1.0:0.5:97.8:0.5:0.2. The reaction assay yield was 94% over 2 steps. (coupling1 assay yield was 98%, coupling 2 assay yield was 96%) The reaction mixture was added slowly to H_2O (300 mL) at 0 °C over 1 h and stirred at 0 °C for 3 h. The product was collected by filtration and dried under vacuum at 40 °C, yielding 14.9 g of **7d** as a white solid (95.3% LCAP, 94.9 wt%) in 93% isolated yield.

mp 96-100 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.00 (s, 1H), 8.15 (d, J = 8.4 Hz, 1H), 7.94 (d, J = 8.3 Hz, 2H), 7.87 (d, J = 7.5 Hz, 2H), 7.74 - 7.60 (m, 6H), 7.55 - 7.09 (m, 12H), 5.09 - 4.39 (m, 2H), 4.33 - 4.22 (m, 1H), 4.13 - 4.10 (m, 2H), 3.30 - 3.23 (m, 2H), 3.17 (s, 3H), 3.05 - 2.93 (m, 3H), 2.88 - 2.80 (m, 4H), 2.76 - 2.68 (m, 1H), 1.57 (s, 9H), 1.51 - 1.41 (m, 2H), 1.20 - 1.13 (m, 1H), 1.02 - 0.95 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.82, 171.61, 170.84, 164.70, 155.72, 144.09, 143.69, 143.65, 143.64,

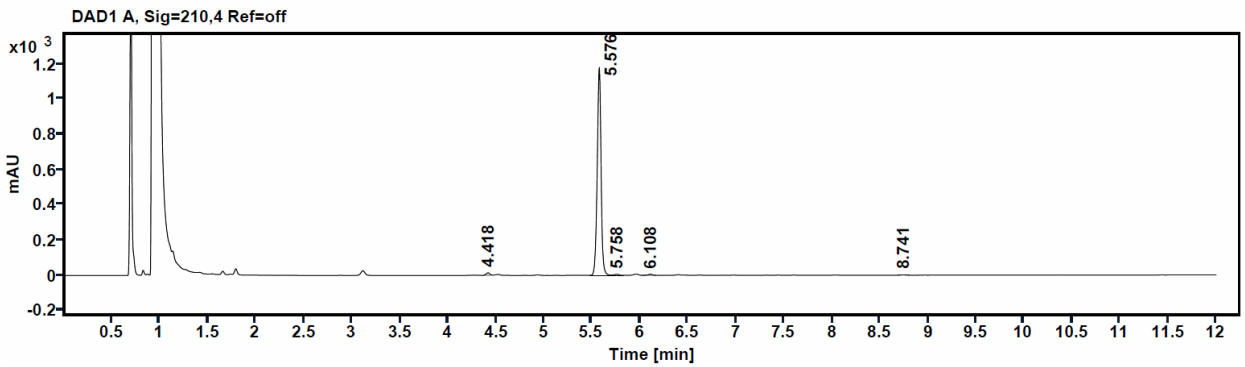
143.62, 140.56, 138.48, 137.82, 136.67, 129.97, 129.79, 129.51, 129.11, 128.60, 128.33, 128.06, 127.50, 126.94, 126.48, 126.40, 126.06, 125.23, 119.96, 80.57, 71.35, 71.23, 65.56, 60.66, 58.39, 57.61, 55.71, 48.03, 46.44, 37.08, 33.60, 32.60, 27.72, 24.80. HRMS (ESI): m/z calcd for $C_{51}H_{56}N_3O_9$ $[M+H]^+$: 854.4011, found: 854.4021. FTIR (neat): 3283, 2930, 1706, 1634, 1292, 1163, 1114, 739, 700 cm^{-1}.

HPLC of **3** formation (reaction mixture)



Compound ID	Time	Area	Height	Area%
3	5.009	2310.7837	635.8	99.5
1	5.568	12.6702	3.3	0.5
3 over-addition	4.868	-	-	0.0
Total	-	-	-	100.0

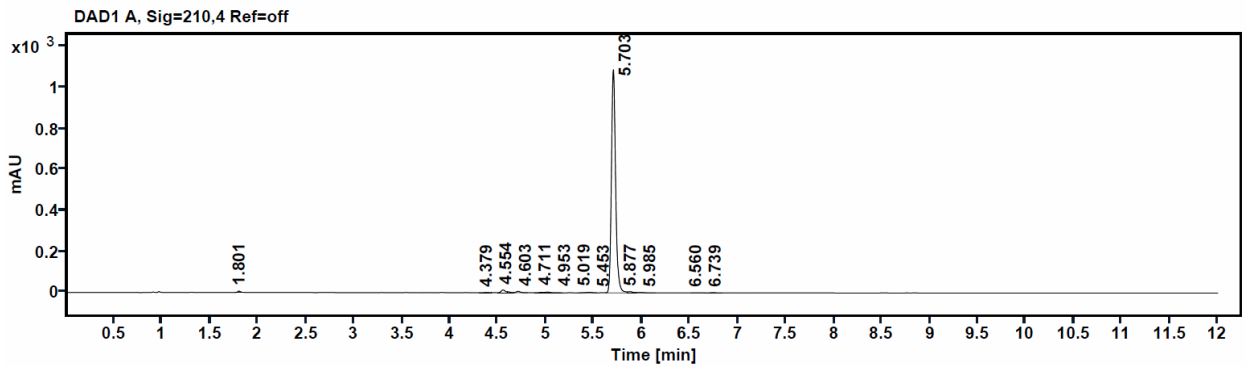
HPLC of **7d** formation (reaction mixture)

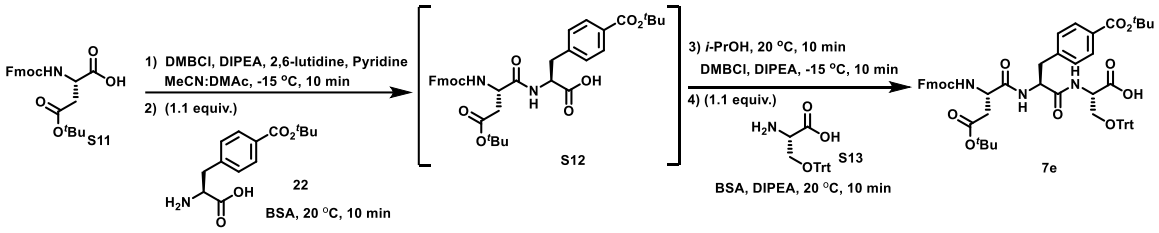


Compound ID	Time	Area	Height	Area% ratio
3	4.418	34.2469	13.9	1.0
7d	5.576	3317.7144	1179.2	97.8

***epi*-7d**	5.758	18.1892	6.2	0.5
***epi* -3-INT**	6.108	18.4381	6.5	0.5
7d-over-addition	8.741	5.4088	2	0.2
Total	-	-	-	100.0

HPLC of isolated **7d**

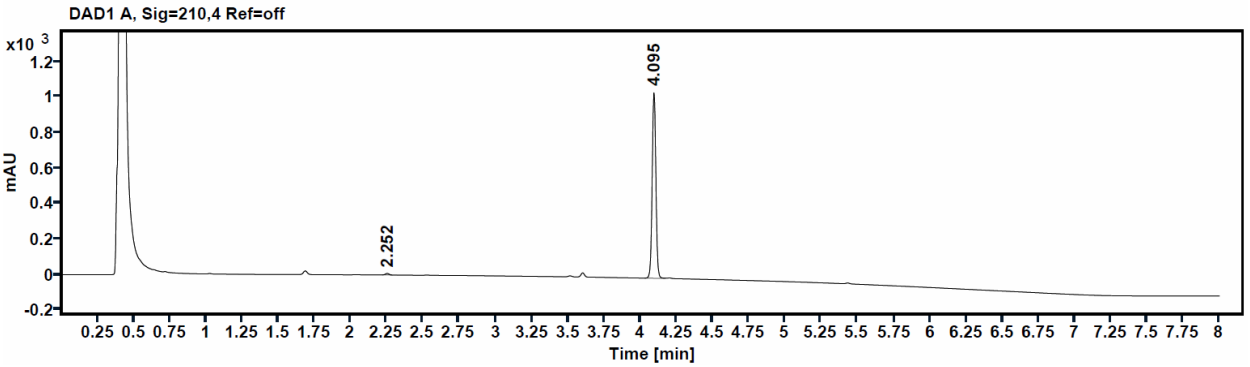




Synthesis of Fmoc-L-Asp(O*t*Bu)-L-Phe(4-CO$_2$*t*Bu)-L-Ser(Trt)-OH (7e) (**Method A**). In a 40 mL vial, **Fmoc-L-Asp(O*t*Bu)-OH (S11)** (500 mg, 1.22 mmol), pyridine (20 µL, 0.24 mmol) and 2,6-lutidine (142 µL, 1.22 mmol) were dissolved in DMAc/MeCN (1:1, 2.5 mL). The solution was cooled to -15 °C, then DMBCl (179 µL, 1.28 mmol) and DIPEA (207 µL, 1.22 mmol) were added. The reaction mixture was stirred at -15 °C for 10 min and monitored by HPLC (**Method 6**), which showed the ratio of **S11:S11-INT** (mixed anhydride intermediate quenched by BnNH$_2$) was 0.6:99.4. The reaction mixture was added to a DMAc and MeCN solution of **L-Phe(4-CO$_2$*t*Bu)-OH (22)** and BSA at 20 °C. The solution was prepared by mixing **22** (355 mg, 1.34 mmol) and BSA (594 µL, 2.43 mmol) in DMAc/MeCN (1:1, 2.5 mL), stirring at 40 °C for 0.5 h, and then cooling to 20 °C before use. The reaction mixture was stirred at 20 °C for 10 min and monitored by HPLC (**Method 6**), which showed the ratio of **S11:S12:S12-over-addition** was 0.8:99.2:0.0. Then *i*-PrOH (369 µL, 4.86 mmol) was added into the reaction mixture at 20 °C and stirred for 10 min. The reaction was cooled to -15 °C, DMBCl (222 µL, 1.58 mmol) and DIPEA (227 µL, 1.34 mmol) were added and stirred at -15 °C for 10 min, then monitored by HPLC (**Method 6**). HPLC showed

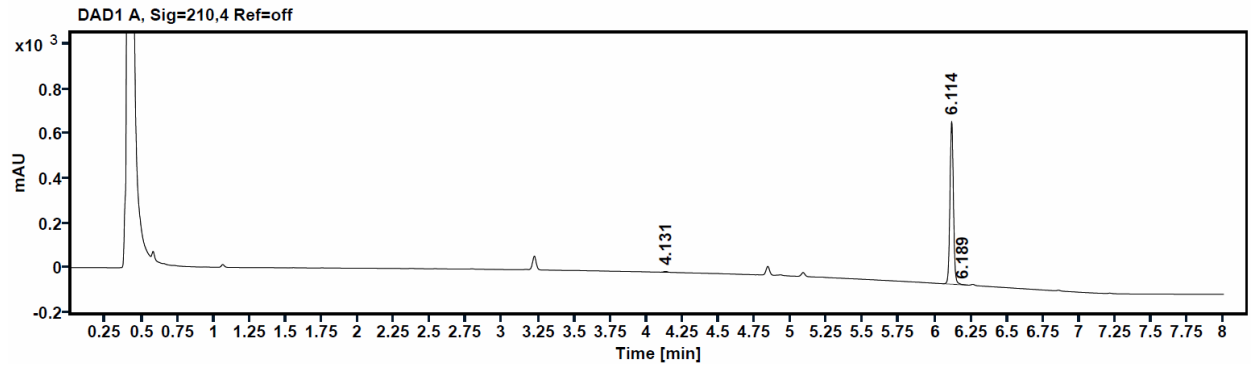
the ratio of **S12:(S12-INT+*epi*-S12-INT)** (mixed anhydride quenched by $BnNH_2$) was 0.3:99.7. The reaction mixture was added to a DMAc and MeCN solution of **L-Ser(Trt)-OH (S13)**, BSA and DIPEA at 20 °C. The solution was prepared by mixing **S13** (507 mg, 1.46 mmol) and BSA (891 µL, 3.65 mmol) in DMAc/MeCN (1:1, 2.5 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C, adding DIPEA (145 µL, 0.85 mmol) before use. The reaction mixture was stirred at 20 °C for 10 min and monitored by HPLC (**Method 6**), which showed the ratio of **S12:7e:*epi*-7e:*epi*-S12-INT:7e-over-addition** was 0.5:99.4:0.1:0.0:0.0. The assay yield of the reaction was 94% over two steps.

HPLC of **S12** formation (reaction mixture)

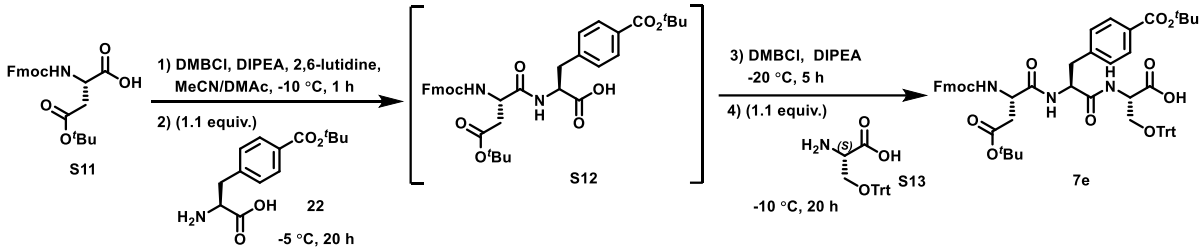


Compound ID	Time	Area	Height	Area% ratio
S11	2.252	15.2435	8.9	0.8
S12	4.095	1851.6898	1049.1	99.2
S12-over-addition	5.443	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of **7e** formation (reaction mixture)



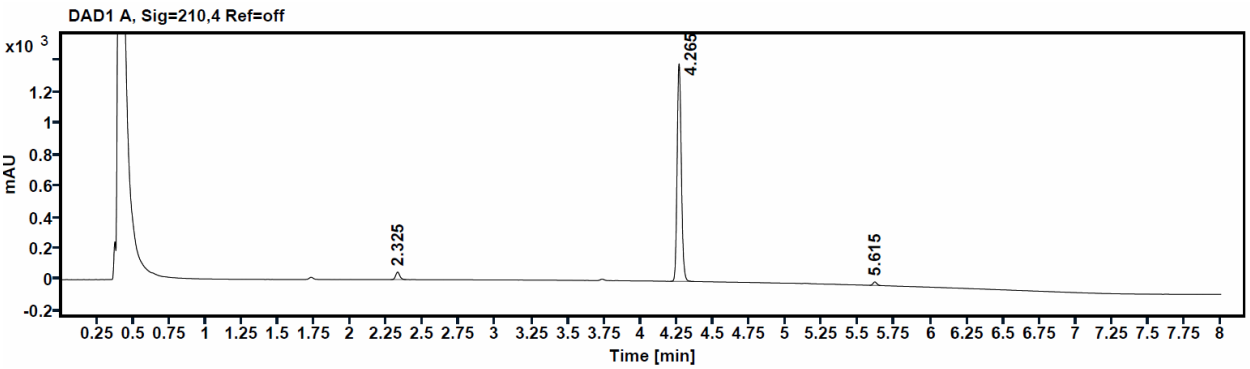
Compound ID	Time	Area	Height	Area% ratio
S12	4.131	6.4275	4.0	0.5
***epi*-S12-INT**	5.355	0.0	0.0	0.0
7e	6.114	1219.9703	732.7	99.4
***epi*-7e**	6.189	1.2284	1.3	0.1
7e-over-addition	6.890	0.0	0.0	0.0
Total	-	-	-	100.0



Synthesis of Fmoc-L-Asp(O*t*Bu)-L-Phe(4-CO$_2$*t*Bu)-L-Ser(Trt)-OH (**7e**) (**Method B)**: In a 250 mL 3-neck round bottom flask, **Fmoc-L-Asp(O*t*Bu)-OH (S11)** (20.00 g, 48.61 mmol) and 2,6-lutidine (5.21 g, 48.61 mmol) were dissolved in DMAc/MeCN (1:1, 100 mL). Then the solution was cooled to -10 °C, DMBCl (6.87 g, 51.04 mmol) and DIPEA (6.28 g, 48.61 mmol) were added to the reaction mixture. The reaction mixture was stirred at -10 °C for 1 h and monitored by HPLC **(Method 6)**, which showed the ratio of **S11:S11-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 1.5:98.5. **L-Phe(4-CO$_2$*t*Bu)-OH (22)** (14.19 g, 53.47 mmol) was added to the reaction solution at -10 °C. The reaction mixture was stirred at -5 °C for 20 h and monitored by HPLC **(Method 6)**, which showed the ratio of **S11:S12:S12-over-addition** was 3.2:95.4:1.4. The reaction mixture was cooled to -20 °C, then DMBCl (8.51 g, 63.19 mmol) and DIPEA (6.91 g, 53.47 mmol) were added to the reaction mixture. The reaction mixture was stirred at -20 °C for 5 h and monitored by HPLC **(Method 6)**, which showed a ratio of **S12:(S12-INT+*epi*-S12-INT)** was 0.8:99.2. **L-Ser(Trt)-OH (S13)** (18.58 g, 53.47 mmol) was added to the reaction mixture at -20 °C. The reaction mixture was stirred at -10 °C for 20 h and monitored by HPLC **(Method 6)**, which showed a ratio of **S12:7e:*epi*-7e:*epi*-S12-INT:7e-over-addition** was 0.9:97.5:0.3:0.0:1.3. The assay yield of the reaction was 90% over two steps. This reaction mixture was added dropwise to H_2O (500 mL) at 5 °C over 2 h and then stirred at 5 °C for 1 h. The solid was collected by filtration and washed with H_2O (100 mL). The solid was dried under N_2 overnight and slurried with THF:MTBE:*n*-Hexane=1:10:20 (500 mL) at 0 °C for 2 h, then the solid was collected by filtration and dried to provide 42.86 g of **7e** as a white solid (91.4% LCAP, 90.3 wt%) in 81% isolated yield.

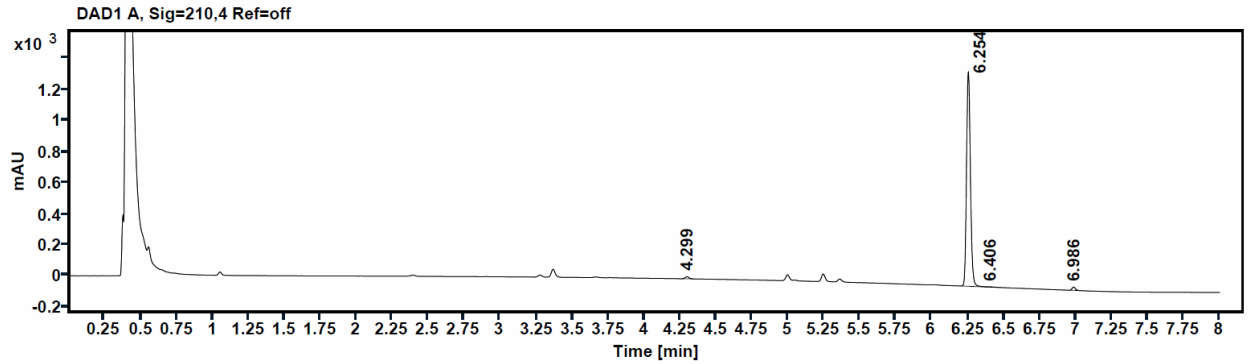
mp 115 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ(ppm) 12.95 (s, 1H), 8.59 (d, J = 8.0 Hz, 1H), 7.89 (d, J = 7.6 Hz, 3H), 7.75 (d, J = 8.0 Hz, 2H), 7.71 - 7.67 (m, 3H), 7.43 - 7.30 (m, 15H), 7.29 - 7.24 (m, 6H), 4.75 (td, J = 8.8, 4.4 Hz, 1H), 4.53 - 4.49 (m, 1H), 4.37 (td, J = 8.8, 4.8 Hz, 1H), 4.30 - 4.18 (m, 3H), 3.27 - 3.21 (m, 2H), 3.12 - 3.07 (m, 1H), 2.96 - 2.90 (m, 1H), 2.58 (dd, J = 16.0, 4.8 Hz, 1H), 2.44 - 2.37 (m, 1H), 1.44 (s, 9H), 1.32 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6): δ(ppm) 169.74, 168.72, 168.68, 167.60, 163.28, 154.01, 142.11, 141.99, 141.73, 140.95, 139.05, 127.90, 127.73, 127.05, 126.63, 126.22, 125.98, 125.42, 123.60, 118.45, 84.29, 78.71, 78.44, 64.15, 61.62, 51.39, 50.72, 49.73, 44.93, 36.04, 35.73, 26.06, 25.99. HRMS (ESI): m/z calcd for $C_{59}H_{61}N_3NaO_{11}$ $[M+Na]^+$: 1010.4198, found: 1010.4212. FTIR (neat): 3316, 3063, 2929, 1708, 1509, 1291, 1156, 1103, 846, 758, 741, 704, 631 cm^{-1}

HPLC of **S12** formation (reaction mixture)



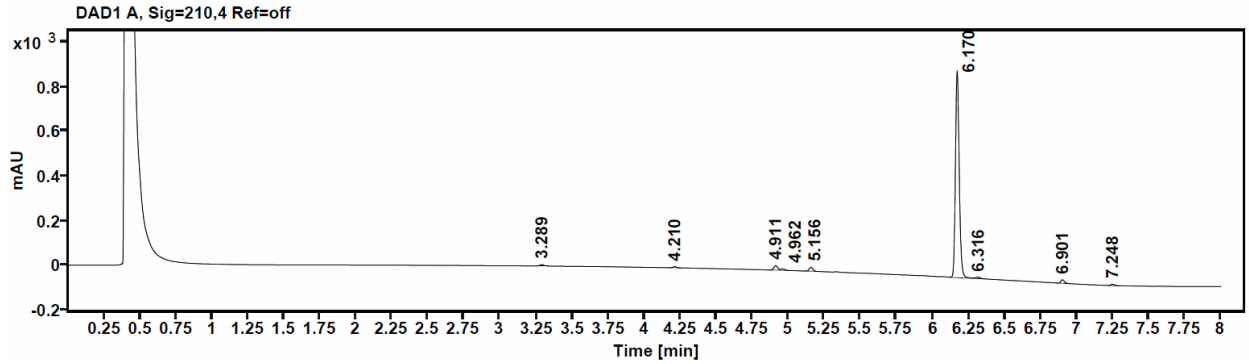
Compound ID	Time	Area	Height	Area%
S11	2.325	94.1907	48.2	3.2
S12	4.265	2800.1421	1398.8	95.4
S12-over-addition	5.615	41.9259	22.8	1.4
Total	-	-	-	100.0

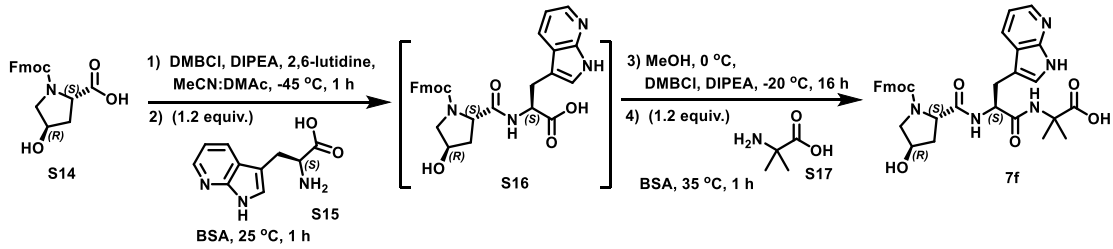
HPLC of **7e** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S12	4.299	23.8067	13.3	0.9
***epi*-S12-INT**	5.523	0.0	0.0	0.0
7e	6.254	2608.8508	1390.9	97.5
***epi*-7e**	6.406	7.3603	2.2	0.3
7e-over-addition	6.986	34.417	20.7	1.3
Total	-	-	-	100.0

HPLC of isolated **7e**



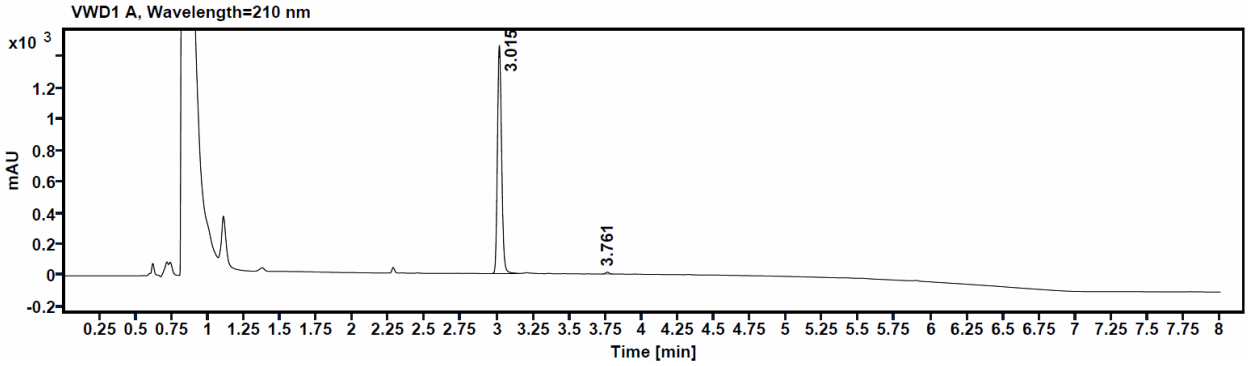


Synthesis of Fmoc-Pro(*t*4OH)-7-aza-Trp-Aib-OH (7f) (Method B). In a 100 mL 3-neck round bottom flask, **Fmoc-Pro(*t*4OH)-OH (S14)** (10.00 g, 28.30 mmol) and 2,6-lutidine (3.03 g, 28.30 mmol) were dissolved in DMAc/MeCN (1:1, 50 mL). Then the solution was cooled to -45 °C, DMBCl (4.19 g, 31.13 mmol) and DIPEA (3.66 g, 28.30 mmol) were added to the reaction mixture. The reaction mixture was stirred at -45 °C for 1 h and monitored by HPLC (**Method 4**), which showed the ratio of **S14:S14-INT** was 1.5:98.5. The reaction mixture was added slowly into a solution of **7-aza-Trp (S15)** and BSA in DMAc and MeCN at 25 °C over 1 h. which was prepared by stirring **S15** (6.97 g, 33.96 mmol) and BSA (11.52 g, 56.60 mmol) in DMAc/MeCN (1:1, 50 mL) at 50 °C for 1 h until clear, then cooling to 25 °C. The reaction mixture was stirred at 25 °C for 1 h and monitored by HPLC (**Method 4**), which showed the ratio of **S14:S16:S16-over-addition** was 0.7:99.3:0.0. MeOH (2.27 g, 70.75 mmol) was added into the reaction mixture at 0 °C and stirred for 30 min, then the reaction mixture was cooled to -20 °C. DMBCl (5.52 g,

41.03 mmol) and DIPEA (4.94 g, 38.20 mmol) were added to the reaction mixture and stirred for 16 h at -20 °C and monitored by HPLC (**Method 4**). HPLC showed the ratio of **S16:S16-INT:*epi*-S16-INT** (mixed anhydride quenched by $BnNH_2$) was 1.6:98.4:0.0. The reaction solution was added slowly into a solution of **H-Aib-OH (S17)** and BSA in DMAc at 35 °C over 1 h. Which was prepared by mixing **S17** (3.50 g, 33.96 mmol) and BSA (11.51 g, 56.6 mmol) in DMAc (50 mL), stirring at 50 °C for 1 h until clear, then cooling to 35 °C. The reaction mixture was stirred at 35 °C for 1 h and monitored by HPLC (**Method 4**), which showed the ratio of **S16**:**7f**:***epi*-7f**:**(S16-INT+*epi*-S16-INT)**:**7f-over-addition** was 2.5:96.5:0.0:1.0:0.0. The assay yield of the reaction was 91% over two steps. The reaction mixture was added slowly into a solution of KH_2PO_4 (7.70 g, 56.60 mmol) in H_2O (200 mL) at -10 °C, then extracted with 2-MeTHF (100 mL). After phase separation, the organic phase was concentrated to afford the crude product, which was slurried in isopropyl acetate (IPAc) (200 mL) at 0 °C for 1 h. The solid was collected by filtration and dried under vacuum to provide 14.4 g of **7f** as a white solid (98.1% LCAP, **Method 4**) in 80% isolated yield.

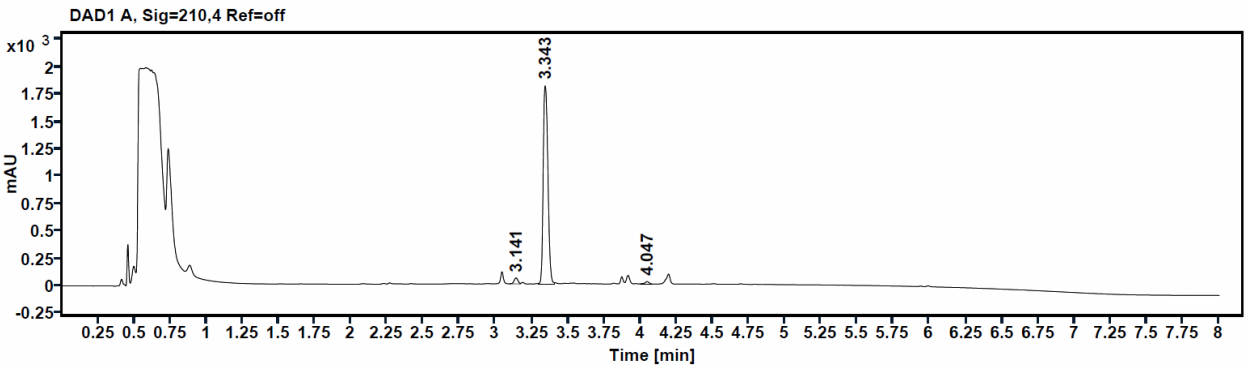
mp 135 °C, ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 11.61 - 11.51 (m, 1H), 8.31 - 7.83 (m, 6H), 7.68 - 7.51 (m, 2H), 7.46 - 7.23 (m, 5H), 7.13 - 6.96 (m, 1H), 4.70 - 4.46 (m, 1H), 4.36 - 3.72 (m, 6H), 3.58 - 3.33 (m, 3H), 3.19 - 2.90 (m, 2H), 1.72 - 1.58 (m, 1H), 1.34 - 1.29 (s, 3H), 1.12 (d, J = 6.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 175.79, 171.91, 170.58, 155.03, 144.23, 144.19, 143.80, 141.20, 128.21, 128.04, 127.65, 125.63, 121.65, 120.68, 120.44, 110.42, 110.19, 74.71, 67.21, 55.38, 53.45, 53.25, 47.03, 33.15, 25.86, 21.39, 21.32. HRMS (ESI): m/z calcd for $C_{34}H_{36}N_5O_7$ $[M+H]^+$: 626.2609, found: 626.2630. FTIR (neat): 3283, 2925, 1648, 1536, 1420, 1351, 1171, 1129, 995, 756, 739, 720, 621 cm^{-1}.

HPLC of **S16** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S16-over-addition	2.750	0.0	0.0	0.0
S16	3.015	2946.5161	1468.3	99.3
S14	3.761	19.7902	11.9	0.7
Total	-	-	-	100.0

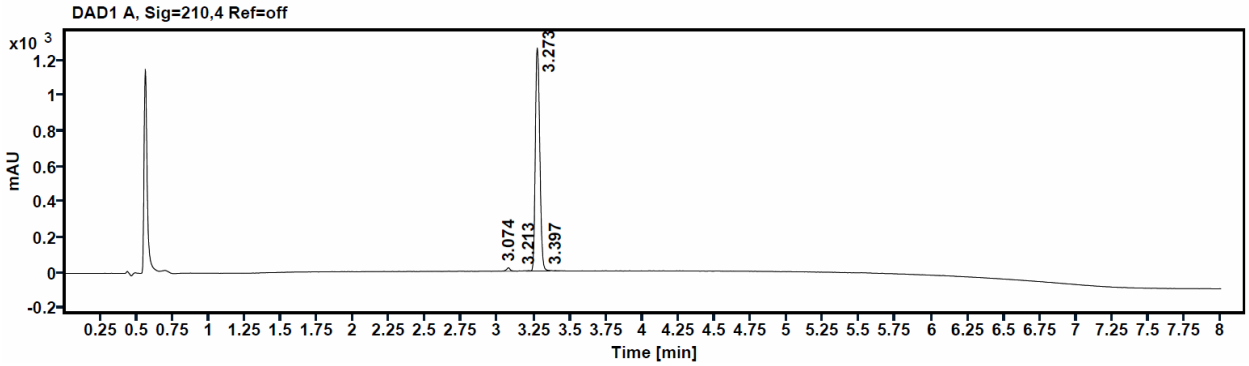
HPLC of **7f** formation (reaction mixture)

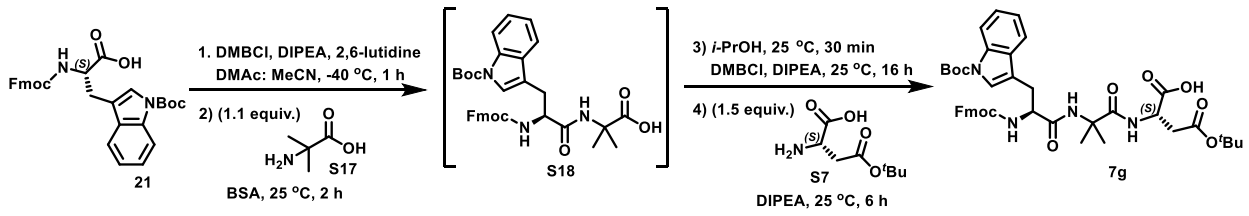


Compound ID	Time	Area	Height	Area% ratio
S16	3.141	104.4301	55.2	2.5
7f	3.343	4081.3945	1812.4	96.5
***epi*-7f**	3.357	0.0	0.0	0.0
7f-over-addition	3.600	0.0	0.0	0.0
S16-INT + *epi*-S16-INT	4.047	42.5424	22	1.0*
Total	-	-	-	100.0

*Note: Contains 0.49% ***epi*-S16-INT**

HPLC of isolated **7f**



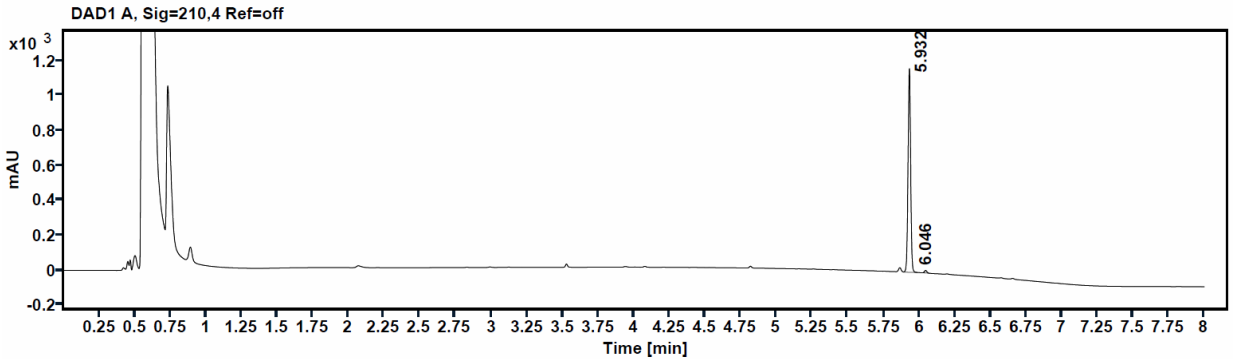


Synthesis of Fmoc-Trp(Boc)-Aib-L-Asp(O*t*Bu)-OH (7g) (Method B). In a 100 mL 3-neck round bottom flask, **Fmoc-Trp(Boc)-OH (21)** (5.00 g, 9.50 mmol) and 2,6-lutidine (1.02 g, 9.50 mmol) were dissolved

in DMAc/MeCN (1:1, 25 mL). Then the solution was cooled to -40 °C, DMBCl (1.34 g, 9.97 mmol) and DIPEA (1.23 g, 9.50 mmol) were added to the reaction mixture. The reaction mixture was stirred at -40 °C for 1 h and monitored by HPLC **(Method 4)**, which showed the ratio of **21**:**21-INT** was 0.9:99.1 (quenched by $BnNH_2$). The reaction solution was added slowly into a solution of **H-Aib-OH (S17)** and BSA in DMAc and MeCN at 25 °C, which was prepared by stirring **S17** (1.08 g, 10.44 mmol) and BSA (3.86 g, 18.99 mmol) in DMAc/MeCN (1:1, 25 mL) at 50 °C for 16 h until clear, then cooling to 25 °C. The reaction mixture was stirred at 25 °C for 2 h and monitored by HPLC (**Method 4**), which showed the ratio of **21:S18:S18-over-addition** was: 1.1:98.9:0.0. Then *i*-PrOH (2.28 g, 37.98 mmol) was added into the reaction mixture at 25 °C and stirred for 30 min. DMBCl (2.68 g, 19.94 mmol) and DIPEA (2.70 g, 20.89 mmol) were added into the reaction mixture. The reaction mixture was stirred at 25 °C for 16 h and monitored by HPLC **(Method 4)**, which showed the ratio of **S18:S18-INT** (mixed anhydride quenched by $BnNH_2$) was 2.2:97.8. **L-Asp(O*t*Bu)-OH (S7)** (2.69 g, 14.24 mmol) and DIPEA (2.45 g, 18.99 mmol) was added into the reaction mixture at 25 °C. The reaction mixture was stirred at 25 °C for 6 h and monitored by HPLC (**Method 4**), which showed the ratio of **S18:7g:7g-over-addition** was 3.8:95.5:0.7. The assay yield of the reaction was 91% over two steps. The reaction solution was added slowly into H_2O (250 mL, containing 3 equiv. HCl) at 5 °C for 2 h and stirred at 5 °C for 1 h, the solid was collected by filtration and dried under N_2 for overnight. The crude product was slurried with MTBE:*n*-heptane (1:20, 100 mL) at 0 °C for 1 h. The solid was collected by filtration and dried under N_2 for overnight to provide 7.10 g of **7g** as a white solid (93.6% LCAP, **Method 4**, 90.0 wt%) in 86% isolated yield

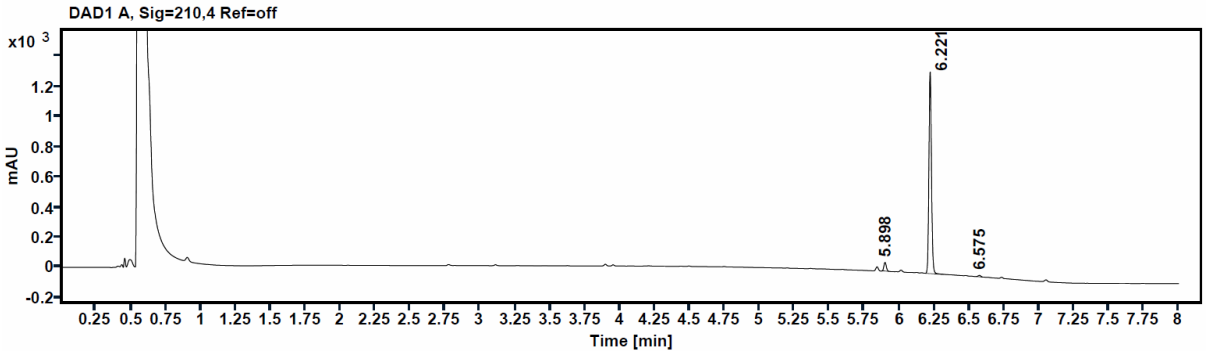
mp 115-118 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.87 (s, 1H), 8.41 (s, 1H), 8.04 (d, J = 8.2 Hz, 1H), 7.87 (d, J = 7.6 Hz, 2H), 7.81 (d, J = 7.5 Hz, 1H), 7.77 (d, J = 7.8 Hz, 1H), 7.66 (d, J = 7.5 Hz, 2H), 7.59 (s, 1H), 7.53 (d, J = 8.2 Hz, 1H), 7.40 (td, J = 7.6, 7.6, 2.7 Hz, 2H), 7.33 (t, J = 7.6, 7.6 Hz, 1H), 7.29 - 7.22 (m, 3H), 4.53 (q, J = 7.0, Hz, 1H), 4.37 - 4.31 (m, 1H), 4.26-4.13 (m, 3H), 3.11 (dd, J = 14.8, 5.0 Hz, 1H), 2.99 (dd, J = 14.8, 9.6 Hz, 1H), 2.62 (dd, J = 16.2, 7.2 Hz, 1H), 2.47 (d, J = 5.5 Hz), 1.58 (s, 9H), 1.35 (s, 6H), 1.31 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.39, 172.21, 171.05, 169.31, 155.99, 148.93, 143.70, 143.47, 140.57, 134.55, 130.17, 127.53, 126.92, 126.89, 125.25, 125.22, 124.22, 124.03, 122.30, 119.99, 119.50, 116.50, 114.51, 83.32, 79.96, 65.69, 55.87, 54.55, 48.72, 46.48, 37.03, 30.87, 27.54, 26.71, 25.29, 24.10, 21.97, 13.87. HRMS (ESI): m/z calcd for $C_{43}H_{51}N_4O_{10}$ $[M+H]^+$: 783.3600, found: 783.3635. FTIR (neat): 3317, 2978, 1731, 1659, 1520, 1451, 1367, 1255, 1152, 1086, 1032, 1017, 853, 758, 739, 539 cm^{-1}

HPLC of **S18** formation (reaction mixture)



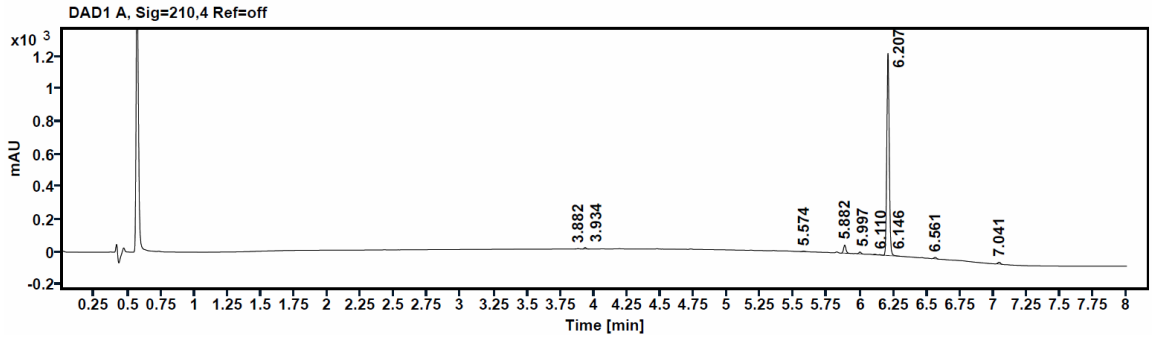
Compound ID	Time	Area	Height	Area% ratio
S18-over-addition	5.812	0.0	0.0	0.0
S18	5.932	1471.5404	1177.1	98.9
21	6.046	16.5697	14.6	1.1
Total	-	-	-	100.0

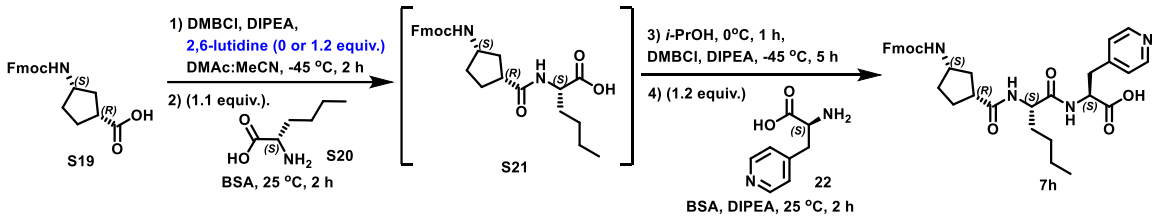
HPLC of **7g** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S18	5.898	67.6981	57.6	3.8
7g	6.221	1713.0706	1351.5	95.5
7g-over-addition	6.575	12.9367	9.6	0.7
Total	-	-	-	100.0

HPLC of isolated **7g**





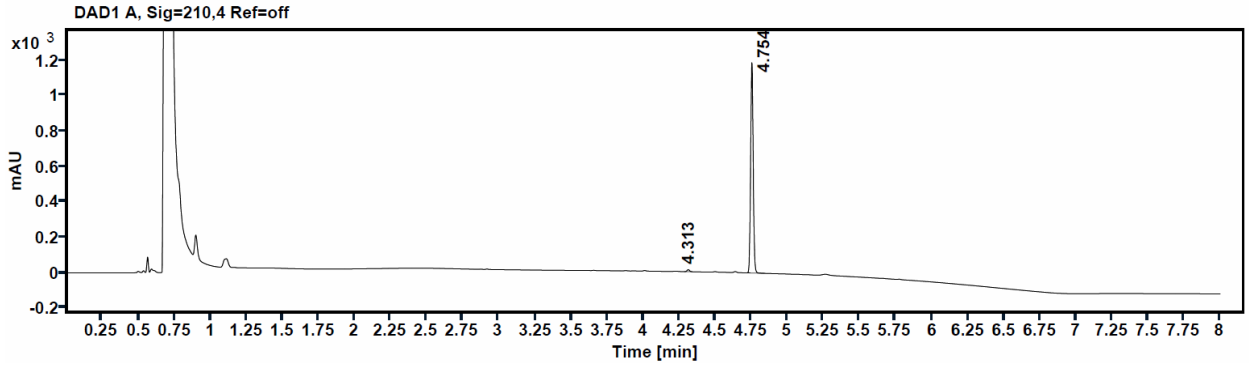
Synthesis of Fmoc-bhcLeu-L-Nle-L-Ala(4-PYRI)-OH (7h) (Method B).

With 2,6-lutidine: In a 50 L 3-neck round bottom flask, **Fmoc-bhcLeu-OH (S19)** (2000.0 g, 5.69 mol) and 2,6-lutidine (609.8 g, 5.69 mol) were dissolved in DMAc/MeCN (1:2, 20 L). The solution was cooled to -45 °C, then DMBCl (804.4 g, 5.98 mol) and DIPEA (735.6 g, 5.69 mol) were added to the reaction mixture. The reaction mixture was stirred at -45 °C for 2 h and monitored by HPLC (**Method 4**), which showed the ratio of **S19:S19-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.9:99.1. The reaction mixture was added slowly into a solution of **L-Nle-OH (S20)** and BSA in DMAc and MeCN at 25 °C over 1 h, which was prepared by mixing **S20** (821.3 g, 6.26 mol) and BSA (1736.7 g, 8.54 mol) in DMAc/MeCN (1:2, 10.0 L), stirred at 50 °C for 1 h until clear, then cooling to 25 °C. The reaction mixture was stirred at 25 °C for 2 h and monitored by HPLC (**Method 4**), which showed the ratio of **S19:S21:S21-over-addition** was: 0.9:99.1:0.0. Then *i*-PrOH (1024.5 g, 17.07 mol) was added to the reaction mixture and stirred at 0°C for 1 h. The reaction mixture was cooled to -45 °C, DMBCl (991.5 g, 7.40 mol) and DIPEA (809 g, 6.26 mol) were added to the reaction mixture and stirred for 5 h at -45 °C and then monitored by HPLC (**Method 4**). HPLC showed the ratio of **S21:S21-INT:*epi*-S21-INT (**mixed anhydride quenched by $BnNH_2$) was 1.2:98.8:0.0. The reaction solution was added slowly into a solution of **L-Ala(4-PYRI)-OH (22)**, BSA and DIPEA in DMAc at 25 °C over 1 h, which was prepared by mixing of **22** (1135.0 g, 6.83 mol), BSA (2662.6 g, 13.09 mol) in DMAc (10.0 L), stirred at 50 °C for 1 h until clear, cooling to 25 °C, and then adding DIPEA (147.11 g, 1.14 mol). The reaction mixture was stirred at 25 °C for 2 h and monitored by HPLC (**Method 4**), which showed the ratio of **S21:7h:*epi*-7h:*epi*-S21-INT:7h-over-addition** was 2.6:96.1:0.8:0.1:0.4. The assay yield of the reaction was 92% over two steps. The reaction

mixture was cooled to 0 °C. A mixture solution of IPAc (50.0 L) and MeOH (729.4 g, 22.77 mol) were added slowly into the reaction mixture over 3 h, and the reaction mixture was stirred at 0 °C for 3 h. The solid was collected by filtration and slurried with MeOH (40.0 L) at 0 °C for 3 h. The solid was collected by filtration and dried under vacuum to provide 3103.8 g of **7h** as a white solid (97.6% LCAP, **Method 4**, 95.5 wt%) in 85% isolated yield.

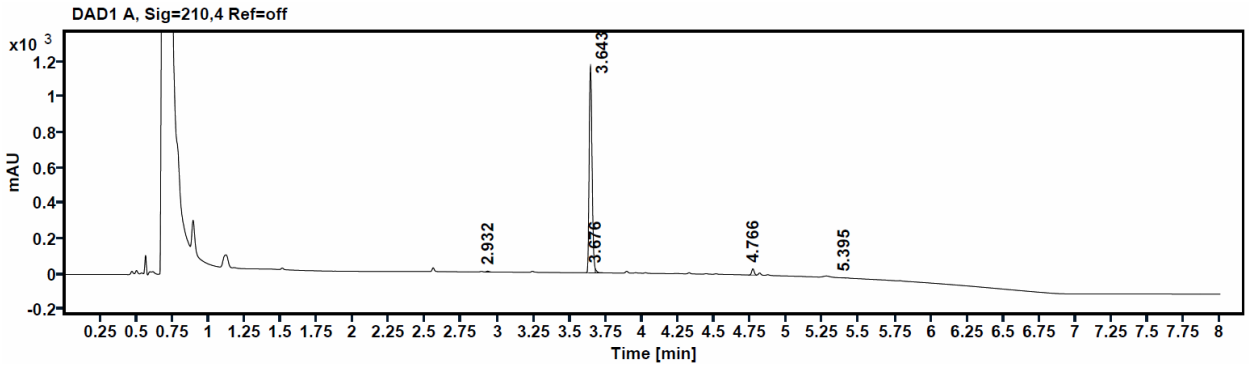
mp 215-218 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ(ppm) 12.86 (s, 1H), 8.46-8.38 (m, 2H), 8.19 (d, J = 8.0 Hz, 1H), 7.89 (d, J = 7.2 Hz, 3H), 7.69 (d, J = 7.2 Hz, 2H), 7.45 - 7.38 (m, 3H), 7.32 (t, J = 7.6 Hz, 2H), 7.27 - 7.19 (m, 2H) , 4.49 (td, J = 8.8, 4.8 Hz, 1H), 4.27 - 4.18 (m, 4H), 3.80 (h, J = 7.6 Hz, 1H), 3.09 (dd, J = 14.4, 5.2 Hz, 1H), 2.96 - 2.90 (m, 1H), 2.71 (p, J = 8.0 Hz, 1H), 2.04 - 1.98 (m, 1H), 1.80 - 1.67 (m, 3H), 1.62 - 1.41 (m, 4H), 1.27 - 1.18 (m, 4H), 0.83 (t, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6): δ(ppm) 175.39, 172.91, 172.33, 155.96, 149.71, 146.98, 144.39, 141.18, 128.07, 127.51, 125.67, 125.08, 120.58, 65.73, 52.78, 52.68, 47.20, 42.34, 36.88, 36.22, 32.46, 32.22, 27.85, 27.41, 22.33, 14.35. HRMS (ESI): m/z calcd for $C_{35}H_{41}N_4O_6$ $[M+H]^+$: 613.3021, found: 613.3060. FTIR (neat): 3302, 2956, 2869, 1684, 1638, 1535, 1294, 1250, 1221, 1028, 735, 664 cm^{-1}

HPLC of **S21** formation (reaction mixture)



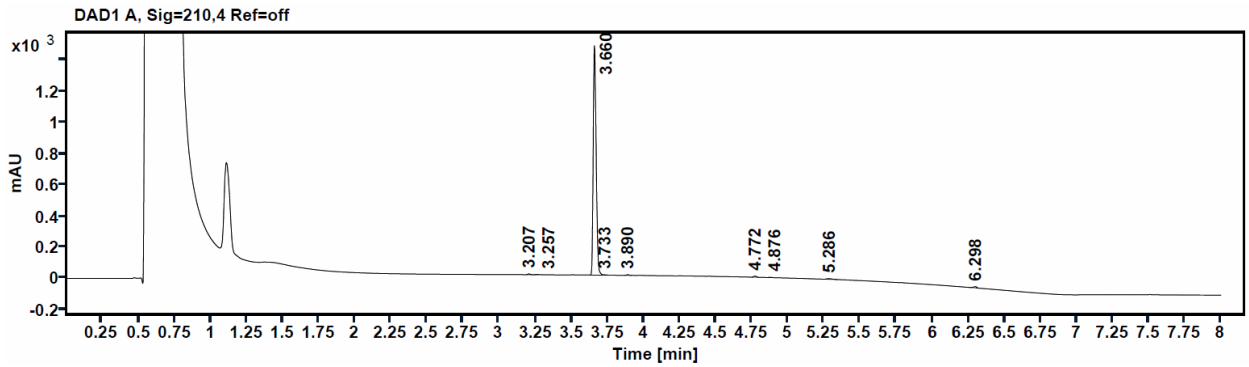
Compound ID	Time	Area	Height	Area% ratio
S19	4.313	13.8981	11.5	0.9
S21	4.754	1468.34	1197.5	99.1
S21-over-addition	5.105	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of **7h** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
7h-over-addition	2.932	6.1949	5.0	0.4
7h	3.643	1603.5524	1184.6	96.1
***epi*-7h**	3.676	13.8062	20.8	0.8
S21	4.766	43.4400	35.7	2.6
***epi* -S21-INT**	5.395	1.8789	1.5	0.1
Total	-	-	-	100.0

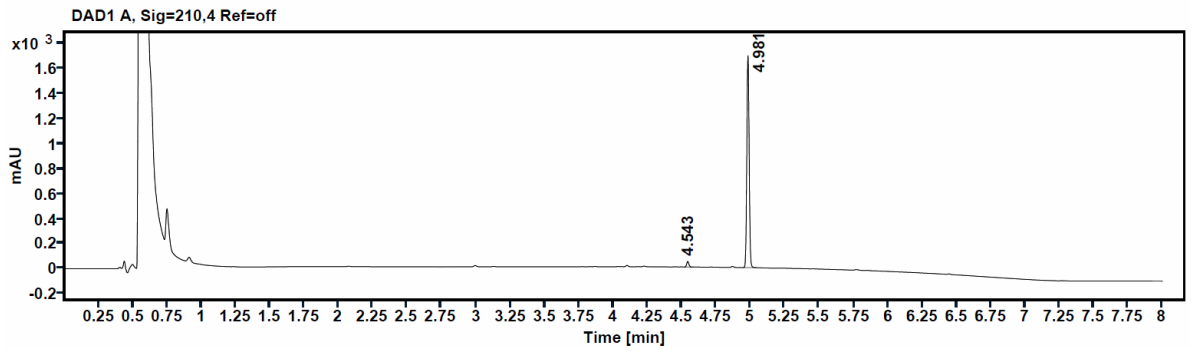
HPLC of isolated **7h**



No 2,6-lutidine: In a 50 mL 3-neck round bottom flask, Fmoc-bhcLeu-OH (**S19**) (1.00 g, 2.85 mmol) was dissolved in DMAc/MeCN (1:1, 5 mL). The solution was cooled to -45 °C, then DMBCl (0.42 g, 3.14 mmol) and DIPEA (0.44 g, 3.42 mmol) were added to the reaction mixture. The reaction mixture was stirred at -45 °C for 2 h and monitored by HPLC (**Method 4**), which showed the ratio of **S19:S19-INT** was 2.6:97.4. The reaction mixture was added slowly into a solution of **L-Nle-OH (S20)** and BSA in DMAc and MeCN at 25 °C over 1 h, which was prepared by mixing **S20** (0.41 g, 3.14 mmol) and BSA (0.87 g, 4.27 mmol) in DMAc/MeCN (1:1, 5 mL), stirred at 50 °C for 1 h until clear, then cooling to 25 °C. The reaction mixture was stirred at 25 °C for 2 h and monitored by HPLC (**Method 4**), which showed the ratio

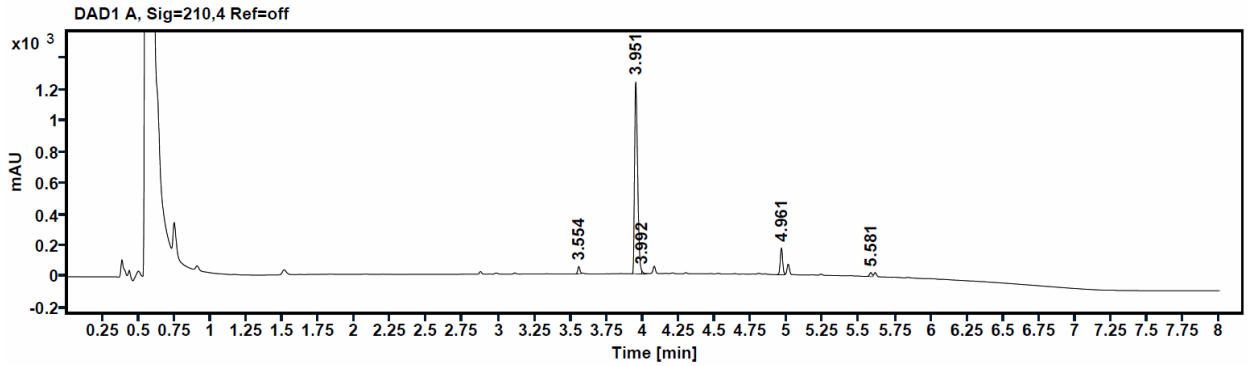
of **S19:S21:S21-over-addition** was: 2.5:97.5:0.0. Then *i*-PrOH (0.51 g, 8.55 mmol) was added to the reaction mixture at 0 °C and stirred for 1 h. The mixture was cooled to -45 °C. DMBCl (0.65 g, 4.85 mmol) and DIPEA (0.55 g, 4.28 mmol) were added to the reaction mixture, then stirred for 5 h at -45 °C and monitored by HPLC (**Method 4**). HPLC showed the ratio of **S21:S21-INT:*epi*-S21-INT** was 5.4:92.9:1.7. The reaction solution was added slowly into a solution of **L-Ala(4-PYRI)-OH (22)**, BSA and DIPEA in DMAc at 25 °C over 1 h, which was prepared by mixing of **22** (0.66 g, 3.99 mmol), BSA (1.39 g, 6.84 mmol) in DMAc (5 mL), stirred at 50 °C for 1 h until clear, cooling to 25 °C, and then adding DIPEA (0.18 g, 1.43 mmol) before use. The reaction mixture was stirred at 25 °C for 2 h and monitored by HPLC (**Method 4**), which showed the ratio of **S21:7h:*epi*-7h:*epi*-S21-INT:7h-over-addition** was: 9.3:85.9:0.7:1.7:2.4. The assay yield of the reaction was 82% over two steps.

HPLC of **S21** formation (reaction mixture)

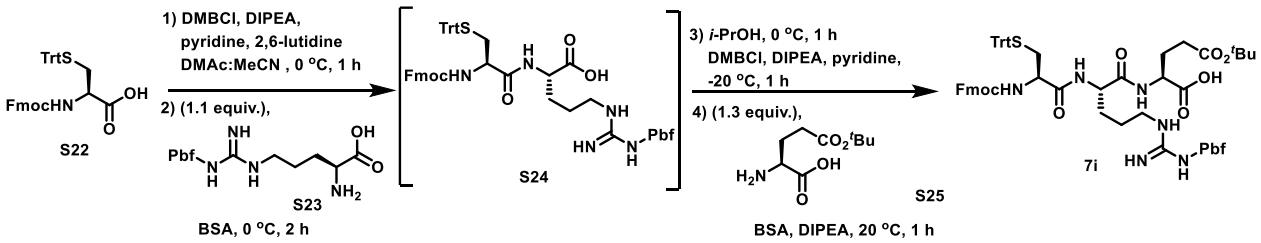


Compound ID	Time	Area	Height	Area% ratio
S19	4.543	50.2801	45.4	2.5
S21	4.981	1987.8292	1710.2	97.5
S21-over-addition	5.349	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of **7h** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
7h-over-addition	3.555	51.6130	48.4	2.4
7h	3.951	1823.8807	1239.7	85.9
***epi*-7h**	3.992	15.0580	22.5	0.7
S21	4.961	197.9647	173.4	9.3
***epi* -S21-INT**	5.581	35.4610	26.5	1.7
Total	-	-	-	100.0



Synthesis of Fmoc-L-Cys(Trt)-L-Arg(Pbf)-L-Glu(5-CO_2*t*Bu)-OH (**7i**) (**Method A**). In a 100 mL 3-neck round bottom flask, **Fmoc-L-Cys(Trt)-OH (S22)** (10.00 g, 17.07 mmol), pyridine (0.27 g, 3.41 mmol) and 2,6-lutidine (1.83 g, 17.07 mmol) were dissolved in DMAc/MeCN (1:1, 50 mL). The solution was cooled to 0 °C, DMBCl (2.41 g, 17.93 mmol) and DIPEA (2.21 g, 17.07 mmol) were added into the reaction mixture. The reaction mixture was stirred at 0 °C for 1 h and monitored by HPLC (**Method 6**) and SFC-MS (Chiral **Method 1**), which showed the ratio of **S22:S22-INT:*epi*-S22-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.6:99.4:0.0. A solution of L-Arg(Pbf)-OH **(S23)** and BSA in DMAc and MeCN was prepared by mixing **S23** (8.01 g, 18.78 mmol) and BSA (6.95 g, 34.15 mmol) in DMAc/MeCN (1:1, 300 mL), stirring at 25 °C for 1 h. This solution was added slowly to the reaction mixture at 0 °C over 1 h. The reaction mixture was stirred at 0 °C for 2 h and monitored by HPLC (**Method 6**) and SFC-MS (Chiral **Method 2**), which showed the ratio of **S22:(S24+*epi*-S24):S24-over-addition** was 0.9:99.1:0.0. Then *i*-PrOH (4.10 g, 68.29 mmol) was added into the reaction mixture at 0 °C and stirred for 1 h, then cooling to -20 ºC, DMBCl (2.99 g, 22.19 mmol), pyridine (0.27 g, 3.41 mmol) and DIPEA (2.21 g, 17.07 mmol) were added into the reaction mixture and stirred at -20 °C for 1 h, monitored by HPLC (**Method 9**). HPLC showed the ratio of **S24:S24-INT:*epi*-S24-INT** (mixed anhydride quenched by $BnNH_2$) was 0.8:99.1:0.1. The reaction mixture was added dropwise into a solution of **L-Glu(5-CO_2*t*Bu)-OH (S25)**, BSA and DIPEA in DMAc and MeCN over 1 h at 20 °C. The solution was prepared by mixing **S25** (4.51 g, 22.20 mmol) and BSA (10.42 g, 51.22 mmol) in DMAc/MeCN (1:1, 50 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C, then adding DIPEA (0.44 g, 3.41 mmol) before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 9**), which showed the ratio of **S24:7i:*epi*-7i:*epi*-**

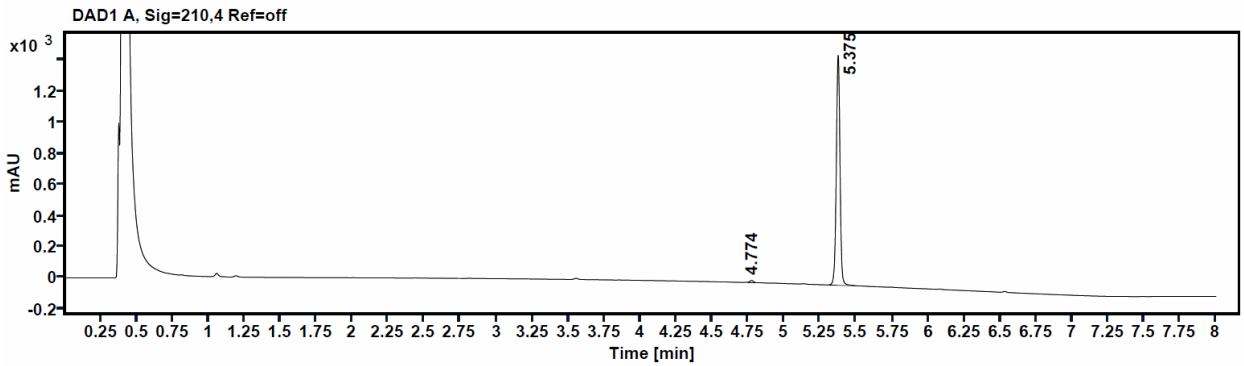
S24-INT:7i-over-addition was 1.8:97.6:0.1:0.2:0.3. The assay yield of the reaction was 92% over two steps. The reaction solution was slowly added dropwise into 4% KH_2PO_4 solution (200 mL) at 20 °C over 1 h. Then extracted with ethyl acetate (EtOAc) (100 mL). Upon phase separation, the organic phase was washed with 5% NaCl solution (50 mL), dried with anhydrous Na_2SO_4, filtered, concentrated, and slurried with MeCN (200 mL) at 50 °C for 16 h. The solid was collected by filtration and dried under N_2 for overnight to provide 17.8 g of **7i** as a white solid (95.8% LCAP, 95.0 wt%) in 84% isolated yield (**Method 9**).

mp 125-130°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.66 (br s, 1H), 8.11 (d, J = 7.6 Hz, 1H), 7.88 (d, J = 7.6 Hz, 2H), 7.75 - 7.04 (m, 3H), 7.65 (d, J = 8.4 Hz, 1H), 7.39 (t, J = 7.6 Hz, 2H), 7.33 - 7.28 (m, 12H), 7.26 - 7.20 (m, 5H), 6.64 - 6.39 (m, 3H), 4.34 - 4.13 (m, 5H), 4.00 - 3.96 (m, 1H), 3.01 - 2.96 (m, 2H), 2.92 (s, 2H), 2.46 (s, 3H), 2.44 - 2.36 (m, 4H), 2.20 (t, J = 7.6 Hz, 2H), 1.99 - 1.98 (m, 5H), 1.94 - 1.89 (m, 1H), 1.77 - 1.68 (m, 1H), 1.65 - 1.59 (m, 1H), 1.50 - 1.44 (m, 1H), 1.38 (s, 6H), 1.34 (s, 10H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 172.92, 171.42, 170.96, 170.33, 169.51, 157.44, 156.06, 155.61, 144.27, 143.79, 143.66, 140.69, 137.29, 134.20, 131.44, 129.10, 128.03, 127.63, 127.06, 126.74, 125.30, 124.30, 120.08, 116.26, 86.25, 79.73, 65.95, 65.85, 59.75, 53.84, 51.84, 51.13, 46.58, 42.46, 40.19, 33.83, 31.18, 29.64, 28.28, 27.69, 26.36, 24.89, 20.75, 18.95, 17.60, 14.08, 12.27. HRMS (ESI): m/z calcd. for $C_{65}H_{74}N_6O_{11}S_2$ $[M+H]^+$: 1179.4930, found: 1179.4884. FTIR (neat): 3330, 2969, 2926, 1720, 1702, 1653, 1543, 1525, 1510, 1446, 1366, 1243, 1152, 1090, 1034, 848, 740, 701 cm^{-1}.

Chiral Method 1: CHIRALPAK IC-U 3.0 × 50 mm, 1.6μm; co-solvent: 20 mM NH_3 in *i*-PrOH; gradient: 50-50% (co-solvent) in 3 min, flow rate: 1.5 mL/min; detection wavelength: 254 nm.

Chiral Method 2: CHIRALPAK IC-U 4.6 × 50 mm, 3.0μm; co-solvent: 20 mM NH_3 in MeOH; gradient: 45-45% (co-solvent) in 5.5 min, flow rate: 3.0 mL/min; detection wavelength: 254 nm.

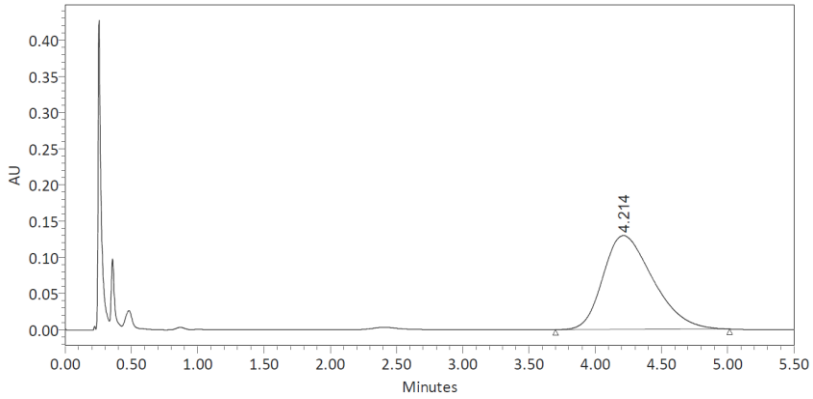
HPLC of **S24** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S22	4.774	25.3535	14.7	0.9
S24+*epi*-S24	5.375	2648.1509	1489.2	99.1*
S24-over-addition	5.972	0.0	0.0	0.0
Total	-	-	-	100.0

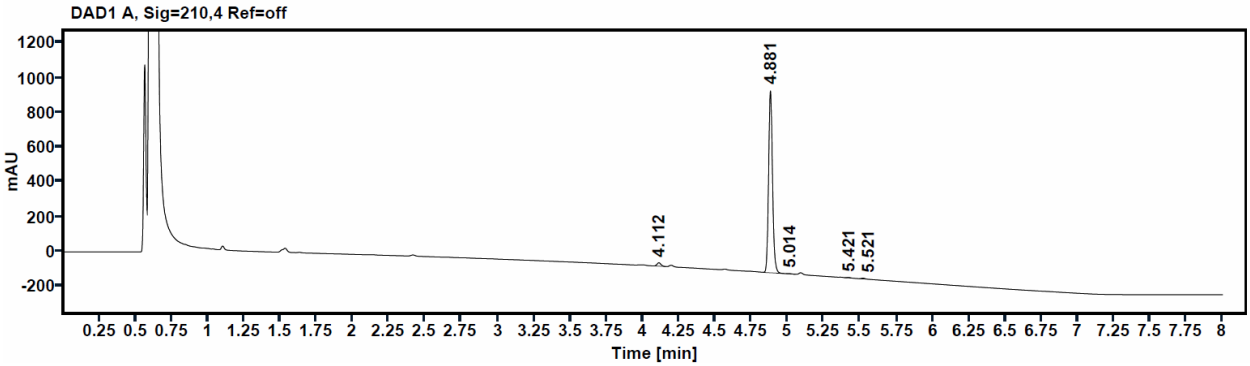
*Note: Contains 0.0% ***epi*-S24**

SFC-MS (Chiral) of **S24** chiral purity



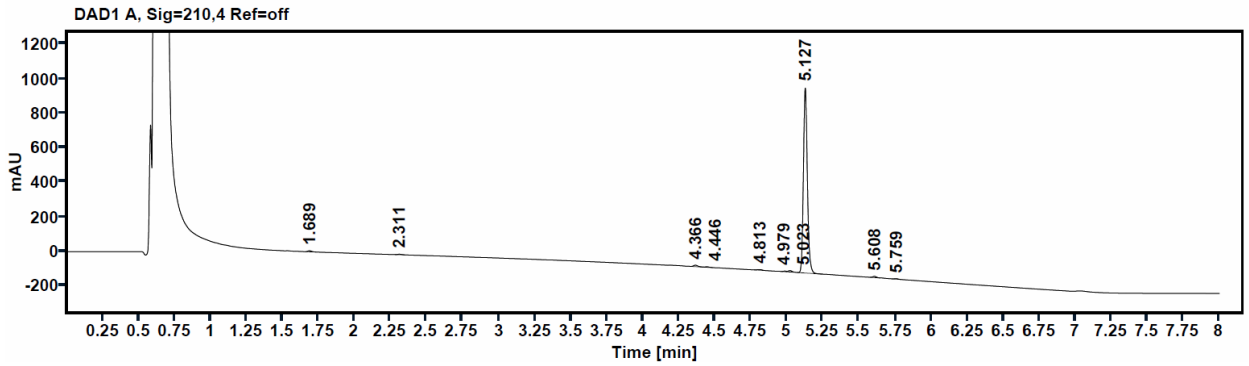
Compound ID	Time	Area	Height	Area% ratio
***epi*-S24**	3.357	0.0	0.0	0.0
S24	4.214	3388721	129690	100.0
Total	-	-	-	100.0

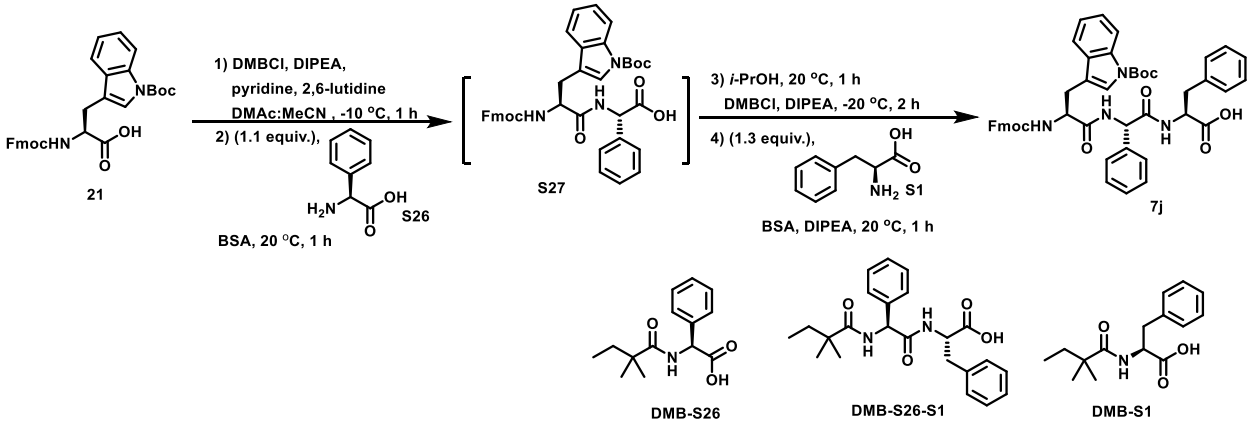
HPLC of **7i** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S24	4.112	36.1512	19.6	1.8
7i	4.881	1967.9197	1048.1	97.6
***epi*-7i**	5.014	2.5315	1.5	0.1
***epi* -S24-INT**	5.421	4.2093	2.6	0.2
7i-over-addition	5.521	6.3454	4.2	0.3
Total	-	-	-	100.0

HPLC of isolated **7i**



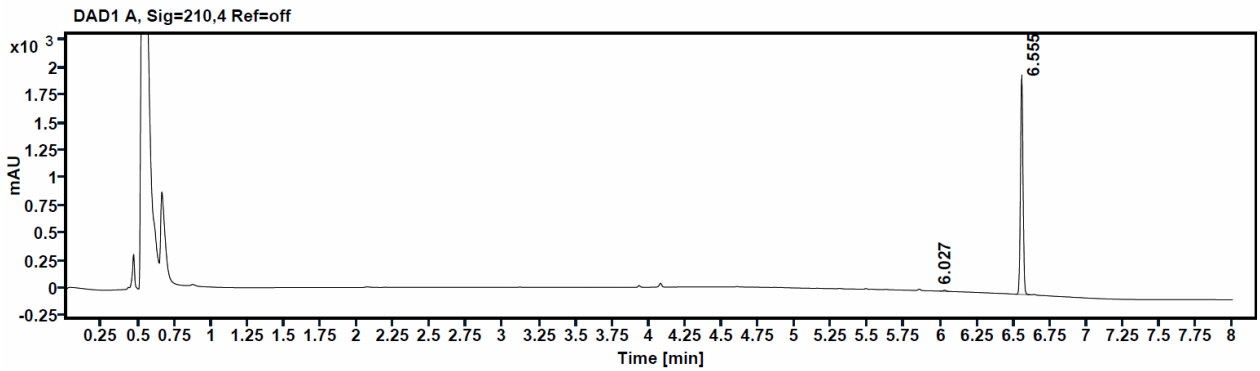


Synthesis of Fmoc-L-Trp(Boc)-L-Phg-L-Phe-OH (**7j**) (**Method A**). In a 100 mL 3-neck round bottom flask, **Fmoc-L-Trp(Boc)-OH (21)** (10.00 g, 18.99 mmol), pyridine (0.30 g, 3.80 mmol) and 2,6-lutidine (2.03 g, 18.99 mmol) were dissolved in DMAc/MeCN (1:1, 50 mL). The solution was cooled to -10 °C, DMBCl (2.68 g, 19.94 mmol) and DIPEA (2.45 g, 18.99 mmol) were added into the reaction mixture. The reaction mixture was stirred at -10 °C for 1 h and monitored by HPLC (**Method 6**), which showed the ratio of **21:21-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.6:99.4. The reaction mixture was

added slowly over 1 h to a DMAc and MeCN solution of **L-Phg-OH (S26)** and BSA at 20 °C. The solution was prepared by mixing **S26** (3.16 g, 20.89 mmol) and BSA (11.59 g, 56.97 mmol) in DMAc/MeCN (1:1, 50 mL), stirring at 50 °C for 1 h, and then cooling to 20 °C before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **21:S27:*epi*-S27:S27-over-addition:DMB-S26** was 0.6:98.1:0.0:0.0:1.3. Then *i*-PrOH (6.85 g, 113.94 mmol) was added to the reaction mixture. The reaction mixture was stirred at 20 °C for 1 h. The reaction was cooled to -20 ºC, DMBCl (3.32 g, 24.69 mmol), pyridine (0.30 g, 3.80 mmol) and DIPEA (2.45 g, 18.99 mmol) were added to the reaction mixture. The reaction mixture was stirred at -20 °C for 2 h, then monitored by HPLC (**Method 10**). HPLC showed the ratio of **S27:S27-INT:*epi*-S27-INT:DMB-S26-INT** (mixed anhydride quenched by $BnNH_2$) was 0.7:97.1:0.1:2.1. The reaction mixture was added slowly over 1 h to a DMAc and MeCN solution of **L-Phe-OH (S1),** BSA and DIPEA at 20 °C. The solution was prepared by mixing **S1** (4.08 g, 24.69 mmol) and BSA (11.59 g, 56.97 mmol) in DMAc/MeCN (1:1, 50 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C, then adding DIPEA (0.49 g, 3.80 mmol) before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 6**), which showed the ratio of **S27:7j:*epi*-7j:*epi*-S27-INT:7j-over-addition:DMB-S26-S1:DMB-S1** was 2.0:92.6:0.3:0.0:0.1:2.8:2.2. The assay yield of the reaction was 94% over two steps. The reaction solution was added dropwise into 4% KH_2PO_4 solution (200 mL) at 20 ºC over 1 h. Then extracted with EtOAc (100 mL). Upon phase separation, the organic phase was washed with 5% NaCl solution (50 mL), dried with anhydrous Na_2SO_4, filtered and concentrated, then slurried with MeCN (200 mL) at 40 ºC for 16 h. The solid was collected by filtration and dried under N_2 for overnight to provide 13.4 g of **7j** as a white solid (95.5% LCAP, 94.8 wt%) in 83% isolated yield (**Method 6**).

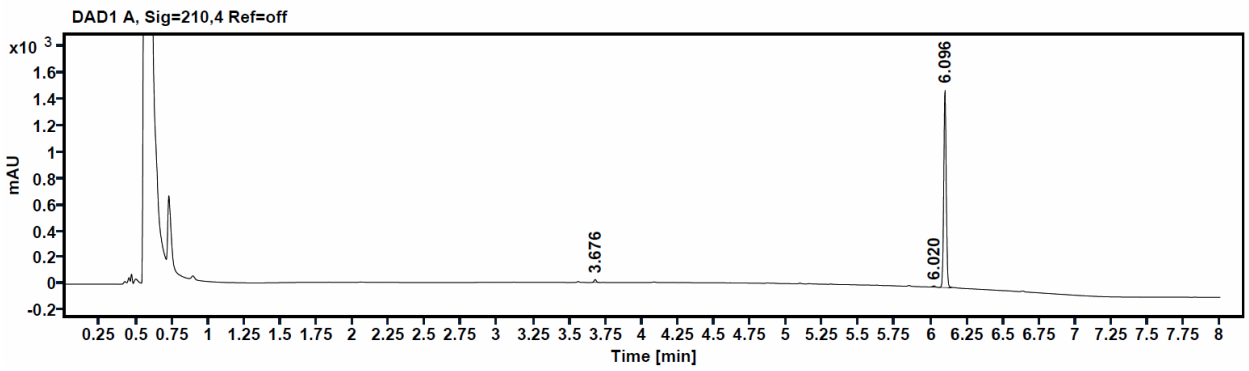
mp 236-237 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.80 (br s, 1H), 8.63 (dd, J = 17.2, 8.0 Hz, 2H), 8.02 (d, J = 8.4 Hz, 1H), 7.85 (d, J = 7.6 Hz, 2H), 7.79 (dd, J = 16.0, 8.0 Hz, 2H), 7.61 (s, 1H), 7.57 (t, J = 6.4 Hz, 2H), 7.46 - 7.28 (m, 8H), 7.25 - 7.14 (m, 8H), 5.68 - 5.66 (m, 1H), 4.56 - 4.51 (m, 1H), 4.48 - 4.43 (m, 1H), 4.17 - 4.09 (m, 2H), 3.07 (td, J = 14.0, 5.6 Hz, 2H), 2.99 - 2.91 (m, 2H), 1.99 (s, 1H), 1.56 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 172.42, 171.09, 169.39, 155.89, 149.06, 143.70, 143.65, 140.65, 140.62, 138.35, 137.35, 134.65, 130.33, 129.19, 128.13, 127.58, 127.47, 127.20, 127.01, 126.98, 126.35, 125.27, 125.18, 124.25, 124.15, 122.38, 120.08, 120.06, 119.71, 116.91, 114.61, 83.43, 65.78, 55.91, 54.35, 53.65, 46.52, 36.74, 27.65, 27.41. HRMS (ESI): m/z calcd. for $C_{48}H_{46}N_4O_8$ $[M+H]^+$: 807.3388, found: 807.3371. FTIR (neat): 3276, 3056, 3029, 1687, 1638, 1525, 1450, 1366, 1252, 1221, 1154, 1085, 1029, 732, 695 cm^{-1}.

HPLC of **21-INT** formation (reaction mixture)



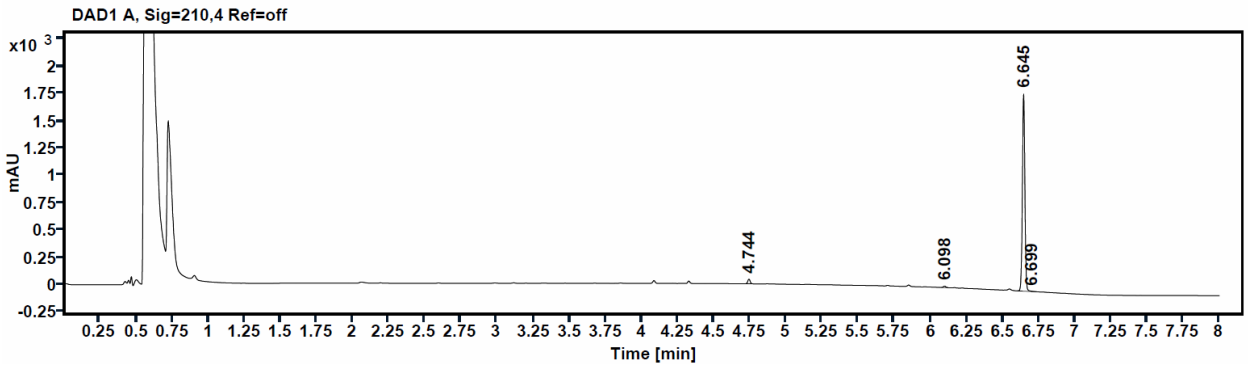
Compound ID	Time	Area	Height	Area% ratio
21	6.027	13.4991	9.9	0.6
21-INT	6.555	2431.2986	2000.8	99.4
Total	-	-	-	100.0

HPLC of **S27** formation (reaction mixture)



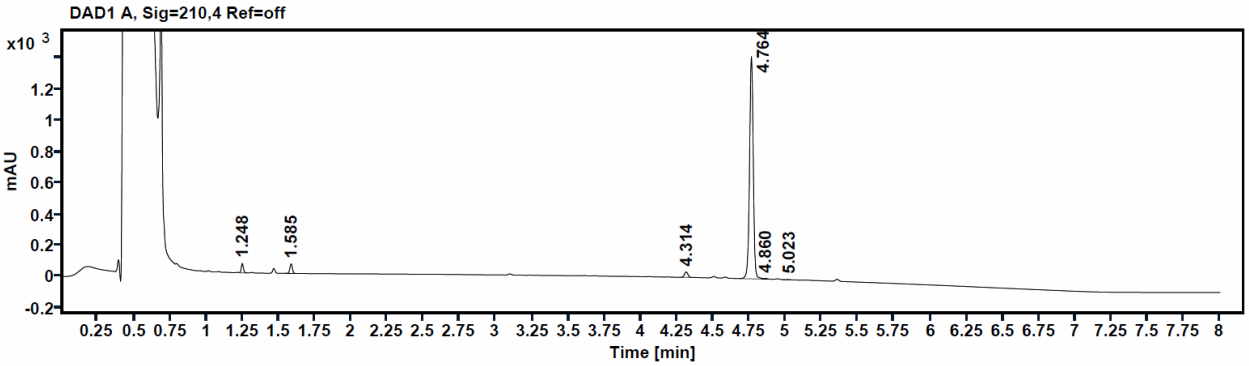
Compound ID	Time	Area	Height	Area% ratio
DMB-S26	3.676	23.2970	22.6	1.3
21	6.02	11.1415	9.4	0.6
S27	6.096	1811.7123	1508.5	98.1
***epi*-S27**	6.134	0.0	0.0	0.0
S27-over-addition	6.201	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of **S27-INT** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
DMB-S26-INT	4.744	47.5033	43.0	2.1
S27	6.098	14.9828	13.4	0.7
S27-INT	6.645	2170.4792	1814.6	97.1
***epi*-S27-INT**	6.699	2.9400	3.0	0.1
Total	-	-	-	100.0

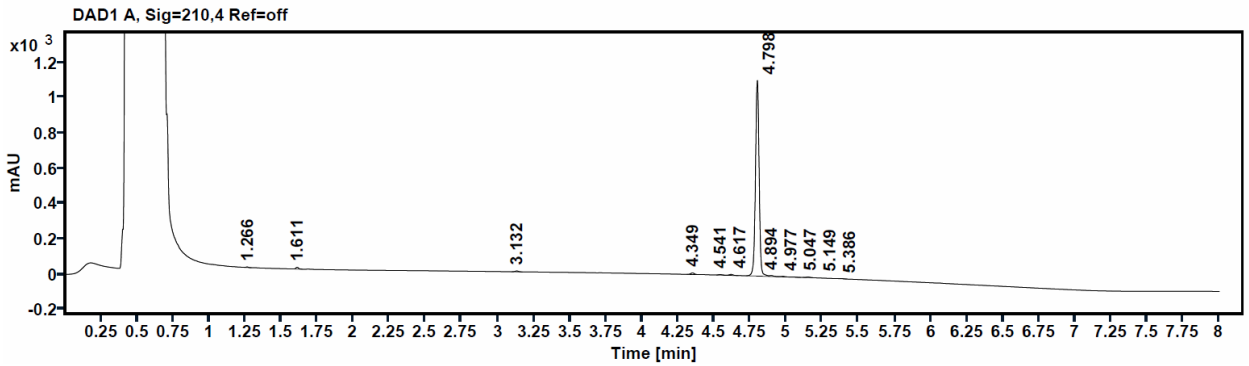
HPLC of **7j** formation (reaction mixture)



Compound ID	Time	Area	Height	Area%
DMB-S1	1.248	59.2133	59.6	2.2
DMB-S26-S1	1.585	75.7035	62.0	2.8
S27	4.314	56.0467	34.6	2.0

7j	4.764	2536.0354	1434.8	92.6
epi-7j	4.860	7.9868	4.6	0.3
7j-over-addition	5.023	3.7174	1.4	0.1
***epi*-S27-INT**	5.251	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of isolated **7j**

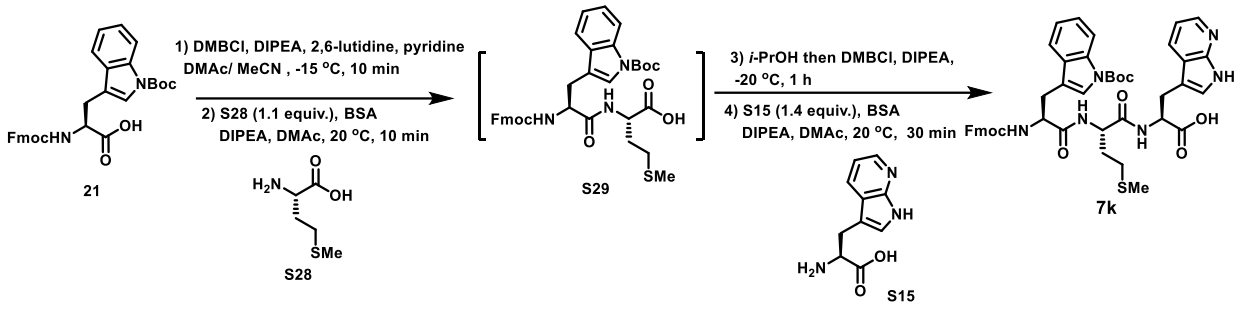


Compound ID	Time	Area	Height	Area%
DMB-S1	1.266	3.7625	3.4	0.2
DMB-S26-S1	1.611	12.7125	10.6	0.6
S27	4.349	16.0654	9.0	0.8
7j	4.798	1954.7271	1113.7	95.5
others	-	-	-	2.9
Total	-	-	-	100.0

Fate and rejection of DMB related byproducts.

	S26	**DMB-S26**	**DMB-S26-INT**	**S1**	**DMB-S1**	**DMB-S26-S1**
Stage 2	N/A	1.3%	N/A	N/A	N/A	N/A
Stage 3	N/A	N/A	2.1%	N/A	N/A	N/A
Stage 4	N/A	N/A	N/A	N/A	2.2%	2.8%
Isolated solid	N/A	N/A	N/A	N/A	0.2%	0.6%

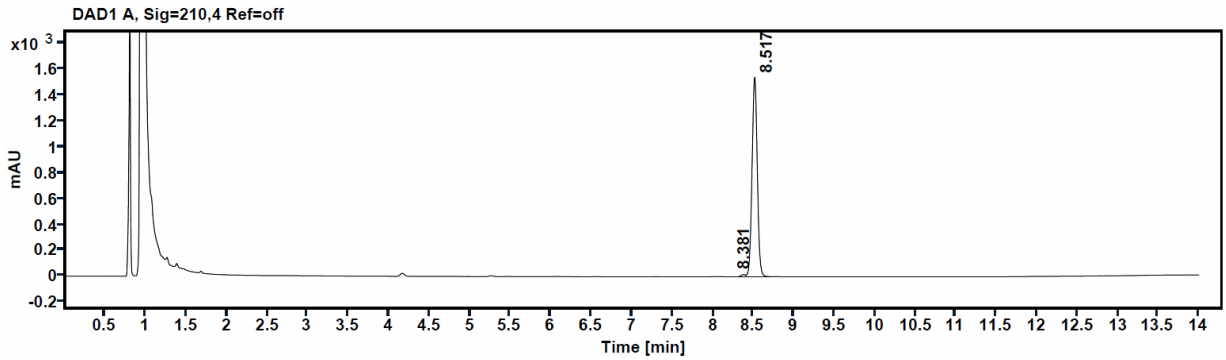
The data show that excess AA_1 from the 1st coupling was capped by DMBCl in the 2nd coupling to form DMB-AA1, which indeed continued to react with AA_2 to form DMB-AA_1-AA_2, an n−1 deletion byproduct. Excess DMBCl was then quenched by AA_2 to form DMB-AA_2, itself an n−2 deletion byproduct. These byproducts were well rejected during crystallization of the final product.



Synthesis of Fmoc-L-Trp(Boc)-L-Met-L-7-aza-Trp-OH (7k) (Method A).

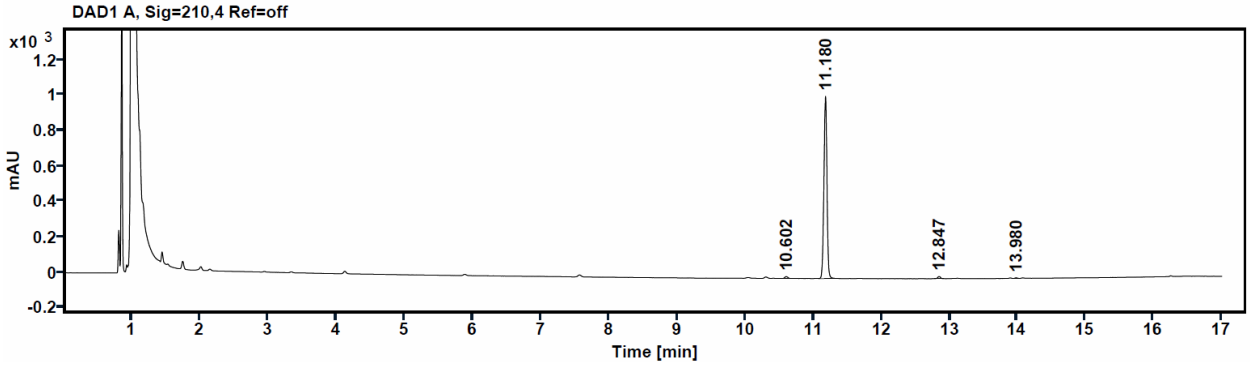
In a 250 mL 3-neck round bottom flask, **Fmoc-L-Trp(Boc)-OH (21)** (10.00 g, 19.00 mmol), pyridine (0.30 g, 3.80 mmol) and 2,6-lutidine (2.24 g, 20.90 mmol) were dissolved in DMAc/MeCN (1:1, 60 mL). The solution was cooled to -15 °C, then DMBCl (2.81 g, 20.90 mmol) and DIPEA (2.70 g, 20.90 mmol) were added. The reaction mixture was stirred at -15 °C for 10 min and monitored by HPLC (**Method 11**), which showed the ratio of **21:21-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 1.0:99.0. The reaction mixture was added to a DMAc solution of **L-Met -OH (S28)** and BSA at 20 °C. The solution was prepared by mixing **S28** (3.12 g, 20.90 mmol) and BSA (12.75 g, 62.70 mmol) in DMAc (30 mL), stirring at 50 °C for 1 h, and then cooling to 20 °C and DIPEA (0.74 g, 5.70 mmol) was added before use. The reaction mixture was stirred at 20 °C for 10 min and monitored by HPLC (**Method 11**), which showed the ratio of **21:S29:S29-over-addition** was 1.0:99.0:0.0. Then *i*-PrOH (7.55 g, 125.40 mmol) was added into the reaction mixture at 20 °C and stirred for 10 min. MeCN (30 mL) was added. The reaction was cooled to -20 °C, DMBCl (3.58 g, 26.60 mmol) and DIPEA (0.74 g, 5.70 mmol) were added and stirred at -20 °C for 1 h, then monitored by HPLC (**Method 12**). HPLC showed the ratio of **S29:S29-INT:*epi*-S29-INT** (mixed anhydride quenched by $BnNH_2$) was 1.6:98.1:0.3. The reaction mixture was added to a DMAc solution of **L-7-aza-Trp-OH (S15)**, BSA and DIPEA at 20 °C. The solution was prepared by mixing **S15** (5.46 g, 26.60 mmol) and BSA (17.39 g, 85.50 mmol) in DMAc (30 mL), stirring at 50 °C for 1 h, and then cooling to 20 °C, adding DIPEA (2.21 g, 17.10 mmol) before use. The reaction mixture was stirred at 20 °C for 30 min and monitored by HPLC (**Method 12**), which showed the ratio of **S29:7k:*epi*-7k:*epi*-S29-INT:7k-over-addition** was 1.1:97.8:0.0:0.2:0.9. The reaction assay yield was 95% over two steps.

HPLC of **S29** formation (reaction mixture)

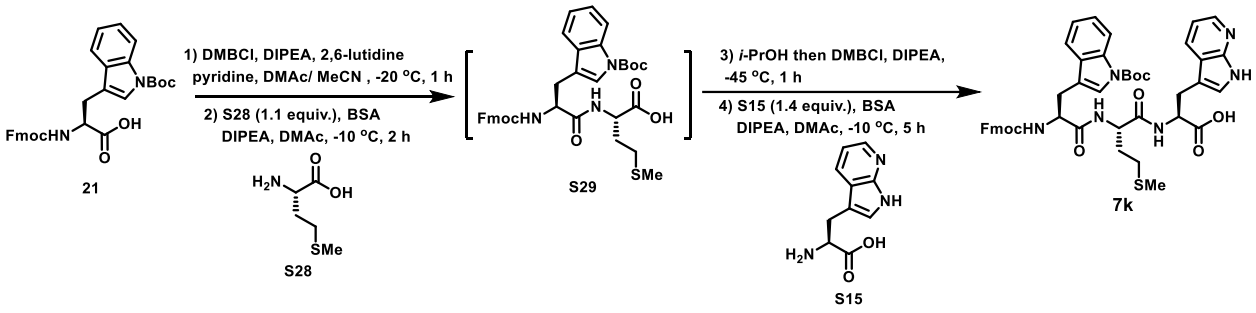


Compound ID	Time	Area	Height	Area% ratio
21	8.381	68.9379	18	1.0
S29	8.517	6965.3569	1546.4	99.0
S29-over-addition	9.282	-	-	0.0
Total	-	-	-	100.0

HPLC of **7k** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
7k-over-addition	10.602	30.4888	10.2	0.9
7k	11.180	3178.7405	1028.4	97.8
***epi*-7k**	10.850	-	-	0.0
S29	12.847	35.0851	12.3	1.1
***epi*-S29-INT**	13.98	6.8851	3.1	0.2
Total	-	-	-	100.0



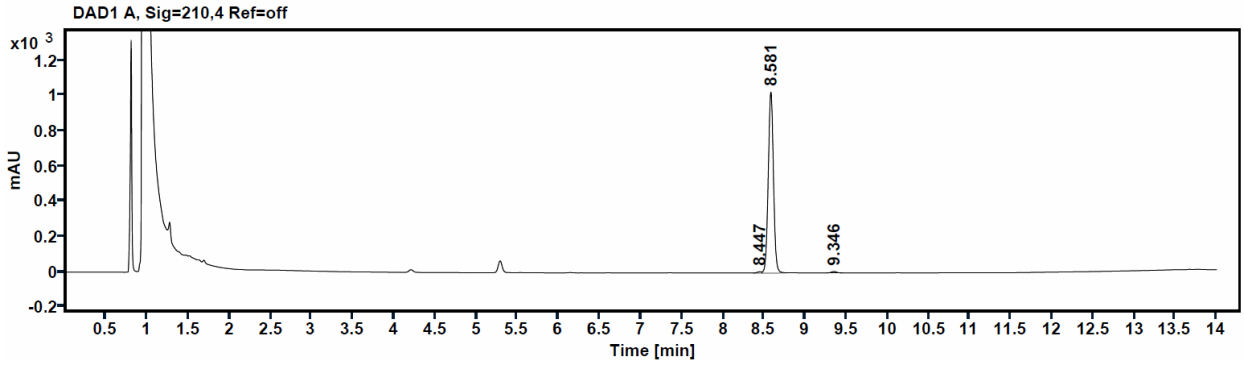
Synthesis of Fmoc-Trp(Boc)-L-Met-L-7-aza-Trp-OH (7k) (Method B)

In a 250-mL 3-neck round bottom flask, **Fmoc-L-Trp(Boc)-OH (21)** (10.00 g, 19.00 mmol) dissolved in DMAc/MeCN (1:1, 60 mL). After the solution was cooled to -20 °C, 2,6-lutidine (2.24 g, 20.90 mmol), pyridine (0.30 g, 3.80 mmol) and DIPEA (2.70 g, 20.90 mmol) were added. Then DMBCl (2.81 g, 20.90 mmol) was added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC **(Method 11)**, which showed the ratio of **21:21-INT** was 0.8:99.2. The reaction mixture was added to a DMAc solution of **L-Met -OH (S28)** and BSA at 20 °C over 10 min. The solution was prepared by mixing **S28** (3.12 g, 20.90 mmol) and BSA (12.75 g, 62.70 mmol) in DMAc (30 mL), stirring at 50 °C for 1 h, and then cooling to 20 °C before use. Then DIPEA (0.74 g, 5.70 mmol) was added to the reaction mixture. The reaction mixture was stirred at -10 °C for 2 h and monitored by HPLC **(Method 11)**, which showed the ratio of **21:S29:S29-over-addition** was 0.6:98.5:0.9. Then *i*-PrOH (7.55 g, 125.4 mmol) was added to the solution at 0 °C and stirred for 1 h. MeCN (30 mL) was added. Then the mixture was cooled to -45 °C, DMBCl (3.58 g, 26.60 mmol) and DIPEA (0.74 g, 5.70 mmol) were added and stirred at -45 °C for 1 h and monitored by HPLC **(Method 12)**, HPLC showed the ratio of **S29:S29-INT:*epi*-S29-INT** (mixed anhydride quenched by $BnNH_2$) was 0.9:98.9:0.2. A BSA solution of **S15**, which was prepared by stirring **S15** (5.46 g, 26.60 mmol) and BSA (7.22 g, 35.40 mmol) in DMAc (30 mL) at 50 °C for 1 h, was added to the reaction at -45 °C over 10 min. DIPEA (2.21 g, 17.10 mmol) were added to the reaction at -45 °C. The reaction mixture was stirred at -10 °C for 5 h and monitored by HPLC **(Method 12)**, which showed the ratio of **S29:7k:*epi*-7k:*epi*-S29-INT:7k-over-addition** was 0.9:98.1:0.0:0.3:0.7. The reaction assay yield was 95% over two steps. The reaction mixture was added slowly to MeCN:H_2O (1:1, 800 mL) at 20 °C over 1 h, and the mixture was stirred at 20 °C for 3 h. The product was collected by filtration and dried under vacuum at 40 °C, yielding 15.4 g of **7k** as a white solid (96.0% LCAP, 94.8 wt%) in 91% isolated yield.

mp 210 °C. ^{1}H NMR (400 MHz, DMSO-d_6+D_2O) δ (ppm) δ 8.26 - 8.17 (m, 1H), 8.14 (d, J = 4.8 Hz, 1H), 7.97 - 7.93 (m, 2H), 7.80 (d, J = 7.6 Hz, 2H), 7.65 (d, J = 8.0 Hz, 1H), 7.52 (s, 1H), 7.47 (d, J = 7.6 Hz, 2H), 7.36 - 7.26 (m, 5H), 7.21 - 7.13 (m, 3H), 7.04 (dd, J = 8.0, 4.8 Hz, 1H), 4.49 - 4.44 (m, 1H), 4.39 - 4.35 (m, 2H), 4.20 - 4.14 (m, 1H), 4.10 - 4.05 (m, 2H), 3.16 (dd, J = 15.2, 5.6 Hz, 1H), 3.09 - 2.99 (m, 2H), 2.92 - 2.86 (m, 1H), 2.38 - 2.33 (m, 2H), 1.94 (s, 3H), 1.90 - 1.79 (m, 2H), 1.49 (s, 9H). ^{13}C NMR (101

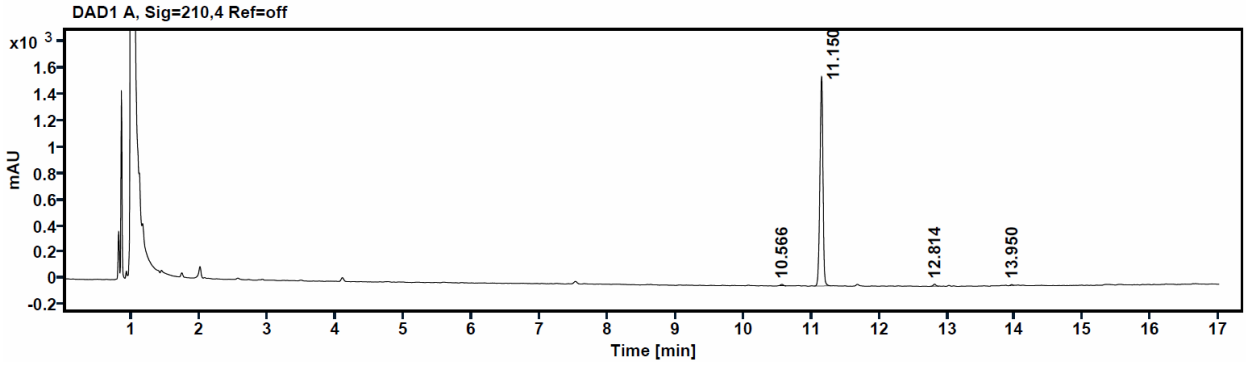
MHz, DMSO-d_6) δ (ppm) 173.04, 171.43, 171.01, 155.92, 149.07, 147.94, 143.72, 143.68, 141.91, 140.68, 140.65, 134.68, 130.33, 127.62, 127.03, 125.29, 125.20, 124.56, 124.29, 124.12, 122.41, 120.12, 119.87, 119.65, 116.86, 115.02, 114.65, 108.75, 83.45, 65.77, 54.37, 52.94, 51.99, 46.56, 32.21, 29.33, 27.66, 27.27, 26.90, 14.66. HRMS (ESI): m/z calcd for $C_{46}H_{49}N_6O_8S$ $[M+H]^+$: 845.3327, found: 845.3366. FTIR (neat): 3278, 1728, 1694, 1633, 1534, 1450, 1366, 1256, 1221, 1159, 1087, 757, 736, 684 cm^{-1}.

HPLC of **S29** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
21	8.447	29.2774	7.4	0.6
S29	8.581	4524.4116	1026.4	98.5
S29-over-addition	9.346	40.2152	8.7	0.9
Total	-	-	-	100.0

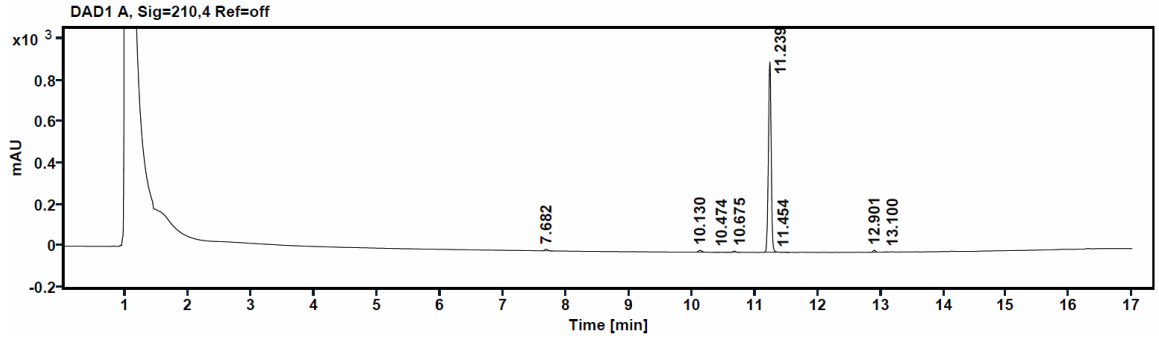
HPLC of **7k** formation (reaction mixture)

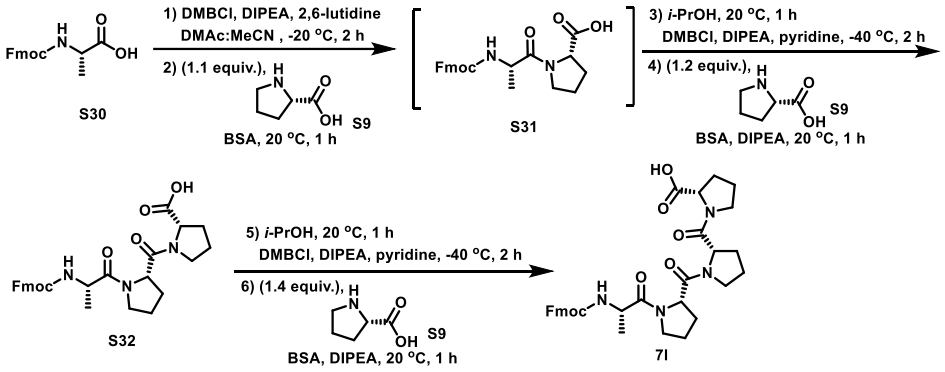


Compound ID	Time	Area	Height	Area% ratio
7k-over-addition	10.566	37.2155	12.3	0.7
7k	11.150	4917.335	1598.8	98.1

***epi*-7k**	10.821	-	-	0.0
S29	12.814	44.5679	15.6	0.9
***epi*-S29-INT**	13.95	13.7287	6.5	0.3
Total	-	-	-	100.0

HPLC of isolated **7k**



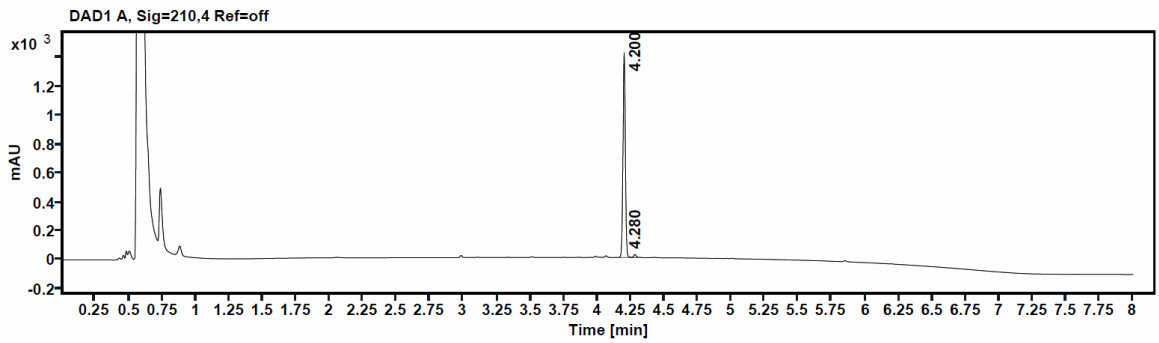


Synthesis of Fmoc-L-Ala-L-Pro-L-Pro-L-Pro-OH (7l) (Method B). In a 250 mL 3-neck round bottom flask, **Fmoc-L-Ala-OH (S30)** (5.00 g, 16.06 mmol) and 2,6-lutidine (1.72 g, 16.06 mmol) were dissolved in DMAc/MeCN (1:1, 25 mL). The solution was cooled to -20 °C, DMBCl (2.27 g, 16.84 mmol) and DIPEA (2.08 g, 16.06 mmol) were added into the reaction mixture. The reaction mixture was stirred at -20 °C for 2 h and monitored by HPLC (**Method 4**), which showed the ratio of **S30:S30-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 1.0:99.0. The reaction mixture was added slowly over 1 h to a DMAc and MeCN solution of **L-Pro-OH (S9)** and BSA at 20 °C. The solution was prepared by mixing **S9** (1.98 g, 17.19 mmol) and BSA (6.53 g, 32.12 mmol) in DMAc/MeCN (1:1, 25 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 4**), which showed the ratio of **S30:S31:S31-over-addition** was 1.4:98.6:0.0. Then *i*-PrOH (3.86 g, 64.24 mmol) was added into the reaction mixture. The reaction mixture was stirred at 20 °C for 1 h. The reaction was cooled to -40 °C, DMBCl (2.92 g, 21.68 mmol), pyridine (0.25 g, 3.21

mmol) and DIPEA (2.49 g, 19.27 mmol) were added into the reaction mixture. The reaction mixture was stirred at at -40 °C for 2 h and monitored by HPLC (**Method 4**). HPLC showed the ratio of **S31:S31-INT** (mixed anhydride quenched by $BnNH_2$) was 1.4:98.5. The reaction mixture was added slowly over 1 h to a DMAc and MeCN solution of **L-Pro-OH (S9)**, BSA and DIPEA at 20 °C. The solution was prepared by mixing **S9** (2.22 g, 19.27 mmol) and BSA (9.80 g, 48.19 mmol) in DMAc/MeCN (1:1, 25 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C, then adding DIPEA (1.04 g, 8.03 mmol) before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 4**), which showed the ratio of **S31:S32:*epi*-S32:S32-over-addition** was 2.0:98.0:0.0:0.0. Then *i*-PrOH (5.79 g, 96.37 mmol) was added into the reaction mixture. The reaction mixture was stirred at 20 °C for 1 h. The reaction was cooled to -40 ºC, DMBCl (3.46 g, 25.70 mmol), pyridine (0.25 g, 3.21 mmol) and DIPEA (2.08 g, 16.06 mmol) were added into the reaction mixture. The reaction mixture was stirred at -40 °C for 2 h and monitored by HPLC (**Method 4**). HPLC showed the ratio of **S32:S32-INT** (mixed anhydride quenched by $BnNH_2$) was 1.6:98.4. The reaction mixture was added slowly over 1h to a DMAc and MeCN solution of **L-Pro-OH (S9)**, BSA and DIPEA at 20 °C. The solution was prepared by mixing **S9** (2.59 g, 22.49 mmol) and BSA (13.07 g, 64.25 mmol) in DMAc/MeCN (1:1, 25 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C, then adding DIPEA (1.04 g, 8.03 mmol) before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 4**), which showed the ratio of **S32:7l:*epi*-7l:7l-over-addition** was 3.8:95.6:0.0:0.6. The reaction assay yield was 91% over three steps. The reaction solution was slowly added dropwise into 0.05M HCl aq. solution (150 mL) at 10 ºC over 0.5 h. Then extracted with EtOAc (100 mL). Upon phase separation, the organic phase was washed with 5% NaCl solution (100 mL), dried with anhydrous Na_2SO_4, filtered and concentrate. The residuum was dissolved in THF (25 mL) and added dropwise into MTBE (150 mL) at 20 ºC over 1 h and stirred at 20 ºC for 2 h. The solid was collected by filtration and dried under N_2 for overnight to provide 7.91 g of **7l** as a white solid (94.2% LCAP, **Method 4**, 93.0 wt%) in 76% isolated yield.

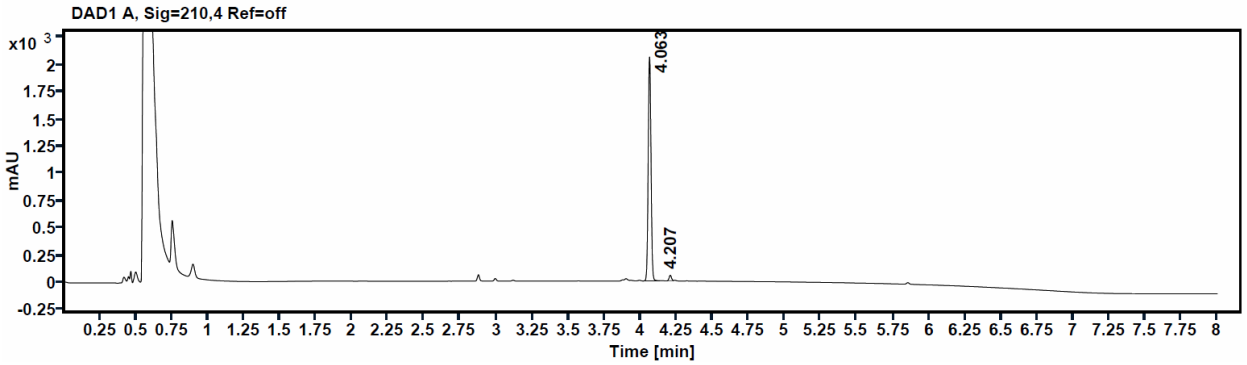
mp 120-125 ℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.37 (br s, 1H), 7.89 (d, J = 7.2 Hz, 2H), 7.73 - 7.57 (m, 2H), 7.51 (d, J = 7.6 Hz, 1H), 7.41 (t, J = 7.6 Hz, 2H), 7.33 (t, J = 7.6 Hz, 2H), 4.60-4.46 (m, 2H), 4.33 - 4.25 (m, 2H), 4.23 -4.16 (m, 2H), 3.70- 3.59 (m, 3H), 3.52 - 3.43 (m, 3H), 3.28 - 3.05 (m, 1H), 2.18 - 2.05 (m, 3H), 1.96 - 1.73 (m, 9H), 1.18 - 0.97 (m, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 173.35, 170.30, 169.70, 169.41, 155.67, 143.88, 143.85, 140.75, 127.66, 127.09, 125.31, 120.13, 65.56, 58.39, 57.47, 57.39, 47.87, 46.68, 46.56, 46.48, 46.13, 28.44, 27.53, 27.44, 24.56, 24.39, 24.31, 16.66. HRMS (ESI): m/z calcd. for $C_{33}H_{38}N_4O_7$ $[M+H]^+$: 603.2813, found: 603.2836. FTIR (neat): 3393, 3261, 2973, 2877, 1716, 1706, 1635, 1540, 1522, 1508, 1472, 1447, 1322, 1245, 1195, 1159, 1105, 1074, 1041, 761, 742 cm^{-1}.

HPLC of **S31** formation (reaction mixture)



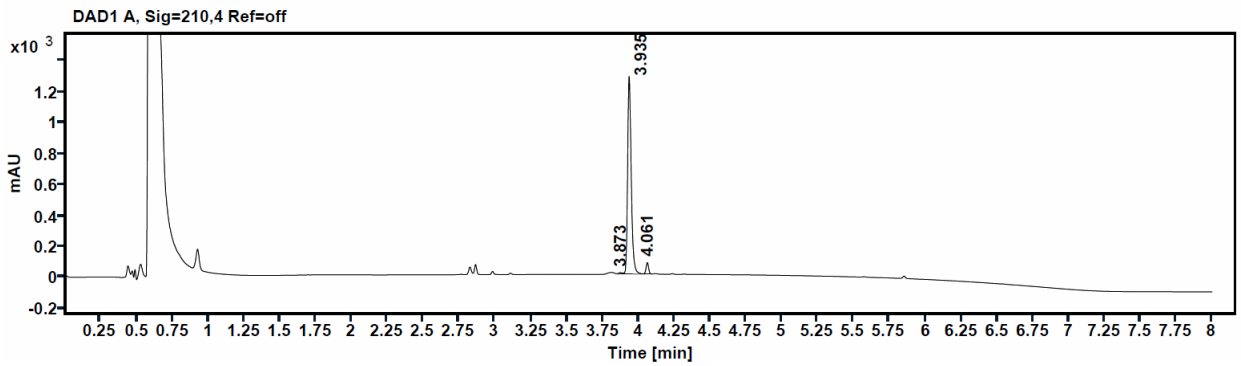
Compound ID	Time	Area	Height	Area% ratio
S31-over-addition	4.056	0.0	0.0	0.0
S31	4.200	1754.4222	1429.8	98.6
S30	4.280	25.1394	21.7	1.4
Total	-	-	-	100.0

HPLC of **S32** formation (reaction mixture)



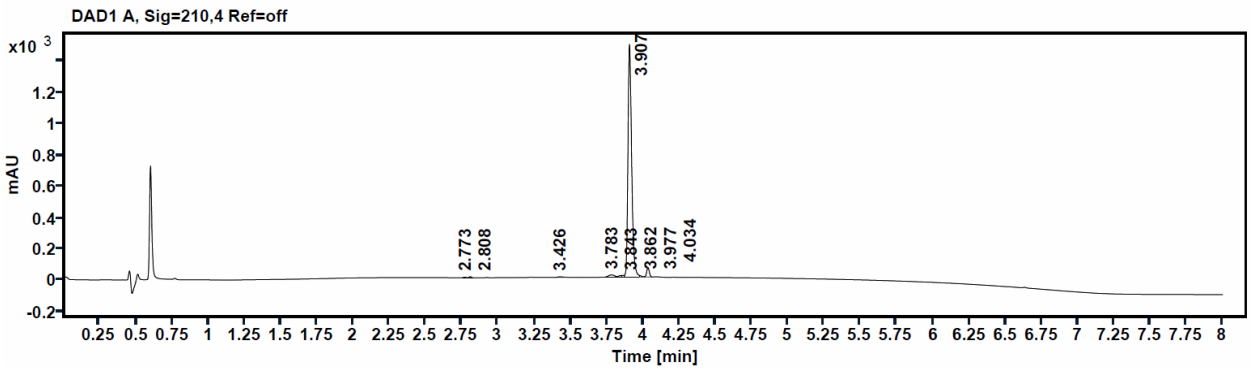
Compound ID	Time	Area	Height	Area% ratio
S32-over-addition	3.936	0.0	0.0	0.0
S32	4.063	2770.208	2073.5	98.0
***epi*-S32**	4.147	0.0	0.0	0.0
S31	4.207	57.2438	51.1	2.0
Total	-	-	-	100.0

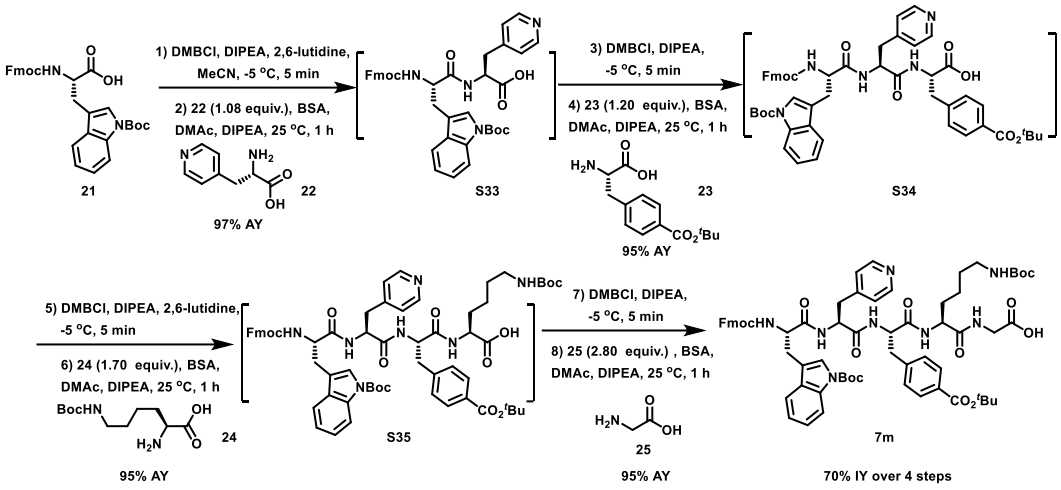
HPLC of **7l** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
7l-over-addition	3.873	14.9138	8.5	0.6
7l	3.935	2228.5852	1282.9	95.6
***epi*-7l**	3.985	0.0	0.0	0.0
S32	4.061	88.1434	73.9	3.8
Total	-	-	-	100.0

HPLC of isolated **7l**





Synthesis of Fmoc-Trp(Boc)-Pal-Phe(4-CO_2*t*Bu)-Lys(Boc)-Gly-OH (7m) (Method A). To a 50 mL three-neck jacketed vessel were added compound **21** (5.00 g, 9.50 mmol) and MeCN (30 mL). After the solution was cooled to -5 °C, 2,6-lutidine (1.02 g, 9.50 mmol), DMBCl (1.34 g, 9.97 mmol) and DIPEA (1.23 g, 9.50 mmol) were added. The reaction mixture was stirred at -5 °C for 5 min and monitored by HPLC (**Method 7**), which showed the ratio of **21**:**21-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.45:99.55. The reaction solution was added into a solution of **22** and BSA in DMAc at 25 °C in one portion, which was prepared by stirring **22** (1.70 g, 10.25 mmol), BSA (5.79 g, 28.49 mmol) and DIPEA (1.23 g, 9.50 mmol) in DMAc (30 mL) at 50 °C for 1 h and then cooled to 25 °C. The reaction mixture was stirred at 25 °C for 1 h and monitored by HPLC (**Method 7**). The HPLC showed the reaction assay yield was 97%, and the ratio of **21**:**S33**:**S33-over-addition** was 2.15:97.6:0.21. Then *i*-PrOH (3.42 g, 56.97 mmol) was added to the reaction and stirred for 2 h at 25 °C.

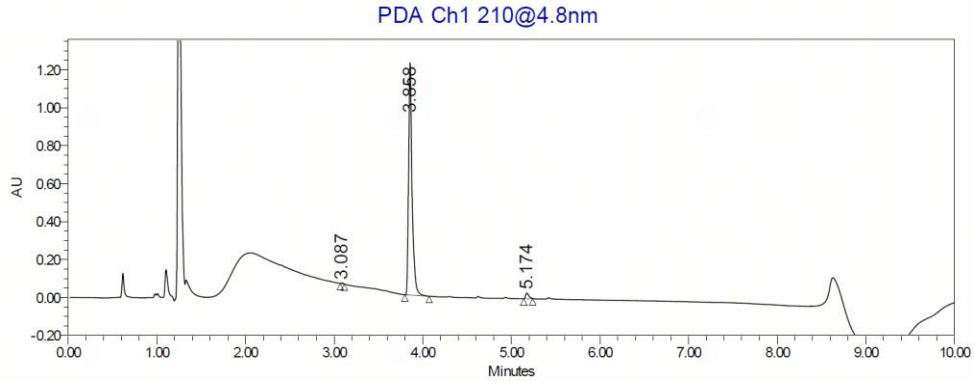
The above **S33** reaction solution was cooled to -5 °C, DIPEA (0.37 g, 2.85 mmol) and DMBCl (1.53 g, 11.39 mmol) were added. The reaction mixture was stirred at -5 °C for 5 min and monitored by HPLC (**Method 7**), which showed the ratio of **S33**:**S33-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.46:99.56. The reaction mixture was quickly transferred in one portion into another 250 mL three-neck jacketed vessel containing **23**, which was pre-prepared from stirring **23** (3.02 g, 11.39 mmol), DMAc (20 mL), DIPEA (1.23 g, 9.50 mmol) and BSA (5.79 g, 28.49 mmol) at 50 °C for 1 h and then cooled to 25 °C, and the resulting reaction solution was stirred for 1 h at 25 °C. The reaction was monitored by HPLC (**Method 7**). The HPLC showed the reaction assay yield was 95% and the ratio of **S33**:**S34**:***epi*-S34**:***epi*-S33-INT**:**S34-over-addition** was 1.59:96.89:0.97:0:0.54. Then *i*-PrOH (3.42 g, 56.97 mmol) was added to the reaction and stirred for 2 h at 25 °C.

The above **S34** reaction solution (9.50 mmol) was cooled to -5 °C, DIPEA (0.49 g, 3.80 mmol),2, 6-lutidine (0.41 g, 3.80 mmol) and DMBCl (2.04 g, 15.19 mmol) were added. The reaction mixture was stirred at -5 °C for 5 min and monitored by HPLC (**Method 7**), which showed the ratio of **S34**:**S34-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.0:100.0. The reaction solution was added in one portion

into a solution of **24** and BSA in DMAc, which was prepared by stirring **24** (3.98 g, 16.14 mmol), DIPEA (1.23 g, 9.50 mmol) and BSA (9.08 g, 44.63 mmol) in DMAc (24 mL) at 50 °C for 1 h and then cooled to 25 °C. The reaction mixture was stirred at 25 °C for 1 h and monitored by HPLC (**Method 7**). The HPLC showed the reaction assay yield was 95%, and the ratio of **S34**:**S35**:***epi*-S35**:***epi*-S34-INT**:**S35-over-addition** was 1.50:98.25:0.21:0:0.04. Then *i*-PrOH (5.36 g, 89.25 mmol) was added to the reaction and stirred for 2 h at 25 °C, which was used for the next step directly.

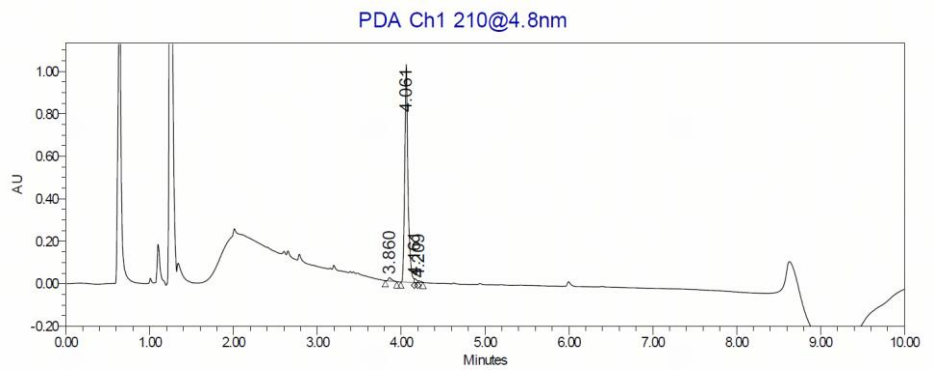
The above **S35** solution was cooled to -5 °C, DMBCl (4.60 g, 34.18 mmol) and DIPEA (4.17 g, 32.28 mmol) were added. The reaction mixture was stirred at -5 °C for 5 min and monitored by HPLC (**Method 7**), which showed the ratio of **S35**:**S35-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 1.02:98.98. The reaction solution was added into a solution of **25** and BSA in DMAc in one portion, which was prepared by stirring **25** (2.00 g, 26.59 mmol), DIPEA (1.23 g, 9.50 mmol) and BSA (96.58 g, 474.75 mmol) in DMAc (24 mL) at 50 °C for 1 h and then cooled to 25 °C. The reaction mixture was stirred at 25 °C for 1 h and monitored by HPLC (**Method 7**). The HPLC showed the reaction assay yield was 95% and the ratio of **S35**:**7m**:***epi*-7m**:***epi*-S35-INT**:**7m-over-addition** was 1.07:98.43:0.50:0:0. The reaction was added into MeCN:H_2O (3:1, 180 mL) and stirred for 16 h at 20 °C. The solid was collected by filtration and washed with MeCN:H_2O (3:1, 25 mL), dried under vacuum at 40 °C to provide 8.42 g of **7m** (98.3% HPLC purity (**Method 7**) and 95.3 wt%) as a white solid in 70% isolated yield (**Method 7**) over 4 steps.

HPLC of **S33** formation (reaction mixture)



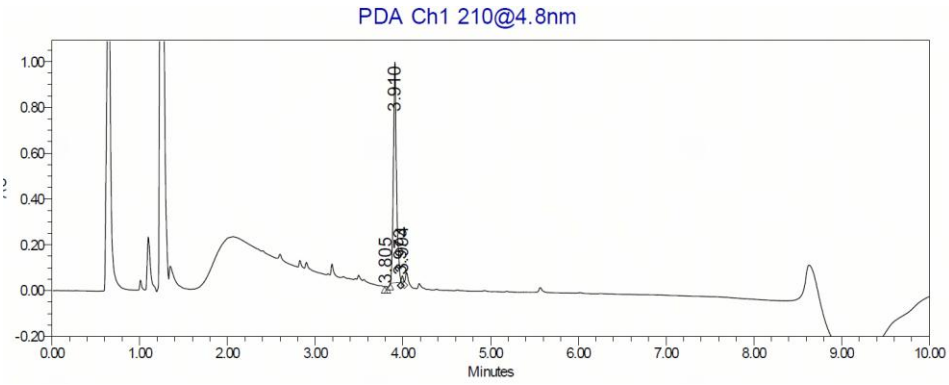
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S33-over-addition	3.087	73293	29614	0.21
S33	3.858	3324627	1225950	97.64
21	5.174	7181	29614	2.15
Total	-	-	-	100.00

HPLC of **S34** formation (reaction mixture)



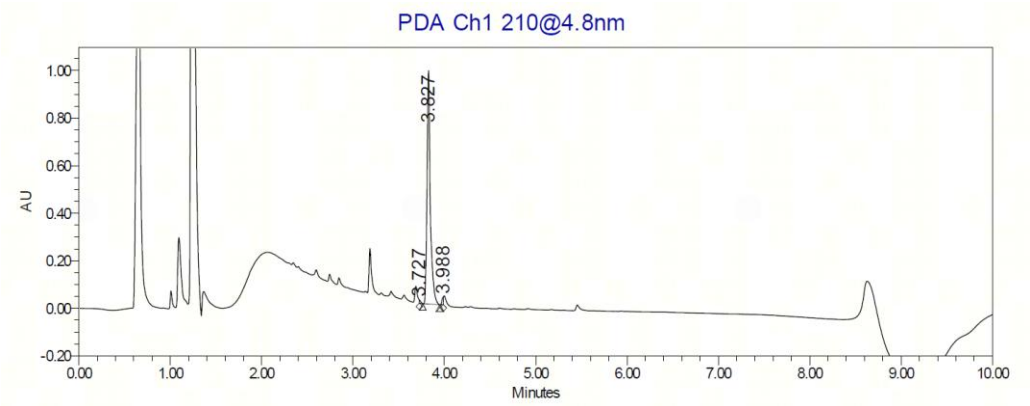
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S34-over-addition	4.209	15420	7204	0.54
S34	4.061	2744093	1024568	96.89
***epi*-S34**	4.161	27533	16516	0.97
***epi*-S33-INT**	3.905	-	-	-
S33	3.860	45046	15567	1.59
Total	-	-	-	100.00

HPLC of **S35** formation (reaction mixture)



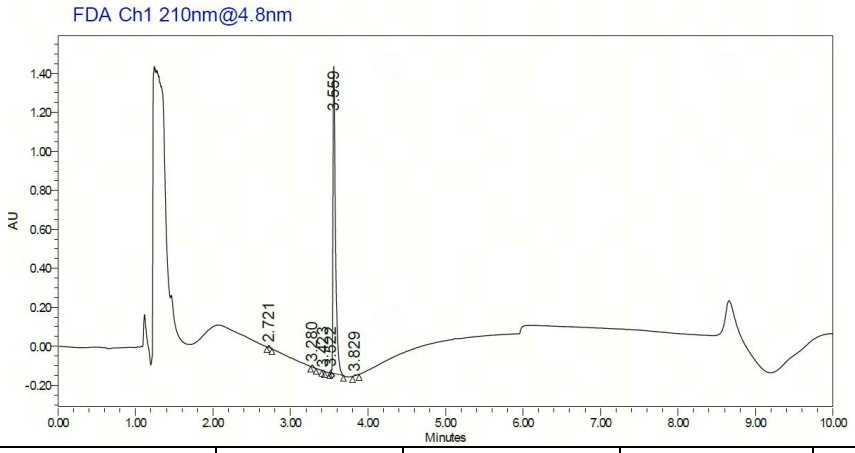
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S35-over-addition	3.805	997	932	0.04
S35	3.910	2342135	963436	98.25
***epi*-S35**	3.973	5065	17741	0.21
***epi*-S34-INT**	3.751	-	-	-
S34	3.994	35747	27189	1.50
Total	-	-	-	100.00

HPLC of **7m** formation (reaction mixture)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
7m-over-addition	3.631	-	-	-
7m	3.827	2447148	982083	98.43
***epi*-7m**	3.727	12440	13076	0.50
***epi*-S35-INT**	4.022	-	-	-
S35	3.988	26704	31186	1.07
Total	-	-	-	100.00

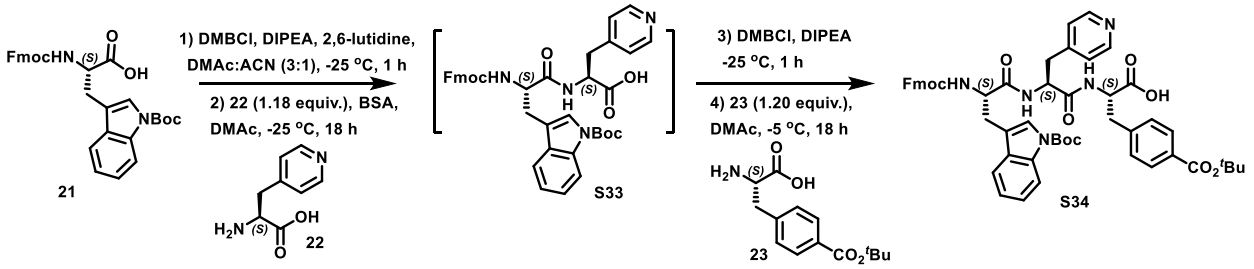
HPLC of isolated **7m**



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
7m-over-addition	3.377	-	-	-
7m	3.559	3534166	1574662	98.29
***epi*-7m**	3.466	-	-	-
***epi*-S35-INT**	3.740	-	-	-
S35	3.829	8255	5136	0.23
Others	-	-	-	1.48
Total	-	-	-	100.00

Reducing AA particle size by milling enables successful couplings

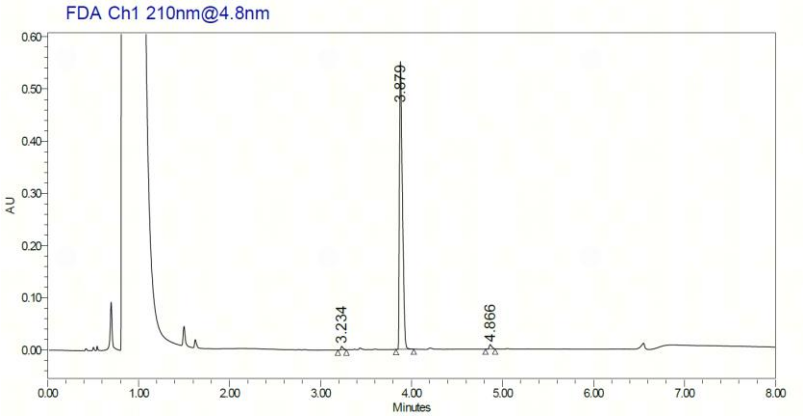
In the absence of BSA, milling of amino acid **23** provided a favorable reaction profile. In contrast, unmilled **23** resulting in lower reaction yield due to increased levels of unreacted starting material and oxazolone formation.



Synthesis of Fmoc-Trp(Boc)-Pal-pCO_2*t*BuPhe-OH (S34) using milled AA 23 (Method B). To a 500 mL three-neck jacketed vessel, compound **21** (50.00 g, 94.95 mmol) was added into DMAc/MeCN (3:1, 200 mL). After the solution was cooled to -25 °C, 2,6-lutidine (5.09 g, 47.48 mmol), DMBCl (15.34 g, 113.94 mmol), and DIPEA (13.50 g, 104.45 mmol) were added. The reaction mixture was stirred at -25 °C for 1 h and monitored by HPLC (**Method 7**), which showed the ratio of **21**:**21-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.99:99.01. The reaction solution was slowly added into a pre-cooled (-25 °C) solution of **22** and BSA in DMAc at -25 °C over 0.5 h, which was prepared by stirring **22** (18.62 g, 112.04 mmol) and BSA (38.63 g, 189.90 mmol) in DMAc (200 mL) at 50 °C for 1 h and then cooled to -25 °C. The reaction mixture was stirred at -25 °C for 18 h and monitored by HPLC. The HPLC showed the ratio of **21:S33**:***epi*-S33**:**S33-over-addition** was 1.32:97.78:0:0.90. Then *i*-PrOH (14.27 g, 237.38 mmol) was added to the reaction and stirred for 2 h at 0 °C. The reaction mixture was filtered, and the filtrate was directly used for the next stage. The above **S33** reaction solution was cooled to -25 °C, and DMBCl (20.45 g, 151.92 mmol) and DIPEA (18.41 g, 142.43 mmol) were added. The reaction mixture was stirred at -25 °C for 1 h and monitored by HPLC, which showed the ratio of **S33**:**S33-INT** was 1.87:98.13. To another 1000 mL three-neck jacketed vessel, milled **23** (PSD_D50 = 203 μm) (30.23 g, 113.94 mmol) was added to DMAc (200 mL). After the mixture was cooled to -5 °C, the **S33**-mixed anhydride solution was added over 0.5 h and stirred for 18 h at -5 °C. The reaction was monitored by HPLC, which showed that the reaction assay yield was 93% over two steps The ratio of **S33**:**S34**:***epi*-S34**:**S34-over-addition** was 2.37:95.34:1.64:0.65. (Note: if unmilled **23** was used, only 88% AY was obtained. The ratio of **S33**:**S34**:***epi*-S34**:**S34-over-addition** was 3.27:93.44:2.45:0.86). The reaction solution of **S34** was added to MeCN:H_2O (1:1, 1500 mL) over 0.5 h and stirred for 24 h at 15-25 °C. The solid was filtered, washed with MeCN:H_2O (1:1, 250 mL), and dried under an N_2 stream to provide 81.06 g **S34** as a white solid in a 90% isolated yield and 98.5% HPLC purity. mp 189 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.75 (br s, 2H), 8.52 (br d, *J* = 7.5 Hz, 2H), 8.02 (br d, *J* = 8.1 Hz, 1H), 7.73-7.90 (m, 15h), 7.51-7.67 (m, 4H), 7.28-7.41 (m, 5H),

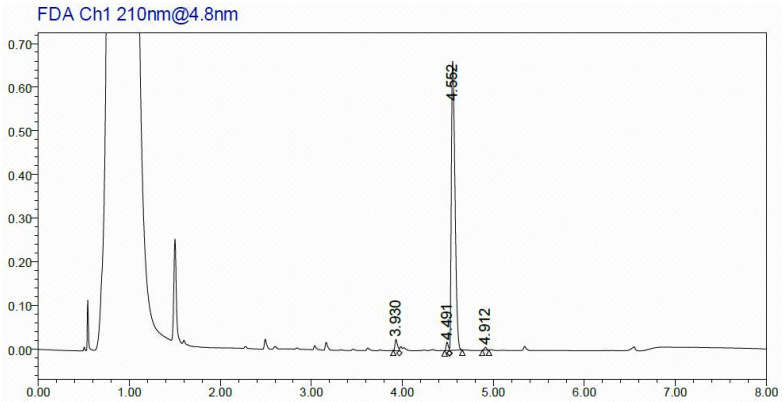
7.15-7.27 (m, 3H), 4.80-4.91 (m, 1H), 4.46-4.61 (m, 1H), 4.27-4.38 (m, 1H), 4.03-4.23 (m, 3H), 3.33 (br dd, J = 13.4, 4.2 Hz, 1H), 3.15-3.23 (m, 1H), 3.07 (ddd, J = 17.3, 13.9, 9.3 Hz, 2H), 2.85-3.00 (m, 2H), 1.55 (s, 9H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.8, 172.0, 170.4, 165.3, 157.3, 156.2, 149.5, 144.2, 144.1, 143.1, 142.4, 141.1, 141.1, 135.1, 130.7, 130.2, 129.8, 129.4, 128.0, 127.4, 125.8, 125.6, 124.7, 124.6, 120.5, 120.0, 117.2, 83.9, 80.8, 66.2, 54.8, 53.8, 52.6, 47.0, 40.8, 38.1, 37.0, 28.1, 28.1, 27.6. HRMS (ESI): m/z calcd. for $C_{53}H_{56}N_5O_{10}$ $[M+H]^+$: 922.4022, found: 922.4017. FTIR (neat): 3082, 2976, 1709, 1639, 1160, 741, 473 cm^{-1}.

HPLC of **S33** formation



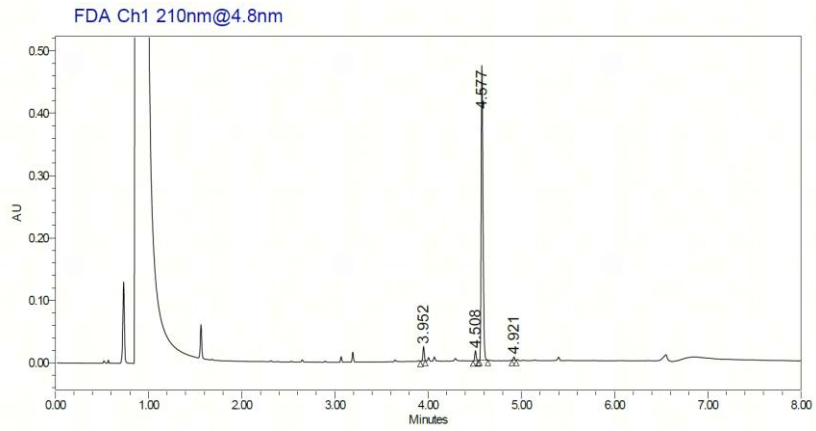
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S33-over-addition	3.234	11505	6776	0.90
S33	3.879	1256488	551184	97.78
***epi*-S33**	3.749	-	-	-
21	4.866	17045	8421	1.32
Total	-	-	-	100.00

HPLC of **S34** formation (from milled **23**)

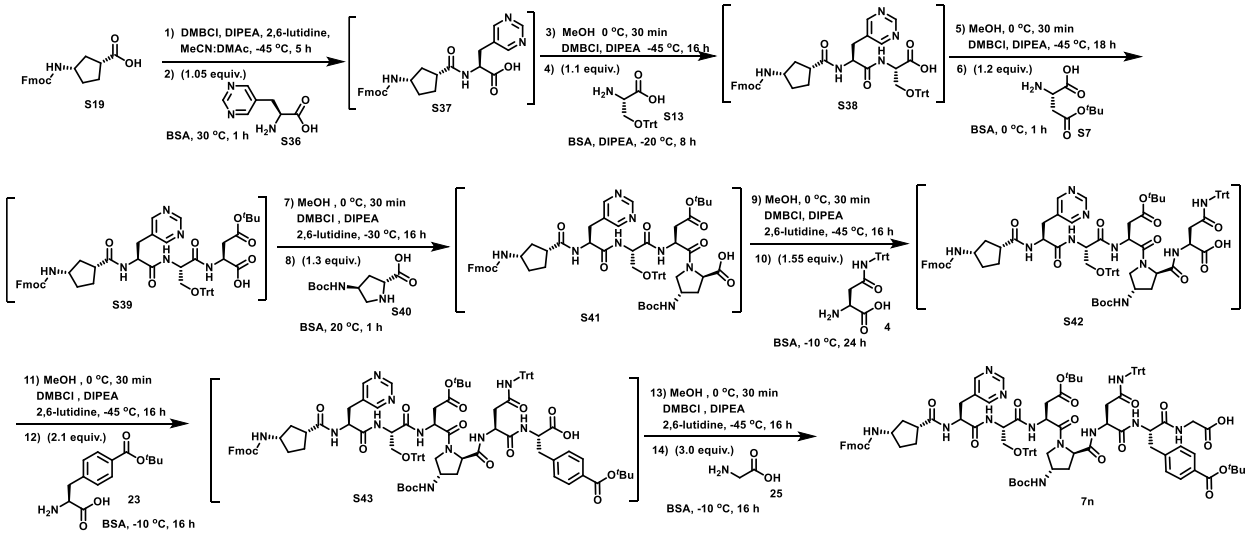


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S34-over-addition	4.912	12571	6869	0.65
S34	4.552	1842177	661114	95.34
***epi*-S34**	4.491	31680	18854	1.64
S33	3.930	45884	25190	2.37
Total	-	-	-	100.00

HPLC of **S34** formation (from unmilled **23**)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S34-over-addition	4.921	5886	5622	0.9
S34	4.577	649108	472154	93.4
***epi*-S34**	4.508	16987	16147	2.5
S33	3.952	22733	22890	3.3
Total	-	-	-	100.0



Synthesis of Fmoc-bhcLeu-3,5-pyrimi-Ala-L-Ser(Trt)-L-Asp(O*t*Bu)-D-Pro(*t*4NHBoc)-L-Asn(Trt)-L-Phe(O*t*Bu)-Gly-OH (7n) (Method B). In a 1000 mL 3-neck round bottom flask, **Fmoc-bhcLeu-OH (S19)** (10.00 g, 28.46 mmol) and 2,6-lutidine (3.05 g, 28.46 mmol) were dissolved in DMAc/MeCN (1:1, 50 mL). Then the solution was cooled to -45 °C, DMBCl (4.02 g, 29.88 mmol) and DIPEA (3.68 g, 28.46 mmol) were added into the reaction mixture. The reaction mixture was stirred at -45 °C for 5 h and monitored by HPLC (**Method 4**), which showed the ratio of **S19:S19-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.9:99.1. The reaction mixture was added slowly into a solution of **3,5-pyrimi-Ala-OH (S36)** and BSA in DMAc and MeCN at 30 °C over 1 h, which was prepared by mixing **S36** (5.00 g, 29.88 mmol) and BSA (11.58 g, 56.92 mmol) in DMAc/MeCN (1:1, 50 mL), stirred at 50 °C for 1 h until clear, then cooling to 30 °C. The reaction mixture was stirred at 30 °C for 1 h and monitored by HPLC (**Method 4**), which showed the ratio of **S19:S37:S37-over-addition** was: 0.7:99.3:0.0. Then MeOH (2.28 g, 71.14 mmol) was added into the reaction mixture at 0 °C and stirred for 30 min. The reaction mixture was then cooled to -45 °C. DMBCl (4.60 g, 34.15 mmol) and DIPEA (3.68 g, 28.46 mmol) were added to the reaction mixture. The reaction mixture was stirred at -45 °C for 16 h and monitored by HPLC (**Method 4**), which showed the ratio of **S37:S37-INT:*epi*-S37-INT** was 0.7:99.3:0.0. A solution of **L-Ser(Trt)-OH (S13)** and BSA in DMAc and MeCN, prepared by mixing **S13** (10.88 g, 31.30 mmol) and BSA (11.58 g, 56.92 mmol) in DMAc/MeCN (1:1, 50 mL), stirred at 50 °C for 1 h until clear, cooling to 25 °C, and then adding DIPEA (1.10 g, 8.54 mmol). The resulting mixture was added into the reaction solution at -45 °C and stirred at -20 °C for 8 h, then monitored by HPLC (**Method 13**), which showed the ratio of **S37:S38:*epi*-S38:*epi*-S37-INT:S38-over-addition** was 1.0:99.0:0.0:0.0:0.0. The assay yield of the reaction was 90% over two steps. To the **S38** reaction mixture, MeOH (2.28 g, 71.14 mmol) was added at 0 °C and stirred for 30 min. The reaction mixture was cooled to -45 °C. DMBCl (6.51 g, 48.38 mmol) and DIPEA (5.15 g, 39.84 mmol) were added, and the mixture was stirred at -45 °C for 18 hours. The reaction was monitored by HPLC (**Method 13**), which showed the ratio of **S38:(S38-INT+*epi*-S38-INT)** was 1.3:98.7. The reaction

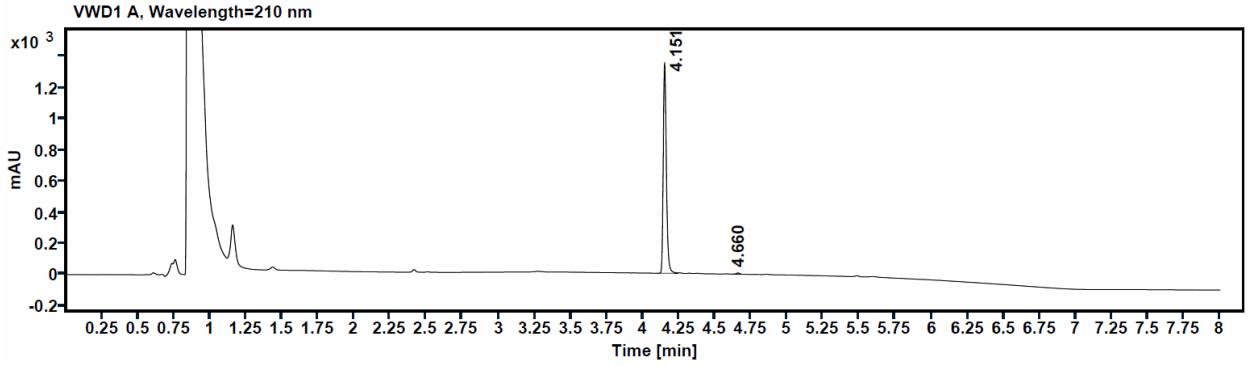
solution was added slowly into a solution of **L-Asp(O*t*Bu)-OH (S7)**, BSA in DMAc and MeCN at 0 °C over 1 h, which was prepared by mixing of **S7** (6.46 g, 34.14 mmol), BSA (11.58 g, 56.92 mmol) in DMAc/MeCN (1:1, 50 mL), stirred at 50 °C for 1 h until clear, then cooling to 0 °C. The reaction mixture was stirred at 0 °C for 1 h and monitored by HPLC (**Method 13**). HPLC showed the ratio of **S38:S39:*epi*-S39:(S38-INT +*epi*-S38-INT):S39-over-addition** was 2.0:97.1:0.0:0.6:0.3. Then MeOH (2.28 g, 71.14 mmol) was added to the reaction mixture at 0 °C and stirred for 30 min, then cooling to -30 °C. DMBCl (5.75 g, 42.67 mmol), DIPEA (5.52 g, 42.67 mmol) and 2,6-lutidine (1.52 g, 14.23 mmol) were added into the reaction mixture and stirred at -30 °C for 16 h, monitored by HPLC (**Method 13**). HPLC showed the ratio of **S39: S39-INT:*epi*-S39-INT** was 1.3:98.7:0.0. The reaction solution was added slowly into a solution of **D-Pro(*t*4NHBoc)-OH (S40)**, BSA in DMAc and MeCN at 20 °C over 1 h, which was prepared by mixing of **S40** (8.52 g, 37.00 mmol), BSA (11.58 g, 56.92 mmol) in DMAc/MeCN (1:1, 50 mL), stirred at 50 °C for 1 h until clear, then cooling to 20 °C. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 13**). HPLC showed the ratio of **S39:S41:*epi*-S41:*epi*-S39-INT:S41-over-addition** was 1.2:98.8:0.0:0.0:0.0. Then MeOH (2.28 g, 71.14 mmol) was added to the reaction mixture at 0 °C and stirred for 30 min. The reaction mixture was cooled to -45 °C. DMBCl (7.66 g, 56.92 mmol), DIPEA (7.36 g, 56.92 mmol), and 2,6-lutidine (4.88 g, 45.53 mmol) were added into the reaction mixture at -45 °C and stirred for 16 h, monitored by HPLC (**Method 13**). HPLC showed the ratio of **S41:S41-INT:*epi*-S41-INT** was 1.8:98.2:0.0. A solution of **L-Asn(Trt)-OH (4)**, BSA in DMAc and MeCN, which was prepared by mixing of **4** (16.52 g 44.11 mmol), BSA (11.58 g, 56.92 mmol) in DMAc/MeCN (1:1, 50 mL), stirred at 50 °C for 1 h until clear, then cooling to 20 °C, was added into the reaction mixture at -45 °C and stirred at -10 °C for 24 h, monitored by HPLC (**Method 14**). HPLC showed the ratio of **S41:S42:*epi*-S42:*epi*-S41-INT:S42-over-addition** was 1.7:98.3:0.0:0.0:0.0. MeOH (2.28 g, 71.2 mmol) was added to the reaction mixture at 0 °C and stirred for 30 min. The reaction mixture was cooled to -45 °C. DMBCl (8.43 g, 62.61 mmol), DIPEA (8.09 g, 62.61 mmol), and 2,6-lutidine (4.88 g, 45.53 mmol) were added into the reaction mixture at -45 °C and stirred for 16 h, monitored by HPLC (**Method 14**). **S42:S42-INT:*epi*-S42-INT** was 1.4:98.6:0.0. A solution of **pCO_2*t*BuPhe-OH (23)**, BSA in DMAc and MeCN, which was prepared by mixing of **23** (15.86 g, 59.76 mmol) and BSA (11.58 g, 56.92 mmol) in DMAc/MeCN (1:1, 50 mL), stirred at 50 °C for 1 h until clear, then cooling to 20 °C, was added into the reaction mixture at -45 °C and stirred at -10 °C for 16 h, monitored by HPLC (**Method 14**). HPLC showed the ratio of **S42:S43:*epi*-S43:*epi*-S42-INT:S43-over-addition** was 1.0:98.6:0.0:0.0:0.4. Then MeOH (2.28 g, 71.14 mmol) was added to the reaction mixture at 0 °C and stirred for 30 min. The reaction mixture was cooled to -45 °C. DMBCl (9.58 g, 71.14 mmol), DIPEA (9.20 g, 71.14 mmol), and 2,6-lutidine (4.88 g, 45.53 mmol) were added into the reaction mixture at -45 °C and stirred for 16 h, monitored by HPLC (**Method 14**). HPLC showed the ratio of **S43:S43-INT:*epi*-S43-INT** (mixed anhydride quenched by $BnNH_2$) was 1.3:98.7:0.0. A solution of **Gly-OH (25)**, BSA in DMAc and MeCN, which was prepared by mixing of **25** (6.41g, 85.37

mmol) and BSA (34.73 g, 170.75 mmol) in DMAc/MeCN (1:1, 50 mL), stirred at 50 °C for 1 h until clear, then cooling to 24 °C, was added into the reaction mixture at -45 °C and stirred at -10 °C for 16 h, monitored by HPLC (**Method 14**). HPLC showed the ratio of **S43:7n:*epi*-7n:*epi*-S43-INT:7n-over-addition** was 1.3:98.5:0.2:0.0:0.0. The assay yield was 73.0% over seven couplings. The reaction mixture was added slowly to KH_2PO_4 (7.70 g, 56.60 mmol) in H_2O (500 mL) at -10 °C and extracted with ethyl acetate (300 mL). After phase separation, the organic phase was concentrated to afford crude product. The crude product was added in *n*-Heptane (300 mL) and stirred at 0 °C for 1 h. The solid was collected by filtration and dried under vacuum to yield 74.3 g of crude **7n** as a white solid (53.7% LCAP, 50.6 wt%) in an isolated yield of 71% over 7 steps (**Method 14**). Starting from **S19**, 1588.7 grams of reagents/solvents were used above to synthesize 74.3 g (50.6 wt%) of **7n**, giving a synthesis PMI of 42.3.

A sample of the crude product was purified by preparative HPLC with the following conditions: column, C8 column, mobile phase, MeCN in H_2O (0.05% TFA), 70-80% gradient in 60 min; detection wavelength: 210 nm to provide **7n** as a white solid in 96.7 % HPLC purity (**Method 14**) for ^{1}HNMR, ^{13}CNMR, and FTIR test.

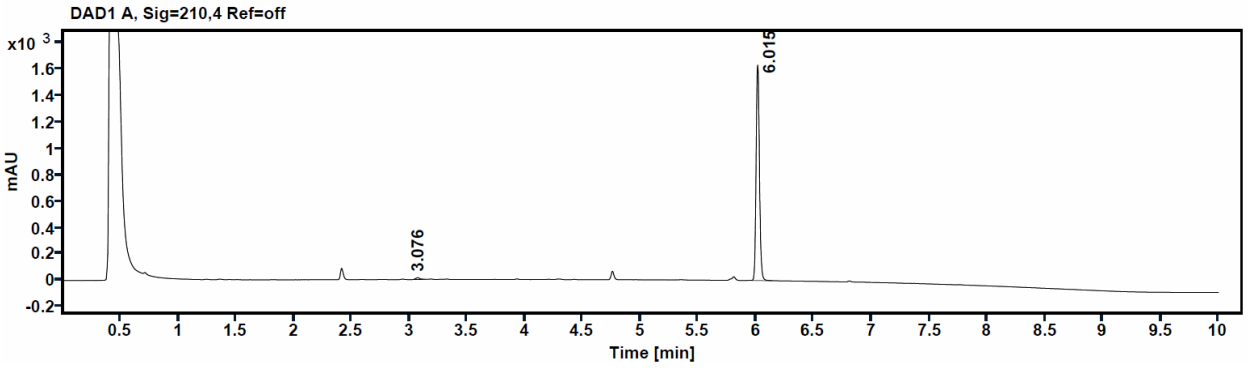
mp 135 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (s, 1H), 8.65 (s, 2H), 8.19 - 8.03 (m, 3H), 7.89 (d, J = 7.5 Hz, 2H), 7.82 (d, J = 7.9 Hz, 2H), 7.69 (d, J = 7.5 Hz, 2H), 7.45 - 7.28 (m, 22H), 7.25 - 7.15 (m, 21H), 5.07 (s, 1H), 4.89 (s, 1H), 4.60 - 4.36 (m, 4H), 4.29 - 4.18 (m, 3H), 3.79 - 3.71 (m, 3H), 3.50 (s, 3H), 3.25 - 3.06 (s, 6H), 2.82 - 2.56 (m, 4H), 1.86 - 1.58 (m, 8H), 1.54 (s, 9H), 1.43 (s, 9H), 1.25 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.64, 170.34, 169.62, 168.40, 167.71, 163.83, 156.23, 155.31, 154.31, 143.55, 142.81, 142.71, 142.27, 139.55, 128.26, 128.21, 127.79, 127.36, 127.17, 126.71, 126.45, 126.29, 125.91, 125.77, 125.18, 124.07, 118.94, 84.98, 79.32, 78.61, 76.87, 68.19, 64.16, 51.19, 51.02, 49.49, 46.65, 45.56, 40.91, 36.60, 35.77, 35.35, 33.38, 30.58, 27.08, 26.66, 26.41, 25.51. HRMS (ESI): m/z calcd for $C_{101}H_{117}N_{12}O_{19}$ $[M+H]^+$: 1873.8552, found: 1873.8513. FTIR (neat): 3310, 3058, 2975,2934, 1639, 1492, 1448, 1367, 1291, 1202, 1157, 1114, 1033, 901, 848, 743, 698 cm^{-1}.

HPLC of **S37** formation (reaction mixture)



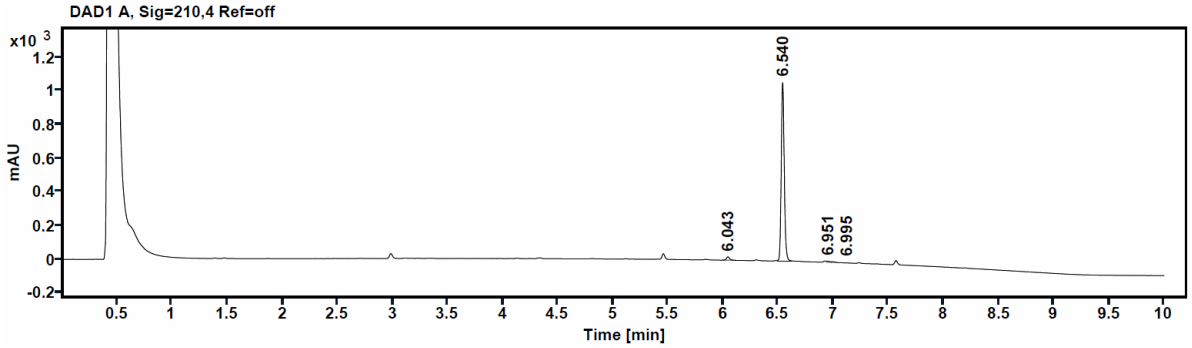
Compound ID	Time	Area	Height	Area%
S37-over-addition	3.642	-	-	0.0
S37	4.151	2042.4821	1362.3	99.3
S19	4.660	14.7077	8.7	0.7
Total	-	-	-	100.0

HPLC of **S38** formation (reaction mixture)



Compound ID	Time	Area	Height	Area%
S37	3.076	33.3921	13.7	1.0
***epi*-S37-INT**	4.401	-	-	0.0
S38	6.015	3288.9507	1641.3	99.0
***epi*-S38**	6.071	-	-	0.0
S38-over-addition	8.850	-	-	0.0
Total	-	-	-	100.0

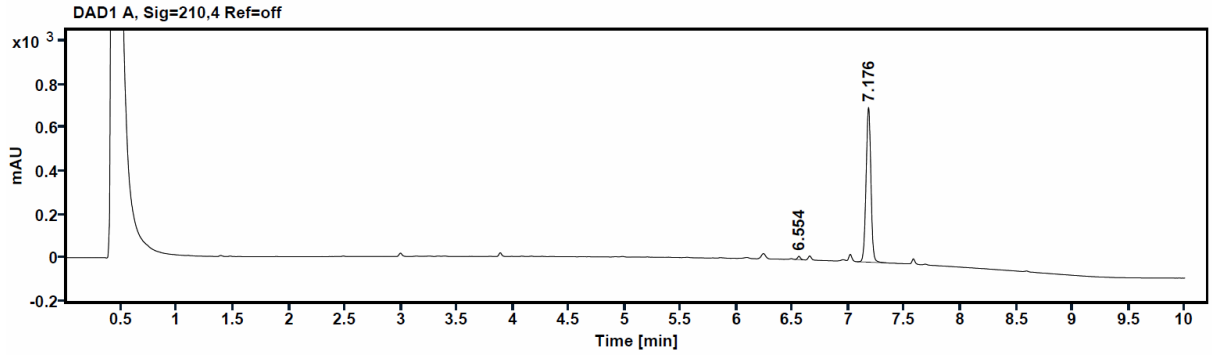
HPLC of **S39** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S38	6.043	41.5572	20.1	2.0
S39	6.540	2005.054	1061.7	97.1
***epi*-S39**	6.621	-	-	0.0
S38-INT + ***epi*-S38-INT**	6.951	12.4394	7.1	0.6*
S39-over-addition	6.995	6.1846	3.5	0.3
Total	-	-	-	100.0

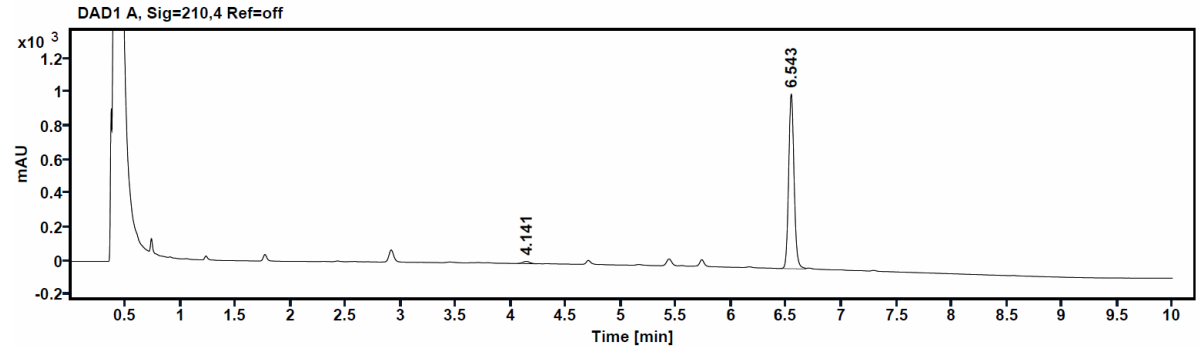
*Note: Contains 0.3% ***epi*-S38-INT**

HPLC of **S41** formation (reaction mixture)



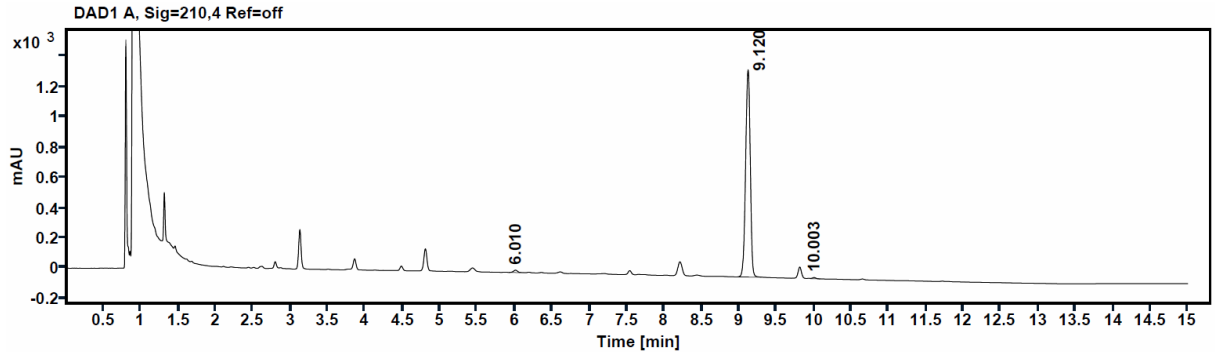
Compound ID	Time	Area	Height	Area%
S39	6.554	26.6871	15.2	1.2
S41	7.176	2141.1597	717.2	98.8
***epi*-S41**	7.250	-	-	0.0
***epi*-S39-INT**	7.610	-	-	0.0
S41-over-addition	7.862	-	-	0.0
Total	-	-	-	100.0

HPLC of **S42** formation (reaction mixture)



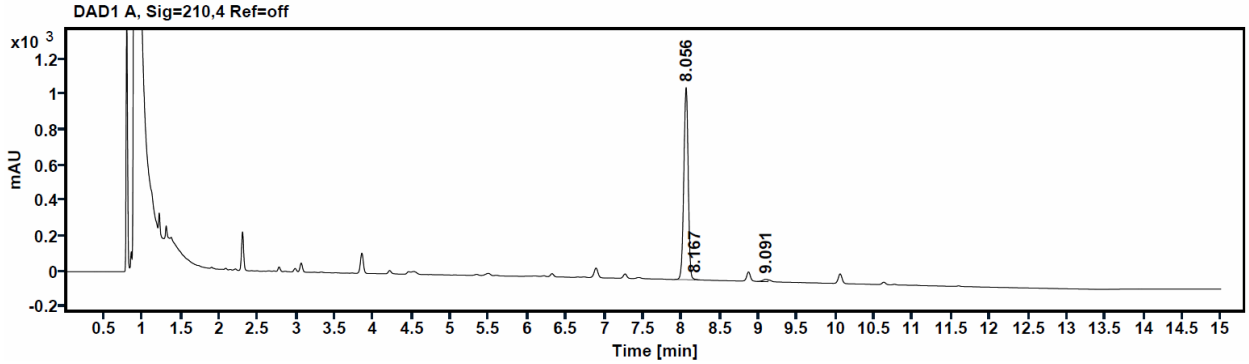
Compound ID	Time	Area	Height	Area%
S41	4.141	60.4312	13.6	1.7
***epi*-S41-INT**	5.251	-	-	0.0
S42	6.543	3547.7007	1035.6	98.3
***epi*-S42**	6.577	-	-	0.0
S42-over-addition	8.732	-	-	0.0
Total	-	-	-	100.0

HPLC of **S43** formation (reaction mixture)



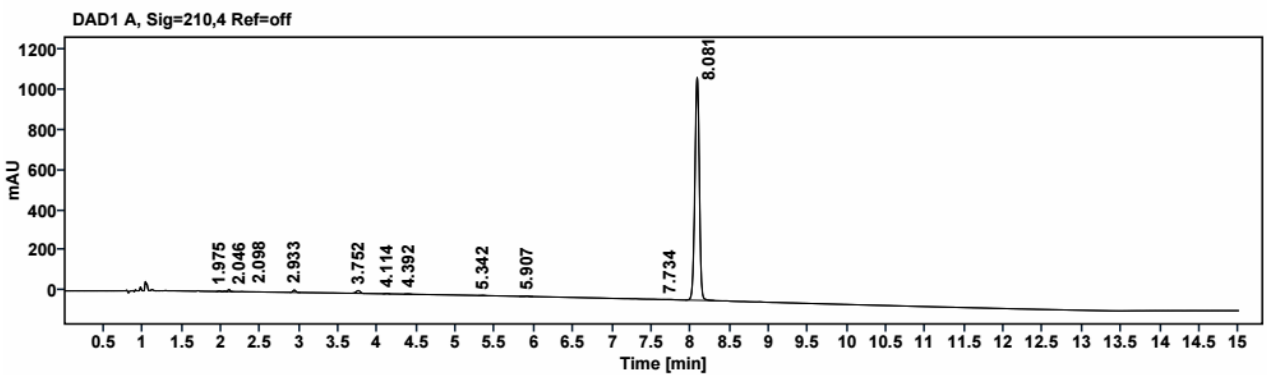
Compound ID	Time	Area	Height	Area%
S42	6.010	59.0004	15.7	1.0
***epi*-S42-INT**	7.890	-	-	0.0
S43	9.120	5947.7583	1374.8	98.6
***epi*-S43**	9.200	-	-	0.0
S43-over-addition	10.003	27.0953	8.1	0.4
Total	-	-	-	100.0

HPLC of **7n** formation (reaction mixture)

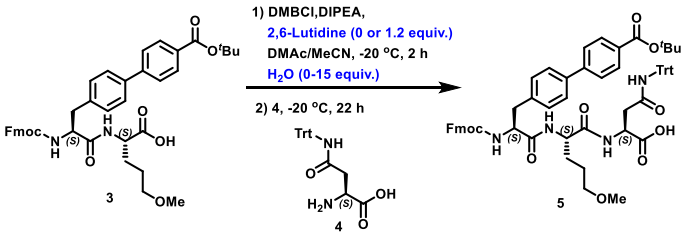


Compound ID	Time	Area	Height	Area%
7n-over-addition	7.058	-	-	0.0
7n	8.056	4311.6816	1085.2	98.5
***epi*-7n**	8.167	7.1047	4.2	0.2
S43	9.091	58.0738	13.5	1.3
***epi*-S43-INT**	9.501	-	-	0.0
Total	-	-	-	100.0

HPLC of isolated **7n**



Supplementary Table 10. Water tolerance.



Entry	Stage 1: mixed anhydride formation					Stage 2: amide coupling					
	Condition		HPLC result (LCAP, 210 nm)			HPLC result (LCAP, 210 nm)					
	Reagents/ solvents	H_2O (equiv.)	**3**	**3-mixed anhydride**	**3-oxazolone**	**3**	**3-mixed anhydride**	**5**	***epi*-5**	**3-oxazolone**	**6**
1	DMBCl (1.1 equiv.) DIPEA (1.0 equiv.) **2,6-lutidine (1.2 equiv.)**/ DMAc/MeCN (1:1, 10V)	**0**	1	97.5	1.5	0.5	0.2	97.6	0.3	1.0	0.1
2		**2**	0.8	96.8	2.4	0.5	0.5	96.9	0.5	1.4	0.2
3		**5**	0.7	96.8	2.6	0.6	0.7	96.3	0.6	1.5	0.3
4		**10**	0.7	96.8	2.6	0.7	0.7	95.8	0.6	1.9	0.3
5		**15**	0.6	96.7	2.7	0.6	2.6	93.5	0.7	2.2	0.4
6	DMBCl (1.1 equiv.) DIPEA (1.2 equiv.)/ **No 2,6-lutidine** / DMAc (10 V)	**0**	2.6	93.1	4.3	1.2	0.1	93.8	0.6	3.5	0.4
7		**2**	2.9	87.8	9.3	2.7	0.3	89.7	1.1	6.1	0
8		**5**	3.6	84.6	11.9	3.0	0.5	87.6	1.5	7.3	0.1
9		**10**	3.7	80.7	15.6	3.2	0.6	84.4	2.1	9.7	0
10		**15**	4.1	74.7	21.3	3.7	0.7	79.7	3.1	12.9	0

Procedure:

Entry 1-5:

In a 20-mL vial, **3** (500 mg, 0.72 mmol) was dissolved in DMAc/MeCN (1:1, 5 mL). After the solution was cooled to -20 °C, DIPEA (93.3 mg, 0.72 mmol) and 2,6-lutidine (92.1 mg, 0.86 mmol) were added, along with H_2O (0-10.8 mmol). Then DMBCl (106.9 mg, 0.79 mmol) was added, and the reaction mixture was stirred at -20 °C for 2 h. **4** (297.3 mg, 0.79 mmol) was then added to the reaction mixture. The reaction mixture was stirred at -20 °C for 22 h.

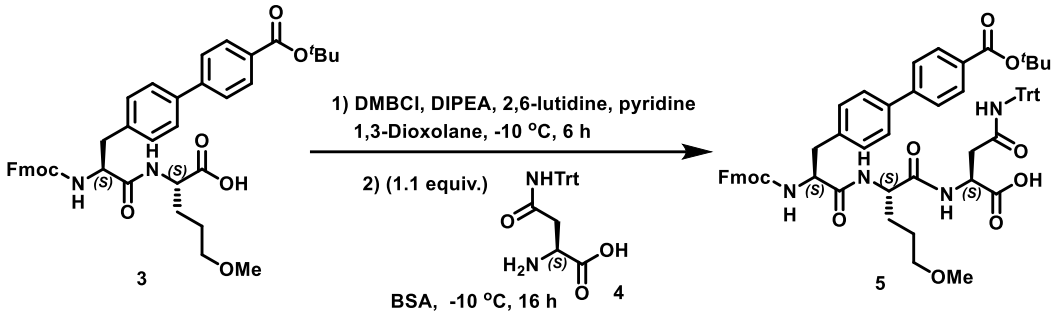
Entry 6-10:

In a 20-mL vial, **3** (500 mg, 0.72 mmol) was dissolved in DMAc (5 mL). After the solution was cooled to -20 °C, DIPEA (93.3 mg, 0.86 mmol) and H_2O (0-10.8 mmol) were added. Then DMBCl (109.8 mg, 0.81

mmol) was added, and the reaction mixture was stirred at -20 °C for 2 h. **4** (297.3 mg, 0.79 mmol) was then added to the reaction mixture. The reaction mixture was stirred at -20 °C for 22 h.

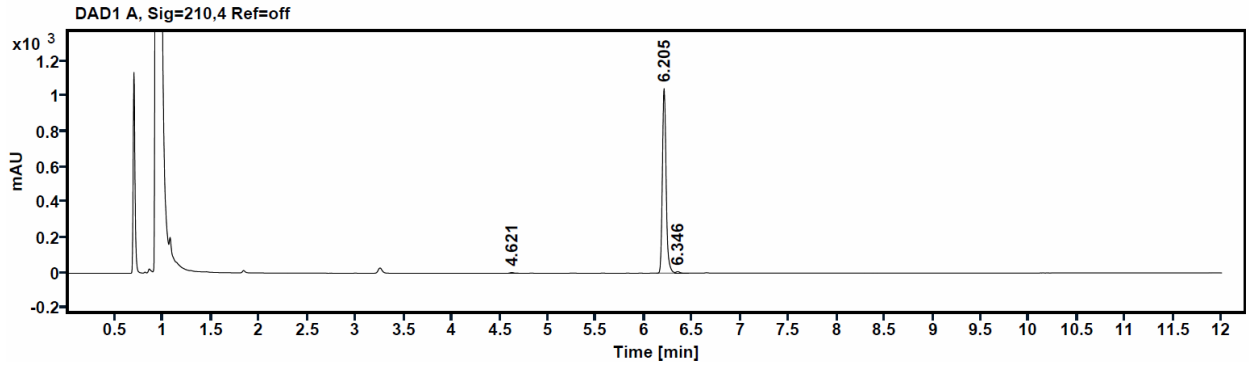
Alternative solvent and activation systems

1,3-Dioxolane as a more sustainable solvent



Synthesis of Fmoc-Bip-4-CO_2*t*Bu-5-OMe-NVA-L-Gln(Trt)-OH (5) in 1,3-dioxolane. In a 20 mL vial, **3** (1.00 g, 1.44 mmol) was dissolved in 1,3-dioxolane (5 mL). The solution was cooled to -10 °C, DIPEA (186.55 mg, 1.44 mmol) and 2,6-lutidine (154.66 mg, 1.44 mmol) and pyridine (22.83 mg, 0.29 mmol) and DMBCl (203.1 mg, 1.51 mmol) was added into the reaction mixture. The reaction mixture was stirred at -10 °C for 6 h and monitored by HPLC (**Method 1**), which showed the ratio of **3**:**3-INT**:***epi*-3-INT** was 0.4:99.2:0.4 (0.5 h), 0.4:99.1:0.5 (2 h), 0.4:98.7:0.9 (6 h). A solution of **4** and BSA in 1,3-dioxolane, which was prepared by mixing **4** (594.45 mg, 1.58 mmol) and BSA (293.01 mg, 1.44 mmol) in 1,3-dioxolane (5 mL), stirred at 50 °C for 20 min until clear, then cooling to 20 °C, was slowly added into the reaction mixture at -10 °C over 5 min. The reaction mixture was stirred at -10 °C for 16 h, and the reaction solution was monitored by HPLC (**Method 1**), which showed the ratio of **3**:**5**:***epi*-5**:***epi*-3-INT**:**6** was 0.7:98.1:0.8:0.4:0.0.

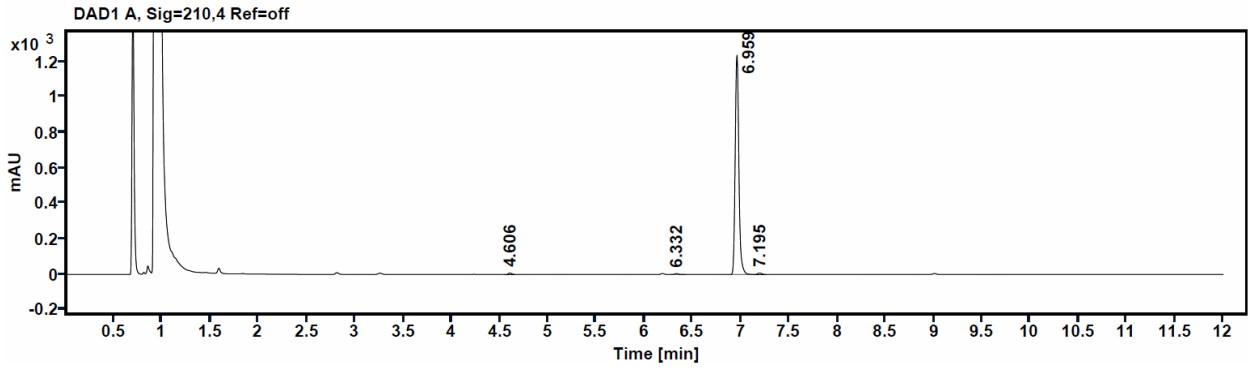
HPLC of **3-INT** formation (reaction mixture)



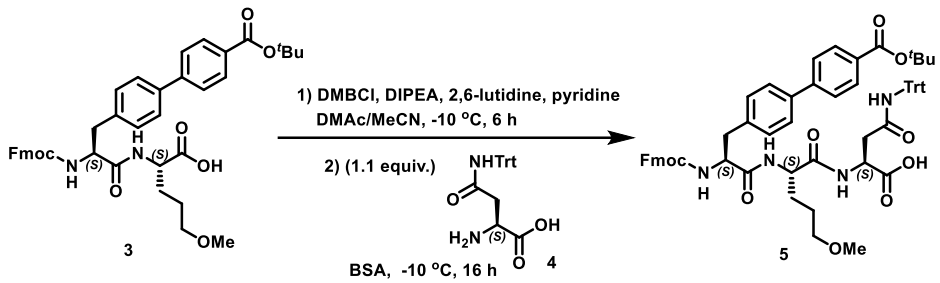
Compound ID	Time	Area	Height	Area% ratio

3	4.621	11.3318	4.2	0.4
3-INT	6.205	2777.9478	1046.6	98.7
***epi*-3-INT**	6.346	25.4764	8.8	0.9
Total	-	-	-	100.0

HPLC of **5** formation (reaction mixture)



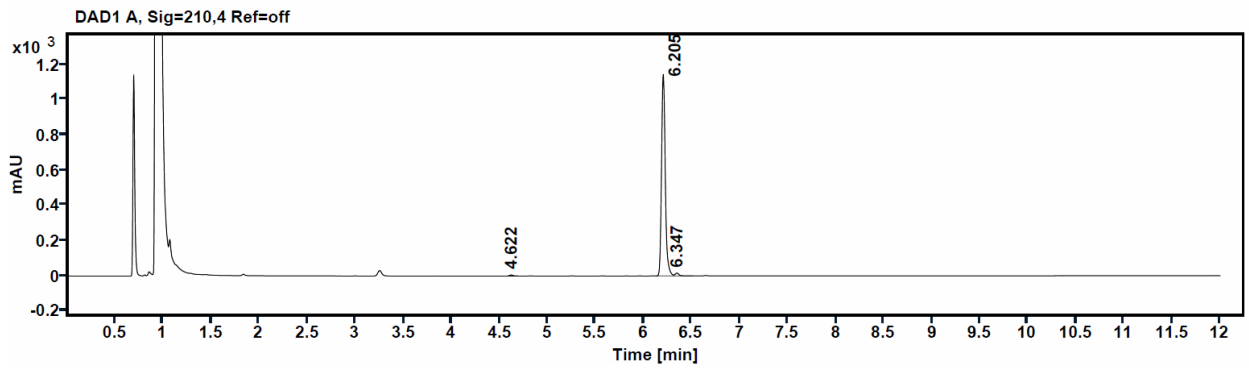
Compound ID	Time	Area	Height	Area% ratio
3	4.606	22.1493	8.8	0.7
***epi*-3-INT**	6.332	14.4071	5.5	0.4
5	6.959	3214.9097	1239.0	98.1
***epi*-5**	7.195	26.6335	8.9	0.8
6	9.007	0	0	0.0
Total	-	-	-	100.0



Synthesis of Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-L-Gln(Trt)-OH (5) in DMAc/MeCN. In a 20 mL vial, **3** (1.00 g, 1.44 mmol) was dissolved in DMAc/MeCN (1:1, 5 mL). The solution was cooled to -10 °C, DIPEA (186.55 mg, 1.44 mmol) and 2,6-lutidine (154.66 mg, 1.44 mmol) and pyridine (22.83 mg, 0.29

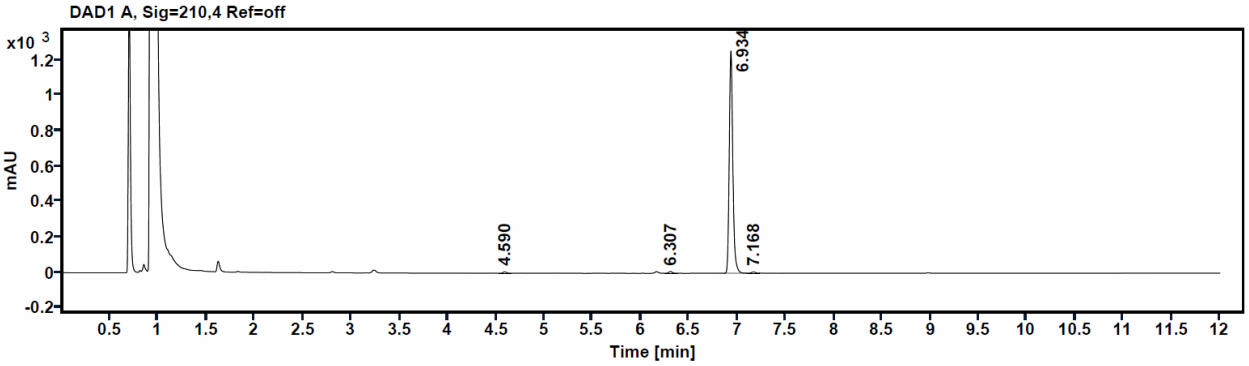
mmol) and DMBCl (203.1 mg, 1.51 mmol) was added into the reaction mixture. The reaction mixture was stirred at -10 °C for 6 h and monitored by HPLC (**Method 1**), which showed the ratio of **3:3-INT:*epi*-3-INT** was 0.5:99.0:0.5 (0.5 h), 0.4:98.8:0.7 (2 h), 0.5:98.0:1.5 (6 h). A solution of **4** and BSA in DMAc:MeCN, which was prepared by mixing **4** (594.45 mg, 1.58 mmol) and BSA (293.01 mg, 1.44 mmol) in DMAc/MeCN (1:1, 5 mL), stirred at 50 °C for 20 min until clear, then cooling to 20 °C, was slowly added into the reaction mixture at -10 °C over 5 min. The reaction mixture was stirred at -10 °C for 16 h, and the reaction solution was monitored by HPLC (**Method 1**), which showed the ratio of **3:5:*epi*-5:*epi*-3-INT:6** was 0.6:97.9:0.7:0.8:0.0.

HPLC of **3-INT** formation (reaction mixture)



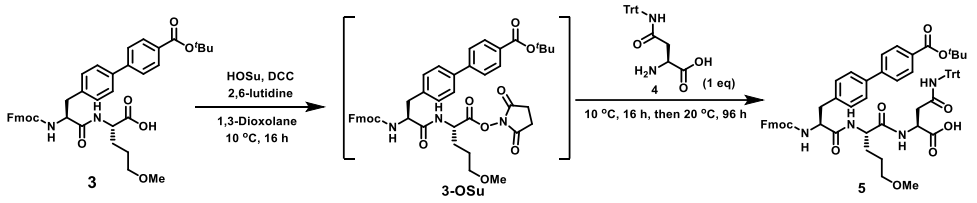
Compound ID	Time	Area	Height	Area% ratio
3	4.622	16.4721	6.2	0.5
3-INT	6.205	3046.2937	1145.1	98.0
***epi*-3-INT**	6.347	46.5696	16.3	1.5
Total	-	-	-	100.0

HPLC of **5** formation (reaction mixture)



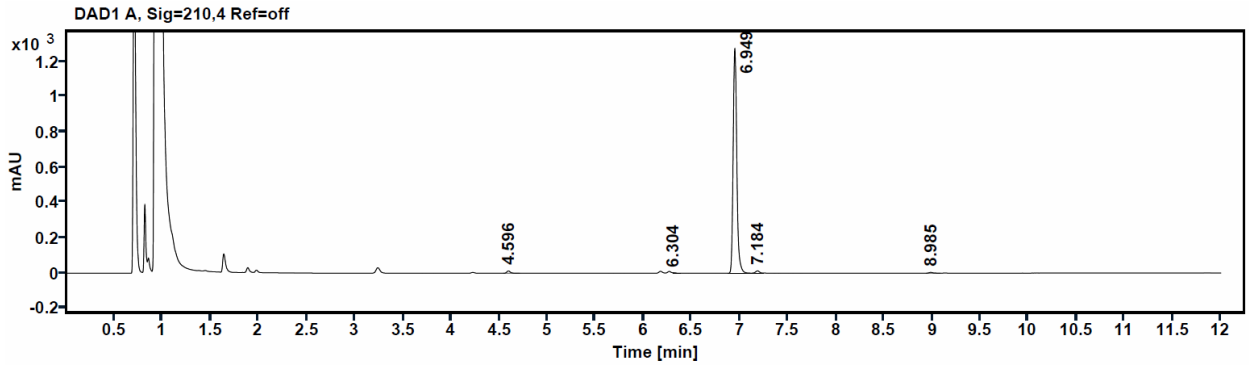
Compound ID	Time	Area	Height	Area% ratio
3	4.59	21.0987	8.4	0.6
***epi*-3-INT**	6.307	26.7691	10.1	0.8
5	6.934	3239.5273	1257.3	97.9
***epi*-5**	7.168	22.9141	7.8	0.7
6	9.007	0	0	0.0
Total	-	-	-	100.0

Improved DCC/HOSu activation system



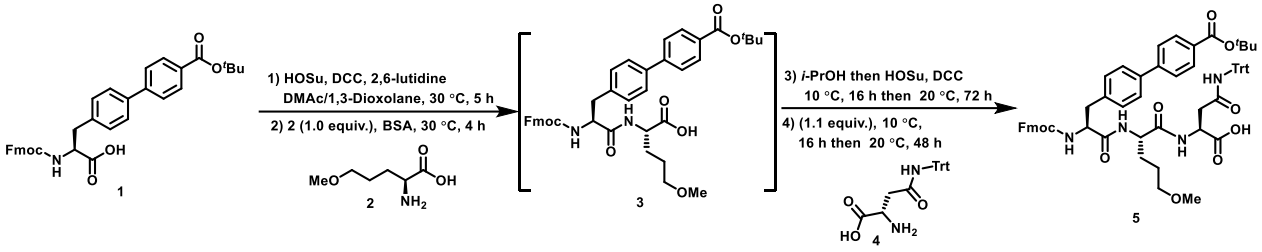
Synthesis of Fmoc-Bip-4-CO_2*t*Bu-5-OMe-NVA-L-Gln(Trt)-OH (5). In an 8 mL vial, **3** (1.00 g, 1.44 mmol), HOSu (181.84 mg, 1.58 mmol) and 2,6-lutidine (154.31 mg, 1.44 mmol) were dissolved in 1,3-Dioxolane (10 mL). The solution was cooled to 10 °C, DCC (311.97 mg, 1.51 mmol) was added into the reaction mixture. The reaction mixture was stirred at 10 °C for 16 h and monitored by HPLC (**Method 1**), which showed the ratio of **3:3-INT:*epi*-3-INT** (OSu ester intermediate quenched by $BnNH_2$) was 0.3:98.4:1.3. Then **4** (539.19 mg, 1.44 mmol) was added into the reaction mixture. The reaction mixture was stirred at 10 °C for 16 h and then stirred at 20 °C for 96 h, the reaction solution was monitored by HPLC (**Method 1**), which showed the ratio of **3:5:*epi*-5:*epi*-3-INT:6** was 1.0:97.4:1.1:0.2:0.3. The assay yield of the reaction was 93%.

HPLC of **5** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
3	4.596	34.2585	13.3	1.0
***epi*-3-INT**	6.304	5.6413	2.6	0.2
5	6.949	3306.0105	1276.8	97.4
***epi* -5**	7.184	37.8755	13.2	1.1
6	8.985	10.804	4.3	0.3
Total	-	-	-	100.0

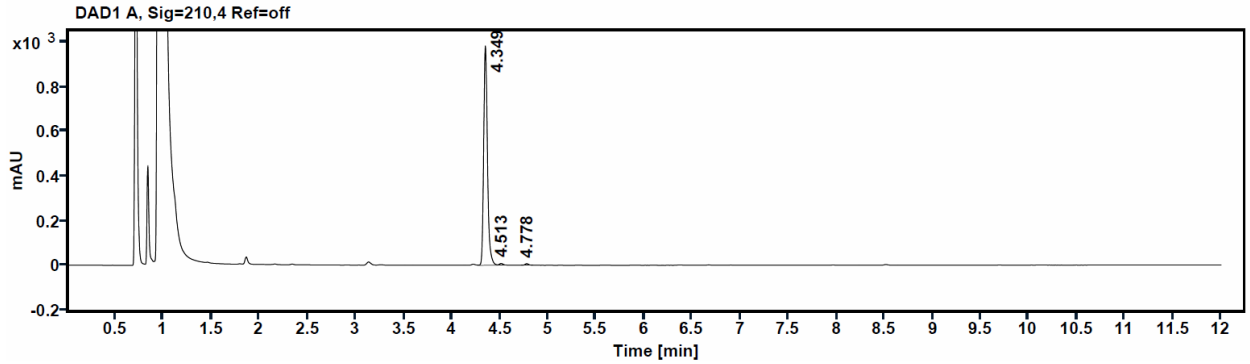
Continuous Elongation with DCC/HOSu method to prepare 5.



Synthesis of Fmoc-Bip-4-CO_2*t*Bu-5-OMe-NVA-L-Gln(Trt)-OH (5). In a 40 mL vial, **Fmoc-Bip-4-CO_2*t*Bu-OH (1)** (1.00 g, 1.77 mmol), HOSu (0.22 g, 1.95 mmol) and 2,6-lutidine (0.19 g, 1.77 mmol) were dissolved in DMAc/1,3-dioxolane (1:1, 5 mL). Then DCC (0.38 g, 1.86 mmol) was added into the reaction mixture over 10 min. The reaction mixture was stirred at 30 °C for 5 h and monitored by HPLC (**Method 1**), which showed the ratio of **1:1-INT:*epi*-1-INT** (-OSu ester intermediate quenched by $BnNH_2$) was 0.6:98.8:0.6. A solution of **2** and BSA in DMAc/1,3-dioxolane was prepared by mixing **2** (0.26 g, 1.77 mmol) and BSA (0.72 g, 3.54 mmol) in DMAc/1,3-dioxolane (1:1, 5 mL), stirring at 50 °C for 1 h, then cooling to 30 °C. This solution was added slowly to the reaction mixture at 30 °C over 10 min. The reaction mixture was stirred at 30 °C for 4 h and monitored by HPLC (**Method 1**), which showed the ratio of **1:3:*epi*-3:3-over-addition** was 0.7:98.4:0.9:0.0. Then *i*-PrOH (0.42 g, 7.08 mmol) was added into the reaction mixture at 0 °C and stirred for 1 h; the mixture was then heated to 10 °C, HOSu (0.31 g, 2.66 mmol) and DCC (0.41 g, 1.99 mmol) were added into the reaction mixture. The reaction mixture was stirred at 10 °C for 16 h, then stirred at 20 °C for 72 h and monitored by HPLC (**Method 1**), which showed the ratio of **3:3-INT:*epi*-3-INT** (-OSu ester intermediate quenched by $BnNH_2$) was 0.9:97.2:1.9. Then **4** (0.72 g, 1.91 mmol) was added into the reaction mixture. The reaction mixture was stirred at 10 °C for 16 h, then stirred at 20 °C for 48 h and monitored by HPLC (**Method 1**), which showed the ratio of **3:5:*epi*-5:*epi*-3-INT:6** was 0.8:95.4:2.4:0.3:1.1. The assay yield was 89% over two couplings.

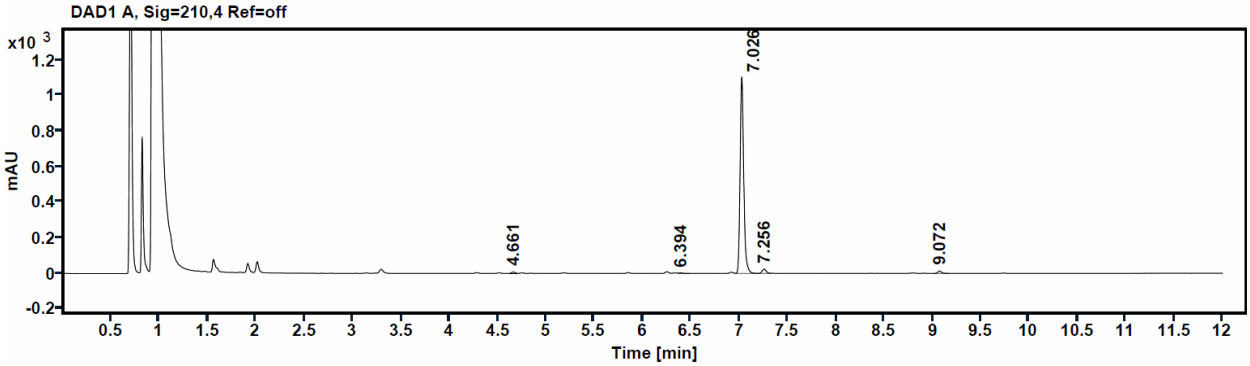
Method 1: SB-Aq 4.6 × 100 mm, particle size 1.8 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 55-95% (solvent B) in 11 min, hold 1 min; flow rate: 1.2 mL/min; detection wavelength: 210 nm.

HPLC of **3** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
3-over-addition	4.125	0.0	0.0	0.0
3	4.349	2655.4036	988.3	98.4
***epi*-3**	4.513	23.9315	8.6	0.9
1	4.778	19.7389	7.5	0.7
Total	-	-	-	100.0

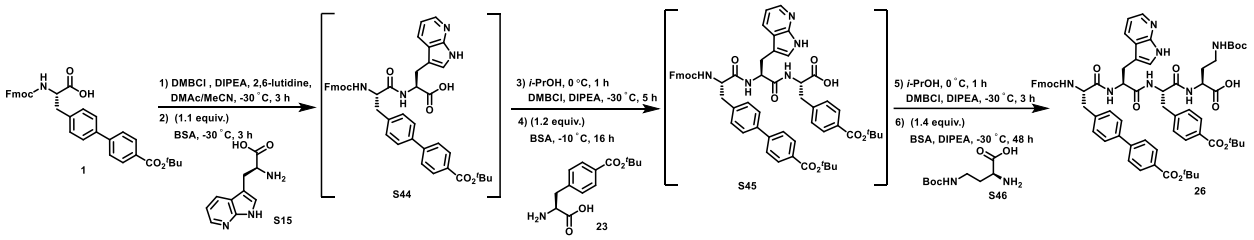
HPLC of **5** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
3	4.661	22.6629	9	0.8
***epi*-3-INT**	6.394	9.7561	3.6	0.3
5	7.026	2870.8364	1106.2	95.4

***epi* -5**	7.256	71.5305	24.4	2.4
6	9.072	33.815	13.6	1.1
Total	-	-	-	100.0

Experimental procedures for **8-10**



Synthesis of Fmoc-Bip-4-CO_2*t*Bu-7-aza-Trp-4-CO_2*t*BuPhe-L-Dab(Boc)-OH (26). In a 1000 mL 3-neck round bottom flask, **Fmoc-Bip-4-CO_2*t*Bu-OH (1)** (20.00 g, 35.48 mmol) and 2,6-lutidine (3.80 g, 35.48 mmol) were dissolved in DMAc/MeCN (1:1, 100 mL). The solution was cooled to -30 °C, and DMBCl (5.25 g, 39.03 mmol) and DIPEA (4.59 g, 35.48 mmol) were added to the reaction mixture. The reaction mixture was stirred at -30 °C for 3 h and monitored by HPLC **(Method 1)**, which showed the ratio of **1:1-INT** was 0.8:99.2. A solution of **7-aza-Trp-OH (S15)** and BSA in DMAc, which was prepared by mixing **S15** (8.01 g, 39.03 mmol) and BSA (14.44 g, 70.97 mmol) in DMAc (100 mL), stirred at 50 °C for 1 h until clear, then cooling to 20 °C, was added slowly into the reaction mixture at -30 °C over 1 h. The reaction mixture was stirred at -30 °C for 3 h and monitored by HPLC **(Method 1)**, which showed the ratio of **1:S44:S44-over-addition** was:0.5:99.5:0.0. Then *i*-PrOH (8.53 g, 141.93 mmol) was added to the reaction mixture at 0 °C and stirred for 1 h. The mixture was then cooled to -30 °C. DMBCl (6.69 g, 49.68 mmol) and DIPEA (5.96 g, 46.13 mmol) were added to the reaction mixture, and stirred for 5 hours at -30 °C. The reaction was monitored by HPLC **(Method 1)**, which showed the ratio of **S44:(S44-INT + *epi*-S44-INT)** was 1.1:98.9 (contains 0.4% ***epi*-S44-INT**). **S44-INT and *epi*-S44-INT** were quenched by $BnNH_2$ and monitored by HPLC **(Method 2)**. A solution of **4-CO_2*t*BuPhe-OH (23)** and BSA in DMAc and MeCN, which was prepared by mixing **23** (11.30 g, 42.58 mmol) and BSA (14.44 g, 70.97 mmol) in DMAc/MeCN (1:1, 100 mL), stirred at 50 °C for 1 h until clear, then cooling to 20 °C, was added slowly into the reaction mixture at -30 °C over 1 h. The reaction mixture was stirred at -10 °C for 16 h and monitored by HPLC **(Methods 1 and 3)**, which showed the ratio of **S44:(S45+*epi*-S45):(S44-INT+*epi*-S44-INT):S45-over-addition** was 1.2:98.0 (**S45:*epi*-S45** ratio is **100:0** monitored by HPLC **Method 3**): 0.8:0.0. Then *i*-PrOH (8.53 g, 141.93 mmol) was added to the reaction mixture at 0 °C, and stirred at 0 °C for 1 h. Then the reaction mixture was cooled to -30 °C. DMBCl (7.16 g, 53.22 mmol) and DIPEA (6.42 g, 49.68 mmol) were added to the reaction mixture at -30 °C and stirred for 3 h and monitored by HPLC **(Method 1).** HPLC

showed the ratio of **S45:(S45-INT+*epi*-S45-INT)** was 0.8:99.2. **S45-INT and *epi*-S45-INT** were quenched by $BnNH_2$ and monitored by HPLC **(Method 4)**. A solution of **L-Dab(Boc)-OH (S46)**, BSA and DIPEA in DMAc and MeCN, which was prepared by mixing **S46** (10.84 g, 49.68 mmol) and BSA (14.44 g, 70.97 mmol) in DMAc/MeCN (1:1, 100 mL), stirred at 50 °C for 1 h until clear, cooling to 20 °C and then adding DIPEA (2.29 g, 17.74 mmol), was added slowly into the reaction mixture at -30 °C over 10 min. The reaction mixture was stirred at -30 °C for 48 h and monitored by HPLC **(Methods 5 and 6)**, which showed the ratio of **S45:(26+*epi*-26):(S45-INT+ *epi*-S45-INT):26-over-addition** was 1.5:97.7 **(26:*epi*-26** ratio is **99.5:0.5** monitored by HPLC **Method 6)**: 0.5:0.3. The assay yield of the reaction was 89% over three couplings**.** The reaction mixture was warmed to -10 °C. H_2O (200 mL) was added slowly to the reaction mixture over 1 h, and the mixture was stirred at -10 °C for 3 h. The solid was collected by filtration and slurried in H_2O (100 mL) at 10 °C for 3 h. The solid was collected by filtration and dried under vacuum to provide 36.30 g of **26** as a white solid (99.4% LCAP) in an 85% isolated yield over three steps **(Method 5)**.

mp 230-232 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.80 (s, 1H), 11.34 (s, 1H), 8.36 (d, J = 8.0 Hz, 1H), 8.20 (d, J = 8.8 Hz, 2H), 8.15 (dd, J = 4.8, 1.6 Hz, 1H), 7.98 (dd, J = 8.0, 1.6 Hz, 1H), 7.93 (d, J = 8.4 Hz, 2H), 7.86 (d, J = 7.6 Hz, 2H), 7.77 (d, J = 8.0 Hz, 2H), 7.68 (d, J = 8.4 Hz, 2H), 7.62 (t, J = 8.0 Hz, 2H), 7.57 (d, J = 8.8 Hz, 1H), 7.53 (d, J = 8.0 Hz, 2H), 7.40 - 7.33 (m, 6H), 7.30 - 7.22 (m, 3H), 7.00 (dd, J = 8.0, 4.8 Hz, 1H), 6.83 (t, J = 5.6 Hz, 1H), 4.68 - 4.58 (m, 2H), 4.31 - 4.25 (m, 1H), 4.24 - 4.18 (m, 1H), 4.15 - 4.10 (m, 3H), 3.14 - 3.13 (m, 1H), 3.12 - 3.11 (m, 1H), 3.07 - 2.86 (m, 4H), 2.73 (dd, J = 13.6, 10.8 Hz, 2H), 1.94 - 1.87 (m, 1H), 1.77 - 1.68 (m, 1H), 1.57 (s, 9H), 1.47 (s, 9H), 1.37 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.87, 171.81, 171.47, 171.02, 165.38, 165.25, 156.21, 156.03, 148.91, 144.65, 144.26, 144.17, 143.33, 142.70, 141.12, 138.94, 137.21, 130.48, 130.35, 130.07, 129.97, 129.91, 129.26, 128.03, 127.48, 127.15, 126.97, 126.91, 125.80, 124.65, 120.49, 120.15, 115.25, 109.33, 81.11, 80.79, 78.11, 66.15, 56.46, 53.75, 53.61, 50.73, 47.01, 38.08, 37.57, 31.86, 28.70, 28.27, 28.18. HRMS (ESI): m/z calcd for $C_{68}H_{76}N_7O_{13}$ $[M+H]^+$: 1198.5496, found: 1198.5551. FTIR (neat): 3287, 2975, 1710, 1671, 1636, 1609, 1519, 1445, 1415, 1392, 1366, 1289, 1249, 1164, 1112, 1018, 848, 772, 759, 737, 699 cm^{-1}.

Method 1: HALO C18 3.0 × 100 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 10-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 2: HALO C8 4.6 × 150 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), MeOH (solvent B); gradient 70-95% (solvent B) in 15 min, hold 2 min; flow rate: 1.2 mL/min; detection wavelength: 210 nm.

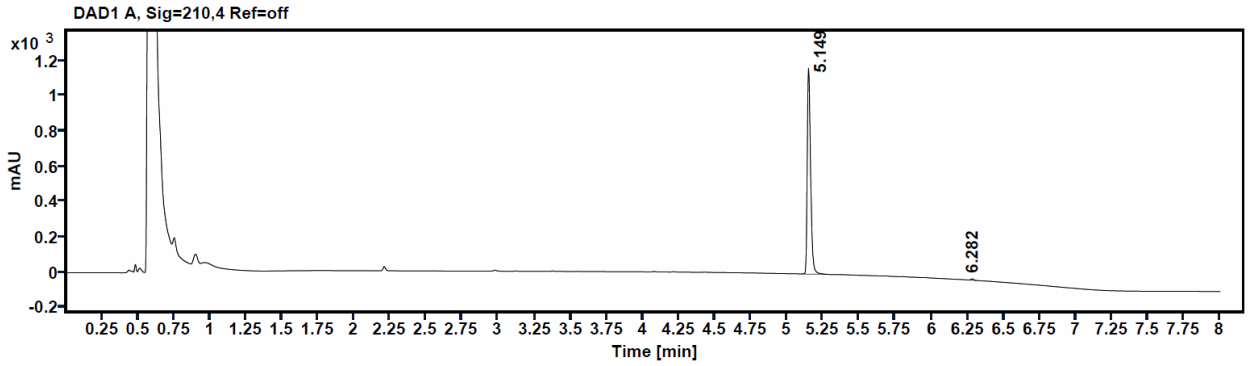
Method 3: XSelect CSH C18 3.0 × 100 mm, particle size 2.5 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 55-70% (solvent B) in 4 min; 70-85% (solvent B) in 10 min; 85-95% (solvent B) in 11 min; hold 1 min; flow rate: 0.8 mL/min; detection wavelength: 210 nm.

Method 4: HALO C8 4.6 × 150 mm, particle size 2.7 μm column; 0.1% TFA (v/v) in H_2O (solvent A), MeOH (solvent B); gradient 70-95% (solvent B) in 10 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 5: HALO phenyl-hexyl 4.6 × 150 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 30-95% (solvent B) in 12 min, hold 3 min; flow rate: 1.2 mL/min; detection wavelength: 210 nm.

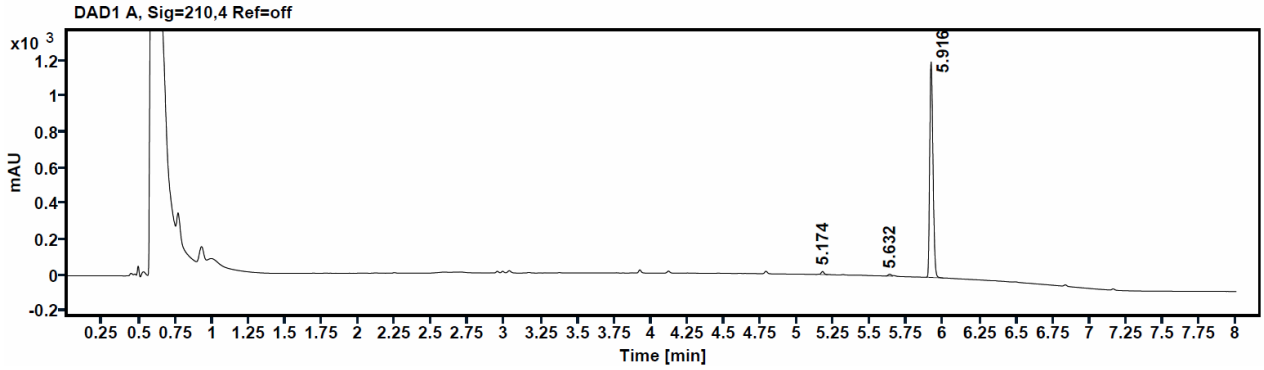
Method 6: HALO phenyl-hexyl 4.6 × 150 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), MeOH (solvent B); gradient 85-90% (solvent B) in 6 min, 90-95% (solvent B) in 14 min, hold 2 min; flow rate: 1.2 mL/min; detection wavelength: 210 nm.

HPLC of **S44** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S44-over-addition	4.654	0.0	0.0	0.0
S44	5.149	1899.1085	1170.0	99.5
1	6.282	10.0559	7.5	0.5
Total	-	-	-	100.0

HPLC of **S45** formation (reaction mixture)

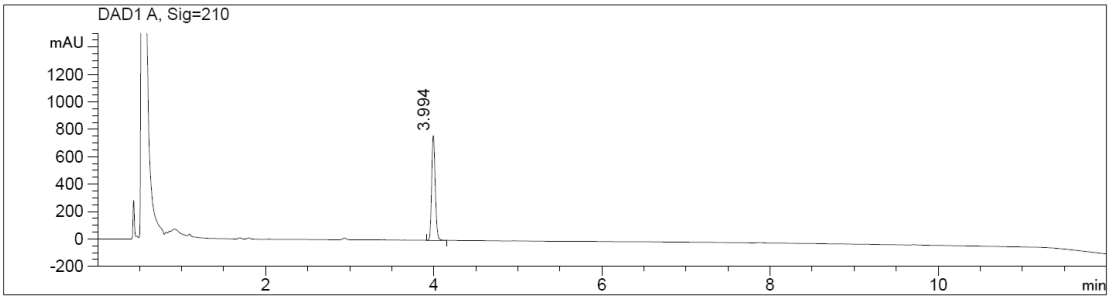


Compound ID	Time	Area	Height	Area% ratio
S44	5.174	22.7648	17.7	1.2
S44-INT + ***epi*-S44-INT**	5.632	15.2215	10.2	0.8*
S45 + ***epi*-S45**	5.916	1868.3599	1212.4	98.0**
S45-over-addition	7.135	0.0	0.0	0.0
Total	-	-	-	100.0

*Note: Contains 0.4% ***epi*-S44-INT**

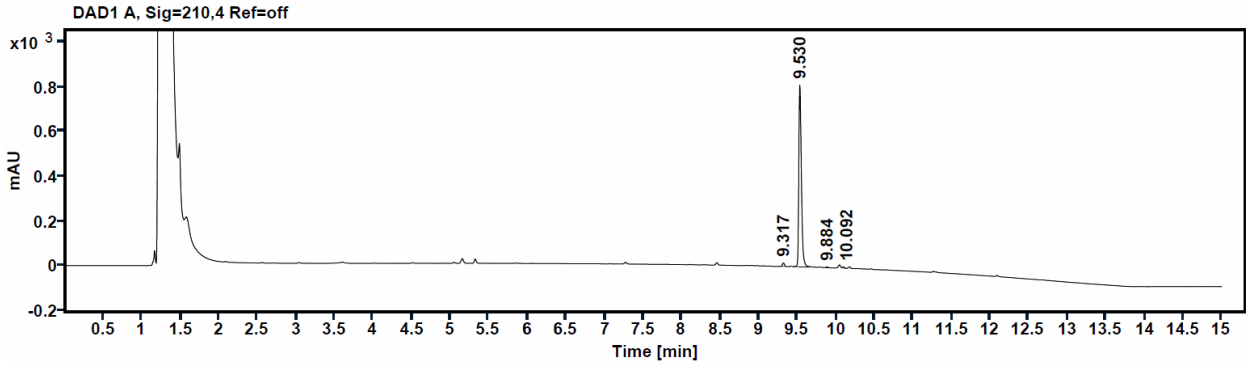
Note: Contains 0.0% ***epi*-S45

HPLC of **S45** chiral purity



Compound ID	Time	Area	Height	Area% ratio
***epi*-S45**	3.834	0.0	0.0	0.0
S45	3.992	2248.2	764.8	100.0
Total	-	-	-	100.0

HPLC of **26** formation (reaction mixture)

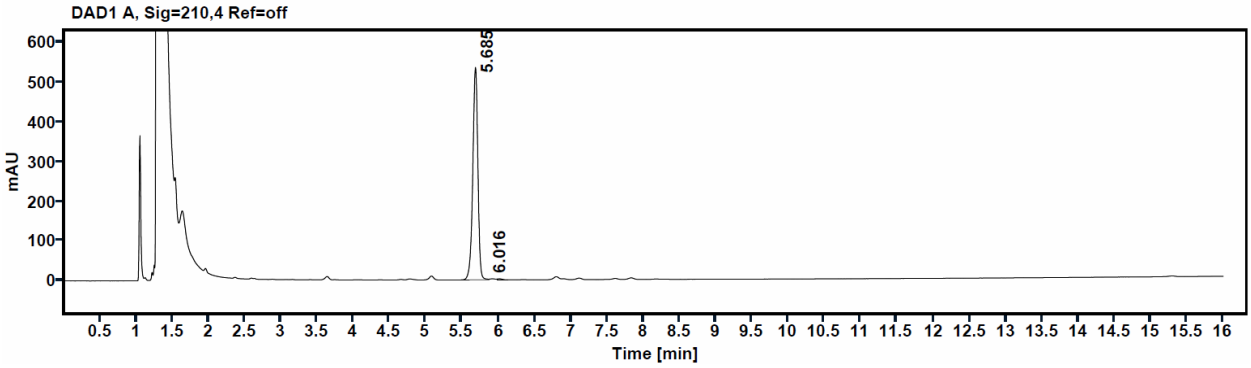


Compound ID	Time	Area	Height	Area% ratio
S45	9.317	28.3462	15.7	1.5
26+ ***epi*-26**	9.530	1873.2677	816.8	97.7*
26-over-addition	9.884	5.5972	3.2	0.3
S45-INT + ***epi*-S45-INT**	10.092	9.5952	5.7	0.5**
Total	-	-	-	100.0

*Note: Contains 0.5% ***epi*-26**

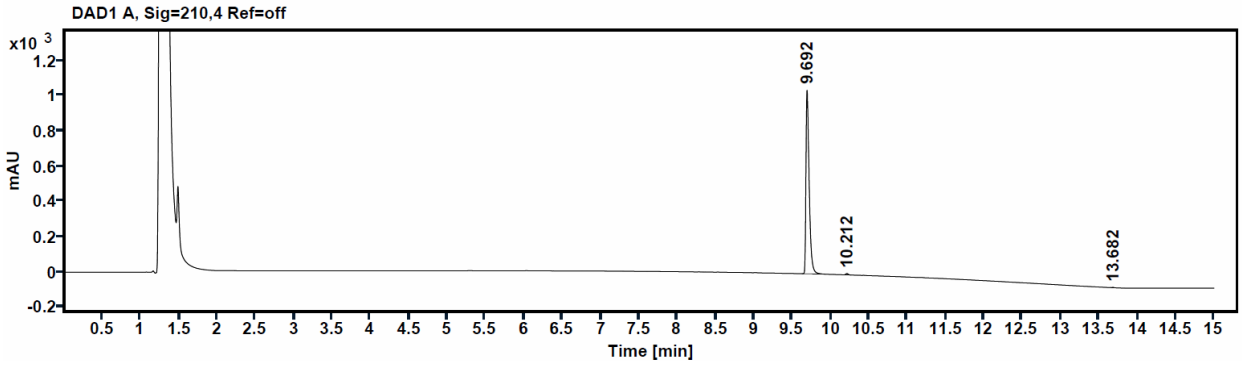
Note: Contains 0.3% ***epi*-S45-INT

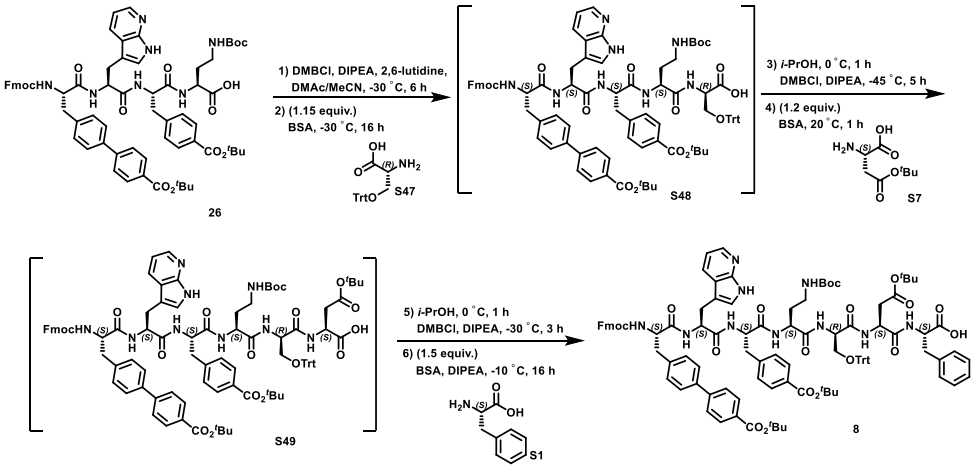
HPLC of **26** chiral purity



Compound ID	Time	Area	Height	Area% ratio
26	5.685	2611.8245	535.9	99.5
***epi*-26**	6.016	12.3492	2.7	0.5

HPLC of isolated **26**





Synthesis of Fmoc-Bip-4-CO$_2$*t*Bu-7-aza-Trp-L-pCO$_2$*t*BuPhe-L-Dab(Boc)-D-Ser(Trt)-L-Asp(O*t*Bu)-L-Phe-OH (8). In a 1000 mL 3-neck round bottom flask, **26** (20.00 g, 16.69 mmol) and 2,6-lutidine (1.79 g, 16.69 mmol) were dissolved in DMAc/MeCN (1:1, 100 mL). The solution was cooled to -30 °C, DMBCl (2.47 g, 18.36 mmol) and DIPEA (2.16 g, 16.69 mmol) were added to the reaction mixture. The reaction mixture was stirred at -30 °C for 6 h and monitored by HPLC **(Methods 1 and 7)**, which showed the ratio of **26:26-INT:*epi* -26-INT** was 99.3:0.7:0.0. **26-INT** and ***epi* -26-INT** were monitored by HPLC **(Method 7)**. A solution of **D-Ser(Trt)-OH (S47)** and BSA in DMAc and MeCN, which was prepared by mixing **S47** (6.67 g, 19.19 mmol) and BSA (5.09 g, 25.03 mmol) in DMAc/MeCN (1:1; 100 mL), stirred at 50 °C for 1 h until clear, then cooling to 20 °C, was added slowly into the reaction mixture at -30 °C over 1 h. The reaction mixture was stirred at -30 °C for 16 h and monitored by HPLC **(Methods 1 and 7)**, which showed the ratio of **26:(S48+*epi*-S48):(26-INT+ *epi*-26-INT):S48-over-addition** was: 0.8:99.2 **(S48:*epi*-S48** ratio is **100.0:0.0** monitored by HPLC **Method 7)**:0.0:0.0. Then *i*-PrOH (2.51 g, 41.72 mmol) was added to the reaction mixture at 0 °C and stirred for 1 h. The reaction mixture was cooled to -45 °C. DMBCl (3.14 g, 23.36 mmol) and DIPEA (2.80 g, 21.70 mmol) were added to the reaction mixture at -45 °C, stirred for 5 h, and monitored by HPLC **(Methods 7 and 8).** HPLC showed the ratio of **S48:S48-INT:*epi*-S48-INT** was 0.6:99.4:0.0. **S48-INT** and ***epi*-S48-INT** were monitored by HPLC **(Method 7).** The reaction solution was added slowly into a solution of **L-Asp(O*t*Bu)-OH (S7)** and BSA in DMAc and MeCN at 20 °C over 1 h,

which was prepared by mixing **S7** (3.79 g, 20.03 mmol) and BSA (5.09 g, 25.03 mmol) in DMAc/MeCN (1:1, 100 mL), stirred at 50 °C for 1 h until clear, then cooled to 20 °C. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC **(Methods 7 and 8)**, which showed the ratio of **S48:(S49 + *epi*-S49):(S48-INT+ *epi*-S48-INT):S49-over-addition** was 1.2:98.4 **(S49:*epi*-S49** ratio is **99.8:0.2** monitored by HPLC **Method 7)**: 0.4:0.0. Then *i*-PrOH (2.51 g, 41.72 mmol) was added to the reaction mixture at 0 °C, and the mixture was stirred for 1 h. The reaction mixture was cooled to -30 °C. DMBCl (4.27 g, 31.71 mmol) and DIPEA (3.88 g, 30.04 mmol) were added, and the mixture was stirred for 3 h, monitored by HPLC **(Methods 8 and 9)**. HPLC analysis revealed a ratio of **S49**:(**S49-INT** + ***epi*-S49-INT)** of 0.6:99.4 (**S49-INT:*epi*-S49-INT** ratio was 99.8:0.2 monitored reported HPLC **Method 9)**. The reaction solution was added slowly into a solution of **L-Phe-OH (S1)**, BSA and DIPEA in DMAc and MeCN at -10 °C over 1 h, which was prepared by mixing **S1** (4.13 g, 25.03 mmol) and BSA (10.19 g, 50.07 mmol) in DMAc/MeCN (1:1, 100 mL), stirred at 50 °C for 1 h until clear, cooling to 20 °C and then adding DIPEA (2.16 g, 16.69 mmol). The reaction mixture was stirred at -10 °C for 16 h and monitored by HPLC **(Methods 7 and 8)**, which showed the ratio of **S49:(8 + *epi*-8):(S49-INT+ *epi*-S49-INT):8-over-addition** was 0.6:99.1 **(8:*epi*-8** ratio is **99.6:0.4** monitored by HPLC **(Method 7)**: 0.3:0.0. The assay yield of the reaction was 89% over three couplings. H_2O (100 mL) was added slowly to the reaction mixture at -10 °C over 1 h. The mixture was stirred at -10 °C for 3 h. The solid was collected by filtration and slurried in H_2O (100 mL) at 10 °C for 3 h. The solid was collected by filtration and dried under vacuum to provide 27.8 g of **8** as a white solid (96.2% LCAP) in 87% isolated yield over three couplings **(Method 10)**. Starting from **1**, 1053.3 grams of reagents/solvents were used above to synthesize 27.8 g (96.2% LCAP) of **8**, giving a PMI of 39.4.

mp 240 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.69 (br s, 1H), 11.30 (s, 1H), 8.48 (d, J = 8.0 Hz, 1H), 8.38 (d, J = 8.0 Hz, 1H), 8.29 (d, J = 8.0 Hz, 2H), 8.14 (dd, J = 4.8, 1.6 Hz, 2H), 8.11-8.05 (m, 1H), 7.98 (d, J = 8.0 Hz, 1H), 7.92 (d, J = 8.0 Hz, 2H), 7.86 (d, J = 8.0 Hz, 2H), 7.76 (d, J = 8.0 Hz, 2H), 7.66 (d, J = 8.0 Hz, 2H), 7.61 (t, J = 8.4 Hz, 2H), 7.54 (d, J = 8.8 Hz, 1H), 7.51 (d, J = 8.0 Hz, 2H), 7.40 - 7.09 (m,, 29H), 6.97 (dd, J = 8.0, 4.6 Hz, 1H), 6.78 (t, J = 4.8 Hz, 1H), 4.76 (dd, J = 13.2, 6.4 Hz, 1H), 4.67 - 4.63 (m, 2H), 4.60 - 4.51 (m, 2H), 4.37 (q, J = 7.6 Hz, 1H), 4.28 - 4.22 (m, 1H), 4.16 - 4.08 (m, 3H), 3.20 - 3.17 (m, 1H), 3.13 - 3.04 (m, 3H), 3.02 - 2.85 (m, 15H), 2.74 - 2.67 (m, 1H), 2.57 (dd, J = 16.4, 6.4 Hz, 1H), 2.31 (dd, J = 16.4, 6.4 Hz, 1H), 1.86 - 1.78 (m, 1H), 1.67 - 1.59 (m, 1H), 1.56 (s, 9H), 1.45 (s, 9H), 1.35 (s, 9H), 1.25 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.93, 169.65, 169.52, 169.41, 169.07, 168.36, 167.23, 163.27, 163.14, 154.07, 153.87, 146.77, 142.55, 142.15, 142.05, 141.86, 141.51, 140.56, 139.00, 136.85, 135.58, 135.06, 128.37, 128.22, 127.96, 127.74, 127.46, 127.16, 126.65, 126.49, 126.16, 125.92, 125.37, 125.31, 125.07, 124.84, 124.79, 123.69, 122.50, 118.38, 118.03, 116.41, 113.13, 107.26, 84.23, 78.99, 78.65, 78.47, 75.98, 64.02, 62.41, 54.32, 52.07, 51.68, 51.39, 50.91, 48.75, 47.40, 44.88, 35.80, 35.74, 35.53, 35.08, 31.43, 26.60, 26.16, 26.06, 25.90. HRMS (ESI): m/z calcd for $C_{101}H_{117}N_{10}O_{19}$

[M+H]$^+$: 1845.8591, found: 1845.8552. FTIR (neat): 3281, 3064, 2976, 2931, 1710, 1682, 1634, 1525, 1448, 1392, 1366, 1290, 1250, 1162, 1112, 847, 758, 739, 696, 630 cm^{-1}

Method 1: HALO C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 10-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

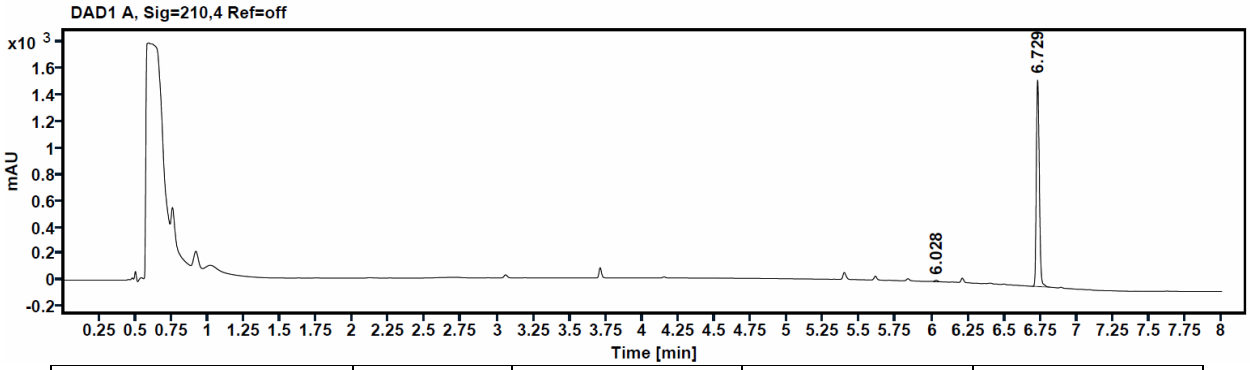
Method 7: HALO C8 4.6 × 150 mm, particle size 2.7 µm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN (solvent B); gradient 50-70% (solvent B) in 4 min; 70-95% (solvent B) in 12 min; hold 3 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

Method 8: HALO C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 30-75% (solvent B) in 2 min, 75-95% (solvent B) in 8 min; hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 9: HALO C8 4.6 × 150 mm, particle size 2.7 µm column; 0.1% TFA (v/v) in H_2O (solvent A), MeOH (solvent B); gradient 85-100% (solvent B) in 10 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 10: Express C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 50-75% (solvent B) in 2 min, 75-95% (solvent B) in 8 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

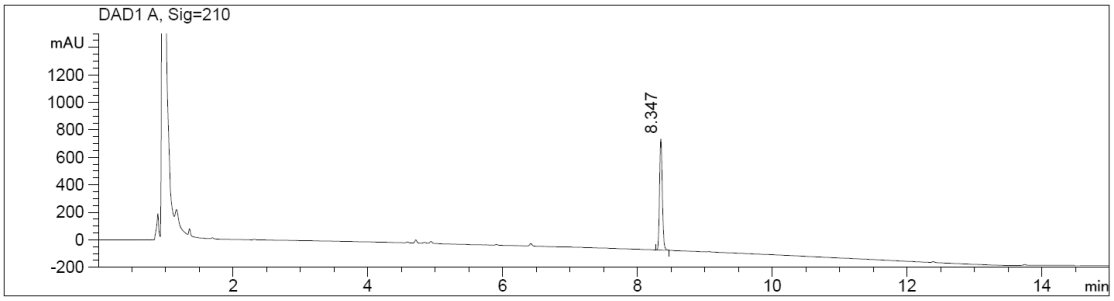
HPLC of **S48** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
26	6.028	17.5	11.8	0.8
26-INT+ *epi*-26-INT	6.359	0.0	0.0	0.0
S48+*epi*-S48	6.729	2237.5	1571.3	99.2*
S48-over-addition	7.215	0.0	0.0	0.0
Total	-	-	-	100.0

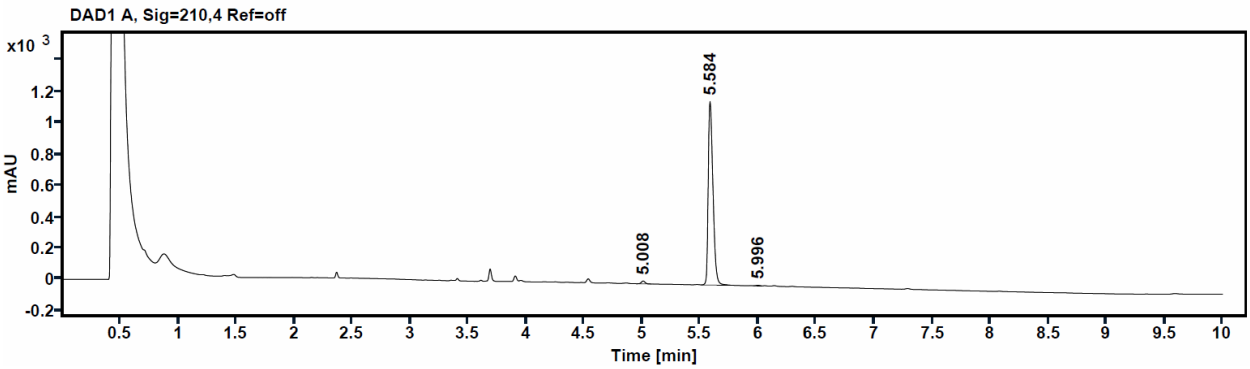
*Note: Contains 0.0% ***epi*-S48**

HPLC of **S48** chiral purity



Compound ID	Time	Area	Height	Area% ratio
S48	8.361	2305.4	809.1	100.0
***epi*-S48**	8.516	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of **S49** formation (reaction mixture)

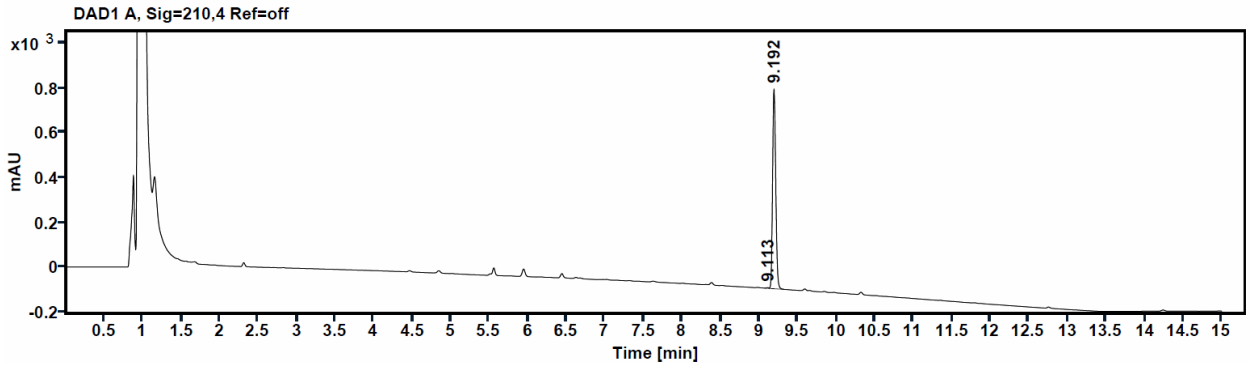


Compound ID	Time	Area	Height	Area% ratio
S48	5.008	42.1697	18.4	1.2
S49+ ***epi*-S49**	5.584	3333.9741	1175.5	98.4*
S48-INT + ***epi*-S48-INT**	5.996	13.0637	4.9	0.4**
S49-over-addition	6.204	0.0	0.0	0.0
Total	-	-	-	100.0

*Note: Contains 0.2% ***epi*-S49**

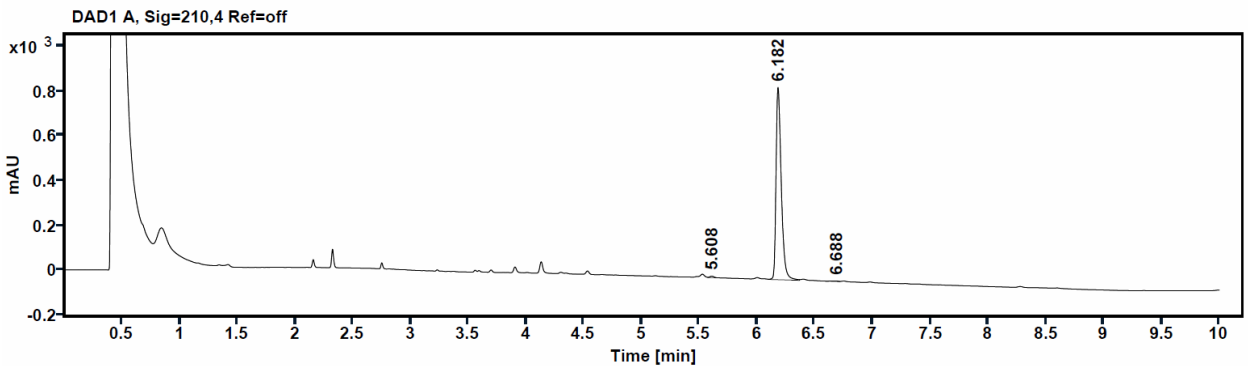
*Note: Contains 0.2% ***epi*-S48-INT**

HPLC of **S49** chiral purity



Compound ID	Time	Area	Height	Area% ratio
***epi*-S49**	9.113	6.3	2.8	0.2
S49	9.192	2538.4	895.3	99.8

HPLC of **8** formation (reaction mixture)

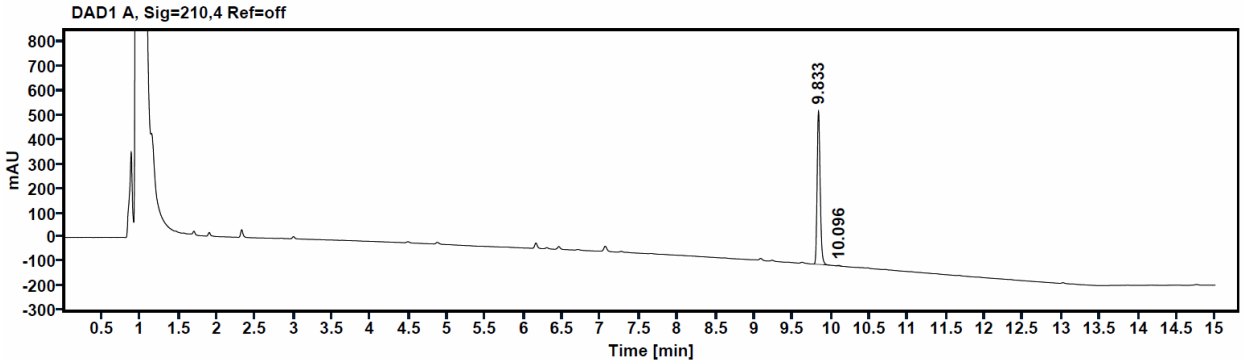


Compound ID	Time	Area	Height	Area% ratio
S49	5.608	15.6486	6.7	0.6
8+ ***epi*-8**	6.182	2707.9253	863.2	99.1*
S49-INT + ***epi*-S49-INT**	6.688	9.4252	2.3	0.3**
8-over-addition	6.411	0.0	0.0	0.0
Total	-	-	-	100.0

*Note: Contains 0.4% ***epi*-S8**

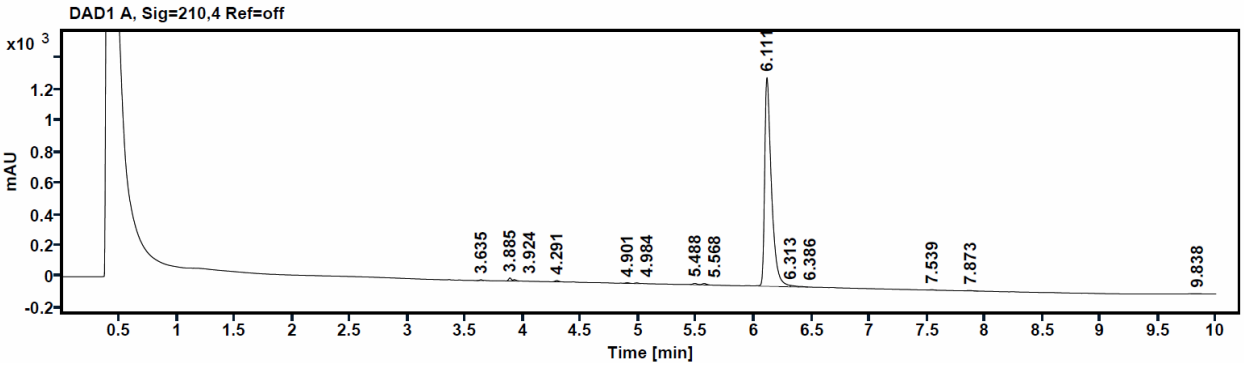
*Note: Contains 0.2% ***epi*-S49-INT**

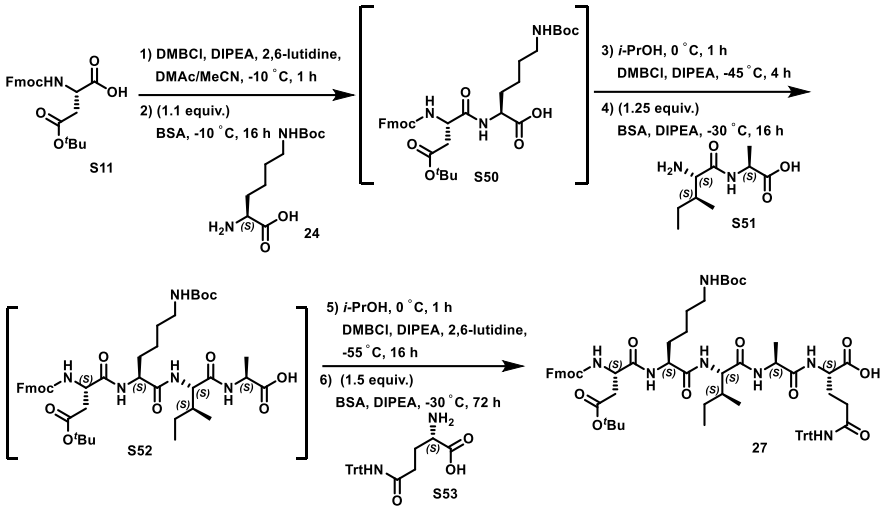
HPLC of **8** chiral purity



Compound ID	Time	Area	Height	Area% ratio
8	9.833	1739.8	633.2	99.6
***epi*-8**	10.096	6.7	2.7	0.4

HPLC of isolated **8**





Synthesis of Fmoc-L-Asp(O*t*Bu)-L-Lys(Boc)-L-Lle-L-Ala-L-Gln(Trt)-OH (27). In a 1000 mL 3-neck round bottom flask, **Fmoc-L-Asp(O*t*Bu)-OH (S11)** (20.00 g, 48.61 mmol) and 2,6-lutidine (5.21 g, 48.61 mmol) were dissolved in DMAc/MeCN (1:1, 100 mL). The solution was cooled to -10 °C, DMBCl (6.87 g, 51.04 mmol) and DIPEA (6.28 g, 48.61 mmol) were added to the reaction mixture. The reaction mixture was stirred at -10 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S11:S11-INT** was 0.7:99.3. A solution of **L-Lys(Boc)-OH (24)** and BSA in DMAc and MeCN, prepared by mixing **24** (13.17 g, 53.47 mmol) and BSA (13.84 g, 68.05mmol) in DMAc (50 mL), stirring at 50 °C for 1 h until clear, cooling to 25 °C, and then adding MeCN (50 mL), was added slowly into the reaction mixture at -10 °C over 30 minutes. The reaction mixture was stirred at -10 °C for 16 h and monitored by HPLC (**Method 1**), which showed the ratio of **S11:S50:S50-over-addition** was 1.1:98.6:0.3. Then *i*-PrOH (7.30 g, 121.52 mmol) was added to the reaction mixture at 0 °C and stirred for 1 h. Then the reaction mixture was cooled to -45 °C. DMBCl (8.51 g, 63.19 mmol) and DIPEA (5.03 g, 38.89 mmol) were added, and the mixture was stirred for 4 h and monitored by HPLC (**Method 1 and Method 2**). HPLC showed the ratio of **S50:(S50-INT+*epi*-S50-INT)** was 0.7:99.3. A solution of **L-Lle-L-Ala-OH (S51)**, BSA and DIPEA in DMAc and MeCN, prepared by mixing **S51** (12.29 g, 60.76 mmol) and BSA (9.89 g, 48.61 mmol) in DMAc (50 mL), stirring at 50 °C for 1 h until clear, cooling to 25 °C, and then adding MeCN (50 mL) and DIPEA (2.51 g, 19.44 mmol), was added slowly into the reaction mixture at -45 °C over 30 minutes. The reaction mixture was stirred at -30 °C for 16 h and monitored by HPLC (**Method 1 and Method 3**), which showed the ratio of **S50:(S52+*epi*-S52):(S50-INT+*epi*-S50-INT):S52-over-addition** was 0.9:97.9 **(S52:*epi*-S52** ratio is **99.7:0.3** monitored by SFC-MS **Method 3)**: 0.9:0.3. Then *i*-PrOH (7.30 g, 121.52 mmol) was added to the reaction mixture at 0 °C and stirred for 1 h. Then the reaction mixture was cooled to -55 °C, DMBCl (11.78 g, 87.50 mmol), DIPEA (5.03 g, 38.89 mmol), and 2,6-lutidine (2.60 g, 24.30 mmol) were added to the reaction mixture and stirred for 16 h at -55 °C. The reaction was monitored by HPLC (**Method 4**), which showed the ratio of **S52:S52-INT:*epi*-S52-INT** was 0.5:98.5:1.0. A solution of **L-Gln(Trt)-OH**

(S53), BSA and DIPEA in DMAc and MeCN, prepared by mixing **S53** (28.32 g, 72.91 mmol) and BSA (19.78 g, 97.22 mmol) in DMAc (50 mL), stirring at 50 °C for 1 h until clear, cooling to 25 °C, and then adding MeCN (50 mL) and DIPEA (3.77 g, 29.17 mmol), was added slowly into the reaction mixture at -55 °C over 30 minutes. The reaction mixture was stirred at -30 °C for 72 h and monitored by HPLC (**Method 4**), which showed the ratio of **S52:27:*epi*-27:*epi*-S52-INT:27-over-addition** was 0.4:98.7:0.5:0.3:0.1. The assay yield of the reaction was 92% over three steps. A solution of aq. KH_2PO_4 (26.46 g, 194.43 mmol in 400 mL H_2O) was added slowly into the reaction mixture over 1 h The resulting mixture was stirred for 2 h at 0 °C. The mixture was filtered, and the filter cake was dried under a nitrogen atmosphere. The crude solid was dissolved in MeOH (400 mL). H_2O (200 mL) was added slowly to the solution over 1 h, and the resulting mixture was stirred for 2 h at 0 °C. The product was collected by filtration and dried under N_2 to provide 49.50 g of **27** as a white solid (98.1% LCAP) with 84% isolated yield over three couplings (**Method 4**).

mp 190 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.36 (s, 1H), δ 8.63 (s, 1H), 8.02 (d, J = 7.6 Hz, 1H), 7.95 (d, J = 7.6 Hz, 1H), 7.90 (d, J = 8.4 Hz, 1H), 7.89 (d, J = 7.6 Hz, 2H), 7.79 (d, J = 8.8 Hz, 1H), 7.70 (t, J = 7.0 Hz, 3H), 7.41 (t, J = 7.5 Hz, 2H), 7.32 (dd, J = 7.5, 1.2 Hz, 2H), 7.30 - 7.27 (m, 2H), 7.26 - 7.24 (m, 5H), 7.21 - 7.20 (m, 1H), 7.19 - 7.18 (m, 1H), 7.17 - 7.14 (m, 4H), 7.16 - 7.14 (m, 2H), 6.71 (t, J = 5.6 Hz, 1H), 4.40 - 4.34 (m, 1H), 4.33 - 4.20 (m, 5H), 4.17 - 4.10 (m, 2H), 2.89 - 2.82 (m, 2H), 2.64 (dd, J = 16.0, 4.8 Hz, 1H), 2.43 (dd, J = 16.0, 9.6 Hz, 1H), 2.37 - 2.31 (m, 2H), 1.92 - 1.83 (m, 1H), 1.74 - 1.64 (m, 2H), 1.63 - 1.55 (m, 1H), 1.52 - 1.46 (m, 1H), 1.43 - 1.39 (m, 1H), 1.36 - 1.35 (m, 19H), 1.32 - 1.28 (m, 1H), 1.19 (d, J = 6.8 Hz, 5H), 1.10 - 0.99 (m, 1H), 0.81 - 0.75 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 173.65, 172.46, 171.75, 171.69, 170.98, 170.75, 169.77, 156.22, 155.97, 145.36, 144.29, 144.17, 141.17, 128.97, 128.11, 127.91, 127.54, 126.76, 125.71, 120.59, 80.58, 77.78, 69.65, 66.22, 57.08, 53.07, 52.16, 51.87, 48.31, 47.06, 37.97, 37.05, 33.06, 32.13, 29.67, 28.72, 28.15, 27.81, 24.70, 23.04, 18.74, 15.75, 11.51. HRMS (ESI): m/z calcd for $C_{61}H_{84}N_7O_{13}$ $[M+H]^+$: 1194.6122, found: 1194.6132. FTIR (neat): 3285, 2930, 1707, 1680, 1627, 1526, 1447, 1366, 1261, 1158, 1036, 766, 740, 698 cm^{-1}

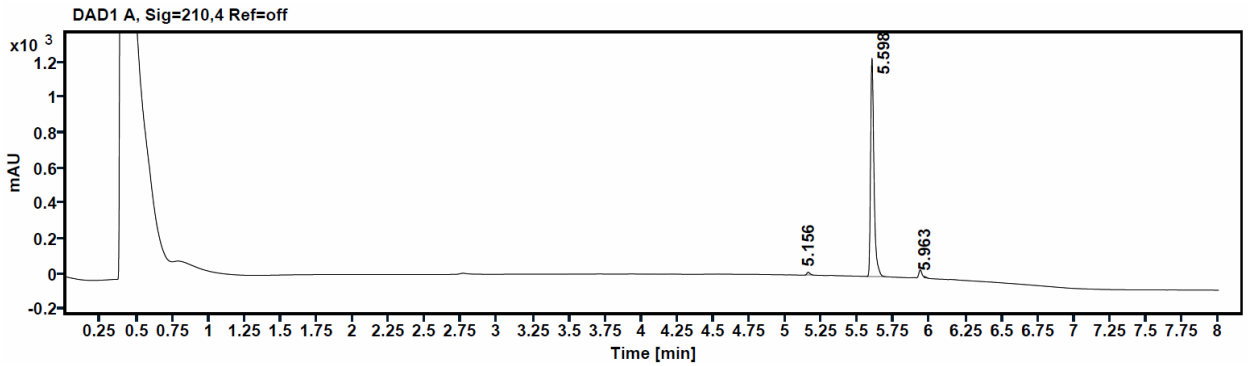
Method 1: Express C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 10-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 2: CHIRALPAK Cellulose SZ 4.6 × 150 mm, particle size 3 µm column; co-solvent: 20 mM NH_3 in MeOH; gradient: 40-70% (solvent B) in 5 min, hold 1 min, flow rate: 2 mL/min; detection wavelength: 210 nm

Method 3: CHIRALPAK IK-3 4.6 × 250 mm, particle size 3 μm column; Co-solvent: 20 mM NH_3 in *i*-PrOH; gradient: 30-40% (solvent B) in 8 min, hold 2 min, flow rate: 2 mL/min; detection wavelength: 210 nm

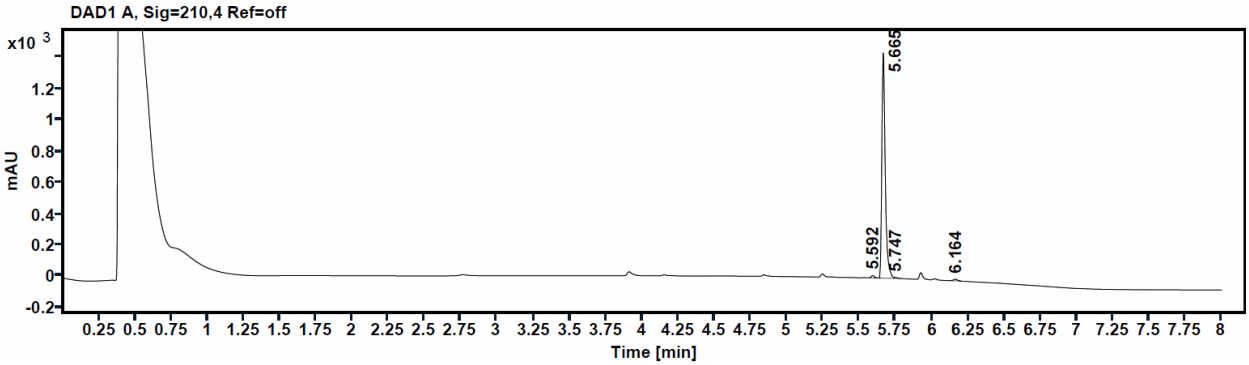
Method 4: Express C18 3.0 × 100 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 50-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

HPLC of **S50** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S11	5.156	22.0363	15.5	1.1
S50	5.598	2004.089	1241.3	98.6
S50-over-addition	5.963	6.4133	6.3	0.3
Total	-	-	-	100.0

HPLC of **S52** formation (reaction mixture)

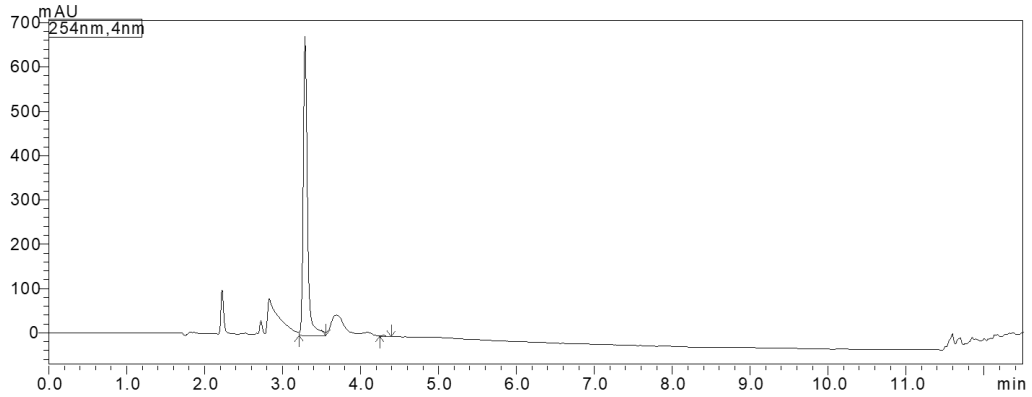


Compound ID	Time	Area	Height	Area% ratio
S50	5.592	22.1255	15.1	0.9
S52+ *epi*-S52	5.665	2364.1775	1456.5	97.9*
S52-over-addition	5.747	8.2586	0.0229	0.3
S50-INT+ *epi*-S50-INT	6.164	20.8046	10.7	0.9**
Total	-	-	-	100.0

*Note: Contains 0.3% ***epi*-S52**

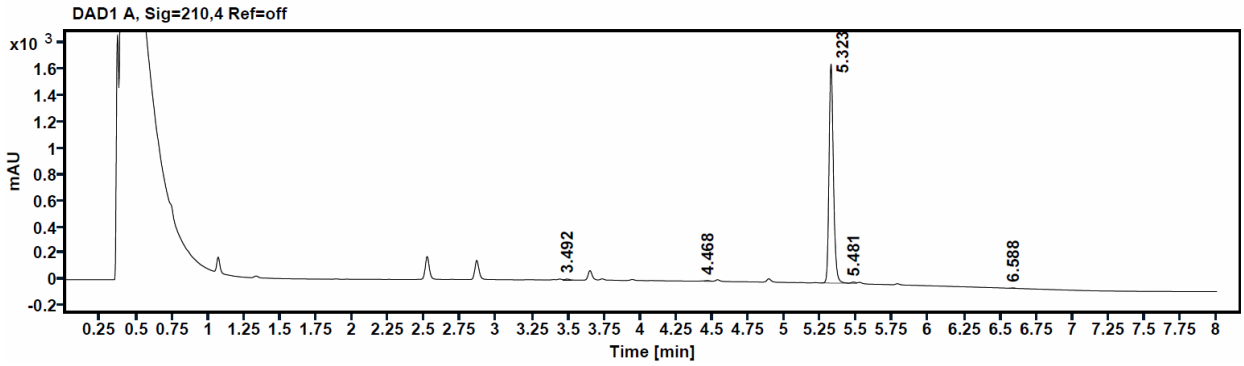
*Note: Contains 0.5% ***epi*-S50-INT**

SFC of S5**2** chiral purity



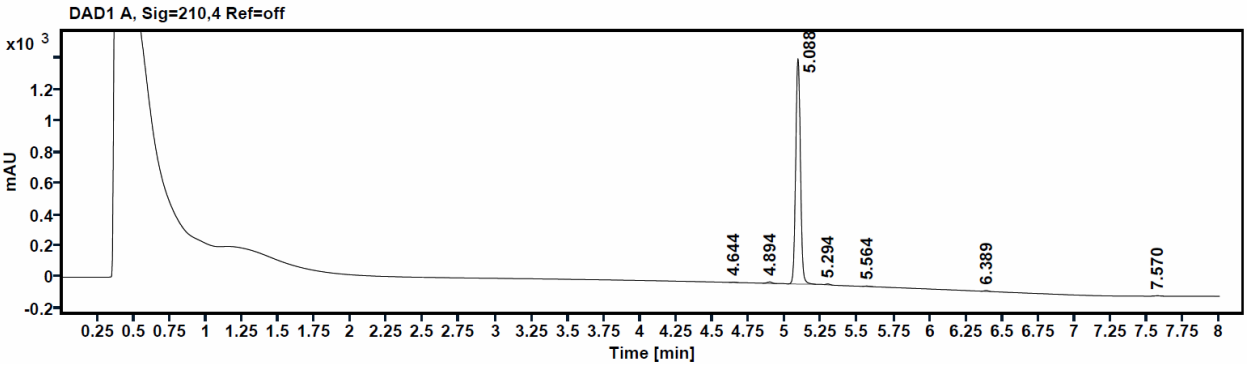
Compound ID	Time	Area	Height	Area% ratio
S52	3.288	2559878	674590	99.7
***epi*-S52**	4.292	7655	2256	0.3
Total	-	-	-	100.0

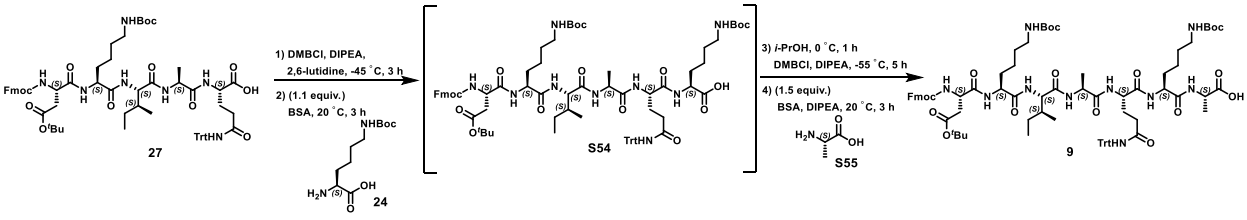
HPLC of **27** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S52	3.492	13.3607	7.3	0.4
***epi*-S52-INT**	4.468	12.4401	5.2	0.3
27	5.323	3534.5225	1672.6	98.7
***epi* -27**	5.481	17.4656	9.3	0.5
27-over-addition	6.588	4.8769	2.8	0.1
Total	-	-	-	100.0

HPLC of isolated **27**





Synthesis of Fmoc-L-Asp(O*t*Bu)-L-Lys(Boc)-L-Lle-L-Ala-L-Gln(Trt)-L-Lys(Boc)-L-Ala-OH (9). In a 2000 mL 3-neck round bottom flask, **27** (20.39 g, 98.1% LCAP, 16.74 mmol) and 2,6-lutidine (1.79 g,

16.74 mmol) were dissolved in DMAc/MeCN (1:1, 200 mL). The solution was cooled to -45 °C, DMBCl (2.37 g, 17.58 mmol) and DIPEA (2.16 g, 16.74 mmol) were added to the reaction mixture. The reaction mixture was stirred at -45 °C for 3 h and monitored by HPLC (**Method 5**), which showed the ratio of **27:27-INT:*epi*-27-INT** was 0.5:99.4:0.1. The reaction mixture was added slowly into a solution of **L-Lys(Boc)-OH (24)** and BSA in DMAc and MeCN at 20 °C over 30 min, which was prepared by mixing **24** (4.54 g, 18.42 mmol) and BSA (4.77 g, 23.44 mmol) in DMAc (100 mL), stirred at 50 °C for 1 h until clear, cooling to 20 °C, and then adding MeCN (100 mL). The reaction mixture was stirred at 20 °C for 3 h and monitored by HPLC (**Method 5 and Method 6**), which showed the ratio of **27:(S54 + *epi*-S54):*epi*-27-INT:S54-over-addition** was: 0.6:98.5 **(S54:*epi*-S54** ratio is **99.7:0.3)**: 0.7:0.2. Then *i*-PrOH (4.03 g, 66.98 mmol) was added into the reaction mixture at 0 °C and stirred for 1 h. Then the reaction mixture was cooled to -55 °C, DMBCl (3.38 g, 25.12 mmol) and DIPEA (2.16 g, 16.74 mmol) were added into the reaction mixture and stirred for 5 h, monitored by HPLC (**Method 7**). HPLC showed the ratio of **S54:S54-INT:*epi*-S54-INT** was 0.4:99.4:0.2. The reaction mixture was added slowly into a solution of **L-Ala-OH (S55)**, BSA and DIPEA in DMAc and MeCN at 20 °C over 30 min, which was prepared by mixing **S55** (2.24 g, 25.11 mmol) and BSA (8.52 g, 41.86 mmol) in DMAc (100 mL), stirred at 50 °C for 5 h until clear, cooling to 20 °C, and then adding DIPEA (1.08 g, 8.37 mmol) and MeCN (100 mL). The reaction mixture was stirred at 20 °C for 3 h and monitored by HPLC (**Method 7**), which showed the ratio of **S54:9:*epi*-9:*epi*-S54-INT:9-over-addition** was 1.4:97.7:0.2:0.1:0.6. The assay yield of the reaction was 88% over 2 steps. The reaction mixture was cooled to 0 °C, then H_2O (480 ml) was added slowly to the reaction mixture over 0.5 h. The result mixture was stirred for 2 h at 0 °C and filtered. The crude product was slurried with MeOH (400 mL) at 0 °C for 1 h, the mixture was filtered, and the filter cake was dried under nitrogen to provide 21.00 g of **9** as a white solid (95.0% LCAP) in 80% isolated yield over two steps (**Method 7**).

Starting from **S11**, 1958.6 grams of reagents/solvents were used above to synthesize 21.0 g (95.0% LCAP) of **9**, giving a PMI of 98.2.

mp 220~223 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ(ppm) 12.50 (br s, 1H), 8.53 (s, 1H), 8.19 (d, J = 7.2 Hz, 1H), 8.01 (d, J = 6.4 Hz, 1H), 7.91-7.87 (m, 4H), 7.77 (dd, J = 14.2, 8.4 Hz, 2H), 7.70 (t, J = 6.8 Hz, 3H), 7.41 (t, J = 7.6 Hz, 2H), 7.31 (t, J = 7.6 Hz, 2H), 7.27 - 7.24 (m, 6H), 7.19 - 7.15 (m, 9H), 6.76 - 6.70 (m, 2H), 4.40 - 4.34 (m, 1H), 4.32 - 4.11 (m, 9H), 2.85 (q, J = 6.8 Hz, 4H), 2.64 (dd, J = 16.0, 4.8 Hz, 1H), 2.43 (dd, J = 16.0, 9.6 Hz, 1H), 2.31 (t, J = 8.0 Hz, 2H), 1.85 - 1.74 (m, 1H), 1.69 - 1.58 (m, 4H), 1.51 - 1.45 (m, 2H), 1.41 - 1.28 (m, 31H), 1.24 (d, J = 7.2 Hz, 6H), 1.19 - 1.18 (m, 5H), 1.07 - 1.01 (m, 1H), 0.83 - 0.75 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6): δ(ppm) 174.41, 172.43, 171.96, 171.78, 171.69, 171.32, 171.00, 170.92, 169.76, 156.22, 155.97, 145.35, 144.28, 144.17, 141.17, 128.95, 128.11, 127.92, 127.53, 126.77, 125.71, 120.59, 80.58, 77.79, 69.61, 66.22, 57.11, 53.07, 52.70, 52.44, 51.86, 48.50, 47.85, 47.05, 37.95, 37.04, 33.14, 32.41, 32.10, 29.77, 29.66, 28.73, 28.15, 24.73, 23.04, 22.91, 18.58, 17.48, 15.75,

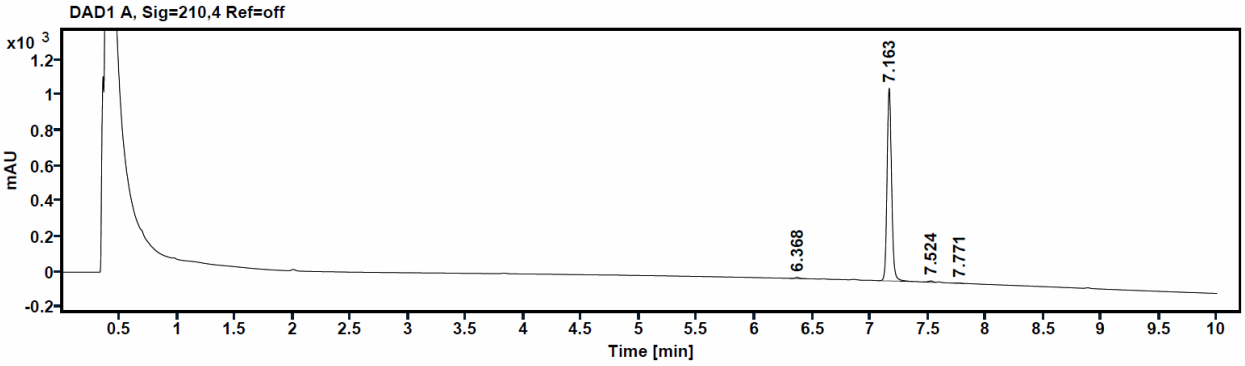
11.49. HRMS (ESI): m/z calcd for $C_{81}H_{109}N_{10}O_{17}$ $[M+H]^+$: 1493.7967, found: 1493.8004. FTIR (neat): 3272, 2932, 1667, 1625, 1525, 1449, 1366, 1250, 1162, 759, 741, 699 cm^{-1}.

Method 5: Express C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 50-95% (solvent B) in 9 min, hold 1 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 6: HALO C8 4.6 × 150 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 60-80% (solvent B) in 3 min, 80-95% (solvent B) in 11 min, hold 1 min; flow rate: 1.2 mL/min; detection wavelength: 210 nm.

Method 7: Express C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 60-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

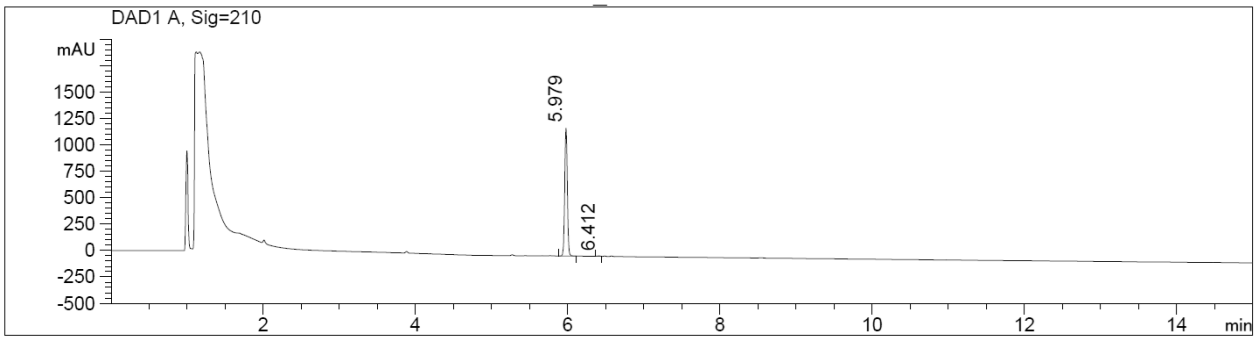
HPLC of **S54** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
27	6.368	19.3769	7	0.6
S54 + *epi*-S54	7.163	2964.4377	1089	98.5*
***epi* -27-INT**	7.524	22.3518	7.5	0.7
S54-over-addition	7.771	4.6976	2	0.2
Total	-	-	-	100.0

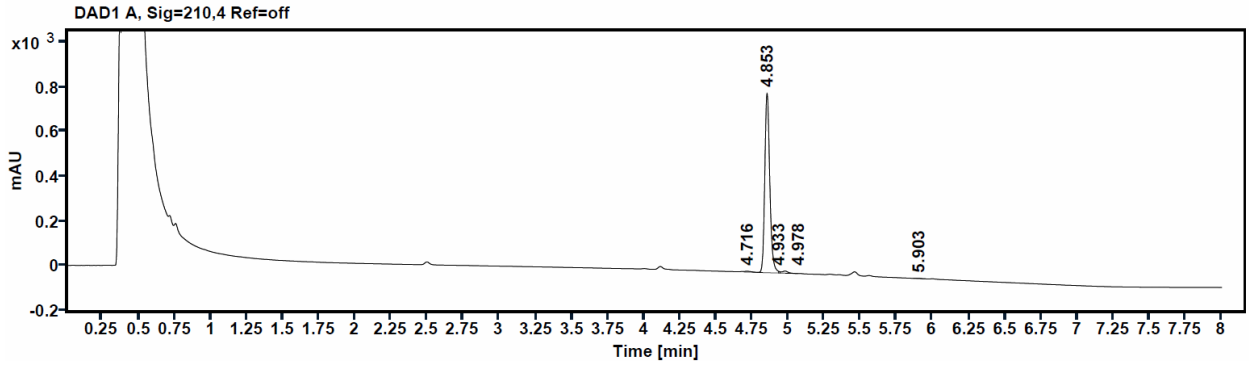
*Note: Contains 0.3% ***epi*-S54**

SFC of **S54** chiral purity



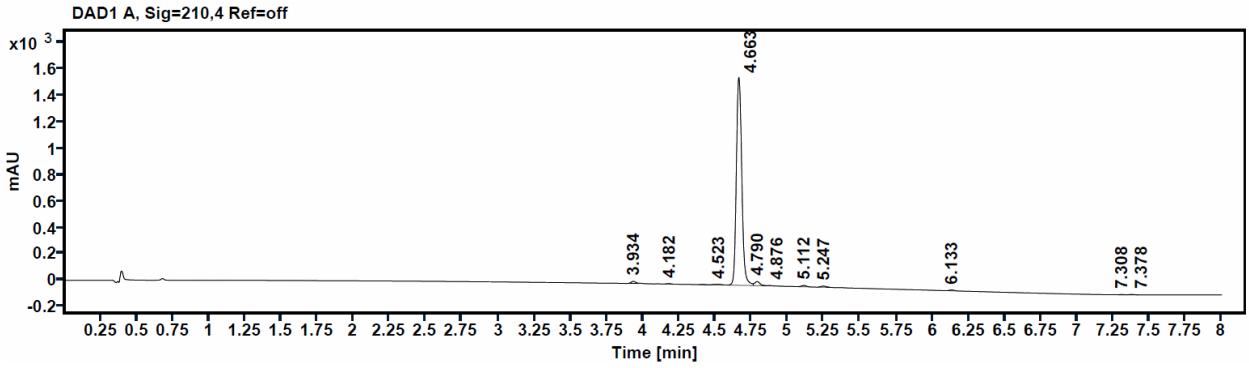
Compound ID	Time	Area	Height	Area% ratio
S54	5.979	2915.0	1212.87	99.7
***epi*-S54**	6.412	9.7	4.02	0.3
Total	-	-	-	100.0

HPLC of **9** formation (reaction mixture)

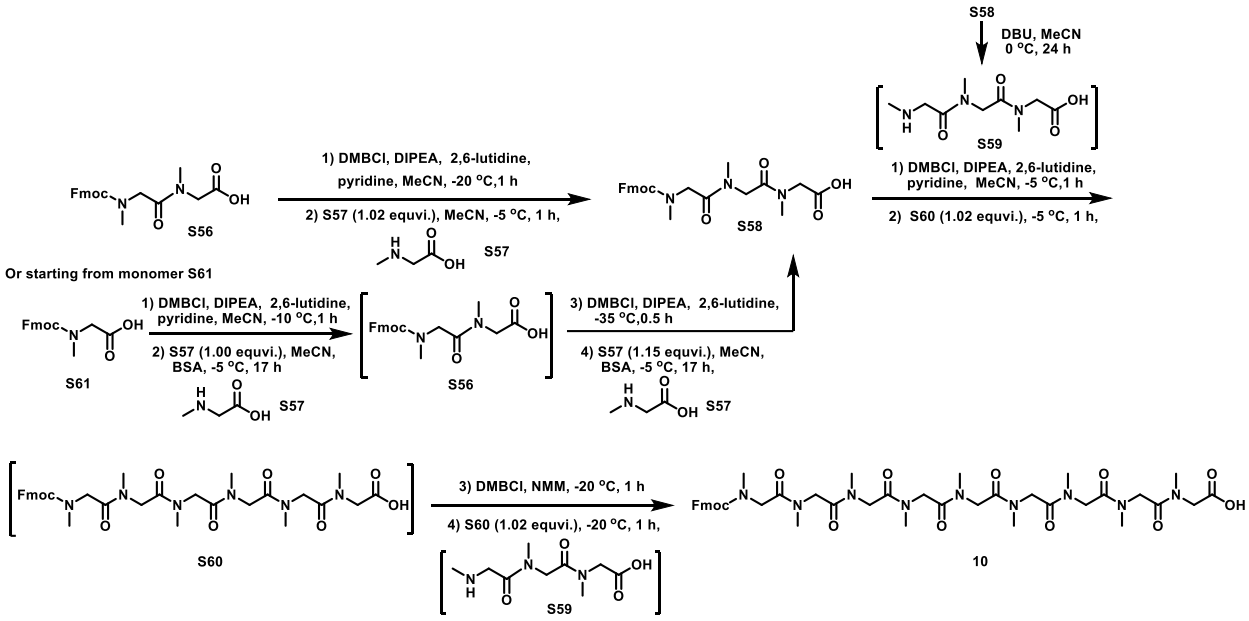


Compound ID	Time	Area	Height	Area% ratio
9-over-addition	4.716	11.2361	3.9	0.6
9	4.853	1937.2657	808.9	97.7
***epi*-9**	4.933	3.6792	7.5	0.2
S54	4.978	28.4085	9.7	1.4
***epi* -S54-INT**	5.903	2.3452	1.1	0.1
Total	-	-	-	100.0

HPLC of isolated **9**



Synthesis of Fmoc-Sar-Sar-Sar-Sar-Sar-Sar-Sar-Sar-Sar-OH (10)



Synthesis of Fmoc-Sar-Sar-Sar-OH (S58). To a 250 mL 3-necked round-bottom flask were added compound **S56** (10.00 g, 26.15 mmol) and MeCN (50 mL). After the solution was cooled to -20 °C, 2,6-lutidine (3.36 g, 31.35 mmol), DIPEA (3.55 g, 27.46 mmol), pyridine (0.21 g, 2.61 mmol) and DMBCl (3.70 g, 27.46 mmol) were added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S56**:**S56-INT** was 0.4:99.6. The resulting solution was added over 10 min into pre-cooled (-5 °C) solution of **S57** and BSA in MeCN, which was prepared by stirring **S57** (2.37 g, 26.60 mmol) and BSA (7.98 g, 39.23 mmol) in MeCN (50 mL) at 45 °C for 3 h and then cooled to -5 °C. The reaction mixture was stirred at -5 °C for 17 h and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 96%, and the ratio of **S56**:**S56-INT**:**S58**:**S58-over-addition** was 0.8:0.2:98.9:0.1. The reaction was quenched with H_2O (10 mL) at 20 °C and stirred for 1 hour. The mixture was then concentrated under vacuum at 20 °C. H_2O (390 mL) and toluene (400 mL) were added, and the

pH was adjusted to 3.0 with 4N HCl. The mixture was stirred at 35 °C for 12 hours (Adding seed crystals can shorten the crystallization time), then at 10 °C for 24 hours. The solid was collected by filtration and dried at 50 °C under vacuum to afford 10.8 g product with 99% purity and 91% isolated yield.

Synthesis of H-Sar-Sar-Sar-OH (S59). To a 250 mL three-neck jacketed vessel were added compound **S58** (10.00 g, 22.05 mmol) and MeCN (150 mL). After the solution was cooled to 0 °C, DBU (6.04 g, 39.69 mmol) was added. The reaction mixture was stirred at 0 °C for 24 h and monitored by HPLC (**Method 2**) which showed the ratio of **S58**:**S59** (quenched by 1 wt% HCl in MeCN) was 0.6:99.4. Wash the reaction with *n*-heptane (100 mL x 11) at 15 °C, stirring for 15 min and standing the mixture for 15 min for each wash. The MeCN mixture solution was used directly in the subsequent step.

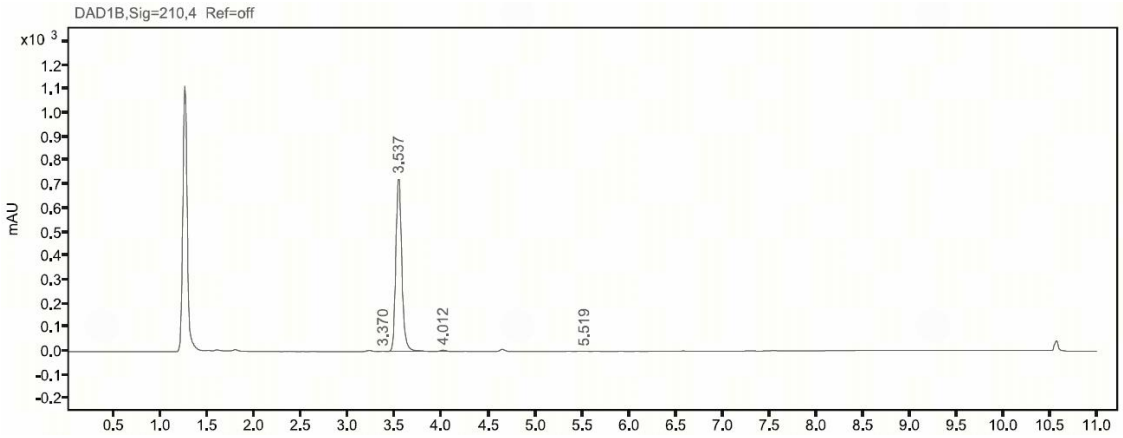
Synthesis of Fmoc-Sar-Sar-Sar-Sar-Sar-Sar-Sar-Sar-Sar-OH (10). To a 250 mL three-neck jacketed vessel were added compound **S58** (10.00 g, 22.05 mmol) and MeCN (50 mL). After the solution was cooled to -5 °C, 2,6-lutidine (2.84 g, 26.46 mmol), DIPEA (2.94 g, 22.71 mmol), pyridine (0.35 g, 4.41 mmol) and DMBCl (3.06 g, 22.71 mmol) were added. The reaction mixture was stirred at -5 °C for 1 h and monitored by HPLC (**Method 3**), which showed the ratio of **S58**:**S58-INT** was 0.7:99.3. The reaction solution was slowly added into a pre-cooled (-5 °C) solution of **S59** (5.20 g, 22.49 mmol) in MeCN (Total 89.7 g solution) at -5 °C. The reaction mixture was stirred at -5 °C for 1 h and monitored by HPLC (**Method 3**). The HPLC showed the reaction assay yield of **S60** was 99%, and the ratio of **S58**:**S58-INT**:**S60**:**S60-over-addition** was 0.8:0.0:99.2:0.0. The mixture of **S60** was directly used for the next step.

The above **S60** solution was cooled to -20 °C, DMBCl (5.64 g, 41.90 mmol) and 4-methylmorpholine (4.24 g, 41.90 mmol) were added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 4**), which showed the ratio of **S60**:**S60-INT** was 0.5:99.5. The reaction solution was slowly added into a pre-cooled (-20 °C) solution of **S59** (5.20 g, 22.49 mmol) in MeCN (Total 89.7 g solution) at -20 °C over 0.5 h. Then the mixture was stirred for 1 h at -20 °C. The reaction was monitored by HPLC (**Method 4**). The HPLC showed the reaction ratio of **S60**:**S60-INT**:**10**:**10-over-addition** was 1.2:0.0:98.5:0.3. The HPLC showed the assay yield was 93% (for two steps). The reaction mixture was added into a suspension of celite (90 g) in MTBE (2500 mL) at 0 °C and filtered to afford **10** as celite complexed solid. The obtained **10** celite complexed solid was treated with MeCN (100 mL) and filtered to afford **10** as MeCN solution with 94.6% LCAP, which could be used in the next step directly.

A portion of the sample was further purified by preparative HPLC to isolate **10** as a solid (**note: moisture-sensitive**), with 100% HPLC purity, for NMR characterization. mp 217.4 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 7.82 (br t, *J* = 7.19 Hz, 2H), 7.60 (d, *J* = 7.50 Hz, 1H), 7.53 (br t, *J* = 8.50 Hz, 1H), 7.24-7.38 (m, 4H), 3.83-4.30 (m, 11H), 3.81-4.05 (m, 10H), 3.76 (br d, *J* = 6.63 Hz, 1H), 2.59-2.93 (m, 27H). ^{13}C NMR (101 MHz, DMSO-d6): δ ppm 168.24, 156.44, 144.32, 141.20, 141.15, 141.07, 128.13, 128.02,

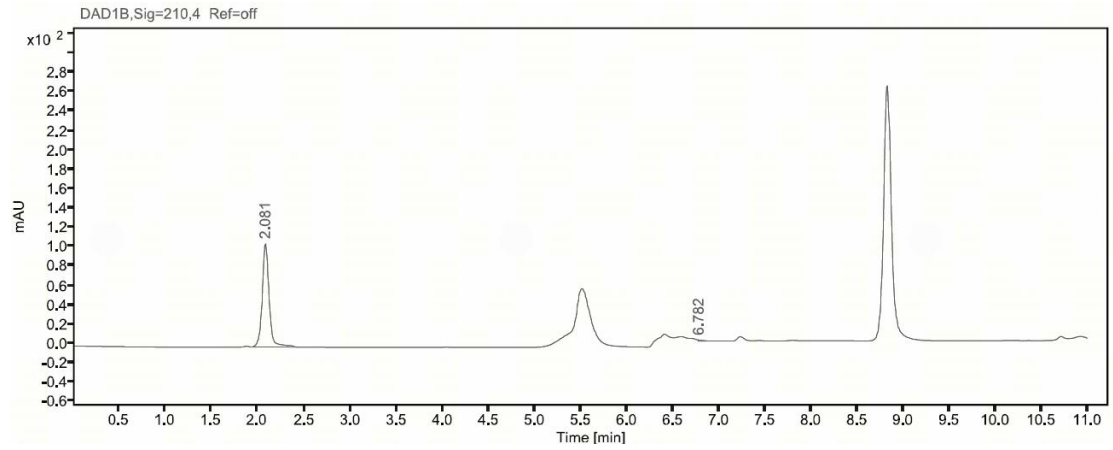
127.63, 125.55, 120.61, 120.46, 67.29, 50.38, 50.33, 50.22, 50.13, 49.52, 47.06, 35.92, 35.66, 35.58, 35.48, 35.36. HRMS (ESI): m/z calcd for $C_{42}H_{58}O_{12}N_9$ $[M+H]^+$: 880.4199, found: 880.4202. FTIR (neat): 3455, 2939, 1640, 1486, 1399, 1207, 1101, 947, 763, 742 cm^{-1}.

HPLC of **S58** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S58	3.537	3239.85	727.32	98.9
S56	4.012	26.07	6.06	0.8
S56-INT	5.519	6.70	1.19	0.2
S58-over-addition	3.370	4.27	1.28	0.1
Total	-	-	-	100.0

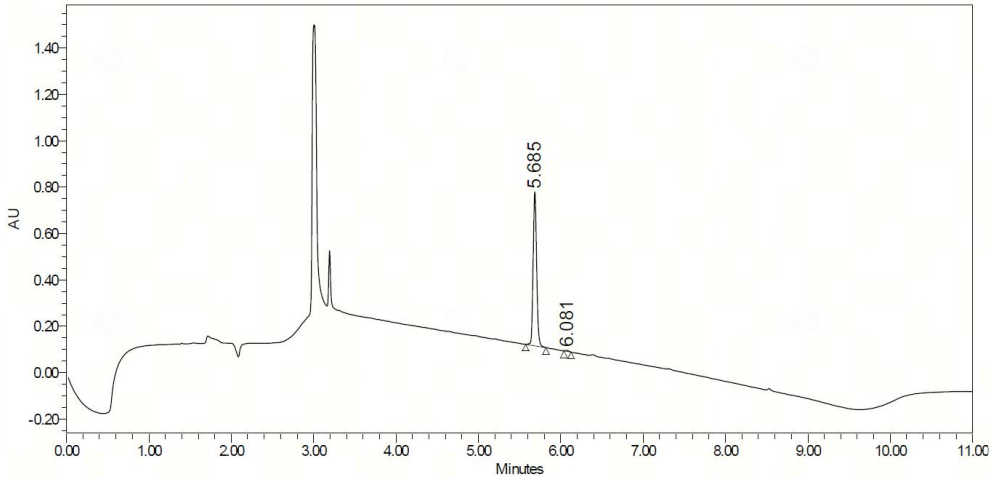
HPLC of **S59** formation*



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
9-Methylene-9H-fluorene	8.820	-	-	-
S58	6.782	3.66	1.12	0.6
S59	2.081	561.23	106.78	99.4
Total	-	-	-	100.00

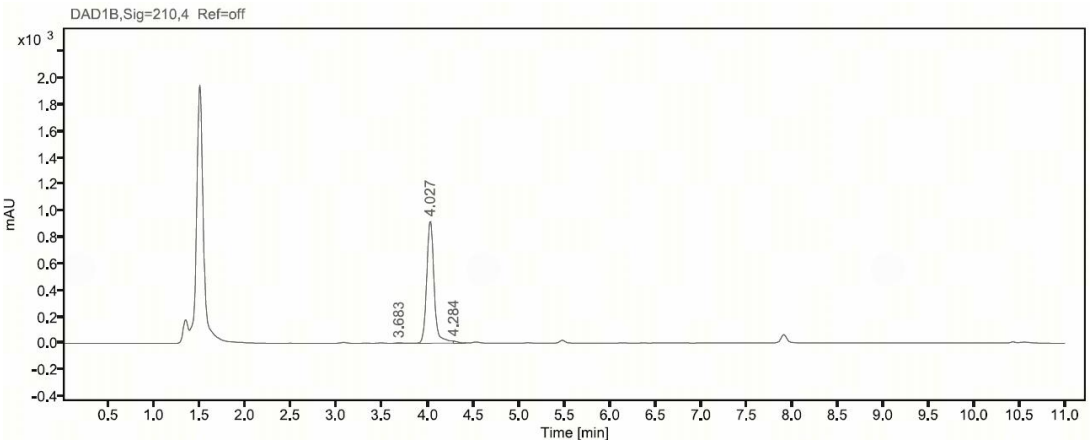
*Broad peaks at 5.6 min and 6.5 min were also observed in blank run.

HPLC of **S60** formation



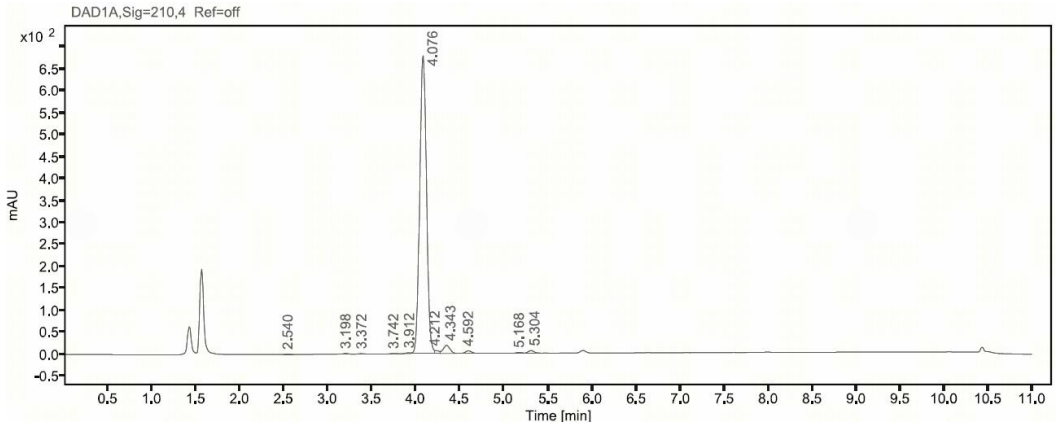
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S58	6.081	15802	5214	0.8
S58-INT	6.875	-	-	-
S60	5.685	1958759	663778	99.2
S60-over-addition	5.51	-	-	-
Total	-	-	-	100.0

HPLC of **10** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	Area% ratio
S60	4.284	61.53	15.87	1.2
S60-INT	4.983	-	-	-
10	4.027	5215.64	920.84	98.5
10-over-addition	3.683	18.23	3.59	0.3
Total	-	-	-	100.0

HPLC of isolated **10** (MeCN solution)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
Blank	1.422			
DMAc	1.571			
S60	4.343	100.10	18.86	2.6
S60-INT	5.032	-	-	-
10	4.078	3636.44	680.04	94.6
10-over-addition	3.742	4.22	0.90	0.1
Others	-	-	-	2.7
Total	-	-	-	100.0

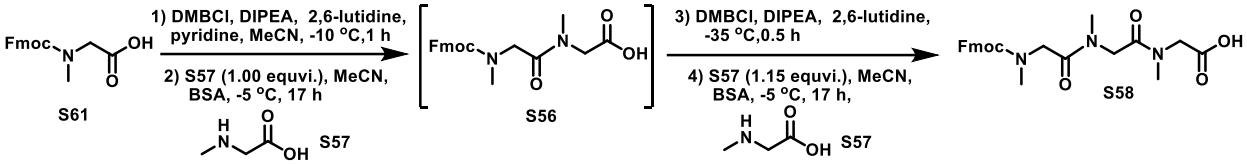
Method 1: Waters Atlantis T3, 4.6 × 150 mm, 3 μm; 0.1% H_3PO_4 in water (v/v) (solvent A), MeCN (solvent B); gradient 5%-95% (solvent B) in 6.0 min, hold 2.0 min; flow rate = 1.0 mL/min; detection wavelength = 210 nm.

Method 2: Waters Xbridge C18, 150 mm × 4.6 mm, 3.5 μm; 0.1% H_3PO_4 in water(v/v) (solvent A), MeCN (solvent B); gradient 5%-85% (solvent B) in 1.0 min, gradient 85%-95% (solvent B) in 3.0 min; hold 1.0 min; flow rate = 0.8 mL/min; detection wavelength = 210 nm.

Method 3: Xselect CSH C18, 4.6 × 150 mm, 3.5 μm column; 0.05% TFA (v/v) in water (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 5%-95% (solvent B) in 7.0 min, hold 2.0 min; flow rate = 1.2 mL/min; detection wavelength = 210 nm.

Method 4: Waters Xbridge C18, 150mm x 4.6 mm, 3.5 μm; 0.1% H_3PO_4 in water(v/v) (solvent A), MeCN (solvent B); gradient 5%-95% (solvent B) in 6.0 min, hold 2.0 min; flow rate = 1.0 mL/min; detection wavelength = 210 nm.

Sar-3 **S58** can also be prepared from Fmoc monomer **S61**

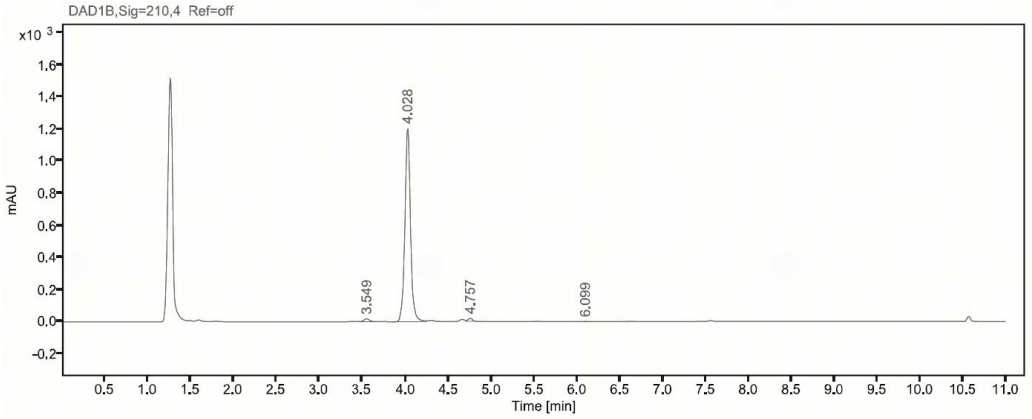


Synthesis of Fmoc-Sar-Sar-Sar-OH (S58). To a 250 mL three-neck jacketed vessel were added compound **S61** (10.00 g, 32.12 mmol) and MeCN (50 mL). After the solution was cooled to -10 °C, 2,6-lutidine (4.13 g, 38.54 mmol), DIPEA (4.32 g, 33.40 mmol), pyridine (0.254 g, 3.21 mmol) and DMBCl (4.50 g, 33.40 mmol) were added. The reaction mixture was stirred at -10 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S61**:**S61-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.5:99.5. The resulting **S61-INT** was added over 10 min into the pre-cooled (-5 °C) solution of **S57** and BSA in MeCN, which was prepared by stirring **S57** (2.86 g, 32.12 mmol) and BSA (9.80 g, 48.18 mmol) in MeCN (50 mL) at 45 °C for 3 h and then cooled to -5 °C. The reaction mixture was stirred at -5 °C for 17 h and monitored by HPLC (**Method 1**). The ratio of **S61**:**S61-INT**:**S56:S56-over-addition** was 1.1:0.1:97.5:1.3. IPA (12.55 g, 208.78 mmol) was added at -5 °C and stirred for 10 h. The resulting **S56** solution was cooled to -35 °C, and 2,6-lutidine (1.72 g, 16.06 mmol), DIPEA (5.19 g, 40.15 mmol) and DMBCl (5.84 g, 43.36 mmol) were added. The reaction mixture was stirred at -35 °C for 0.5 h and monitored by HPLC (**Method 1**), which showed the ratio of **S56**:**S56-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 1.0:99.0. The resulting **S56-INT** solution was added over 10 min into the pre-cooled (-5 °C) solution of **S57** and BSA in MeCN, which was prepared by stirring **S57** (3.29 g, 36.94 mmol) and BSA (32.67 g, 160.6 mmol) in MeCN (50 mL) at 45 °C for 3 h and then cooled to -5 °C. The reaction mixture was stirred at -5 °C for 17 h and monitored by HPLC (**Method 1**). The ratio of **S56**:**S56-INT**:**S58:S58-over-addition** was 2.4:0.4:96.8:0.4. The HPLC showed the reaction assay yield was 90% (two steps). The reaction was quenched with H_2O (10 mL) at 20 °C and stirred for 1 hour. The mixture was then concentrated under vacuum at 20 °C. H_2O (390 mL) and toluene (400 mL) were added, and the pH was adjusted to 3.0 with 4N HCl. The mixture was stirred at 35 °C for 12 hours (Adding seed crystals can shorten the crystallization time), then at 10 °C for 24 hours. The solid was collected by filtration and dried

at 50 °C under vacuum to afford 12.38 g product with 98% purity and 85% isolated yield as a white solid. mp 161.7 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.69 (brs, 1 H), 7.90 (m, 2 H), 7.68 (d, J = 7.38 Hz, 1 H), 7.61 (t, J = 8.57 Hz, 1 H), 7.42 (q, J = 7.13 Hz, 2 H), 7.34 (m, 2 H), 4.30 (m, 2 H), 4.18 (m, 4 H), 4.02 (m, 2 H), 3.92 (m, 1 H), 3.01 (m, 2 H), 2.91 (m, 1 H), 2.81 (m, 6 H). ^{13}C NMR (101 MHz, DMSO-d_6) δ ppm 170.73, 170.69, 170.61, 170.46, 168.70, 168.06, 168.02, 155.99, 155.86, 143.85, 140.73, 140.68, 140.63, 127.67, 127.60, 127.55, 127.16, 125.35, 125.09, 120.15, 120.01, 67.02, 66.83, 66.70, 50.03, 49.98, 49.70, 49.66, 49.64, 49.00, 48.67, 48.61, 46.59, 35.71, 35.63, 35.23, 35.06, 34.93, 34.88, 34.80, 34.72, 34.66. HRMS (ESI): m/z calcd for $C_{24}H_{28}O_6N_3$ $[M+H]^+$: 454.1973, found: 454.1970. FTIR (neat): 3047, 2939, 1743, 1700, 1670, 1619, 1492, 1402, 1242, 1205, 1145, 785, 760, 740, 594 cm^{-1}.

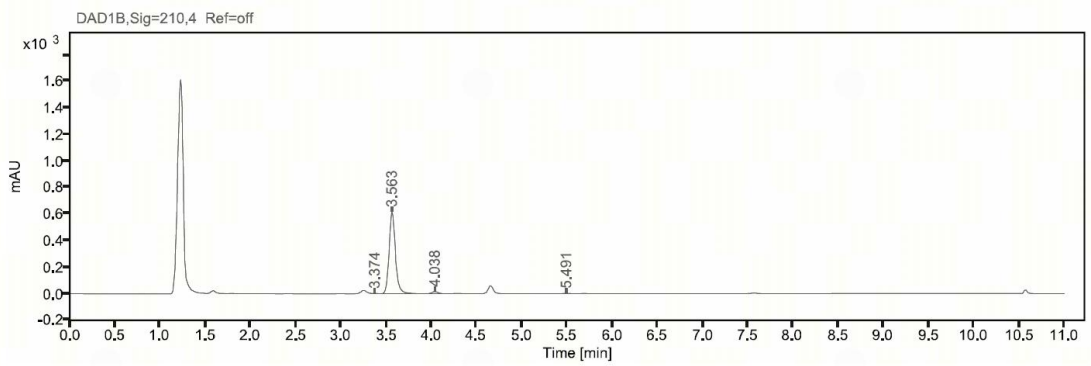
Method 1: Waters Atlantis T3, 4.6 × 150 mm, 3 μm; 0.1% H_3PO_4 in water (v/v) (solvent A), MeCN (solvent B); gradient 5%-95% (solvent B) in 6.0 min, hold 2.0 min; flow rate = 1.0 mL/min; detection wavelength = 210 nm.

HPLC of **S56** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S56	4.028	5509.62	1208.60	97.5
S61	4.757	64.44	17.39	1.1
S61-INT	6.099	2.54	0.70	0.1
S56-over-addition	3.549	76.25	17.23	1.3
Total	-	-	-	100.0

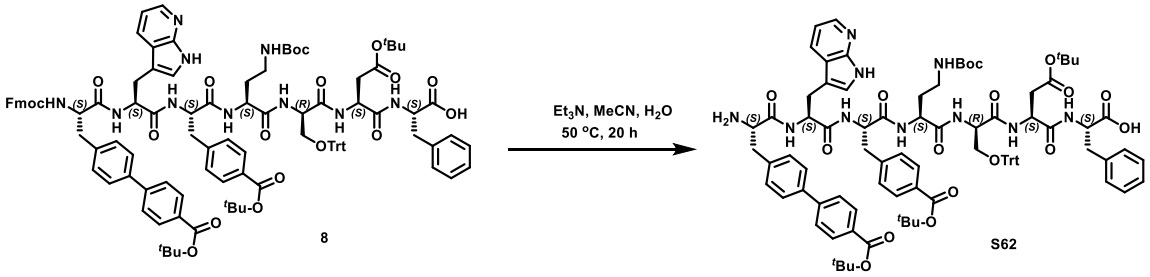
HPLC of **S58** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S58	3.563	3014.0	617.2	96.8
S56	4.038	74.3	16.6	2.4
S56-INT	5.491	12.7	3.3	0.4
S58-over-addition	3.374	13.7	2.9	0.4
Total	-	-	-	100.0

Starting from Fmoc monomer **S61**, 2811 g of reagents/solvents were used above to synthesize 10 g of **10** (as a solution in MeCN), giving a PMI of 281.

Synthesis of a 19-mer by continuous fragment couplings



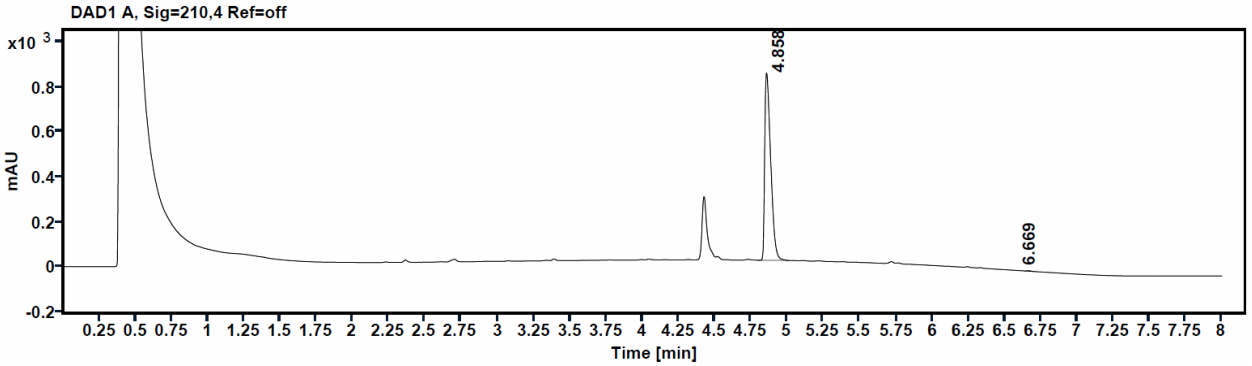
Synthesis of Bip-4-CO$_2$*t*Bu-7-aza-Trp-L-Phe(O*t*Bu)-L-Dab(Boc)-D-Ser(Trt)-L-Asp(O*t*Bu)-L-Phe (S62). Into a 500-mL three-neck jacketed vessel, Fmoc-Bip-4-CO$_2$*t*Bu-7-aza-Trp-L-Phe(O*t*Bu)-L-Dab(Boc)-D-Ser(Trt)-L-Asp(O*t*Bu)-L-Phe (**8**) (20.79 g, 96.2% LCAP, 10.83 mmol) was dissolved in MeCN:H_2O (1:0.1, 200 mL) at 25 °C. Triethylamine (Et_3N) (5.48 g, 54.15 mmol) was added. The reaction mixture was stirred at 50 °C for 20 h and monitored by HPLC (Method 1), which showed the ratio of **8**:**S62** was 0.2:99.8. The reaction mixture was purified by Prep-HPLC under the conditions: Column: C18, 20-40 um, 330 g; Mobile phase A: H_2O, Mobile phase B: MeCN; Flow rate: 100 mL/min; Gradient: 45-75% B in 20 min; Wavelength: 210 nm. The collected fractions were concentrated to remove MeCN and then freeze-

dried to yield 12.67 g of **S62** as a white solid in 70% isolated yield (97.5% LCAP, yield calculated by area%).

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 11.31 (br s, 1H), 9.59 (br s, 1H), 8.91 (br s, 1H), 8.80 (overlap, 1H), 8.74 (overlap, 1H), 8.14 (d, J = 4.8 Hz, 2H), 7.98 - 7.94 (m, 3H), 7.73 (d, J = 8.0 Hz, 4H), 7.50 (d, J = 7.7 Hz, 2H), 7.39 (d, J = 6.7 Hz, 1H), 7.35 - 7.27 (m, 16H), 7.23 (d, J = 7.0 Hz, 3H), 7.19 - 7.10 (m, 8H), 7.06 (d, J = 7.0 Hz, 1H), 6.97 (dd, J = 7.8, 4.7 Hz, 1H), 6.69 (br s, 1H), 4.66 - 4.63 (m, 1H), 4.53 (overlap, 1H), 4.48 (overlap, 1H), 4.41 - 4.37 (m, 1H), 4.29 (br s, 1H), 4.12 - 4.07 (m, 1H), 3.29 (overlap, 1H), 3.13 - 3.05 (m, 5H), 3.01 (d, J = 5.1 Hz, 1H), 2.94 (overlap, 5H), 2.80 - 2.77 (m, 1H), 2.67 - 2.64 (m, 1H), 2.39 - 2.28 (m, 2H), 1.81 (br s, 1H), 1.57 (s, 9H), 1.49 (s, 9H), 1.35 (s, 9H), 1.30 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 173.39, 173.17, 171.11, 170.54, 170.40, 168.66, 168.07, 164.38, 164.22, 154.87, 147.87, 143.70, 143.40, 142.93, 141.58, 138.67, 138.32, 136.00, 129.39, 129.25, 129.03, 128.93, 128.60, 128.17, 127.68, 127.22, 127.02, 126.36, 125.92, 125.03, 123.53, 119.08, 114.14, 108.76, 85.32, 80.04, 79.72, 79.48, 76.94, 62.38, 55.50, 54.88, 52.83, 52.37, 50.05, 49.78, 37.20, 36.60, 36.10, 32.19, 27.65, 27.22, 27.17, 27.03. HRMS (ESI): m/z calcd. for $C_{92}H_{106}N_{10}O_{17}$ $[M+H]^+$: 1623.7810, found: 1623.7877. FTIR (neat): 3278, 3059, 2976, 2931, 1711, 1636, 1606, 1526, 1492, 1448, 1392, 1366, 1292, 1251, 1162, 1114, 1005, 847, 770, 699, 632 cm^{-1}.

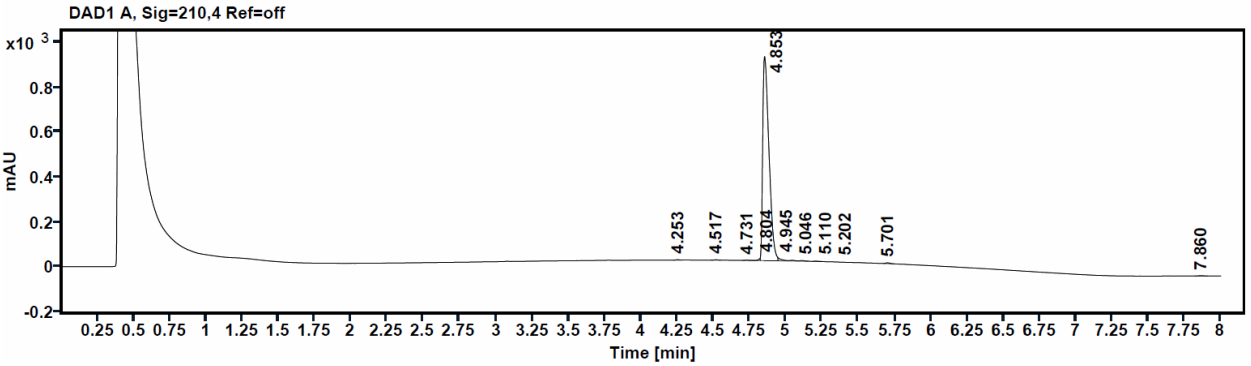
Method 1: HPLC condition: Express C18 3.0 × 100 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 40-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

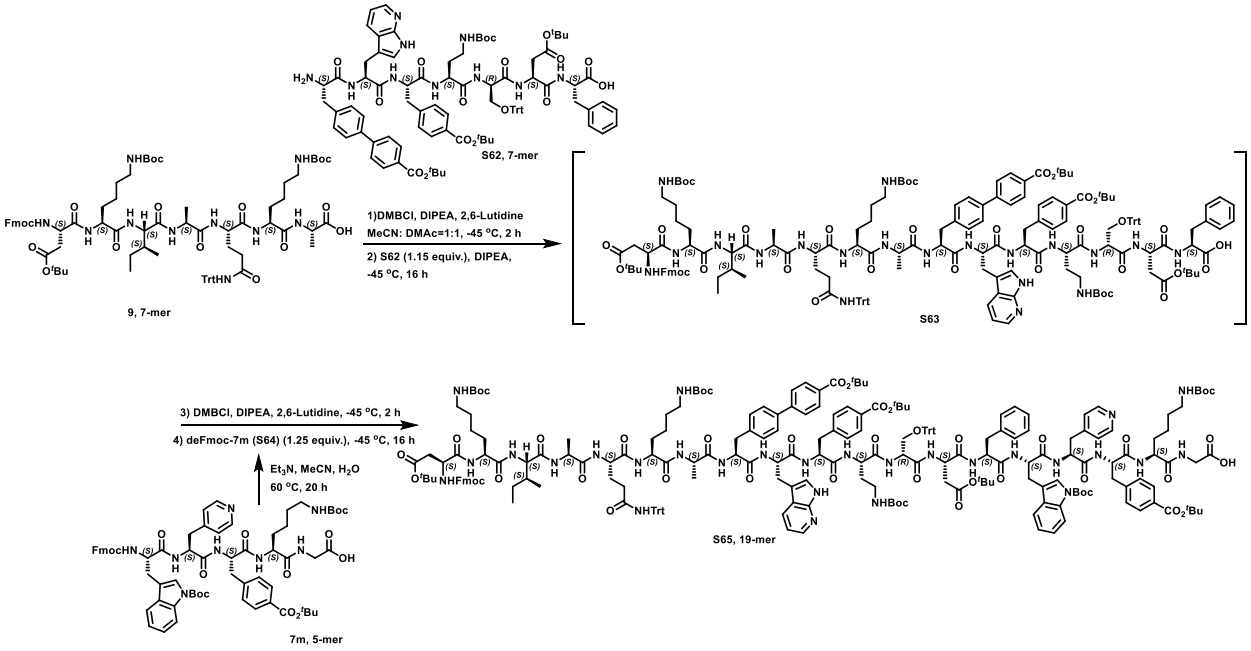
HPLC of **S62** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S62	4.858	2405.5947	836.8	99.8
8	6.669	4.7867	2.6	0.2
Total	-	-	-	100.0

HPLC of isolated **S62**





Synthesis of Fmoc-L-Asp(O*t*Bu)-L-Lys(Boc)-L-Lle-L-Ala-L-Gln(Trt)-L-Lys(Boc)-L-Ala-Bip-4-CO$_2$*t*Bu-7-aza-Trp-L-Phe(O*t*Bu)-L-Dab(Boc)-D-Ser(Trt)-L-Asp(O*t*Bu)-L-Phe-L-Trp(Boc)-L-Ala(4-PYRI)-L-Phe(O*t*Bu)-L-Lys(Boc)-Gly (S65, 19-mer).

Into a 250-mL three-neck jacketed vessel, Fmoc-L-Trp(Boc)-L-Ala(4-PYRI)-L-Phe(O*t*Bu)-L-Lys(Boc)-Gly (**7m**) (10.18 g, 98.2% LCAP, 8.28 mmol) was dissolved in MeCN:H_2O (1:0.1, 100 mL) at 25 °C. Then Et_3N (1.09 g, 10.77 mmol) was added at 25 °C and the reaction mixture was stirred at 60 °C for 20 h. The reaction was monitored by HPLC (**Method 2**), which showed the ratio of **7m:**L-Trp(Boc)-L-Ala(4-PYRI)-L-Phe(O*t*Bu)-L-Lys(Boc)-Gly (**S64)** was 0:95.8. The reaction solution (85.03 g, assumed 100% yield as 8.28 mmol) was cooled down to 25 °C and directly used without isolation.

Into a 250-mL three-neck jacketed vessel, DMBCl (0.60 g, 4.42 mmol) was dissolved in MeCN (30 mL) at 25 °C. After the solution was cooled to -45 °C, a solution of Fmoc-L-Asp(O*t*Bu)-L-Lys(Boc)-L-Lle-L-Ala-L-Gln(Trt)-L-Lys(Boc)-L-Ala **(9)** (6.32 g, 95.0% LCAP, 4.02 mmol), 2,6-lutidine (0.65 g, 6.03 mmol.),

and DIPEA (0.52 g, 4.02 mmol) in DMAc (30 mL) was added at -45 ºC in 10 min. The reaction mixture was stirred at -45 ºC for 2 h and monitored by HPLC, which showed the ratio of **9:9-INT**:***epi*-9-INT** was 1.3:98.0:0.7. Then **S62** (7.51 g, 4.62 mmol) and DIPEA (0.16 g, 1.21 mmol) were added into the reaction mixture at -45 ºC, and stirred at -45 ºC for 16 h then monitored by HPLC (**Method 2**), which showed the ratio of **9:9-INT**:***epi*-9-INT:S63**:***epi*-S63**:**S63-over-addition** was: 1.1:0.9:0.2:97.6:0.1:0.0. DMAc (200 mL) was added to the reaction, and the mixture was filtered. The filtrate was treated by hyperfiltration (membrane: 3 kD, 0.11m^2) and concentrated to 100 mL, then 100 mL DMAc was added, and the solution was subjected to hyperfiltration (membrane: 3 kD, 0.11m^2) to concentrate to ~60 mL.

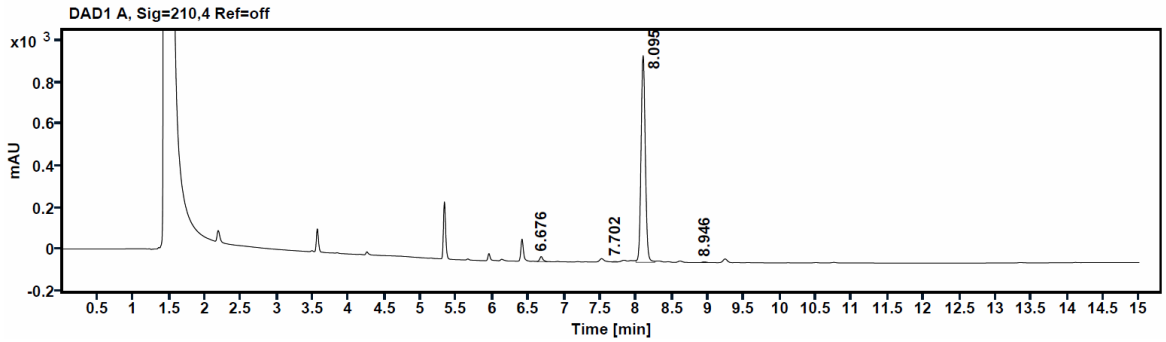
To the above solution, MeCN (30 mL), DMBCl (0.67 g, 5.02 mmol), DIPEA (0.60 g, 4.62 mmol), and 2,6-lutidine (0.65 g, 6.03 mmol) were added at -45 ºC and stirred at -45 ºC for 2 h. HPLC (**Method 3**) showed the ratio of **S63**:**S63-INT**:***epi*-S63-INT** was 1.8:97.8:0.4. The above MeCN solution of **S64** (51.60 g, 5.02 mmol active) was added to the reaction mixture. The reaction mixture was stirred at -45 °C for 16 h and monitored by HPLC (**Method 3**), which showed the ratio of **S63:S65**:***epi*-S65**:**S65-over-addition** was 1.8:97.6:0.3:0.3, and the reaction assay yield was 90% over two couplings. The reaction solution was poured into H_2O (300 mL) at 25 °C. After filtration, the crude product was purified by Prep-HPLC under the conditions: Column: C8, 20-40 μm, 330 g; Mobile Phase A: 5 mM NH_4Ac aq., Mobile Phase B: MeCN:*i*-PrOH= 3: 7; Flow rate:100 mL/min; Gradient: 70-90% B in 40 min; Wavelength: 210 nm. The collected fractions were concentrated to remove most of the organic solvent and then freeze-dried to provide 13.62 g of **S65** with 80% isolated yield over two couplings (96.3% LCAP).

^{1}H NMR (400 MHz, DMSO-d_6, 333.7 K) δ 11.17 (br s, 1H), 8.48 (d, J = 5.3 Hz, 2H), 8.32 - 7.96 (m, 16H), 7.87 - 7.49 (m, 23H), 7.43 - 7.15 (m, 45H), 7.07 - 6.99 (m, 15H), 6.55 - 6.42 (m, 4H), 4.72 - 4.42 (m, 12H), 4.38 - 4.17 (m, 10H), 3.87 - 3.75 (m, 2H), 3.29 - 2.53 (m, 26H), 2.41 - 2.32 (m, 3H), 2.00 - 1.66 (m, 8H), 1.59 - 1.58 (m, 20H), 1.49 - 1.48 (m, 20H), 1.40 - 1.37 (m, 53H), 1.28 - 1.26 (m, 6H), 1.22 (s, 10H), 1.17 - 1.07 (m, 4H), 0.86 - 0.79 (m, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.82, 172.56, 172.25, 171.96, 171.53, 171.19, 171.03, 170.77, 170.54, 170.11, 169.82, 169.51, 165.39, 165.28, 156.31, 156.03, 149.51, 148.59, 147.13, 145.36, 144.70, 144.28, 144.16, 143.99, 143.59, 143.24, 141.21, 138.34, 138.31, 137.69, 137.25, 130.40, 130.11, 129.99, 129.66, 129.31, 128.99, 128.79, 128.27, 128.12, 127.88, 127.53, 126.89, 126.14, 125.70, 120.55, 120.36, 119.89, 118.97, 116.68, 116.01, 109.47, 86.37, 83.75, 81.04, 80.76, 80.71, 80.63, 80.57, 78.10, 77.79, 69.72, 54.22, 52.01, 48.86, 48.80, 47.12, 47.12, 41.13, 40.55, 40.34, 40.13, 39.92, 39.71, 39.50, 39.30, 37.98, 32.53, 32.05, 32.05, 29.76, 29.75, 28.72, 28.28, 28.16, 28.12, 27.91, 24.88, 23.07, 23.05, 23.04, 18.42, 15.78, 11.44. HRMS (ESI): m/z calcd. for $C_{224}H_{280}N_{28}O_{44}$ $[M+2H]^{2+}$: 2033.0261, found: 2033.0179. FTIR (neat): 3281, 2976, 2931, 1655, 1507, 1450, 1366, 1292, 1250, 1161, 1114, 743, 700, 632, 547 cm^{-1}.

Method 2: HPLC condition: XSelect CSH C18 4.6 × 150 mm, particle size 2.5 μm column; 5 mM NH_4Ac aq. (solvent A), MeCN (solvent B); gradient 55%-85% (solvent B) in 3 min, 85-95% (solvent B) in 10 min, hold 5 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

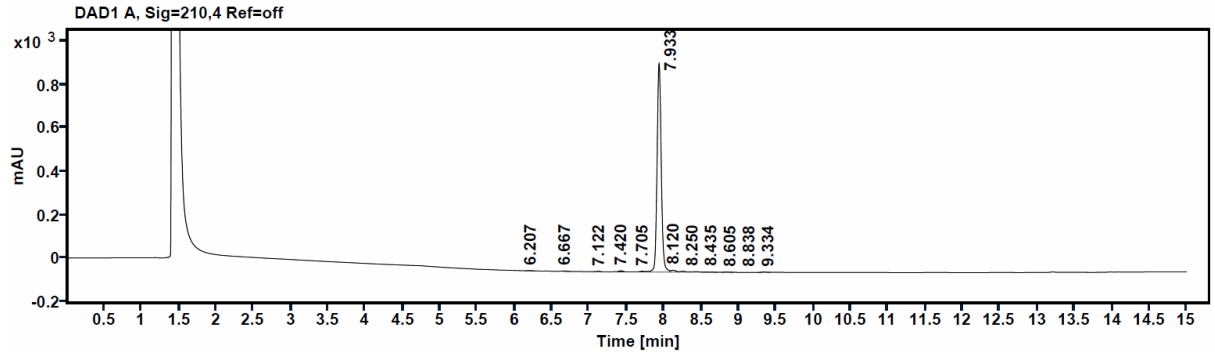
Method 3: HPLC condition: HALO C8 4.6 × 150 mm, particle size 2.7 μm column; 5 mM NH_4Ac aq., pH 9.5 (solvent A), MeCN:*i*-PrOH= 3: 7 (solvent B); gradient 40-75% (solvent B) in 4 min, 75-95% (solvent B) in 15 min, hold 1 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

HPLC of **S65** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S63	6.676	75.3037	24.3	1.8
***epi*-S65**	7.702	11.4556	2.6	0.3
S65	8.095	4115.6538	995.7	97.6
S65-over-addition	8.946	12.9276	3.3	0.3
Total	-	-	-	100.0

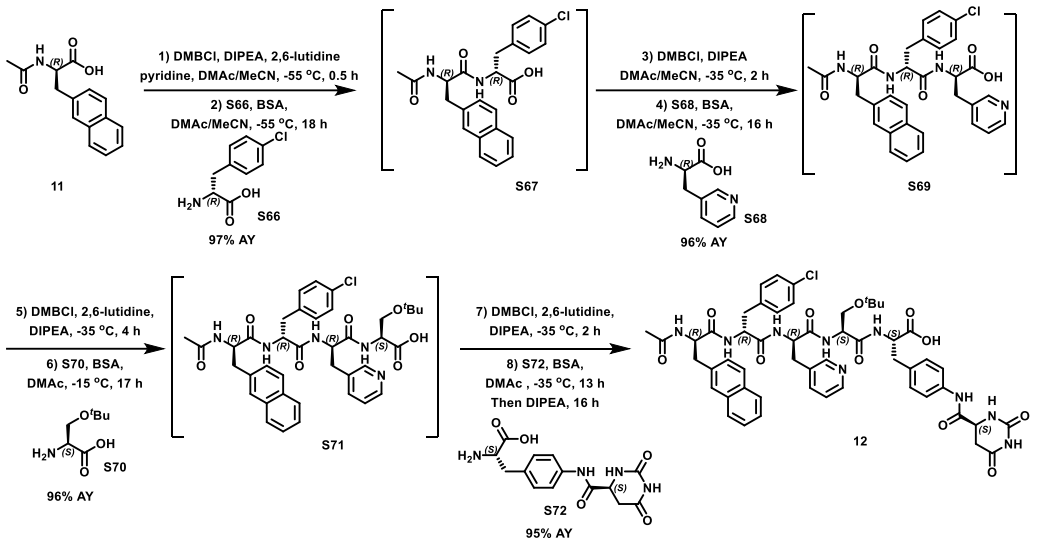
HPLC of isolated **S65**



Experimental procedures and characterizations for Figure 5

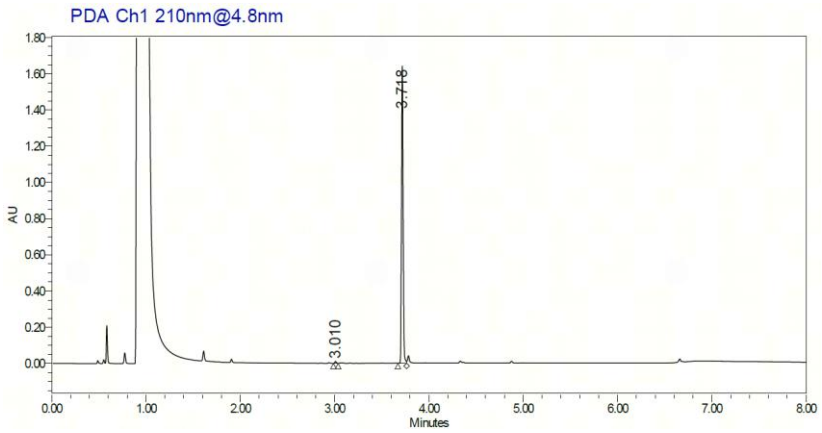
1) Experimental Procedure for Fig. 5a

Total Synthesis of Degarelix



Synthesis of Ac-D-2-Nal-D-Phe(4-Cl)-3-D-Pal-Ser(*t*Bu)-Aph(Hor)-OH (12). To a 2000 mL three-neck jacketed vessel, DMAc/MeCN (1:1, 250 mL) and **11** (50.00 g, 194.33 mmol) were added. After the solution was cooled to -55 °C, DIPEA (25.12 g, 194.33 mmol), 2,6-lutidine (20.82 g, 194.33 mmol), pyridine (3.07 g, 38.87 mmol), and DMBCl (28.77 g, 213.77 mmol) were added in sequence. The reaction mixture was stirred at -55 °C for 0.5 h and monitored by HPLC (**Method 1**), which showed the ratio of **11:11-INT** was 0.30:99.70. A pre-cooled (0 °C) solution of **S66** and BSA in DMAc/MeCN, which was prepared by stirring **S66** (44.62 g, 223.48 mmol) and BSA (79.07 g, 388.67 mmol) in DMAc/MeCN (1:1, 250 mL) at 50 °C for 1 h and then cooled to 0 °C, was added to above solution over 0.5 h. The reaction mixture was stirred at -55 °C for 18 h and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 97%, and the ratio of **11**:**S67**:***epi*-S67**:**S67-over-addition** was: 0.6:99.4:0.0:0.0. Then *i*-PrOH (35.04 g, 583.00 mmol) was added to the reaction and stirred for 2 h at 0 °C. The resulting solution was directly used for the next step.

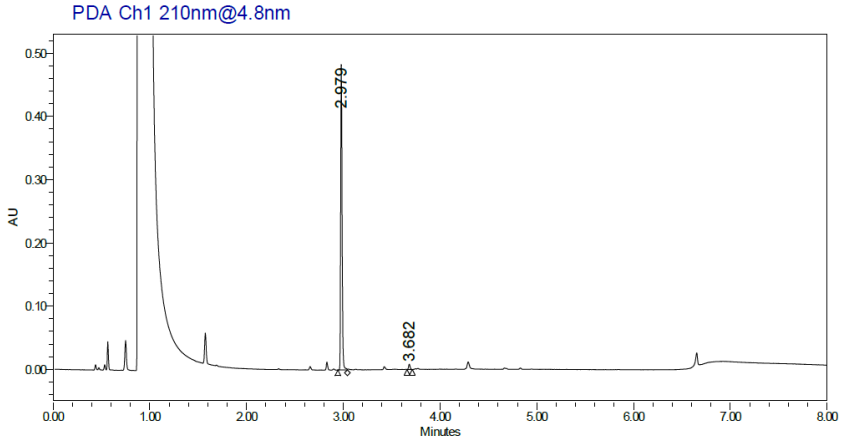
HPLC of **S67** reaction mixture



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	Area% ratio
S67-over-addition	4.330	-	-	0.0
S67	3.718	1959245	1641014	99.4
***epi*-S67**	3.627	-	-	0.0
11	3.010	10741	11532	0.6
Total	-	-	-	100.0

The above **S67** solution was cooled to -35 °C, and DIPEA (32.65 g, 252.63 mmol) and DMBCl (35.31 g, 262.35 mmol) were added in sequence. The reaction mixture was stirred at -35 °C for 2 h and monitored by HPLC (**Method 1**), which showed the ratio of **S67**:**S67-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 1.38:98.62. The pre-cooled (0 °C) solution of **S68** and BSA in DMAc/MeCN, which was prepared by stirring **S68** (40.37 g, 242.91 mmol) and BSA (79.07 g, 388.66 mmol) in DMAc/MeCN (4:1, 250 mL) at 80 °C for 1 h and then cooled to 0 °C, was added to the reaction over 0.5 h. The reaction mixture was stirred at -35 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 96%, and the ratio of **S67**:**S69**:***epi*-S69**:**S69-over-addition** was: 1.75:98.25:0.0:0. Then *i*-PrOH (35.04 g, 583.00 mmol) was added to the reaction and stirred for 2 h at 0 °C. The resulting solution was directly used for the next step.

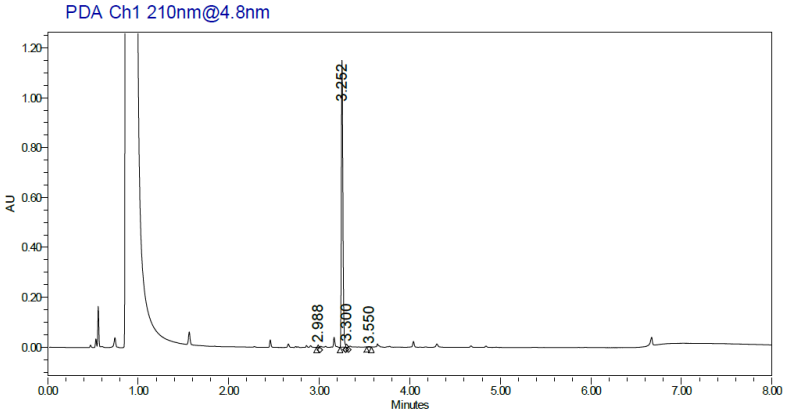
HPLC of **S69** reaction mixture



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S69-over-addition	2.750	-	-	-
S69	2.979	497138	483349	98.25
***epi*-S69**	3.075	-	-	-
S67	3.682	8846	8353	1.75
***epi*-S67-INT**	4.086	-	-	-
Total	-	-	-	100.0

The **S69** reaction solution (194.33 mmol) was cooled to -35 °C, and 2,6-lutidine (6.25 g, 58.30 mmol), DIPEA (37.68 g, 291.50 mmol) and DMBCl (40.54 g, 301.22 mmol) were added in sequence. The reaction mixture was stirred at -35 °C for 4 h and monitored by HPLC (**Method 1**), which showed the ratio of **S69**:**S69-INT** was 0.16:99.84. To another 3000 mL three-neck jacketed vessel, DMAc (250 mL), **S70** (42.29 g, 262.35 mmol), and BSA (79.07 g, 388.66 mmol) were added. The mixture was stirred at 50 °C for 1 h and cooled to -15 °C. The above **S69**-mixed anhydride solution was added to the **S70** and BSA solution over 0.5 h. The reaction mixture was stirred at -15 °C for 17 h and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 96%, and the ratio of **S69**:**S71**:***epi*-S71**:***epi*-S69-INT**:**S71-over-addition** was: 0.59:98.10:1.04:0:0.27. Then *i*-PrOH (35.04 g, 583.00 mmol) was added to the reaction and stirred for 2 h at 0 °C. The resulting solution was directly used for the next step.

HPLC of **S71** reaction mixture



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S71-over-addition	3.550	3704	2505	0.27
S71	3.252	1359031	1148871	98.10
***epi*-S71**	3.300	14361	11204	1.04
S69	2.988	8236	8575	0.59
***epi*-S69-INT**	3.393	-	-	-
Total	-	-	-	100.00

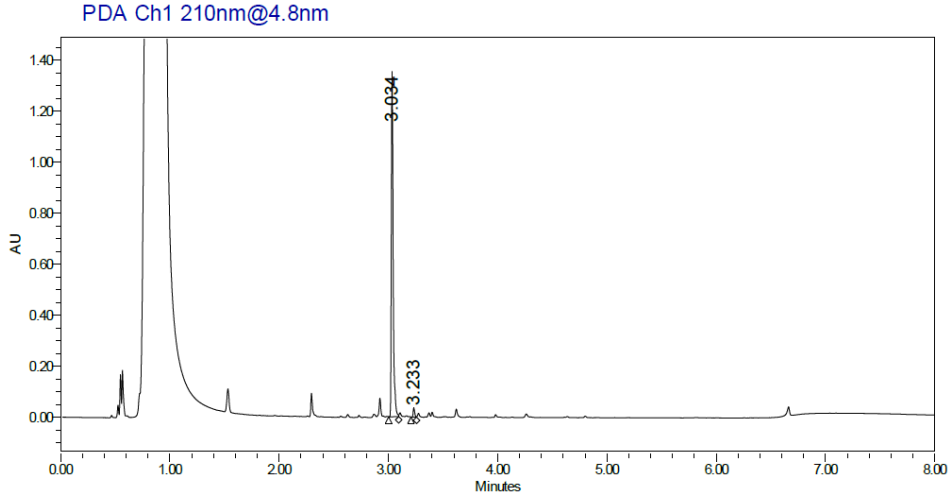
The above **S71** solution (194.33 mmol) was cooled to -35 °C, and 2,6-lutidine (2.08 g, 19.43 mmol), DIPEA (45.21 g, 349.80 mmol), and DMBCl (45.78 g, 340.08 mmol) were added in sequence. The reaction mixture was stirred at -35 °C for 2 h and monitored by HPLC (**Method 1**), which showed the ratio of **S71**:**S71-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 2.45:97.55. Then a pre-cooled (0 °C) solution of **S72**[56] and BSA in DMAc, which was prepared by stirring **S72** (90.26 g, 281.78 mmol) and BSA (118.60 g, 583.00 mmol) in DMAc (300 mL) at 80 °C for 2 h and then cooled to 0 °C, was added at -35 °C over 0.5 h. After addition, the reaction mixture was stirred at -35 °C for 13 h. Extra DIPEA (25.12 g, 194.33 mmol) was added, and the reaction mixture was stirred at -35 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 95% and the ratio of **S71:12**:***epi*-12**:***epi*-S71-INT**:**12-over-addition** was: 2.15:97.85:0:0:0. Then 25 wt% ammonium hydroxide (13.21 g, 194.33 mmol) was added to the reaction and stirred for 2 h at 0 °C. The resulting mixture was filtered at 0 °C to remove the insoluble material, and the filtrate was added to MeCN/H_2O (3:1, 7500 mL) at 10 °C. After adjusting pH 5.0-5.5 with

[56] Compound **S72** was purchased from customized synthesis, and below was the characterization data: ^{1}H NMR of (H-Aph(Hor)-OH) (Dissolve 100 mg sample in 0.7 mL DMSO-d6 and 15 µl TFA): ^{1}H NMR (400 MHz, DMSO-d6) δ 10.25 (s, 1H), 10.04-10.10 (m, 1H), 8.29 (br s, 3H), 7.71 (br s, 1H), 7.50-7.57 (m, J=8.50 Hz, 2H), 7.17-7.23 (m, J=8.50 Hz, 2H), 4.20 (td, J=3.42, 7.04 Hz, 1H), 4.14 (br s, 1H), 3.05 (br d, J=6.38 Hz, 2H), 2.95 (dd, J=7.19, 16.70 Hz, 1H), 2.52-2.59 (m, 1H), 2.48-2.52 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d6) δ 170.8, 169.8, 169.7, 154.3, 138.2, 130.4, 130.4, 119.9, 53.7, 50.8, 35.7, 33.7. HRMS (ESI): m/z calcd for $C_{14}H_{17}N_4O_5$ $[M+H]^+$: 321.1193, found: 321.1210.

4M HCl and stirring for 24 h at 10 °C, the solid was collected by filtration, and the solid was further suspended in H_2O (500 mL), filtered, and this process was repeated, dried under N_2 stream. The solid further was suspended in MTBE (500 mL), filtered, and afforded 188.86 g of **12** as a white solid in 80% isolated yield over 4 steps and 95.3% HPLC purity. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 9.97 (br s, 1H), 8.42 (br s, 1H), 8.39 - 8.35 (m, 1H), 8.30 (br s, 1H), 8.14 (br d, J = 7.9 Hz, 2H), 8.02 - 7.91 (m, 2H), 7.87 - 7.80 (m, 1H), 7.79 - 7.72 (m, 2H), 7.70 - 7.60 (m, 2H), 7.50 - 7.34 (m, 5H), 7.29 - 7.18 (m, 5H), 7.11 (br d, J = 8.3 Hz, 2H), 4.70 (br s, 1H), 4.61 - 4.48 (m, 2H), 4.39 - 4.24 (m, 3H), 3.35 (br s, 2H), 3.10 - 2.71 (m, 9H), 2.68 - 2.58 (m, 1H), 1.68 (s, 3H), 1.08 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ = 175.77, 172.59, 171.30, 170.64, 170.31, 169.30, 169.17, 167.19, 157.65, 150.42, 147.60, 137.42, 136.90, 136.67, 135.81, 132.96, 132.91, 132.33, 131.81, 131.20, 131.00, 129.49, 127.96, 127.87, 127.47, 127.39, 127.35, 125.93, 125.38, 123.18, 118.97, 72.93, 61.83, 54.72, 54.01, 53.76, 53.62, 53.32, 53.19, 37.91, 37.52, 36.79, 36.43, 35.29, 27.18, 22.41. HRMS (ESI): m/z calcd. for $C_{53}H_{59}ClN_9O_{11}$ $[M+H]^+$: 1032.4017, found: 1032.4019. FTIR (neat): 3276, 3066, 1723, 1539, 1191, 714, 470 cm^{-1}.

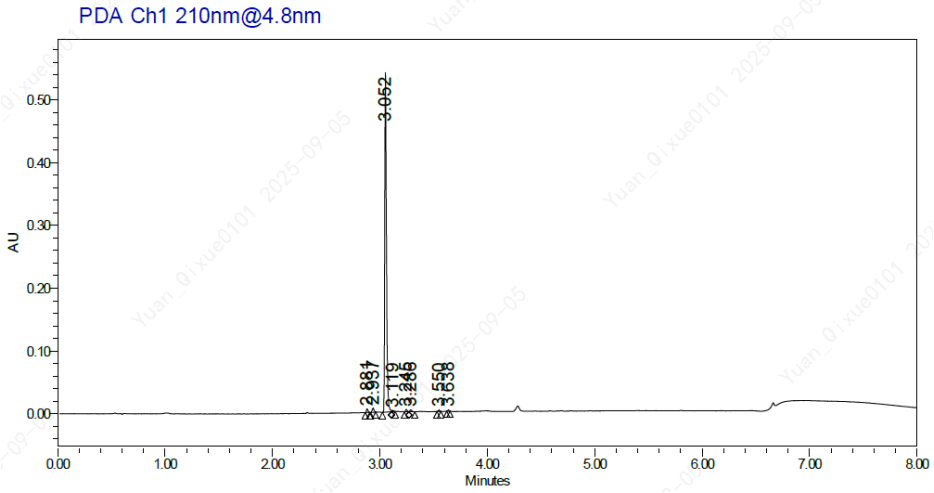
Method 1: HPLC condition: Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-95% (solvent B) in 5.5 min, hold 2.5 min; flow rate = 0.5 mL/min; detection wavelength = 210 nm.

HPLC of **12** reaction mixture

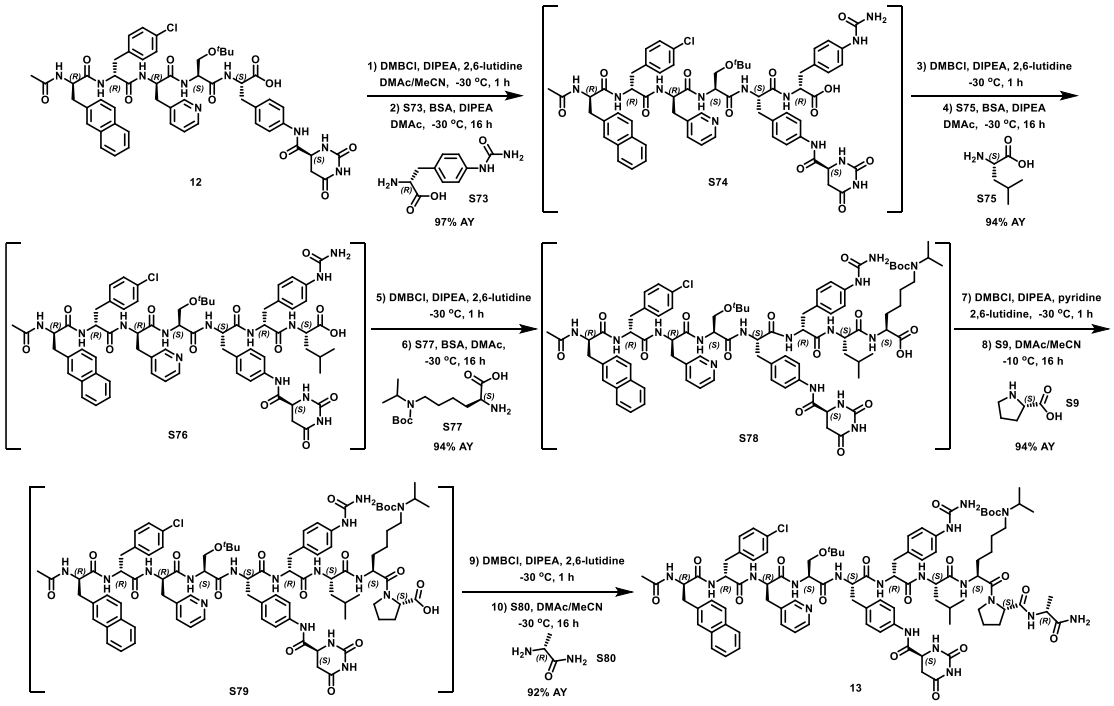


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
12-over-addition	2.813	-	-	-
12	3.034	1564601	1354414	97.85
***epi*-12**	2.993	-	-	-
S71	3.233	34359	35836	2.15
***epi*-S71-INT**	3.670	-	-	-
Total	-	-	-	100.00

HPLC of isolated **12**



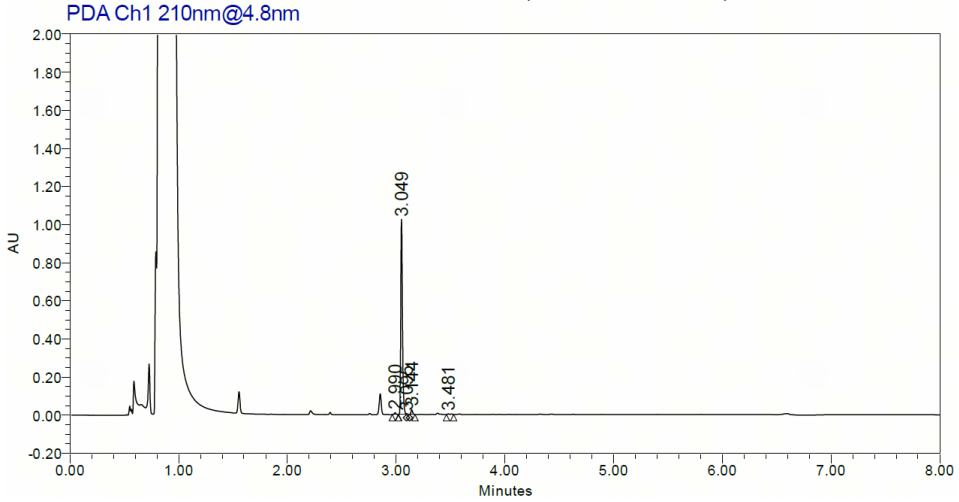
Peak No	RT (min)	Area (mAU*min)	Height (mAU)	area%
12-over-addition	2.820	-	-	-
12	3.052	589212	540296	95.30
***epi*-12**	3.021	-	-	-
S69	3.119	2593	2094	0.42
Total	-	-	-	95.72



Synthesis of Ac-D-2-Nal-D-Phe(4-Cl)-3-D-Pal-Ser(*t*Bu)-Aph(Hor)-D-Phe(4-NHCONH$_2$)-Leu-Lys(BocIPr)-Pro-D-Ala-NH$_2$ (13). To a 250 mL three-neck jacketed vessel, **12** (5.00 g, 4.84 mmol) and

DMAc (50 mL) were added. The solution was distilled azeotropically with MeCN (15 mL x 3) and then MeCN (20 mL) was added. After the solution was cooled to -30 °C, 2,6-lutidine (518.86 mg, 4.84 mmol), DMBCl (749.55 mg, 5.57 mmol), and DIPEA (688.45 mg, 5.33 mmol) were added sequentially. The reaction mixture was stirred at -30 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **12**:**12-INT** was 0.27:99.73. The pre-cooled (-5 °C) solution of **S73**[57] and BSA in DMAc, which was prepared by stirring **S73** (1.17 g, 5.23 mmol) and BSA (1.48 g, 7.26 mmol) in DMAc (25 mL) at 50 °C for 1 h and then cooled to 0 °C, was added to the reaction over 5 min. After stirring for 1 h at -30 °C, DIPEA (625.86 mg, 4.84 mmol) was added. The reaction mixture was stirred at -30 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 97%, and the ratio of **12**:**S74**:***epi*-S74**:***epi*-12-INT**:**S74-over-addition** was: 2.21:96.14:0.27:0.53:0.84. BSA (3.45 g, 16.95 mmol) was added to the reaction and stirred for 2 h at 0 °C. Then *i*-PrOH (2.18 g, 36.32 mmol) was added to the reaction and stirred for 4 h at 20 °C. The resulting solution was directly used for the next step.

HPLC of **S74** formation (reaction mixture)



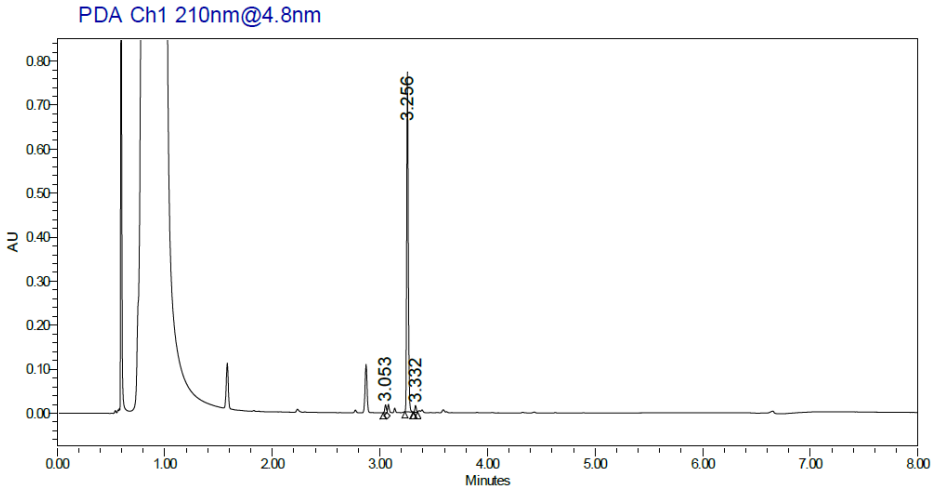
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
BnNH-DMB	2.820	-	-	-
S74-over-addition	2.990	9668	9230	0.84
S74	3.049	1103232	1024972	96.14
***epi*-S74**	3.095	3146	3817	0.27
12	3.140	25368	24501	2.21
***epi*-12-INT**	3.481	6091	3473	0.53

[57] Compound **S73** was purchased from customized synthesis, and below was the characterization data: ^{1}H NMR of H-D-Phe(4-NHCONH$_2$)-OH (Dissolve 100 mg sample in 0.7 mL DMSO-d6 and 70 µl conc. HCl): ^{1}H NMR (400 MHz, DMSO-d6) δ 9.06 (br s, 1H), 8.41 (br s, 3H), 7.27-7.35 (m, *J*=8.50 Hz, 2H), 7.03-7.12 (m, *J*=8.50 Hz, 2H), 3.97-4.10 (m, 1H), 2.95-3.12 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d6) δ 170.7, 156.9, 140.0, 130.2, 127.6, 118.4, 53.8, 35.4. HRMS (ESI): m/z calcd for $C_{10}H_{14}N_3O_3$ $[M+H]^+$: 224.1030, found: 224.1041.

Total	-	-	-	100.00

The above **S74** solution (4.84 mmol) was cooled to -30 °C, then 2,6-lutidine (103.78 mg, 0.97 mmol), DMBCl (912.50 mg, 6.78 mmol) and DIPEA (62.59 mg, 0.49 mmol) were added. The reaction mixture was stirred at -30 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S74**:**S74-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 0.25:99.75. The pre-cooled (-5 °C) solution of **S75** and BSA in DMAc, which was prepared by stirring **S75** (825.73 mg, 6.30 mmol) and BSA (3.45 g, 16.95 mmol) in DMAc (25 mL) at 70 °C for 1 h then cooled to -5 °C, and was added to the reaction over 5 min. After stirring at -30 °C for 2 h, DIPEA (438.10 mg, 3.39 mmol) was added. The resulting mixture was stirred at -30 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 94%, and the ratio of **S74**:**S76**:***epi*-S76**:***epi*-S74-INT**:**S76-over-addition** was 2.14:96.22:0:0:1.64. *i*-PrOH (1.46 g, 24.33 mmol) was added to the reaction and stirred for 12 h at -10 °C. The resulting solution was directly used for the next step.

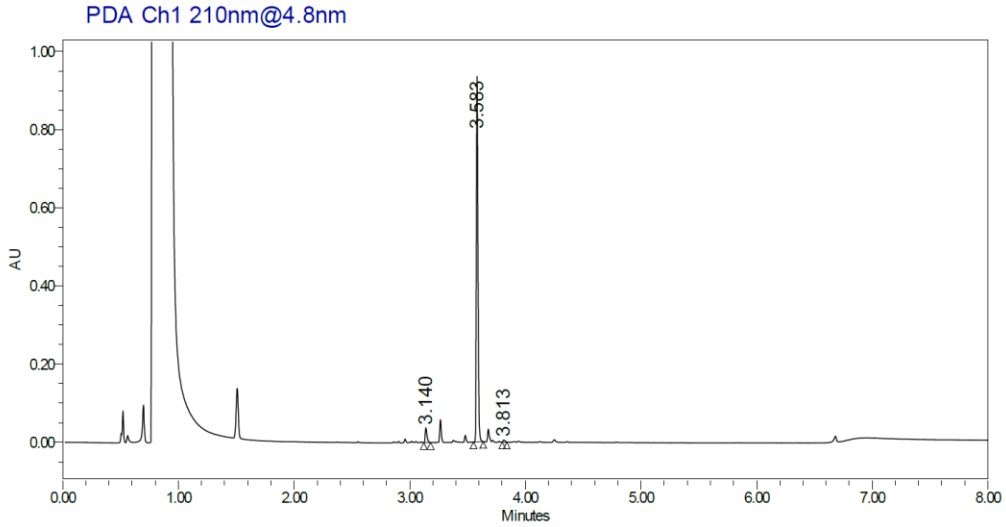
HPLC of **S76** formation (reaction mixture)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
BnNH-DMB	2.820	-	-	-
S76-over-addition	3.332	13296	14952	1.64
S76	3.256	777979	771365	96.22
***epi*-S76**	3.124	-	-	-
S74	3.053	17324	17952	2.14
***epi*-S74-INT**	3.310	-	-	-
Total	-	-	-	100.00

The above **S76** solution (4.84 mmol) was cooled to -30 °C, 2,6-lutidine (103.78 mg, 0.97 mmol), DMBCl (1.24 g, 9.20 mmol), and DIPEA (1.06 g, 8.23 mmol) were added. The reaction mixture was stirred at -30 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S76**:**S76-INT** was 1.99:98.01. Then a pre-cooled (-5 °C) solution of **S77**[58] and BSA in DMAc, which was prepared by stirring **S77** (1.68 g, 5.81 mmol) and BSA (2.46 g, 12.11 mmol) in DMAc (25 mL) at 70 °C for 2 h and cooled to -5 °C, was added at -30 °C over 10 min. The reaction mixture was stirred at -30 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC analysis showed that the reaction assay yield was 94%, and the ratio of **S76**:**S78**:***epi*-S78**:***epi*-S76-INT**:**S78-over-addition** was 4.24:95.01:0.06:0:0.69. *i*-PrOH (1.45 g, 24.21 mmol) was added and stirred for 5 h at 25 °C. The resulting solution was treated by azeotropic distillation with MeCN (20 mL × 3) and used directly for the next step.

HPLC of **S78** formation (reaction mixture)

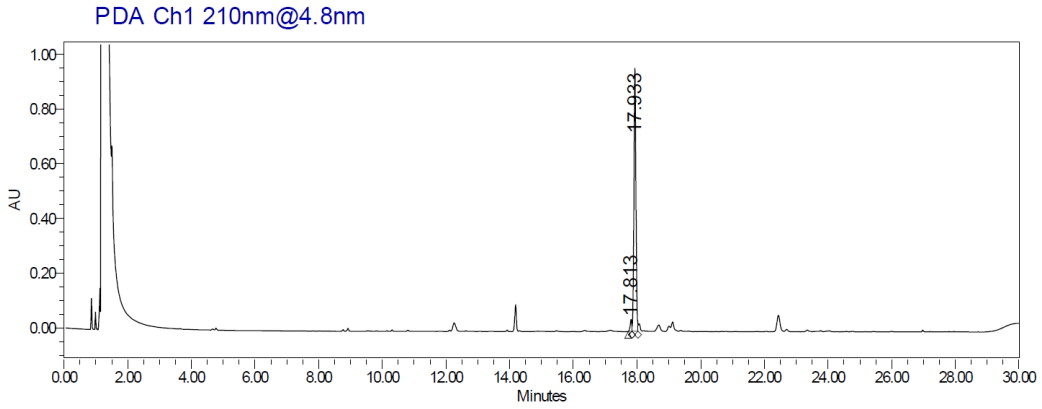


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S78-over-addition	3.813	6684	5027	0.69
S78	3.583	923902	935524	95.01
***epi*-S78**	3.553	557	918	0.06
S76	3.140	41328	38107	4.24
***epi*-S76-INT**	3.527	-	-	-
Total	-	-	-	100.00

[58] Compound **S77** was purchased from customized synthesis, and below was the characterization data: ^{1}H NMR of (H-Aph(Hor)-OH) (Dissolve 100 mg sample in 0.7 mL DMSO-d6 and 15 µl TFA): ^{1}H NMR (400 MHz, DMSO-d6) δ 10.25 (s, 1H), 10.04-10.10 (m, 1H), 8.29 (br s, 3H), 7.71 (br s, 1H), 7.50-7.57 (m, J=8.50 Hz, 2H), 7.17-7.23 (m, J=8.50 Hz, 2H), 4.20 (td, J=3.42, 7.04 Hz, 1H), 4.14 (br s, 1H), 3.05 (br d, J=6.38 Hz, 2H), 2.95 (dd, J=7.19, 16.70 Hz, 1H), 2.52-2.59 (m, 1H), 2.48-2.52 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d6) δ 170.8, 169.8, 169.7, 154.3, 138.2, 130.4, 130.4, 119.9, 53.7, 50.8, 35.7, 33.7. HRMS (ESI): m/z calcd for $C_{14}H_{17}N_4O_5$ $[M+H]^+$: 321.1193, found: 321.1210.

The above **S78** solution (4.84 mmol) was cooled to -30 °C. Pyridine (76.61 mg, 0.97 mmol), 2,6-lutidine (103.78 mg, 0.97 mmol), DIPEA (1.13 g, 8.72 mmol), and DMBCl (1.30 g, 9.68 mmol) were added. The reaction mixture was stirred at -30 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S78**:**S78-INT** (mixed anhydride intermediate quenched by $BnNH_2$) was 1.42:98.58. Then the reaction solution was added into a pre-cooled (-10 °C) mixture of **S9** (1.28 g, 11.14 mmol) in DMAc/MeCN (1:1, 25 mL) over 5 min. The reaction mixture was stirred at -10 °C for 16 h and monitored by HPLC (**Method 2**). The HPLC showed the reaction assay yield was 94%, and the ratio of **S78**:**S79**:***epi*-S79**:**S79-over-addition** was: 3.19:96.81:0.0:0.0. The resulting solution of **S79** was directly used in the next step.

HPLC of **S79** formation (reaction mixture)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S79-over-addition	18.142	-	-	-
S79	17.933	4174805	960882	96.81
***epi*-S79**	18.041	-	-	-
S78	17.813	137489	21426	3.19
Total	-	-	-	100.00

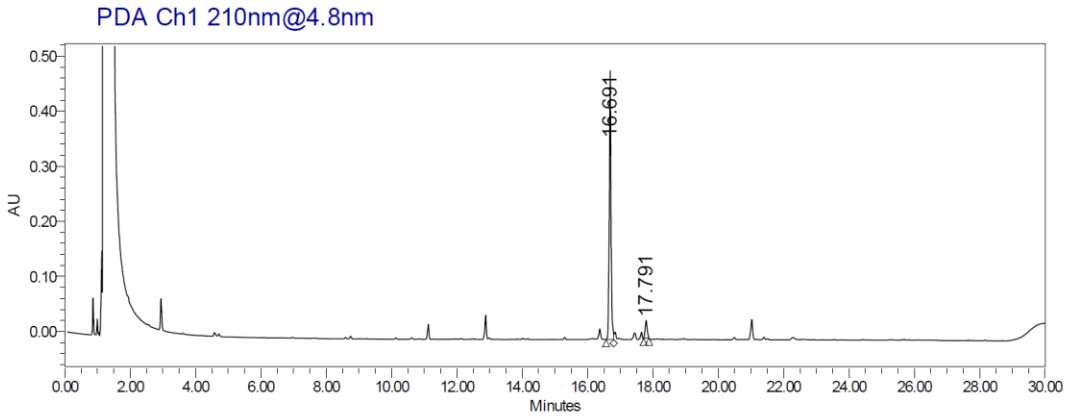
The above **S79** solution (4.84 mmol) was cooled to -30 °C, 2,6-lutidine (103.78 mg, 0.97 mmol), DIPEA (2.32 g, 17.92 mmol), and DMBCl (2.48 g, 18.40 mmol) were added. The reaction mixture was stirred at -30 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S79**:**S79-INT** was 4.30:95.70. Then the reaction solution was added into a pre-cooled (-30 °C) mixture of **S80** (1.49 g, 16.95 mmol) in DMAc/MeCN (1:1, 25 mL) at -30 °C over 5 min. The reaction mixture was stirred at -30 °C for 16 h and monitored by HPLC (**Method 2**). The HPLC showed the reaction assay yield was 92%, and the ratio of **S79**:**13**:**13-over-addition** was: 7.2:92.8:0.0. The H_2O:MeCN (1:1, 5 mL) was added and stirred for 2 h at -5 °C. The reaction solution was transferred into H_2O (250 mL) slowly. Solid was precipitated and filtered

to provide 8.0 g of crude **13** with 79.5% HPLC purity (**Method 1**), 74.7wt% and an isolated yield of 69% over 5 steps.

Method 1: HPLC condition: Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-95% (solvent B) in 5.5 min, hold 2.5 min; flow rate = 0.5 mL/min; detection wavelength = 210 nm.

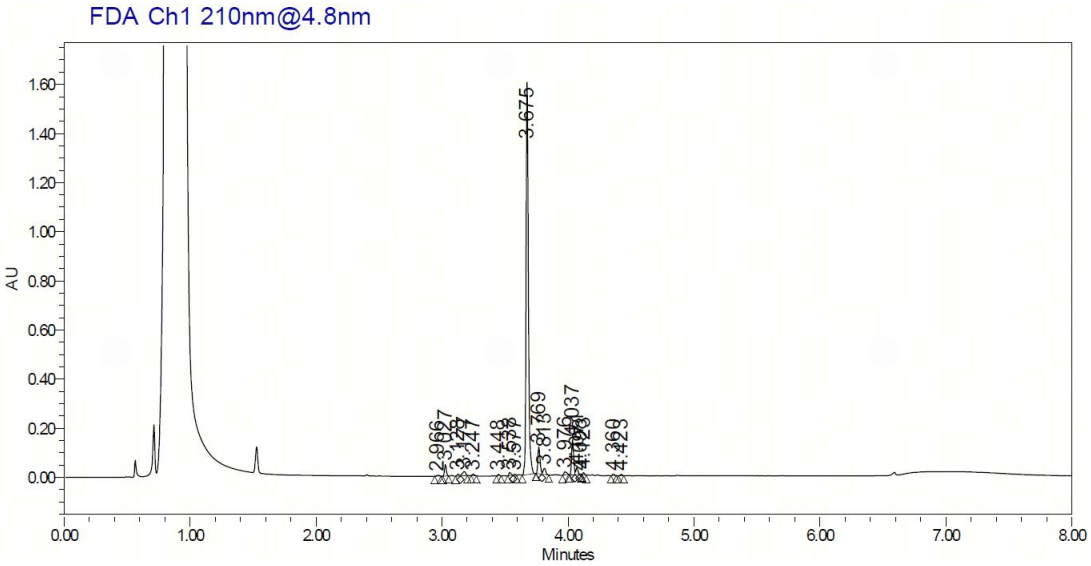
Method 2: HPLC condition: Cortecs UPLC C18+, 2.1 × 150 mm, 1.6 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 20%-55% (solvent B) in 25 min, 55%-95% (solvent B) in 1 min, hold 1 min; flow rate = 0.3 mL/min; detection wavelength = 210 nm.

HPLC of **13** formation (reaction mixture)

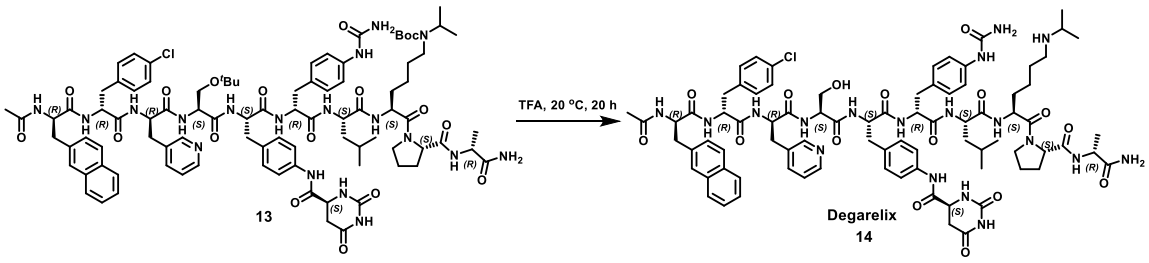


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
13-over-addition	15.548	-	-	-
13	16.691	1786338	488551	92.8
S79	17.791	137725	32487	7.2
Total	-	-	-	100.00

HPLC of crude **13**



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
13-over-addition	3.327	-	-	-
13	3.675	2022452	1598810	79.5
S79	4.037	158272	141651	6.2
Total	-	-	-	85.7



Synthesis of Ac-D-2-Nal-D-Phe(4-Cl)-3-D-Pal-Ser-Aph(Hor)-D-Phe(4-NHCONH$_2$)-Leu-Lys(IPr)-Pro-D-Ala-NH$_2$ (Degarelix, 14). The above 6.0 g crude **13** (4.48 g by assay) was stirred in TFA (30 mL) at 20 °C for 20 h, and monitored by HPLC (**Method 1**), which showed the ratio of **13**:**14** was 0.0:100.0. 3.49 g (by assay) of **14** was obtained and the reaction assay yield was 85%.

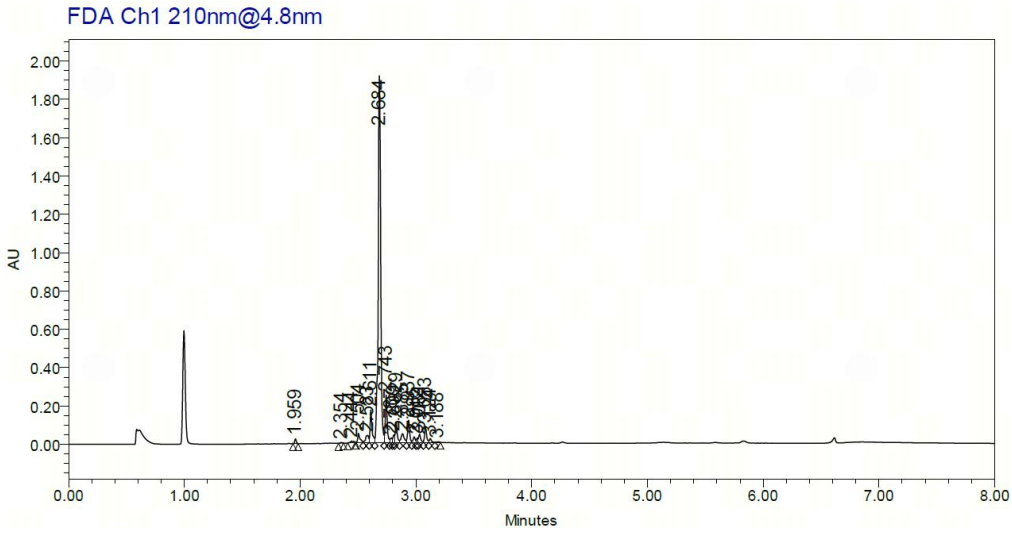
Starting from single AAs, 653 g of reagents/solvents were used in total to synthesize 3.49 g (by assay) of **14**, giving a synthesis PMI of 187.

Most TFA was distilled off under vacuum at room temperature. The resulting reaction mixture was purified by preparative HPLC to yield 3.0 g of **14** (97.15% HPLC purity, 95.4wt%) with an isolated yield of 70%.

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 9.93 (br s, 1H), 8.60 (br s, 1H), 8.43 (br s, 2H), 8.39 - 7.91 (m, 11H), 7.87 - 7.72 (m, 3H), 7.69 - 7.60 (m, 2H), 7.51 - 7.41 (m, 2H), 7.36 (br d, J = 8.3 Hz, 3H), 7.30 - 7.02 (m, 11H), 6.97 - 6.86 (m, 2H), 5.86 (br s, 2H), 4.72 - 4.62 (m, 1H), 4.61 - 4.40 (m, 5H), 4.39 - 4.24 (m, 4H), 4.18 - 4.07 (m, 1H), 3.74 - 3.70 (m, 1H), 3.55 - 3.39 (m, 4H), 3.14 (br s, 1H), 3.08 - 2.69 (m, 12H), 2.67 - 2.55 (m, 3H), 1.98 (dt, J = 6.1, 12.4 Hz, 2H), 1.86 - 1.73 (m, 2H), 1.71-1.62 (m, 4H), 1.61 - 1.39 (m, 6H), 1.31 (br s, 2H), 1.21 (br d, J = 7.1 Hz, 3H), 1.15 (br d, J = 5.6 Hz, 6H), 0.83 (br dd, J = 6.3, 19.3 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 176.23, 174.78, 172.27, 171.82, 171.74, 171.38, 171.34, 171.10, 171.06, 170.89, 170.65, 170.09, 170.06, 169.64, 167.57, 158.14, 158.10, 156.64, 150.80, 147.99, 139.42, 137.61, 137.28, 137.11, 136.21, 133.44, 133.38, 132.91, 132.88, 132.23, 131.61, 131.40, 130.67, 129.90, 129.82, 128.36, 128.28, 127.89, 127.81, 127.78, 126.36, 125.81, 123.63, 119.33, 117.99, 62.18, 60.24, 55.37, 55.16, 54.88, 54.54, 54.22, 54.08, 53.89, 51.63, 50.51, 49.47, 48.54, 47.55, 44.34, 41.63, 38.29, 37.93, 37.38, 37.11, 35.52, 31.06, 29.40, 26.21, 25.16, 24.68, 23.37, 22.83, 22.46, 22.10, 19.49, 18.20. HRMS (ESI): m/z calcd. for Chemical Formula: $C_{82}H_{104}ClN_{18}O_{16}$ $[M+H]^+$: 1631.7561, found: 1631.7577. FTIR (neat): 3280, 3057, 1722, 1515, 1416, 712, 519 cm^{-1}.

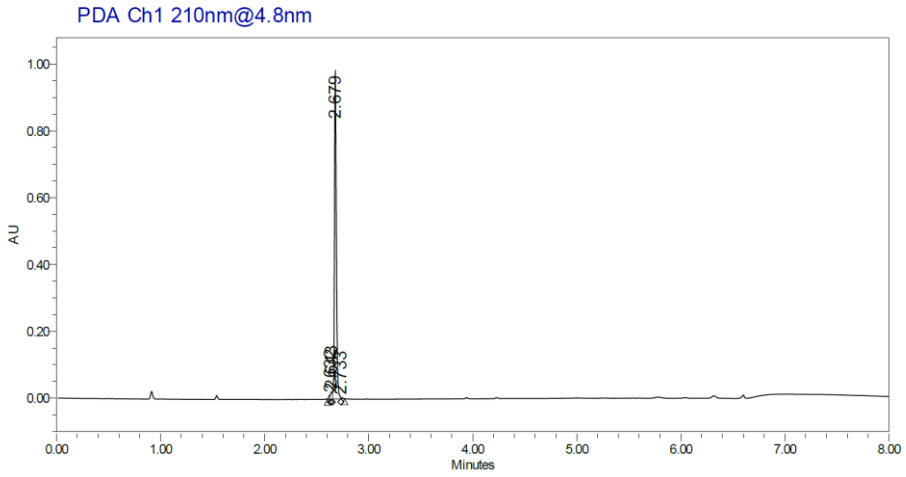
Method 1: HPLC condition: Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-95% (solvent B) in 5.5 min, hold 2.5 min; flow rate = 0.5 mL/min; detection wavelength = 210 nm.

HPLC of **14 (Degarelix)** formation (reaction mixture)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
14 (Degarelix)	2.684	2858379	1912613	68.78
13	3.546	-	-	0
Total	-	-	-	68.78

HPLC of purified **14 (Degarelix)***



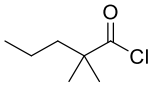
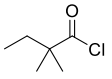
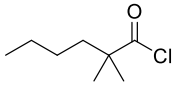
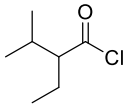
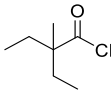
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
14 (Degarelix)	2.679	1230173	984178	97.15
RT2.632	2.632	10179	11809	0.80
RT2.643	2.643	21554	21686	1.71
RT2.733	2.733	4313	4566	0.34
Total	-	-	-	100.00

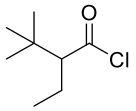
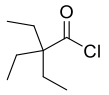
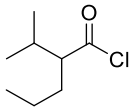
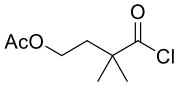
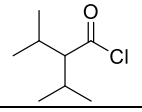
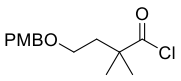
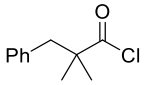
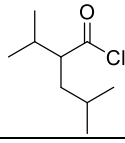
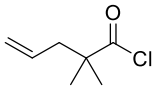
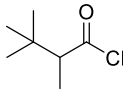
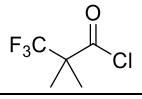
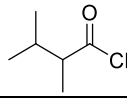
*Early eluting peaks before 2 min were also observed in HPLC blank run.

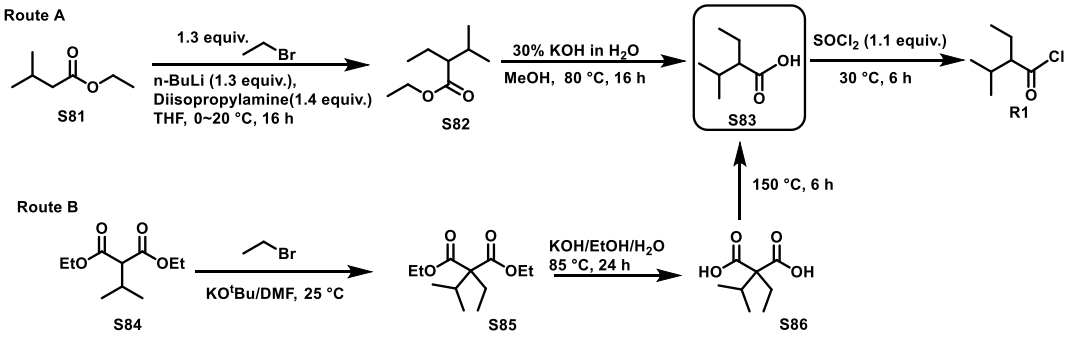
Experimental Procedure for Fig. 5b

Acyl chloride reagents scope and preparation

A series of acyl chloride reagents were prepared and evaluated for the coupling of sterically hindered acids. Commercially available PivCl and DMBCl provided unsatisfactory results, with higher levels of starting acid. In contrast, 2-Ethyl-3-methylbutanoyl chloride (**R1**), 2-ethyl-3,3-dimethylbutanoyl chloride (**R2**), 2-Isopropylpentanoyl chloride (**R3**), and 2-isopropyl-3-methylbutanoyl chloride (**R4**) showed improved conversions.

Reagent #	Name	Structure	Reagent #	Name	Structure
N/A	Pivaloyl chloride **(PivCl)**		**R8**	2,2-Dimethylvaleroyl chloride	
N/A	2,2-Dimethylbutanoyl chloride **(DMBCl)**		**R9**	2,2-Dimethylcaproyl chloride	
R1	2-Ethyl-3-methylbutanoyl chloride		**R10**	2-Ethyl-2-methylbutanoyl chloride	

R2	2-Ethyl-3,3-dimethylbutanoyl chloride		**R11**	2,2-Diethylbutanoyl chloride	
R3	2-Isopropylpentanoyl chloride		**R12**	4-(Acetyloxy)-2,2-dimethylbutanoyl chloride	
R4	2-Isopropyl-3-methylbutanoyl chloride		**R13**	4-((4-Methoxybenzyl)oxy)-2,2-dimethylbutanoyl chloride	
R5	2,2-Dimethyl-3-phenylpropanoyl chloride		**R14**	4-Methyl-2-(propan-2-yl)pentanoyl chloride	
R6	2,2-Dimethylpent-4-enoyl chloride		**R15**	2,3,3-Trimethylbutanoyl chloride	
R7	3,3,3-Trifluoro-2,2-dimethylpropanoyl chloride		**R16**	2,3-Dimethylbutanoyl chloride	



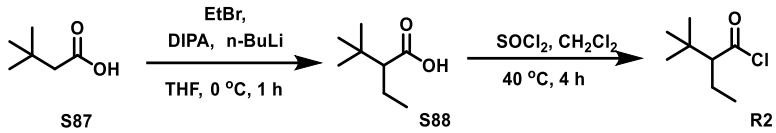
Synthesis of 2-ethyl-3-methylbutanoic acid (S83).

Route A: To a 3 L three-neck jacketed vessel, diisopropylamine (107.0 g, 1.08 mol) was dissolved in THF (500 mL) at 25 °C. The solution was cooled to 5 °C, and n-BuLi (2.5 M, 400 mL, 1.00 mol) was added over 2 h. After cooling the mixture to 0 °C, a solution of ethyl 3-methylbutanoate **(S81)** (100.0 g, 0.77 mol) in THF (500 mL) was added over 1 h, the reaction mixture was stirred at 0 °C for 1 h. A solution of bromoethane (107.0 g, 1.00 mol) in THF (500 mL) was added over 1 h, followed by stirring at 0 °C for 1 h. The reaction mixture was warmed to 20 °C and stirred for 16 h, progress was monitored by GC. Upon completion, HCl aqueous (4 M, 500 mL) was added over 0.5 h to the reaction mixture at 15 °C. The mixture was stirred for 1 h and allowed to stand for 0.5 h. The phases were separated, and the organic layer was collected and concentrated to dryness to provide 115 g of **S82** as a pale yellow oil. The 115 g of **S82** was dissolved in MeOH (460 mL). Aqueous KOH solution (6.8 M, 460 mL) was added, and the mixture was heated to 80 °C and stirred for 16 h and monitored by GC. After completion, the reaction mixture was

cooled to 40 °C and concentrated to remove methanol. The residual aqueous solution was washed with MTBE (575 mL × 2). The aqueous layer was separated, and the pH was adjusted to ~2 at 20 °C using HCl aqueous (6 M). The aqueous phase was extracted with EtOAc (575 mL). The organic layer was collected and concentrated at 45 °C to dryness to provide 79 g of **S83** as a light-yellow oil in 79% isolated yield for 2 steps and 98.4% GC purity.

Route B: In a 3 L three neck jacketed vessel, to the solution of potassium tert-butoxide (66.6 g, 0.59 mol) in DMF (300 mL) at 5 °C, **S84** (100.0 g, 0.50 mol) was charged over 2 h and stirred at 5 °C for another 2 h. After removal of tert-butanol under vacuum at 45 °C, the resulting mixture was cooled to 5 °C and bromoethane (64.7 g, 0.59 mol) was charged at 5 °C over 1 h, then the mixture was warmed to 25 °C and stirred for another 6h till the reaction was completed. The reaction mixture was quenched with water (300 mL) and extracted with MTBE (300 mL) twice. After concentration of combined organic layer, crude **S85** was obtained (115.63 g, 88.4% assay, 0.44 mol) in 90% isolated yield and used directly in the next step. In a 3 L three neck jacketed vessel, to the solution of **S85** (111.2 g, 0.48 mol) in EtOH (440 mL) was charged KOH (162.5 g, 2.9 mol) and water (44 mL). The resulting mixture was heated to 85 °C for 24 h until the reaction was completed. Then the reaction mixture was cooled to 25 °C and diluted with water (550 mL). After removal of EtOH via concentration under vacuum, the solution was extracted with MTBE (2 x 220 mL). The pH of aqueous layer was adjusted to 1.0 with 6N HCl aq. The crude product of **S86** was extracted with MTBE (2 x 220 mL). **S86** was further purified by precipitation from mixture solvent of n-heptane (360 mL) and dichloromethane (85 mL) to afford 75 g product in 90% yield. **S86** (146 g, 0.84 mol) was charged into 500 mL flask and heated to 150 °C for 6 h until the reaction is completed to afford **S83** (109 g, 0.84 mol) in quantitative yield, which was used directly for the next step.

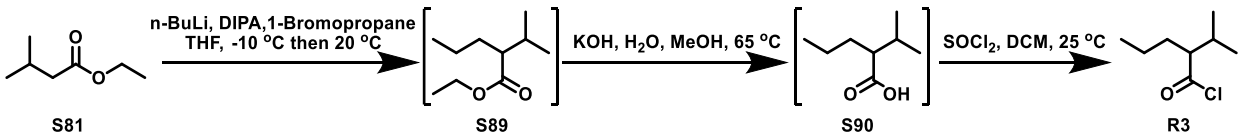
Synthesis of 2-ethyl-3-methylbutanoyl chloride (R1). In a 250-mL three-neck jacketed vessel, 2-ethyl-3-methylbutanoic acid **(S83)** (79.0 g, 0.61 mol) was added at 25 °C. Thionyl chloride (79.7 g, 0.67 mol) was added over 1 h. (Significant HCl off-gassing was observed during the reaction; the addition rate was regulated according to the rate of gas liberation, and a 5 M aqueous NaOH scrubber (1000 mL) was installed to trap the generated HCl.). The reaction mixture was stirred at 30 °C for 6 h and monitored by GC. Upon completion, the mixture was subjected to distillation under reduced pressure (< -95 KPa; reactor internal temperature 70 °C, distillate head-vapour temperature 50 °C) to provide 63.8 g of **R1** as a colourless liquid in 71% isolated yield and 99.3% GC purity. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 2.46 (q, J = 7.6 Hz, 1H), 2.08-1.99 (m, 1H), 1.72 - 1.64 (m, 2H), 1.00 (d, J = 6.8 Hz, 3H), 0.97 - 0.93 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 176.87, 66.12, 30.23, 22.47, 20.85, 19.41, 11.74. FTIR (neat): 2966, 2938, 2876, 1793, 1460, 1390, 1373, 1142, 968, 950, 882, 797, 783, 699 cm^{-1}.



Synthesis of 2-ethyl-3,3-dimethylbutanoic acid (S88). In a 10 L round-bottom flask, diisopropylamine (DIPA) (526.0 g, 5.20 mol) was charged into THF (2.30 L) and the solution was cooled to -30 °C. n-BuLi (2.5 M, 2.00 L, 5.00 mol) was added over 1 h, while maintaining -30 °C and the mixture was stirred for 1 h at -10 °C. The mixture was cooled to -30 °C, and **S87** (232 g, 2.00 mol) was added dropwise to the reaction mixture over 1 h at -30 °C and stirred at -30 °C for 1 h. Ethyl bromide (327.0 g, 3.00 mol) was charged into the reactor over 1 h at -30 °C. The reaction mixture was warmed to 0 °C and stirred for 1 h, then warmed to 20 °C and stirred for 16 h and monitored by ^{1}H NMR. HCl aqueous (4 M, 1.20 L) was added to the reaction mixture at 15 °C. The aqueous mixture was extracted with MTBE (1.20 L x 2). The combined organic layers were concentrated under vacuum at 45 °C to give crude product as a semi-solid. The semi-solid was suspended with CH_3CN (460 mL) at 25 °C for 16 h and then filtered. The filter cake was washed with CH_3CN (110 mL) and dried under vacuum at 40 °C for 16 h to provide 93.0 g of **S88** as a white solid in 32% isolated yield and 99.5% GC purity. mp. 70 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 2.06 (dd, J = 11.6, 3.2 Hz, 1H), 1.69-1.50 (m, 2H), 0.98 (s, 9H), 0.93 (t, J = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 182.18, 58.55, 32.80, 27.97, 20.75, 13.03. FTIR (neat): 3026, 2963, 2926, 2873, 1691, 1475, 1459, 1397, 1365, 1269, 1227, 1184, 956, 931, 861, 787 cm^{-1}.

Synthesis of 2-ethyl-3,3-dimethylbutanoyl chloride (R2) In a 500 mL three-neck jacketed vessel, **S88** (93.00 g, 0.65 mol) was dissolved into methylene chloride (CH_2Cl_2, 270 mL). Thionyl chloride (76.72 g, 0.65 mol) was added over 1 h while maintaining the temperature at 30 °C (Significant HCl off-gassing was observed during the reaction; the addition rate was regulated according to the rate of gas liberation, and a 5 M aqueous NaOH scrubber (1000 mL) was installed to trap the generated HCl.). The reaction mixture was stirred at 40 °C for 4 h and monitored by GC. After reaction completion, CH_2Cl_2 and excess thionyl chloride was distilled off at 50 °C under vacuum (-90 KPa). Pure product was finally distilled out under vacuum (< -95 KPa) at 50 °C to provide 49.0 g of **R2** as a colourless liquid with 47% isolated yield and 97.2% GC purity. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 2.66 (dd, J = 11.6, 3.6 Hz, 1H), 1.79 - 1.58 (m, 2H), 1.07 - 1.01 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 177.41, 70.79, 34.17, 27.85, 22.21, 12.86. FTIR (neat): 2968, 2908, 2876, 1795, 1701, 1459, 1371, 1225, 1191, 1123, 1103, 956, 810, 702 cm^{-1}.

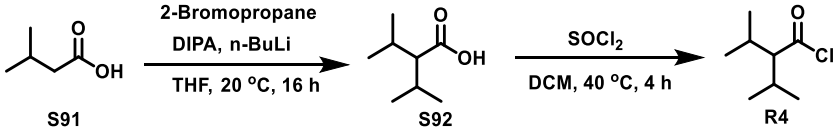
Synthesis of 2-isopropylpentanoyl chloride (R3)



Synthesis of 2-isopropylpentanoyl chloride (R3). To a 1 L three-neck reactor was added anhydrous THF (25 mL) and diisopropylamine (DIPA, 27.2 g, 269 mmol). After the solution was cooled to -10 °C, 2.5 M n-BuLi solution (100 mL, 250 mmol) was charged dropwise into reactor at -10 °C over 1 h. The reaction mixture was further stirred for 1 h. Compound **S81** (25 g, 192 mmol) in anhydrous THF (125 mL) solution was charged dropwise into the reactor at -10 °C over 1 h, then the reaction mixture was further stirred at -10 °C for another 1 h. The 1-Bromopropane (30.7 g, 250 mmol) in anhydrous THF (150 mL) solution was added dropwise into the reactor at -10 °C over 1 h. The reaction mixture was warmed to 20 °C and stirred at 20 °C for 15 h until the completion monitored by GC. 4 M HCl aq. (125 mL) was slowly added into the reactor to quench the reaction. After phases separation, the organic phase was washed with 20 wt% aq. NaCl aq. solution (125 mL) twice. The yield of the organic solution was 61% by GC. The mixture was directly used for the next step as ethyl 2-isopropylpentanoate **(S89)** solution.

To a 2 L three-neck reactor was added KOH (197.9 g, 3528 mmol,), H_2O (304 mL), methanol (760 mL) and ethyl 2-isopropylpentanoate **(S89)** solution (containing 76 g, 441 mmol). The reaction mixture was stirred at 65 ℃ for 25 h then another portion of KOH (49.5 g, 882 mmol) was charged into reactor. The reaction mixture was further stirred at 65 ℃ for 17 h until the completion of the reaction monitored by GC. The reaction mixture was concentrated to remove methanol at 60 ℃ under vacuum. The aqueous phase was washed with MTBE (380 mL) twice, then acidified by 6 M HCl at 5 ℃ until pH reached 2~3. The mixture was extracted with MTBE (760 mL). After concentration, the organic solution was obtained in 91% yield by GC. The mixture was directly used for the next step as 2-isopropylpentanoic acid **(S90)** MTBE solution.

To a 500 mL three-neck reactor, the 2-isopropylpentanoic acid **(S90)** MTBE solution (containing 153.5 g, 1064 mmol) was solvent switched to 2-isopropylpentanoic acid **(S90)** DCM solution (154 mL). $SOCl_2$ (132.9 g, 1117 mmol) in total was added dropwise into the reactor at 25 °C and stirred at 25 ℃ until the completion monitored by GC. After the completion of reaction, DCM was removed by concentration at no higher than 25 °C under vacuum. The head fraction was distilled out at 40~80 °C under about -0.1 MPa, and 163 g **R3** in total was collected at 90~100 °C under about -0.1 MPa in 93.6% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.52-2.58 (m, 1H), 2.01-2.09 (m, 1H), 1.64-1.72 (m, 1H), 1.52-1.59 (m, 1H), 1.41-1.49 (m, 1H), 1.27-1.40 (m, 1H), 0.90-1.03 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$)**:** δ (ppm) 176.9, 64.1, 31.3, 30.4, 20.7, 20.6, 19.3, 13.9. FTIR (neat): 2963, 2875, 1790, 1466, 974, 956, 573 cm^{-1}.



Synthesis of 2-ethyl-3,3-dimethylbutanoic acid (S92). In a 10 L round-bottom flask, diisopropylamine (437.0 g, 4.32 mol) was dissolved in THF (1.70 L), the solution was cooled to -30 °C. n-BuLi (2.5 M, 1.66

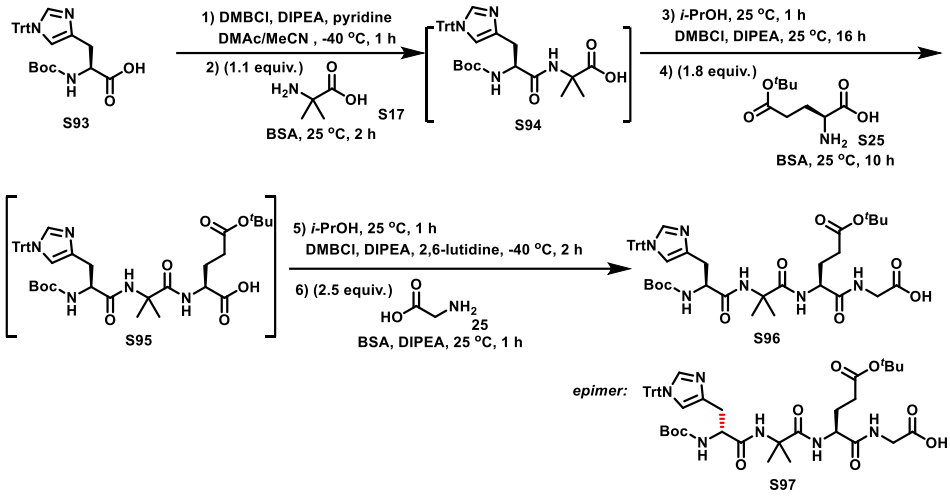
L, 4.15 mol) was added over 1 h while maintaining -30 °C, and the mixture was stirred for 1 h at -10 °C. The mixture was cooled to -30 °C, and **S91** (170.0 g, 1.66 mol) was added dropwise to the reaction mixture over 1 h at -30 °C, followed by stirring at -30 °C for 1 h. 2-Bromopropane (306 g, 2.49 mol) was charged into the reactor over 1 h at -30 °C. The reaction mixture was warmed to 0 °C and stirred for 1 h, then allowed to warm to 20 °C and stirred for 16 h, with reaction progress monitored by GC. HCl aqueous (4 M, 1.70 L) was added to the reaction mixture at 15 °C. The aqueous mixture was extracted twice with EtOAc (1.70 L x 2). The combined organic layers were washed with aqueous HCl aqueous (4 M, 850 mL × 2). The mixture was subjected to distillation under reduced pressure (< -95 Kpa) at 110 °C to remove unreacted **S91** to provide 31.0 g of **S92** as a yellow oil in 13% isolated yield and 96.9% GC purity. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 2.02-1.95 (m, 3H), 0.95 (dd, J = 6.0, 3.6 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 181.47, 58.89, 27.49, 20.93, 19.19. FTIR (neat): 3033, 2961, 2936, 2873, 1701, 1464, 1388, 1373, 1286, 1218, 1117, 989, 924, 748 cm^{-1}.

Synthesis of 2-isopropyl-3-methylbutanoyl chloride (R4) In a 500 mL three-neck jacketed vessel, **S92** (31.0 g, 0.21 mol) was dissolved in CH_2Cl_2 (31 mL). Thionyl chloride (25.0 g, 0.21 mol) was added over 1 h while maintaining the temperature at 30 °C (Significant HCl off-gassing was observed during the reaction; the addition rate was regulated according to the rate of gas liberation, and a 5 M aqueous NaOH scrubber (1000 mL) was installed to trap the generated HCl.). The reaction mixture was stirred at 40 °C for 4 h and monitored by GC. After reaction completion, CH_2Cl_2 and excess thionyl chloride was removed by concentration at 50 °C under vacuum (-90 KPa). Pure product was finally distilled out under vacuum (< -95 KPa) at 50 °C to provide 9 g of **R4** as a colourless oil in 26% isolated yield and 91.3% GC purity. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 2.59 (t, J = 7.6 Hz, 1H), 2.15-1.98 (m, 2H), 1.04-1.00 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm) 176.29, 71.13, 28.86, 20.79, 19.03. FTIR (neat): 2964, 2936, 2876, 1791, 1701, 1466, 1392, 1374, 1163, 1140, 1126, 971, 950, 905, 753 cm^{-1}.

GC Method: CP-Sil 8 CB for Amines 30 m × 0.32 mm 1.00 µm (P/N: CP7596); Injection Temperature: 320 °C; Detector: FID; Detector Temperature: 320 °C; Initial Flow: 2.5 mL/min (constant flow); Split Ratio 20:1; Hydrogen Flow Rate 30 mL/min; Air Flow Rate 300 mL/min; Make up (N_2) 30 mL/min; Injection volume 1 µL; Run Time: 16 min; Needle wash: MeOH.

Total Synthesis of Semaglutide

Fragment 15



Synthesis of Boc-L-His (Trt)-Aib-L-Glu(5-CO_2*t*Bu)-Gly-OH (**S96**). In a 2 L 3-neck round bottom flask, **Boc-L-His (Trt)-OH (S93)** (60.00 g, 120.58 mmol) and pyridine (1.91 g, 24.12 mmol) were dissolved in DMAc/MeCN (2:1, 600 mL). The solution was cooled to -40 °C, DMBCl (17.04 g, 126.61 mmol) and DIPEA (15.58 g, 120.58 mmol) were added into the reaction mixture. The reaction mixture was stirred at -40 °C for 1 h and monitored by HPLC (**Method 1**) and SFC-MS (**Method 2**), which showed the ratio of **S93:S93-INT:*epi*-S93-INT** was 1.6:98.4:0.0. The reaction mixture was added slowly over 1 h to a DMAc solution of **H-Aib-OH (S17)** and BSA at 25 °C, which was prepared by mixing **S17** (13.67 g, 132.64 mmol) and BSA (73.59 g, 361.74 mmol) in DMAc (600 mL), stirring at 50 °C for 16 h, and then cooling to 25 °C before use. The reaction mixture was stirred at 25 °C for 2 h and monitored by HPLC (**Method 1**) and SFC-MS **(Method 3)**, which showed the ratio of **S93:(S94+*epi*-S94):S94-over-addition** was 0.4:99.6 (**S94**:***epi*-S94** ratio is **99.0:1.0**):0.0. Then *i*-PrOH (43.48 g, 723.47 mmol) was added into the reaction mixture at 25 °C and stirred for 1 h. DMBCl (37.33 g, 277.33 mmol) and DIPEA (40.52 g, 313.50 mmol) were added to the reaction mixture. The reaction mixture was stirred at 25 °C for 16 h and monitored by HPLC (**Method 4**). which showed the ratio of **S94:S94-INT** was 1.3:98.7. A solution of **L-Glu(5-CO_2*t*Bu)-OH (S25)** and BSA in DMAc and MeCN was prepared by mixing **S25** (44.10 g, 217.04 mmol) and BSA (98.12 g, 482.31 mmol) in DMAc/MeCN (1:1, 300 mL), stirring at 50 °C for 1 h and then cooling to 25 °C. This solution was added slowly to the reaction mixture at 25 °C over 1 h. The reaction mixture was stirred at 25 °C for 10 h and monitored by HPLC (**Method 4**), which showed the ratio of **S94:S95:S95-over-addition** was 1.4:98.5:0.1. Then *i*-PrOH (30.91 g, 964.63 mmol) was added into the reaction mixture at 25 °C and stirred for 1 h, then cooling to -40 °C, DMBCl (40.57 g, 301.45 mmol), 2,6-lutidine (12.92 g, 120.58 mmol) and DIPEA (31.17 g, 241.16 mmol) were added into the reaction mixture and stirred at -40 °C for 2 h, which was monitored by HPLC (**Method 5**). HPLC showed the ratio of **S95:S95-INT:*epi*-S95-INT** was

0.5:99.5:0.0. The reaction mixture was added slowly over 1 h to a DMAc and MeCN solution of **Gly-OH (25)**, BSA and DIPEA at 25 °C. The solution was prepared by mixing **25** (22.64 g, 301.45 mmol) and BSA (122.65 g, 602.89 mmol) in DMAc/MeCN (1:1, 300 mL), stirring at 50 °C for 1 h, and then cooling to 25 °C, then adding DIPEA (7.79 g, 60.29 mmol) before use. The reaction mixture was stirred at 25 °C for 1 h and monitored by HPLC (**Method 6**), which showed the ratio of **S95:S96:*epi*-S96:S97:S96-over-addition** was 0.5:98.4:0.0:1.1:0.0. The assay yield of the reaction was 93% over three steps. The reaction solution was slowly charged into a solution of KH_2PO_4 (82.04 g, 602.89 mmol) in H_2O (3000 mL) at 20 °C over 0.5 h. Then extracted with IPAc (2400 mL). Upon phase separation, the organic phase was washed with 5% NaCl solution (1200 mL), dried with anhydrous Na_2SO_4, filtered and concentrated, then slurried with IPAc:n-heptane:MeCN (1:4:0.4, 1200 mL) at 20 °C for 16 h (2 wt% crystal seed added). The solid was collected by filtration and dried under N_2 for overnight to provide 83 g of **S96** as a white solid (99.3% LCAP) in 83% isolated yield **(Method 6)**.

mp 125-128°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.58 (d, J = 8.4 Hz, 1H), 8.19 (s, 1H), 7.94 (t, J = 5.6 Hz, 1H),7.42 - 7.36 (m, 9H), 7.25 (s, 1H), 7.10 - 7.08 (m, 6H), 6.86 (d, J = 8.0 Hz, 1H), 6.78 (s, 1H), 4.24-4.19 (m, 1H), 4.08 (q, J = 7.2 Hz, 1H), 3.73 (d, J = 6.0 Hz, 2H), 2.80 (dd, J = 14.4, 7.6 Hz, 1H), 2.71 (dd, J = 14.4, 6.0 Hz, 1H), 2.15 - 2.12 (m, 2H), 2.09 - 2.02 (m, 1H), 1.72 - 1.64 (m, 1H), 1.34 (s, 9H), 1.30 - 1.24 (m, 15H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 173.88, 171.80, 171.67, 171.65, 170.92, 154.99, 142.28, 137.62, 136.46, 129.32, 128.13, 128.03, 119.29, 79.48, 78.21, 74.60, 56.05, 54.97, 51.69, 40.74, 31.45, 31.25, 28.14, 27.67, 26.45, 25.67, 24.60, 22.10. HRMS (ESI): m/z calcd. for $C_{45}H_{56}N_6O_9$ $[M+H]^+$: 825.4182, found: 825.4164. FTIR (neat): 3295, 2976, 2933, 1657, 1521, 1495, 1446, 1366, 1282, 1249, 1224, 1154, 1047, 846, 749, 701 cm^{-1}.

Method 1: CSH Phexyl-Hexyl 4.6 × 100 mm, particle size 2.5 μm column; Phosphate buffer (10 mM NaH_2PO_4, 10 mM Na_2HPO_4) in H_2O (solvent A), MeCN (solvent B); gradient 20-85% (solvent B) in 8 min, hold 2 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

Method 2: CHIRALPAK IA-U 50 × 3.0 mm, 1.6 μm; co-solvent: 20 mM NH_3 in *i*-PrOH; gradient: 10-50% (co-solvent) in 1.2 min, hold 0.7 min, flow rate: 1.5 mL/min; detection wavelength: 254 nm.

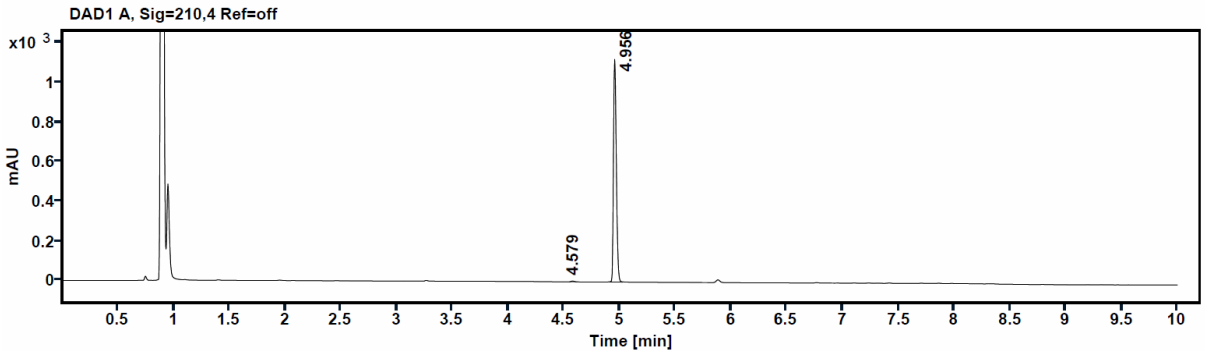
Method 3: CHIRALPAK IM-3 50 × 4.6 mm, 3.0 μm; co-solvent: 20 mM NH_3 in MeOH; gradient: 10-50% (co-solvent) in 1.2 min, hold 0.7 min, flow rate: 3.0 mL/min; detection wavelength: 254 nm.

Method 4: Express C18 3.0 × 100 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 10-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 5: CSH C18 4.6 × 100 mm, particle size 2.5 µm column; Phosphate buffer (10 mM NaH_2PO_4, 10 mM Na_2HPO_4) in H_2O (solvent A), MeCN (solvent B); gradient 60-85% (solvent B) in 8 min, hold 2 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

Method 6: Eclipse Plus C8 4.6 × 100 mm, particle size 1.8 µm column; 10 mM Ammonium Formate in H_2O (solvent A), 0.02% FA (v/v) in MeCN (solvent B); gradient 40-85% (solvent B) in 8 min, hold 2 min; flow rate: 1.2 mL/min; detection wavelength: 210 nm; Column temperature: 20℃.

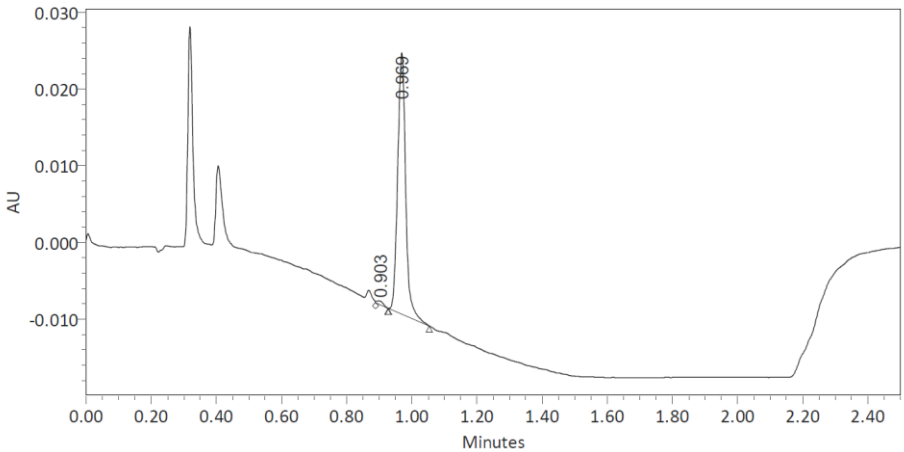
HPLC of **S94** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S93	4.579	8.4765	4.1	0.4
S94 + ***epi*-S94**	4.956	2001.7222	1125.9	99.6*
S94-over-addition	5.179	0.0	0.0	0.0
Total	-	-	-	100.0

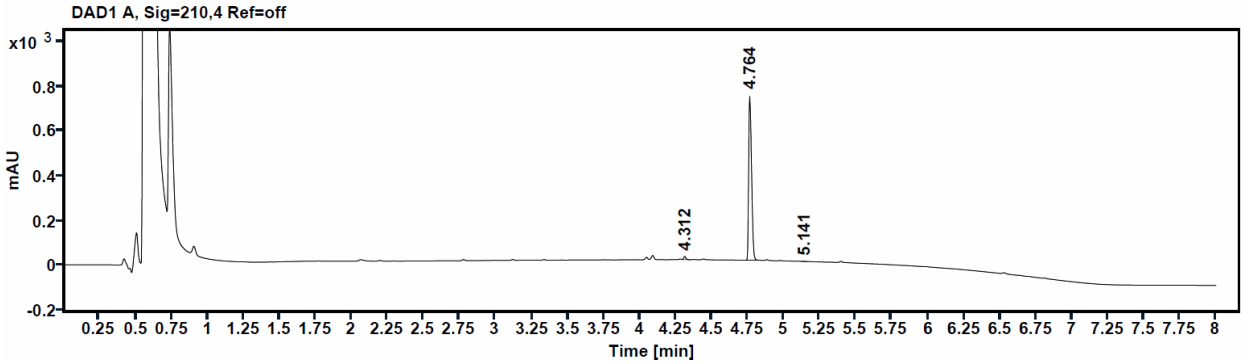
*Note: Contains 1.0% ***epi*-S94**

SFC-MS(Chiral) of **S94** chiral purity



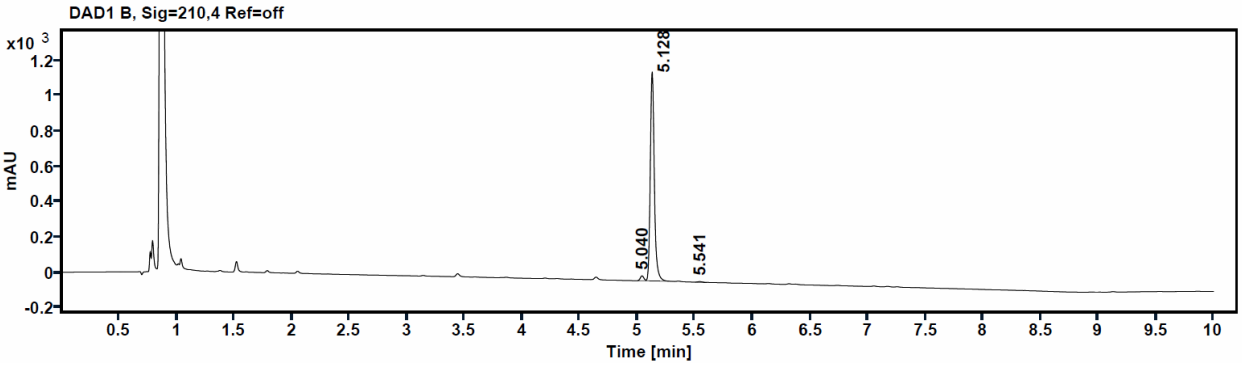
Compound ID	Time	Area	Height	Area% ratio
***epi*-S94**	0.903	630	477	1.0
S94	0.969	58122	34161	99.0
Total	-	-	-	100.0

HPLC of **S95** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S94	4.312	14.6758	14	1.4
S95	4.764	1023.8014	742.6	98.5
S95-over-addition	5.141	1.05	0.76	0.1
Total	-	-	-	100.0

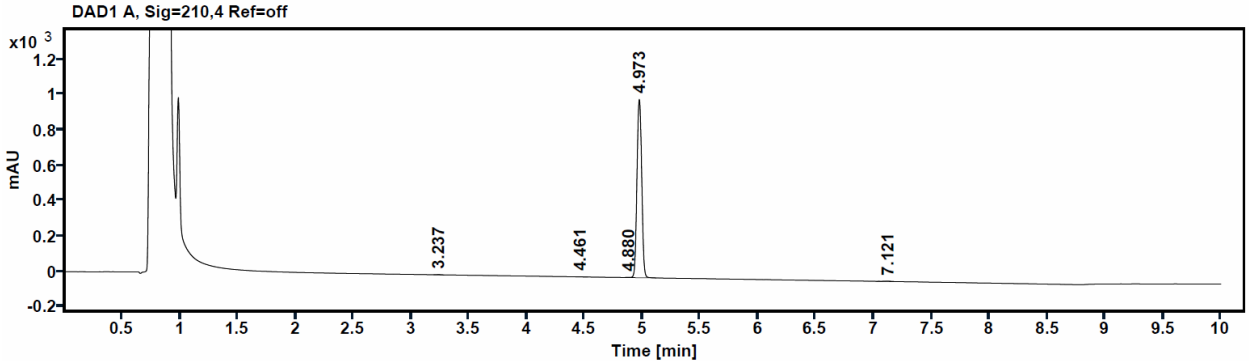
HPLC of **S96** formation (reaction mixture)



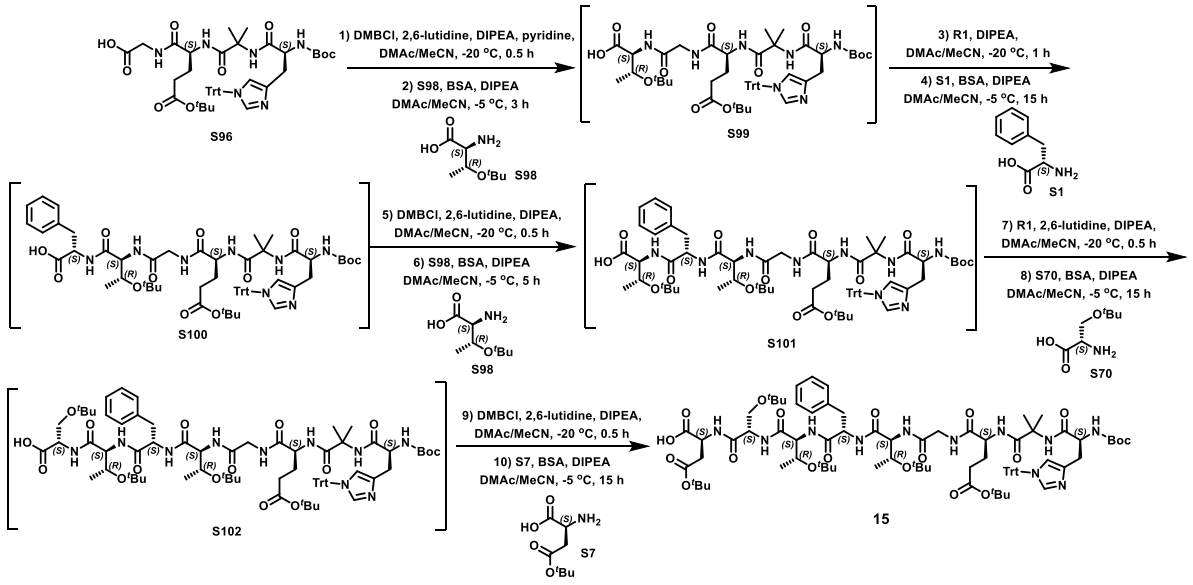
Compound ID	Time	Area	Height	Area% ratio
S96-over-addition	4.832	0.0	0.0	0.0

S97	5.040	32.1839	20.1	1.1
S96	5.128	2878.9785	1184.3	98.4
***epi*-S96**	5.238	0.0	0.0	0.0
S95	5.541	13.6275	4.4	0.5
Total	-	-	-	100.0

HPLC of isolated **S96**



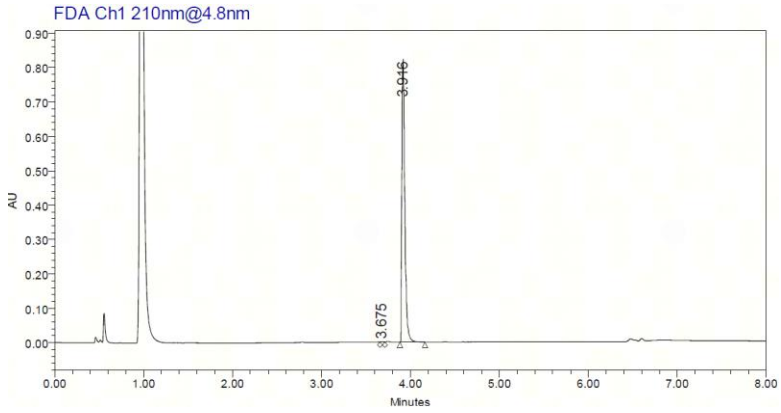
Compound ID	Time	Area	Height	Area%
S97	4.880	3.0942	1.41	0.1
S96	4.973	2866.9690	1008.25	99.3
others	-	-	-	0.6
Total	-	-	-	100.0



Synthesis of Boc-His(Trt)-Val-Glu(O*t*Bu)-Gly-Thr(*t*Bu)-Phe-Thr(*t*Bu)-Ser(*t*Bu)-Ser(*t*Bu)-OH (15)

To a 2000 mL three-neck jacketed vessel was added DMAc/MeCN (1:1, 400 mL) and **S96** (100.00 g, 121.22 mmol). After the solution was cooled to -20 °C, DIPEA (15.67 g, 121.22 mmol), 2,6-lutidine (12.99 g, 121.22 mmol), pyridine (4.79 g, 60.61 mmol) and DMBCl (17.13 g, 127.28 mmol) were added in sequence. The reaction mixture was stirred at -20 °C for 0.5 h and monitored by UPLC (**Method 1**), which showed the ratio of **S96**:**S96-INT** (mixed anhydride intermediate quenched by 1-Phenylethylamine) was 0.0:100.0. The above solution was added into the pre-cooled (-5 °C) solution of **S98**, BSA and DIPEA in DMAc/MeCN over 10 min, which was prepared by stirring **S98** (22.30 g, 127.28 mmol), BSA (49.32 g, 242.44 mmol) and DIPEA (7.83 g, 60.61 mmol) in DMAc/MeCN (1:1, 400 mL) at 50 °C for 1 h and then cooled to -5 °C. The reaction mixture was stirred at -5 °C for 3 h and monitored by UPLC (**Method 1**). The UPLC showed the reaction assay yield was 98%, and the ratio of **S96**:**S99**:**S99-over-addition** was: 0.15:99.85:0.0. Then *i*-PrOH (29.09 g, 484.88 mmol) was added to the reaction and stirred for 16 h at -5 °C. The resulting solution was directly used for the next stage.

UPLC of **S99** formation

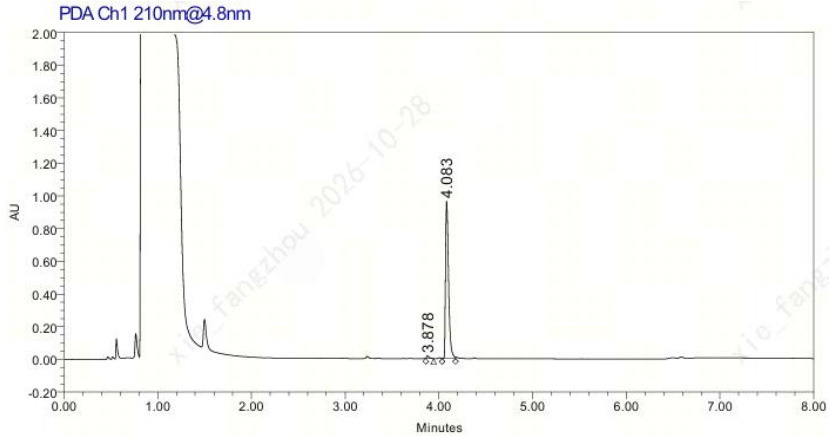


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S99-over-addition	4.210	-	-	-
S99	3.916	1884674	823078	99.85
S96	3.675	2764	1622	0.15
Total	-	-	-	100.00

The above **S99** solution was cooled to -20 °C, DIPEA (14.41 g, 111.52 mmol) and **R1** (25.58 g, 172.13 mmol) were added in sequence. The reaction mixture was stirred at -20 °C for 1 h and monitored by UPLC (**Method 1**), which showed the ratio of **S99**:**S99-INT** (mixed anhydride intermediate quenched by 5% 1-Phenylethylamine in MeCN) was 1.03:98.97. The pre-cooled (0 °C) solution of **S1**, BSA and DIPEA in DMAc:MeCN, which was prepared by stirring **S1** (22.03 g, 133.34 mmol), BSA (73.98 g, 363.66 mmol)

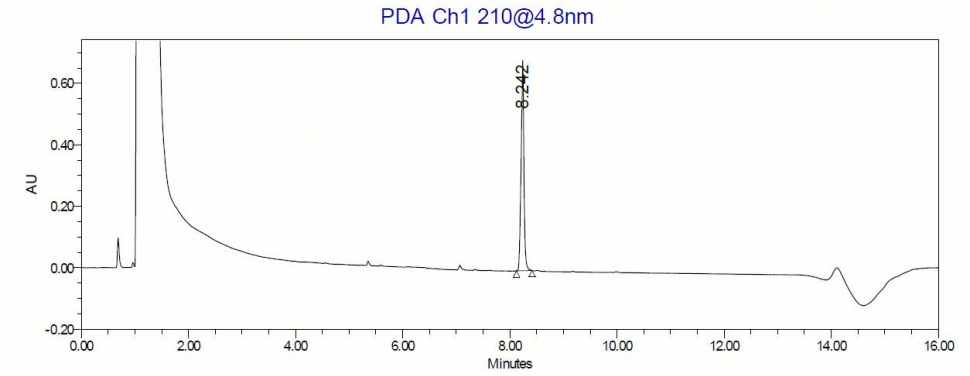
and DIPEA (7.83 g, 60.61 mmol) in DMAc/MeCN (1:1, 400 mL) at 50 °C for 1 h and cooled to 0 °C, was added to the reaction over 10 min. The reaction mixture was stirred at -5 °C for 15 h and monitored by UPLC (**Method 1**). The UPLC showed the ratio of **S99**:**S100**:***epi*-S99-INT**:**S100-over-addition** was 1.49:98.51:0.0:0.0. The reaction assay yield was 97%. Then *i*-PrOH (43.64 g, 727.32 mmol) was added to the reaction and stirred for 18 h at -5 °C.

UPLC of **S100** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S100-over-addition	4.263	-	-	-
S100	4.083	2181419	964177	98.51
S99	3.878	32947	17352	1.49
***epi*-S99-INT**	4.290	-	-	-
Total	-	-	-	100.0

UPLC of **S100** chiral purity (**Method 2**)

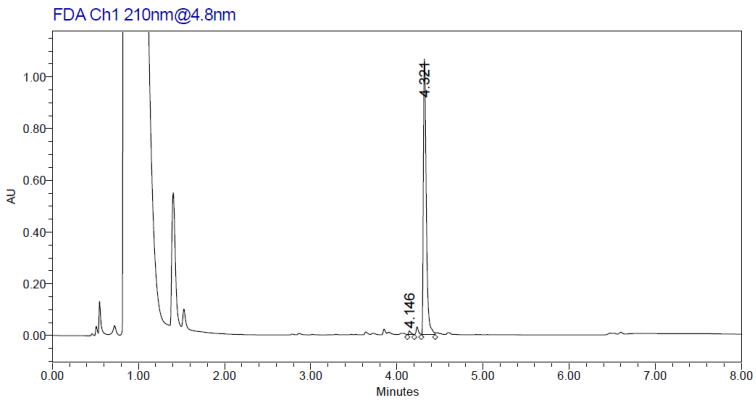


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S100	8.242	2503471	683404	100.0

***epi*-S100**	8.030	-	-	-
Total	-	-	-	100.0

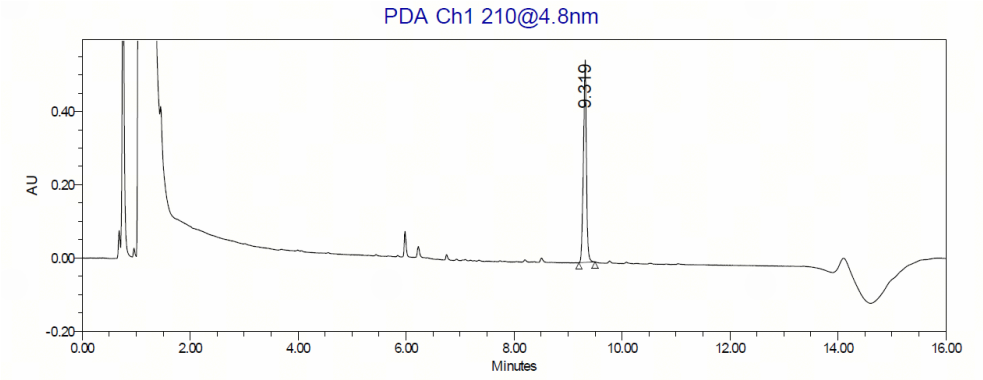
The above **S100** solution (based on 49 g **S96** scale, 59.40 mmol) was transferred to a 2000 mL three-neck jacketed vessel and cooled to -20 °C. DIPEA (8.44 g, 65.34 mmol), 2,6-lutidine (1.27 g, 11.88 mmol) and DMBCl (12.79 g, 95.04 mmol) were added in sequence. The reaction mixture was stirred at -20 °C for 0.5 h and monitored by UPLC (**Method 1**), which showed the ratio of **S100**:**S100-INT** (mixed anhydride intermediate quenched by 5% 1-Phenylethylamine in MeCN) was 1.18:98.82. The above solution was added into a pre-cooled solution of **S98**, BSA and DIPEA in DMAc:MeCN, which was prepared by stirring **S98** (13.01 g, 74.25 mmol), BSA (36.24 g, 178.20 mmol) and DIPEA (3.84 g, 29.70 mmol) in DMAc/MeCN (1:1, 200 mL) at 50 °C for 1 h and then cooled to -5 °C. The reaction mixture was stirred at -5 °C for 5 h and monitored by UPLC (**Method 1**), which showed the ratio of **S100**:**S101**:***epi*-S100-INT**:**S101-over-addition** was 0.97:99.03:0.0:0.0 and the reaction assay yield was 96%. Then *i*-PrOH (21.38 g, 356.4 mmol) was added to the reaction and stirred for 18 h at -5 °C.

UPLC of **S101** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S101-over-addition	4.664	-	-	-
S101	4.321	2491700	1065971	99.03
S100	4.146	24504	13650	0.97
***epi*-S100-INT**	4.552	-	-	-
Total	-	-	-	100.0

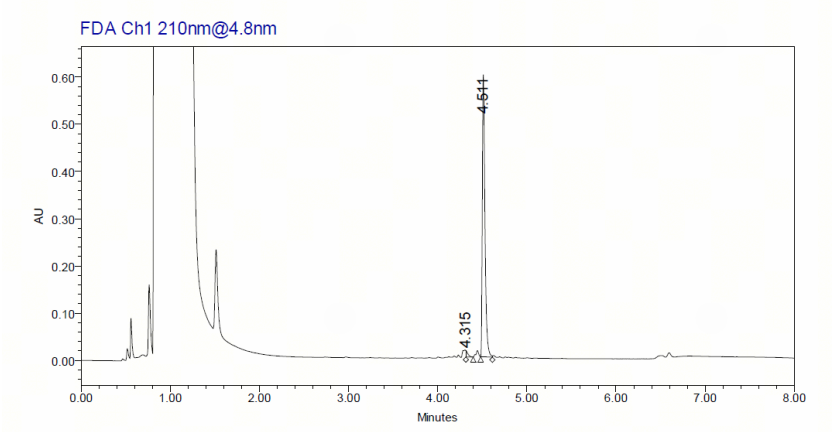
UPLC of **S101** chiral purity (**Method 3**)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S101	9.319	2106816	552791	100.0
***epi*-S101**	9.512	-	-	-
Total	-	-	-	100.0

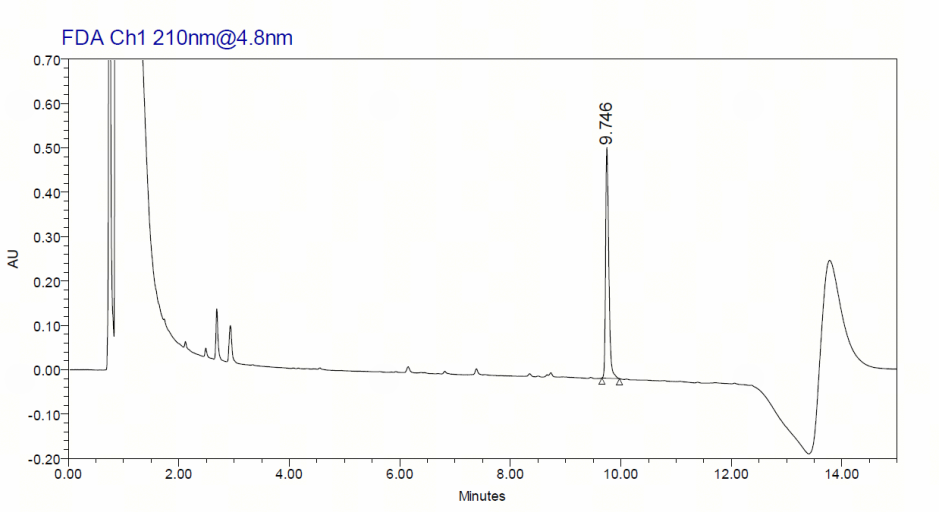
The above **S101** solution was cooled to -20 °C, DIPEA (9.98 g, 77.22 mmol), 2,6-lutidine (1.27 g, 11.88 mmol) and **R1** (20.30 g, 136.61 mmol) were added in sequence. The reaction mixture was stirred at -20 °C for 0.5 h and monitored by UPLC (**Method 1**), which showed the ratio of **S101**:**S101-INT** (mixed anhydride intermediate quenched by 5% 1-Phenylethylamine in MeCN) was 1.0:99.0. The pre-cooled (0 °C) solution of **S70**, BSA and DIPEA in DMAc/MeCN, which was prepared by stirring **S70** (14.36 g, 89.1 mmol), BSA (36.24 g, 178.20 mmol) and DIPEA (3.84 g, 29.70 mmol) in DMAc/MeCN (1:1, 200 mL) at 60 °C for 1 h and cooled to 0 °C, was added to the reaction over 10 min. The reaction mixture was stirred at -5 °C for 15 h and monitored by UPLC (**Method 1**), which showed the ratio of **S101**:**S102**:***epi*-S101-INT**:**S102-over-addition** was: 1.68:98.32:0.0:0.0 and the reaction assay yield was 96%. Then *i*-PrOH (21.38 g, 356.4 mmol) was added to the reaction and stirred for 18 h at -5 °C.

UPLC of **S102** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S102-over-addition	4.731	-	-	-
S102	4.511	1223527	597218	98.32
S101	4.315	20846	13493	1.68
***epi*-S101-INT**	4.451	-	-	-
Total	-	-	-	100.0

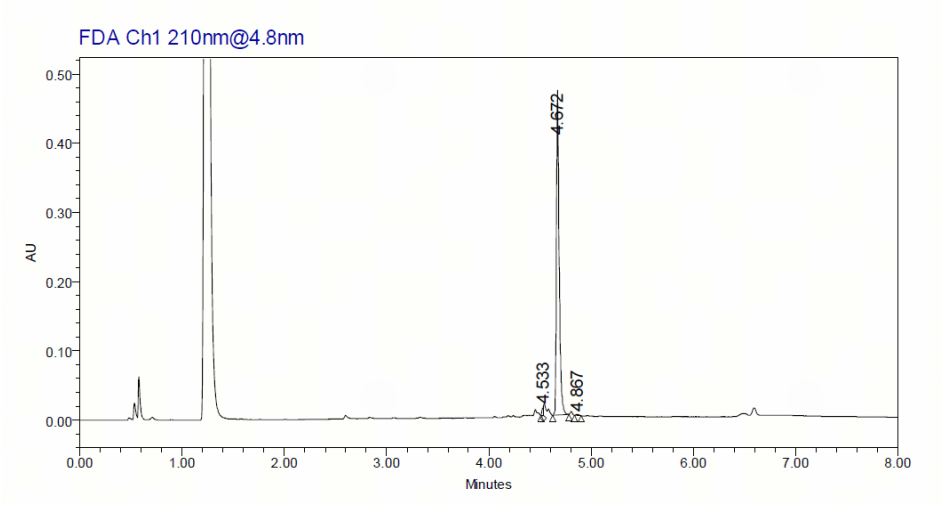
UPLC of **S102** chiral purity (**Method 4**)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S102	9.746	2105731	519683	100.0
***epi*-S102**	9.636	-	-	-
Total	-	-	-	100.0

The above **S102** solution was cooled to -20 °C, DIPEA (19.19 g, 148.50 mmol), 2,6-lutidine (1.27 g, 11.88 mmol) and DMBCl (23.98 g, 178.20 mmol) were added in sequence. The reaction mixture was stirred at -20 °C for 0.5 h and monitored by UPLC (**Method 1**), which showed the ratio of **S102**:**S102-INT** (mixed anhydride intermediate quenched by 5% 1-Phenylethylamine in MeCN) was 1.72:98.28. The pre-cooled (0 °C) solution of **S7**, BSA and DIPEA in DMAc:MeCN, which was prepared by stirring **S7** (22.48 g, 118.8 mmol) and BSA (48.33 g, 237.6 mmol) and DIPEA (3.84 g, 29.70 mmol) in DMAc/MeCN (1:1, 200 mL) at 50 °C for 1 h and cooled to 0 °C, was added to the reaction over 10 min. The reaction mixture was stirred at -5 °C for 15 h and monitored by UPLC (**Method 1**). The UPLC showed the ratio of **S102**:**15**:***epi*-15**:***epi*-S102-INT**:**15-over-addition** was 1.08:98.44:0.10:0.0:0.47 and the reaction assay yield was 94%.

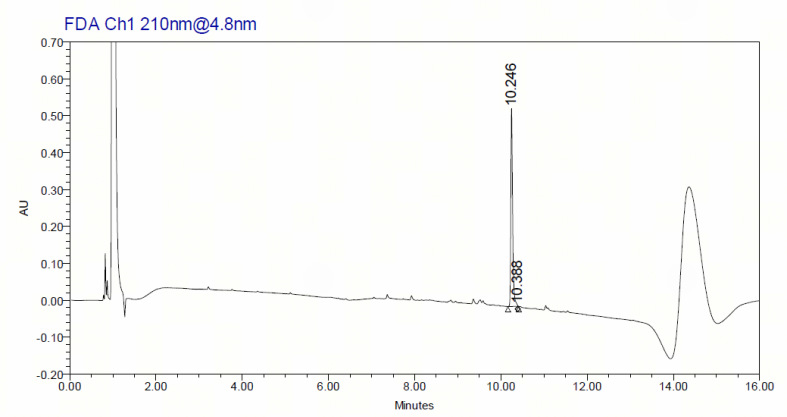
UPLC of **15** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
15-over-addition	4.867	4177	1741	0.47
15	4.672	867366	469237	98.44*
S102	4.533	9539	12233	1.08
***epi*-S102-INT**	4.930	-	-	-
Total	-	-	-	100.00

*Note: Contains 0.10% *epi*-**15** (see supplementary HPLC chromatogram).

UPLC of **15** chiral purity (**Method 5**)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
15	10.246	1565338	536257	99.90
***epi*-15**	10.388	1602	2688	0.10
Total	-	-	-	100.00

The reaction mixture of **15** was added into water (3185 mL) slowly at 0 °C and stirred for 2 h. The solid was filtered and dried at 40 °C for 18 h. The solid was suspended in *i*-PrOH (980 mL) at 50 °C for 5 h and then cooled to 10 °C to allow crystallization. The crystalline solid was filtered, washed with *i*-PrOH (245 mL), and dried for 18 h at 40 °C to provide 82.9 g (86 wt%, 71.3 g by assay) of **15** as a white solid in 75% isolated yield and 95.0% UPLC purity over 5 steps.

Starting from **S93**, 10130 g of reagents/solvents were used to synthesize 71.3 g of **15**, giving a PMI of 142.

mp: 149 °C. ^{1}H NMR (600 MHz, 3:1 CD_3CN/D_2O, 298 K) δ 7.53 (s, 1H), 7.37 – 7.30 (m, 9H), 7.25 – 7.17 (m, 4H), 7.17 – 7.13 (m, 1H), 7.12 – 7.06 (m, 6H), 6.77 (s, 1H), 4.61 (dd, *J* = 8.1, 5.7 Hz, 1H), 4.56 (t, *J* = 5.8 Hz, 1H), 4.34 (dd, *J* = 5.2, 4.0 Hz, 1H), 4.28 (d, *J* = 3.7 Hz, 1H), 4.25 – 4.18 (m, 2H), 4.13 (t, *J* = 6.9 Hz, 1H), 4.03 – 3.94 (m, 2H), 3.84 (d, *J* = 16.7 Hz, 1H), 3.76 (d, *J* = 16.7 Hz, 1H), 3.66 (dd, *J* = 9.3, 4.0 Hz, 1H), 3.47 (dd, *J* = 9.3, 5.2 Hz, 1H), 3.05 (dd, *J* = 14.0, 5.6 Hz, 1H), 2.97 (dd, *J* = 14.0, 8.2 Hz, 1H), 2.90 (dd, *J* = 14.6, 6.1 Hz, 1H), 2.79 (dd, *J* = 14.6, 7.6 Hz, 1H), 2.67 (dd, *J* = 16.5, 5.8 Hz, 1H), 2.63 (dd, *J* = 16.5, 6.0 Hz, 1H), 2.29 – 2.18 (m, 2H), 2.17 – 2.10 (m, 1H), 1.82 (ddt, *J* = 13.8, 10.6, 6.8 Hz, 1H), 1.35 (s, 9H), 1.32 – 1.31 (m, 15H), 1.30 (s, 9H), 1.15 (s, 9H), 1.11 (s, 9H), 0.96 (d, *J* = 5.9 Hz, 6H), 0.95 (s, 9H). ^{13}C NMR (151 MHz, 3:1 CD_3CN/D_2O, 298 K) δ 175.7, 173.4, 173.0, 172.8, 172.7, 171.3, 170.4, 170.4, 170.2, 170.1, 169.6, 156.2, 147.3, 142.0, 138.2, 136.8, 135.0, 129.5, 129.3, 128.5, 128.3, 128.3, 127.8, 126.8, 120.2, 81.4, 80.7, 80.0, 75.7, 75.1, 74.6, 73.8, 66.6, 61.4, 58.1, 58.0, 56.8, 55.0, 54.7, 53.6, 53.0, 49.6, 42.9, 37.5, 37.2, 31.7, 29.3, 27.6, 27.5, 27.4, 27.3, 27.3, 26.6, 26.0, 25.2, 24.0, 18.7, 17.8. HRMS (ESI): m/z calcd for $C_{85}H_{122}O_{19}N_{11}$ $[M+H]^+$: 1600.8913, found: 1600.8916. FTIR (neat): 3292, 2975, 1637, 1497, 1366, 1154, 747, 701 cm^{-1}.

Method 1: HPLC condition: ACQUITY UPLC BEH C18, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-95% (solvent B) in 4.5 min, hold 1 min; flow rate: 0.4 mL/min; detection wavelength = 210 nm/254 nm.

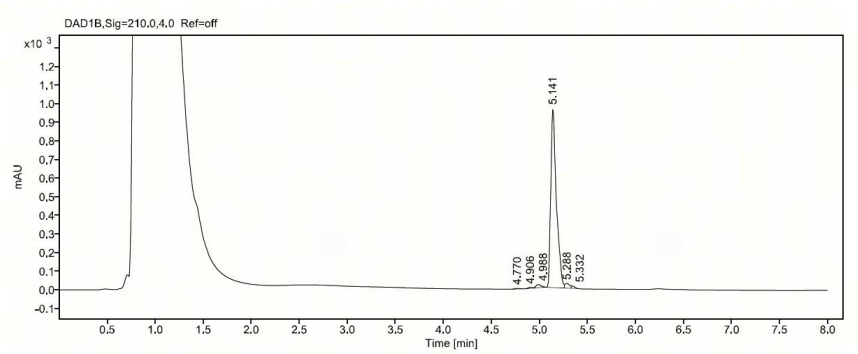
Method 2: HPLC condition: ACQUITY UPLC Peptide CSH C18, 2.1 × 150 mm, 1.7 μm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN (solvent B); gradient 30%-50% (solvent B) in 5 min, gradient 50%-65% (solvent B) in 5 min, gradient 65%-95% (solvent B) in 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

Method 3: HPLC condition: ACQUITY UPLC Peptide CSH C18, 2.1 × 150 mm, 1.7 μm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN (solvent B); gradient 30%-65% (solvent B) in 6 min, gradient 65%-95% (solvent B) in 6 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

Method 4: HPLC condition: ACQUITY UPLC Peptide CSH C18, 2.1 × 150 mm, 1.7 μm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN (solvent B); gradient 50%-70% (solvent B) in 11 min, gradient 70%-95% (solvent B) in 1 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

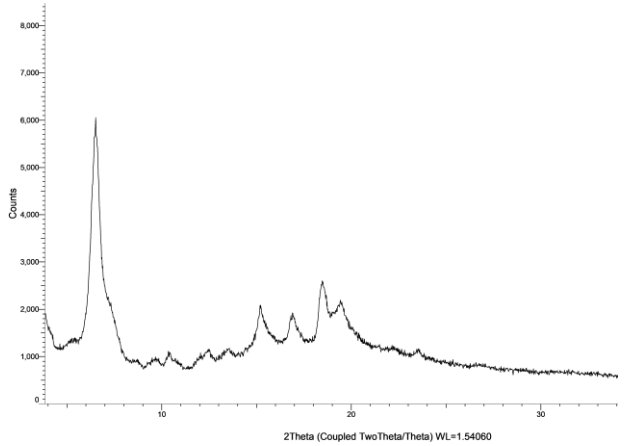
Method 5: HPLC condition: ACQUITY UPLC Peptide CSH C18, 2.1 × 150 mm, 1.7 μm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN (solvent B); gradient 20%-60% (solvent B) in 5 min, gradient 60%-80% (solvent B) in 7 min, gradient 80%-95% (solvent B) in 0.5 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

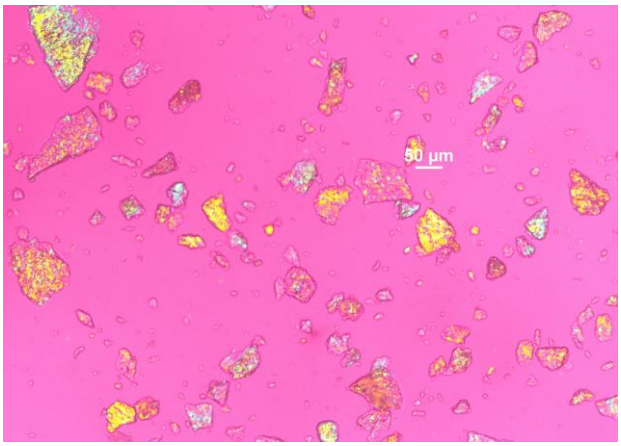
UPLC of isolated **15**



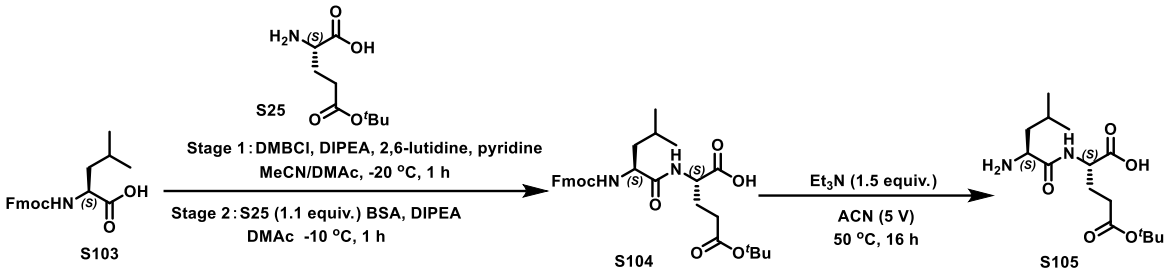
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
15-over-addition	5.332	28.32	12.72	0.69
15	5.141	3889.79	962.93	95.01
S102	4.988	62.23	17.49	1.52
Total	-	-	-	97.22

XRPD (X-ray Powder Diffraction) and PLM (Polarized Light Microscopy) of isolated **15**





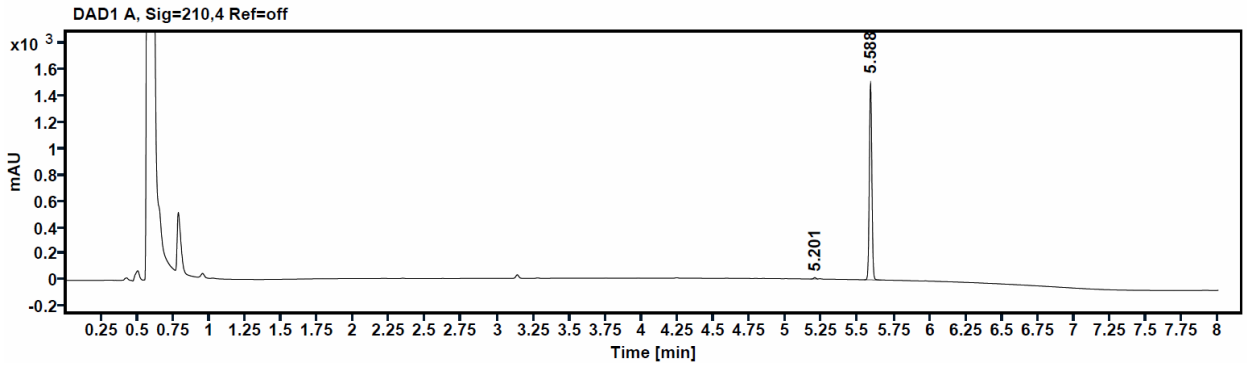
Fragment 16



Synthesis of H-L -Leu-L-Glu(CO_2*t*Bu)-OH (**S105**). In a 500 mL three-neck jacketed vessel, **Fmoc-L-Leu-OH (S103)** (20.00 g, 56.59 mmol) was dissolved in DMAc/MeCN (1:1, 120 mL). After the solution was cooled to -20 °C, 2,6-lutidine (6.67 g, 62.25 mmol), pyridine (0.90 g, 11.32 mmol), and DIPEA (8.03 g, 62.25 mmol) were added. Then DMBCl (8.38 g, 62.25 mmol) was added over 10 min. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1)**, which showed the ratio of **S103:S103-INT** was 0.7:99.3. A solution of **H-L-Glu(CO_2*t*Bu)-OH (S25)** and BSA in DMAc, prepared by stirring **H-L-Glu(CO_2*t*Bu)-OH (S25)** (12.65 g, 62.25 mmol) and BSA (23.02 g, 113.19 mmol) in DMAc (60 mL) at 50 °C for 1 h, was added to the reaction at -20 °C over 10 min. DIPEA (1.46 g, 11.32 mmol) was added. Then the reaction mixture was stirred at -10 °C for 1 h and monitored by HPLC (**Method 1)**, which showed the ratio of **S103:S104:S104-over-addition** was 0.7:99.3:0.0. The reaction mixture was added to water (1000 mL) at 0 °C over 1 h and stirred at 0 °C for 3 h. The product was collected by filtration, and dissolved in EtOAc (120 mL), removed the residual aqueous phase. The organic phase was concentrated to ~ 40 mL, diluted with MeCN (120 mL), and reconcentrated to ~ 40 mL. This solvent exchange procedure was repeated twice. Then Et_3N (8.65 g, 84.89 mmol) was added and the solution was warmed to 50 °C. The reaction mixture was stirred at 50 °C for 16 h and monitored by HPLC (**Method 1)**, which showed the ratio of **S104:S105** was 0.0:100.0. After cooled down to ambient temperature, the product was collected by filtration and then dried under vacuum at 40 °C, yielded 18.5 g of **H-L-Leu-L-Glu(CO_2*t*Bu)-OH** (**S105**) as a white solid (94.6% LCAP, 92.0 wt%) with 95% isolated yield (**Method 3)**.

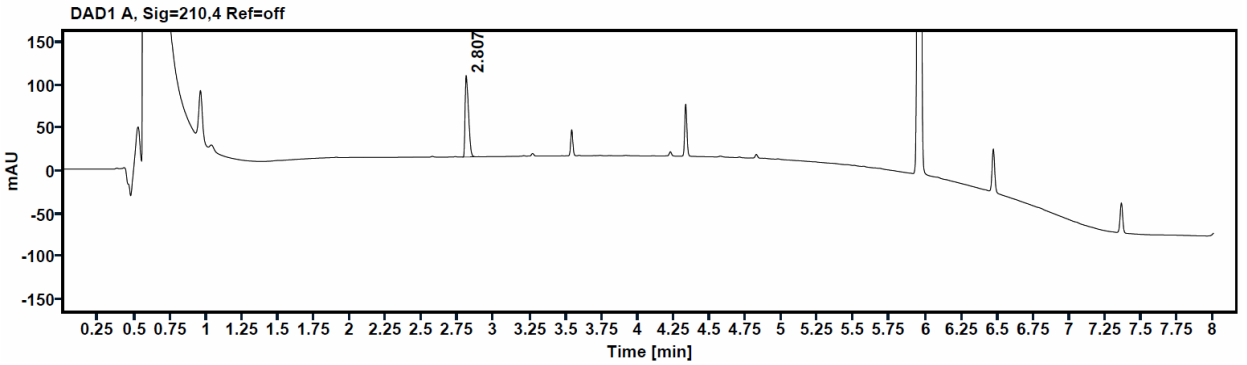
mp 190 °C. ^{1}H NMR (400 MHz, TFA-*d*) δ (ppm) 5.20 (dd, *J* = 8.4, 5.2 Hz, 1H), 4.74 (t, *J* = 7.2 Hz, 1H), 3.02 (t, *J* = 7.2 Hz, 2H), 2.84 - 2.76 (m, 1H), 2.57 - 2.48 (m, 1H), 2.22 - 2.19 (m, 2H), 2.16 - 2.10 (m, 1H), 1.91 (s, 9H), 1.33 (dd, *J* = 8.8, 6.4 Hz, 6H). ^{13}C NMR (101 MHz, FTA-*d*) δ (ppm) 182.09, 178.07, 172.95, 92.88, 92.83, 56.14, 54.89, 42.60, 31.79, 28.49, 28.46, 28.17, 26.68, 23.11, 22.62. HRMS (ESI): m/z calcd for $C_{15}H_{29}N_2O_5$ $[M+H]^+$: 317.2071, found: 317.2077. FTIR (neat): 3364, 2961, 2936, 2871, 1723, 1668, 1577, 1541, 1496, 1445, 1369, 1332, 1248, 1154, 1107, 643, 620 cm^{-1}.

HPLC of **S104** formation (reaction mixture)

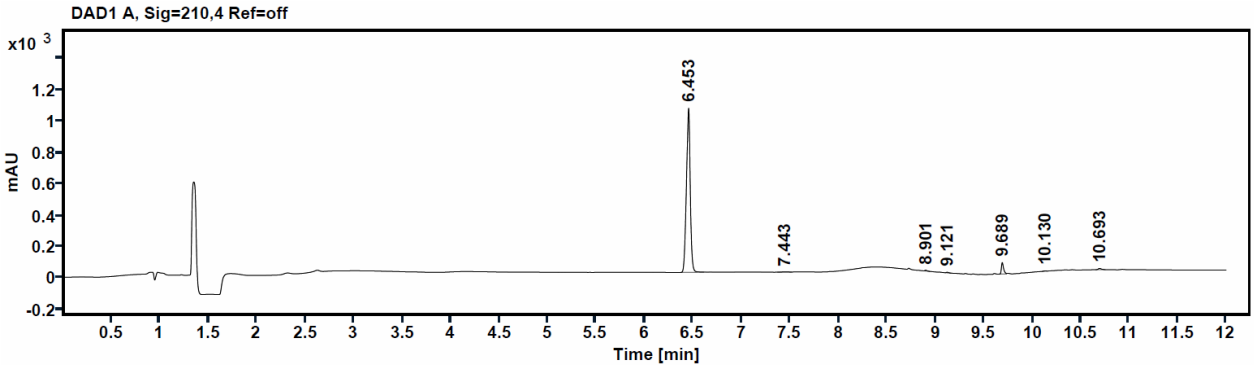


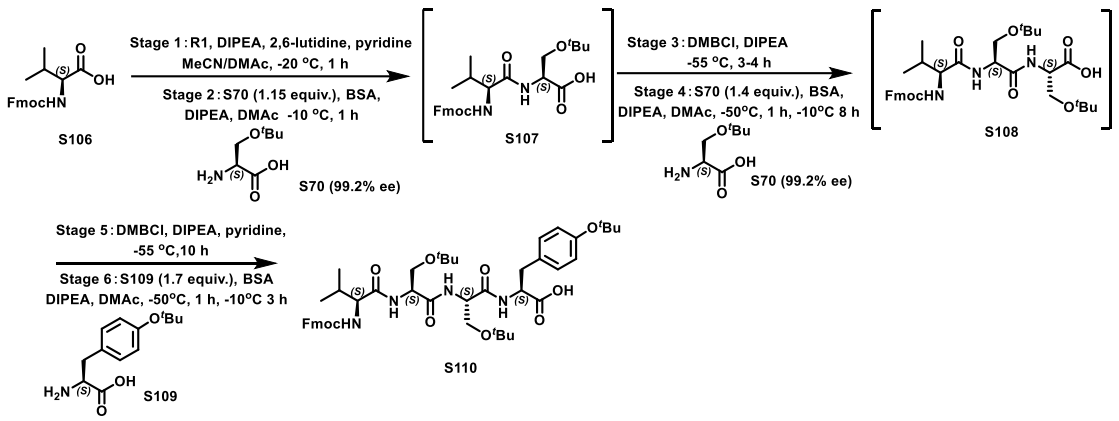
Compound ID	Time	Area	Height	Area%
S103	5.201	13.485	11.5	0.7
S104	5.588	1831.7643	1524.6	99.3
S104-over-addition	5.875	-	-	0.0
Total	-	-	-	100.0

HPLC of **S105** formation (reaction mixture)



HPLC of isolated **S105**



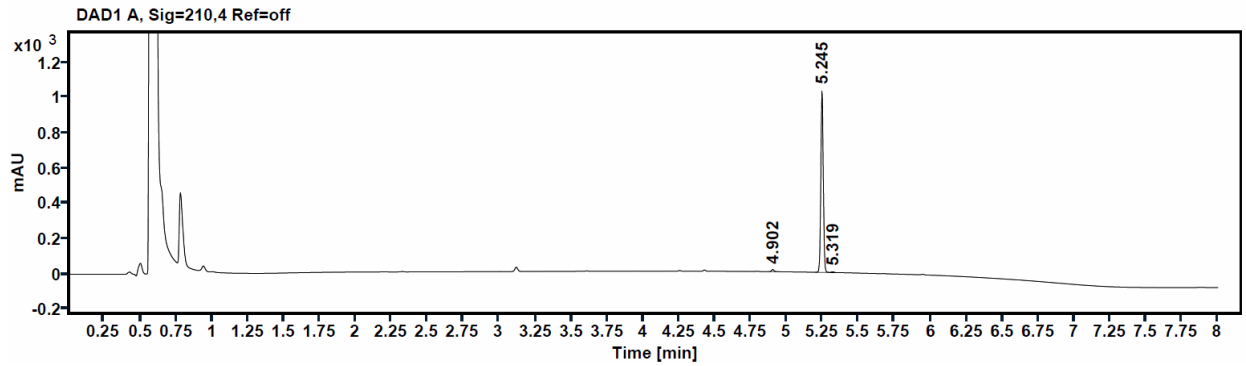


Synthesis of Fmoc-L-Val-L-Ser(O*t*Bu)-L-Ser(O*t*Bu)-L-Tyr(O*t*Bu)-OH (S110). In a 500 mL three-neck jacketed vessel, **Fmoc-L-Val-OH (S106)** (20.00 g, 58.93 mmol) dissolved in DMAc/MeCN (1:1, 120 mL). After the solution was cooled to -20 °C, 2,6-lutidine (6.95 g, 64.82 mmol), pyridine (0.93 g, 11.79 mmol), and DIPEA (8.36 g, 64.82 mmol) were added. Then **R1** (9.60 g, 64.82 mmol) was added over 10 min. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1)**, which showed the ratio of **S106:S106-INT** was 0.3:99.7. A DMAc solution of **H-L-Ser(O*t*Bu)-OH(S70)** and BSA, which was prepared by stirring **H-L-Ser(O*t*Bu)-OH (S70)** (10.92 g, 67.77 mmol) and BSA (23.97 g, 117.86 mmol) in DMAc (60 mL) at 50 °C for 1 h. After cooled to 20 °C, the solution was added to the reaction at -20 °C over 10 min. DIPEA (1.52 g, 11.79 mmol) was added. Then the reaction mixture was stirred at -10 °C for 1 h and monitored by HPLC (**Method 1)**, which showed the ratio of **S106:S107:*epi*-S107:S107-over-addition** was 1.1:98.4:0.5:0.0. Then *i*-PrOH (14.17 g, 235.71 mmol) was added to the solution at 0 °C, and the mixture was stirred for 1 h. Then the mixture was cooled to -55 °C, DMBCl (11.06 g, 82.50 mmol) and DIPEA (6.08 g, 47.14 mmol) were added and stirred for 3-4 h and monitored by HPLC (**Method 2)**, which showed the ratio of **S107:S107-INT:*epi*-S107-INT** was 1.1:98.4:0.5. A DMAc solution of **H-L-Ser(OtBu)-OH (S70)** and BSA, which was prepared by stirring **H-L-Ser(O*t*Bu)-OH(S70)** (13.30 g, 82.50 mmol) and BSA (28.77 g, 141.43 mmol) in DMAc (60 mL) at 50 °C for 1 h. After cooled to 20 °C, the solution was added to the reaction at -50 °C over 10 min. DIPEA (1.52 g, 11.79 mmol) was added. Then the reaction mixture was stirred at -50 °C for 1 h, warmed up to -10 °C and stirred for 8 h, and monitored by HPLC (**Method 2)**, which showed the ratio of **S107:*epi*-S107-INT:S108:*epi*-S108:S108-over-addition** was 1.6:0.0:97.9:0.5:0.0. Then *i*-PrOH (17.00 g, 282.82 mmol) was added to the solution at 0 °C, and the mixture was stirred for 1 h. Then the mixture was cooled to -55 °C, DMBCl (15.00 g, 111.96 mmol) and DIPEA (8.36 g, 64.82 mmol) and pyridine (0.93 g, 11.79 mmol) were added and stirred at -55 °C for 10 h and monitored by HPLC (**Method 2)**, which showed the ratio of **S108:S108-INT:*epi*-S108-INT** was 1.1:98.3:0.6. A DMAc solution of **H-L-Tyr(O*t*Bu)-OH (S109)** and BSA, which was prepared by stirring **S109** (23.77 g, 100.18 mmol) and BSA (40.75 g, 200.35 mmol) in DMAc (60 mL) at 50 °C for 1 h. After cooled to 20 °C, the solution was added to the reaction at -50 °C over 10 min. DIPEA (6.84 g, 53.03 mmol)

was added. Then the reaction mixture was stirred at -50 °C for 1 h, warmed up to -10 °C and stirred for 3 h, and monitored by HPLC (**Method 2)**, which showed the ratio of **S108:*epi*-S108-INT:S110:*epi*-S110:S110-over-addition** was 1.6:0.0:98.0:0.4:0.0. The reaction assay yield was 89% over three steps. The reaction mixture was added to MeCN:water (1:2, 750 mL) at 0 °C over 1 h and stirred at 25 °C for 3 h. The product was collected by filtration and dried under vacuum at 40 °C. The crude product was added to EtOAc (200 mL) at 25 °C and stirred at 60 °C for 16 h. The product was collected by filtration and dried under vacuum at 40 °C, yielding 38.4 g of **S107** as a white solid (97.1% LCAP, 96.0 wt%) in 73% isolated yield (**Method 4)**.

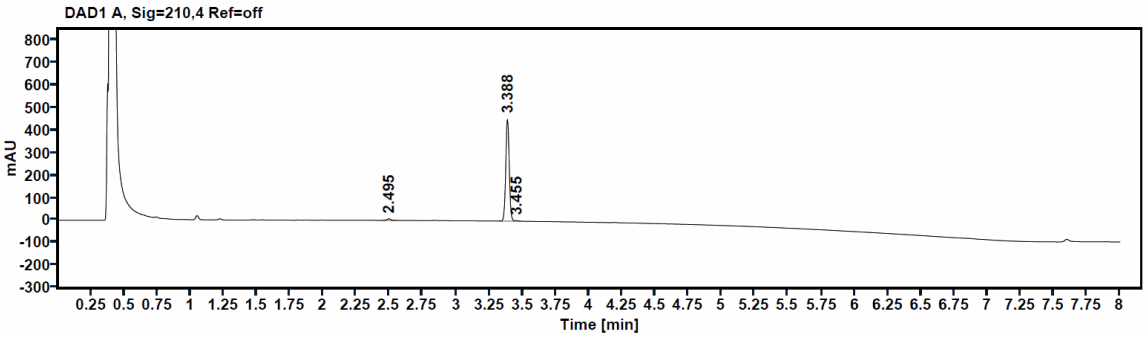
mp 190 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ(ppm) 12.78 (br s, 1H), 7.99 (d, J = 8.0 Hz, 1H), 7.92 (d, J = 8.0 Hz, 1H), 7.89 (d, J = 7.2 Hz, 2H), 7.73 (t, J = 7.2 Hz, 3H), 7.48 (d, J = 8.8 Hz, 1H), 7.41 (t, J = 7.6 Hz, 2H), 7.32 (t, J = 7.6 Hz, 2H), 7.09 (d, J = 8.0 Hz, 2H), 6.84 (d, J = 8.4 Hz, 2H), 4.47 - 4.28 (m, 4H), 4.25 - 4.20 (m, 2H), 3.93 (t, J = 8.0 Hz, 1H), 3.47 - 3.43 (m, 3H), 3.40 - 3.36 (m, 1H), 2.99 (dd, J = 14.4, 5.6 Hz, 1H), 2.88 (dd, J = 14.0, 7.6 Hz, 1H), 2.04 - 1.95 (m, 1H), 1.25 (s, 9H), 1.07 (d, J = 8.4 Hz, 18H), 0.85 (t, J = 7.2 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 172.42, 171.14, 169.39, 169.18, 156.09, 153.61, 143.86, 143.75, 140.71, 140.68, 131.73, 129.62, 127.63, 127.61, 127.06, 125.34, 123.32, 120.09, 120.07, 77.58, 72.94, 72.84, 65.72, 61.74, 61.65, 60.24, 53.39, 53.03, 52.99, 46.68, 36.19, 30.36, 28.53, 27.10, 27.05, 19.24, 18.10. HRMS (ESI): m/z calcd for $C_{47}H_{65}N_4O_{10}$ $[M+H]^+$: 845.4695, found: 845.4707. FTIR (neat): 3280, 2973, 1690, 1668, 1638, 1536, 1508, 1390, 1364, 1292, 1247, 1234, 1191, 1161, 1081, 1031, 896, 874, 757, 738 cm^{-1}.

HPLC of **S107** formation (reaction mixture)



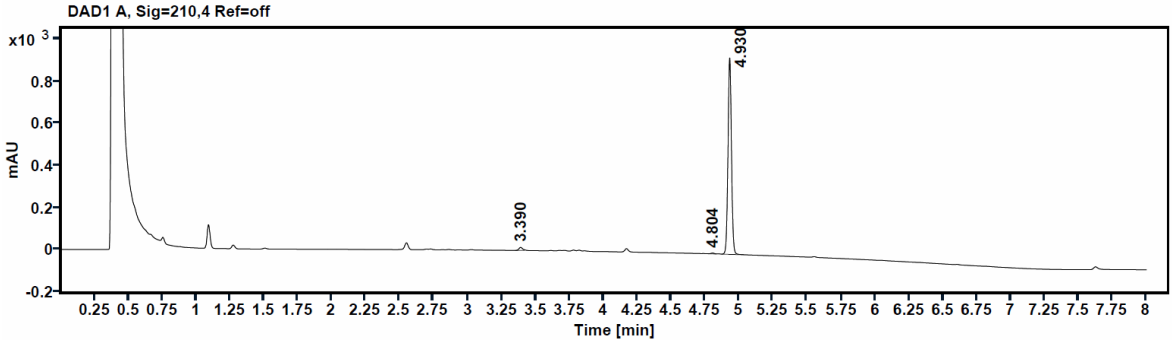
Compound ID	Time	Area	Height	Area% ratio
S106	4.902	13.4798	12.3	1.1
S107	5.245	1219.4553	1034.3	98.4
***epi*-S107**	5.319	6.4206	4.5	0.5
S107-over-addition	5.679	-	-	0.0
Total	-	-	-	100.0

HPLC of **S108** formation (reaction mixture)



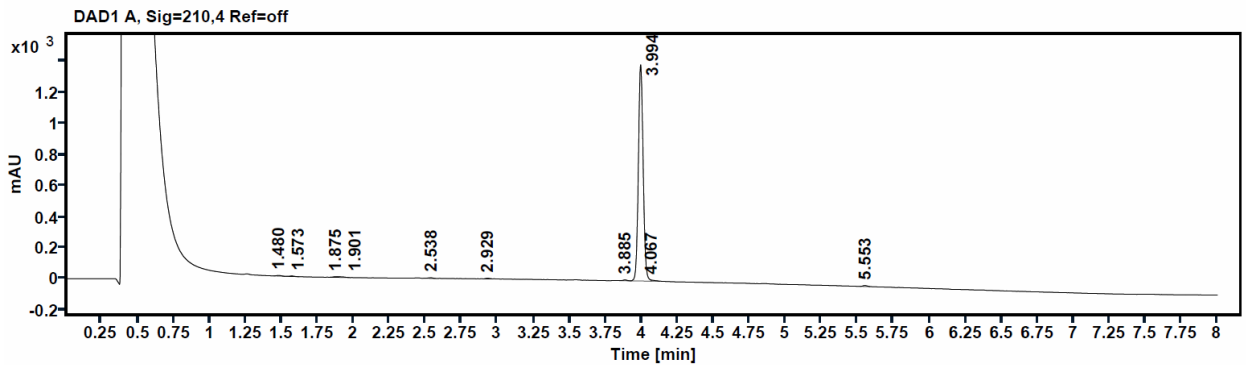
Compound ID	Time	Area	Height	Area%
S107	2.495	13.7318	7.2	1.6
S108	3.388	828.2339	455.6	97.9
***epi*-S108**	3.455	4.0107	2.4	0.5
***epi*-S107-INT**	3.988	-	-	0.0
S108-over-addition	4.208	-	-	0.0
Total	-	-	-	100.0

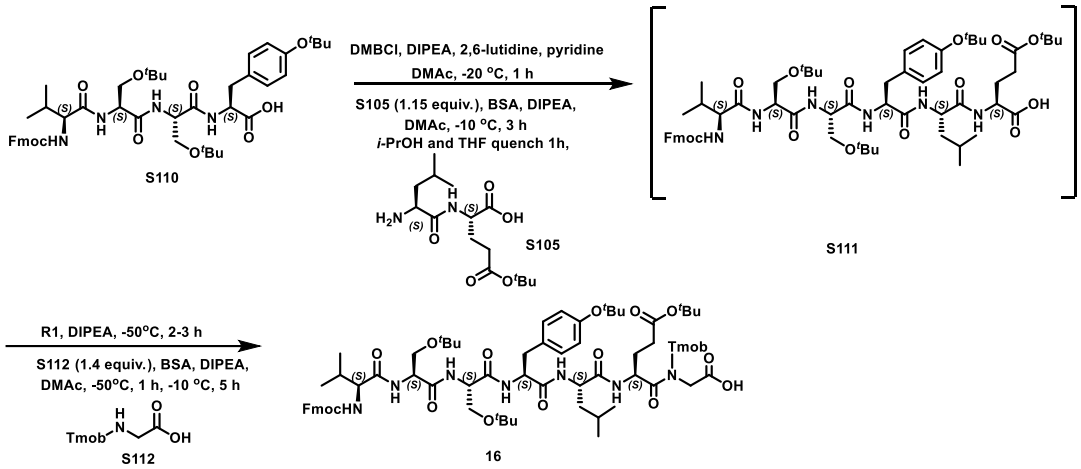
HPLC of **S110** formation (reaction mixture)



Compound ID	Time	Area	Height	Area% ratio
S108	3.390	27.3042	14.5	1.6
***epi*-S108-INT**	4.624	-	-	0.0
***epi*-S110**	4.804	7.4507	4.1	0.4
S110	4.930	1670.4297	940.0	98.0
S110-over-addition	6.086	-	-	0.0
Total	-	-	-	100.0

HPLC of isolated **S110**





Synthesis of Fmoc-L-Val-L-Ser(O*t*Bu)-L-Ser(O*t*Bu)-L-Tyr(O*t*Bu)-L-Leu-L-Glu(CO$_2$*t*Bu) -L-Gly(Tmob)-OH (16). In a 500 mL three-neck jacketed vessel, **Fmoc-L-Val-L-Ser(O*t*Bu)-L-Ser(O*t*Bu)-L-Tyr(O*t*Bu)-OH (S110)** (20.8 g, 97.1% LCAP, 96.0 wt%, 23.67 mmol) was dissolved in DMAc (100 mL). After the solution was cooled to -20 °C, 2,6-lutidine (2.79 g, 26.03 mmol), pyridine (0.37 g, 4.73 mmol), and DIPEA (3.36 g, 26.03 mmol) were added. Then DMBCl (3.49 g, 26.03 mmol) was added over 10 min. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 2)**, which showed the ratio of **S110:S110-INT:*epi*-S110-INT** was 0.2:99.3:0.5. A DMAc solution of **H-L-Leu-L-Glu(CO$_2$*t*Bu)-OH (S105)** and BSA, which was prepared by stirring **H-L-Leu-L-Glu(CO$_2$*t*Bu)-OH (S105)** 9.36 g (94.6% LCAP, 92.0 wt%, 27.22 mmol) and BSA (9.63 g, 47.33 mmol) in DMAc (60 mL) at 50 °C for 1 h. After cooled to 20°C, the solution was added to the reaction at -20 °C over 10 min. Then DIPEA (0.61 g, 4.73 mmol) was added. Then the reaction mixture was stirred at -10 °C for 3 h and monitored by HPLC (**Method 2)**, which showed the ratio of **S110:*epi*-S110-INT:S111:*epi*-S111:S111-over-addition** was 0.6:0.0:98.5:0.6:0.3. Then *i*-PrOH (5.69 g, 94.66 mmol) and THF (100 mL) was added to the solution at 0 °C, and the mixture was stirred for 1 h. Then the mixture was cooled to -50 °C, **R1** (4.91 g, 33.13 mmol) and DIPEA (2.44 g, 18.93 mmol) were added and stirred for 2-3 h, then monitored by HPLC (**Method 2)**, which showed the ratio of **S111:S111-INT:*epi*-S111-INT** was 1.3:98.5:0.2. A DMAc solution

of **H-L-Gly(Tmob)-OH (S112)** and BSA, which was prepared by stirring **H-L-Gly(Tmob)-OH (S112)** (8.46 g, 33.13 mmol) and BSA (13.48 g, 66.26 mmol) in DMAc (60 mL) at 50 °C for 1 h. After cooled to 20 °C, the solution was added to the reaction at -50 °C over 10 min. DIPEA (12.24 g, 9.47 mmol) was added. Then the reaction mixture was stirred at -50 °C for 1 h and stirred at -10 °C for 5 h and monitored by HPLC (**Method 2)**, which showed the ratio of **S111:*epi*-S111-INT:16:*epi*-16:16-over-addition** was 0.9:0.0:98.5:0.3:0.3. The reaction assay yield was 88% over 2 steps. The reaction mixture was added into MeCN:water (1:1.5, 800 mL) at 0 °C over 1 h and stirred at 25 °C for 3 h. The product was collected by filtration and dried under vacuum at 40 °C. The product collected was added to MeCN/water (2:1, 200mL) at 25 °C and stirred at 60 °C for 16 h to allow crystallization. The crystalline solid was collected by filtration and dried for 16 h under vacuum at 40 °C, yielding 27.6 g of **16** as a white solid (96.5% LCAP, 95.4 wt%) with 80% isolated yield. (**Method 5)**.

Starting from **S106**, 2867 grams of reagents/solvents were used to synthesize 27.6 g (95.4 wt%) of **16**, giving a PMI of 109.

mp 215 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ(ppm) 12.52 (br s, 1H), 8.24 (d, J = 8.0 Hz, 1H), 7.92 - 7.83 (m, 4H), 7.78 - 7.69 (m, 4H), 7.47 (d, J = 9.2 Hz, 1H), 7.41 (t, J = 7.6 Hz, 2H), 7.32 (tt, J = 7.2, 1.2 Hz, 2H), 7.06 (d, J = 8.4 Hz, 2H), 6.80 - 6.75 (m, 2H), 6.24 - 6.21 (m, 2H), 5.41 (dt, J = 12.8, 4.4 Hz, 1H), 4.63 - 4.57 (m, 2H), 4.39 - 4.28 (m, 5H), 4.24 - 4.20 (m, 2H), 3.98 (d, J = 17.2 Hz, 1H), 3.92 (t, J = 8.0 Hz, 1H), 3.78 (s, 3H), 3.74 - 3.69 (m, 6H), 3.44 - 3.39 (m, 3H), 3.36 - 3.35 (m, 1H), 3.01 - 2.93 (m, 1H), 2.78 (dd, J = 13.6, 8.0 Hz, 1H), 2.33 - 2.16 (m, 2H), 2.03 - 1.95 (m, 2H), 1.73 - 1.58 (m, 2H), 1.51 - 1.47 (m, 2H), 1.37 - 1.34 (m, 9H), 1.24 - 1.21 (m, 9H), 1.08 (s, 8H), 1.04 (s, 11H), 0.89 - 0.83 (m, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 171.81, 171.34, 171.14, 170.96, 170.41, 170.18, 169.39, 168.86, 161.24, 159.42, 156.08, 153.34, 143.85, 143.75, 140.70, 140.67, 132.02, 129.72, 127.62, 127.06, 125.33, 123.15, 120.07, 103.37, 90.67, 79.59, 77.40, 72.92, 72.83, 65.71, 61.73, 61.59, 60.23, 55.60, 55.56, 55.26, 53.13, 52.99, 51.14, 47.47, 46.67, 44.31, 40.93, 39.99, 36.99, 30.52, 30.34, 28.52, 28.49, 28.04, 27.68, 27.09, 27.00, 24.12, 23.06, 21.59, 19.24, 18.10. HRMS (ESI): m/z calcd for $C_{74}H_{106}N_7O_{18}$ $[M+H]^+$: 1380.7589, found: 1380.7656. FTIR (neat): 3276, 2971, 1730, 1694, 1620, 1523, 1452, 1364, 1230, 1154, 1131, 1100, 1036, 900, 878, 759, 740 cm^{-1}.

Method 1: Express C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 10-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

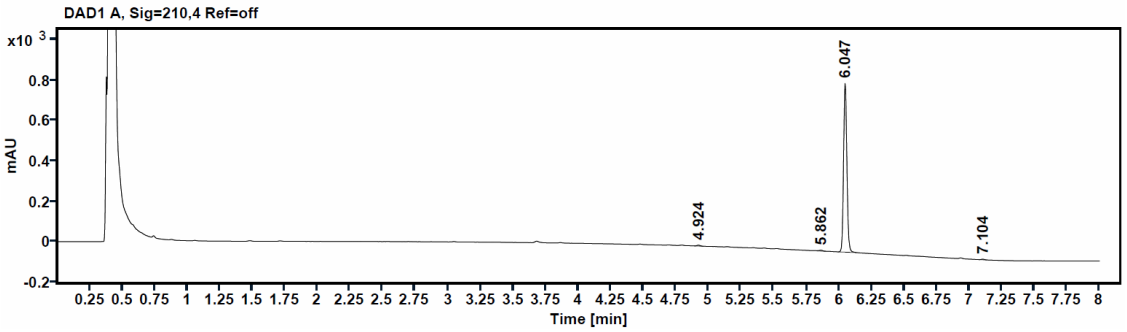
Method 2: Express C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 50-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 3: Express C18 3.0 × 100 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 5-30% (solvent B) in 6 min, gradient 30-90% (solvent B) in 8 min, hold 4 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

Method 4: Express C18 3.0 × 100 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 60-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

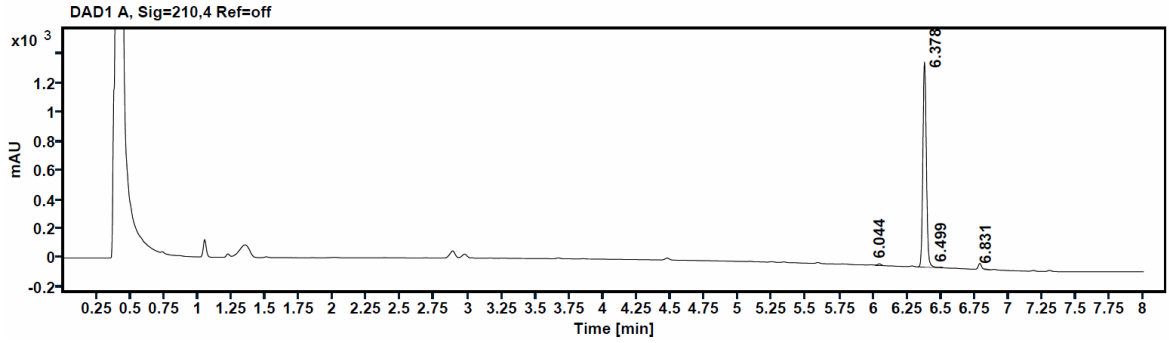
Method 5: Express C18 3.0 × 100 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 70-95% (solvent B) in 6 min, hold 4 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

HPLC of **S111** formation (reaction mixture)



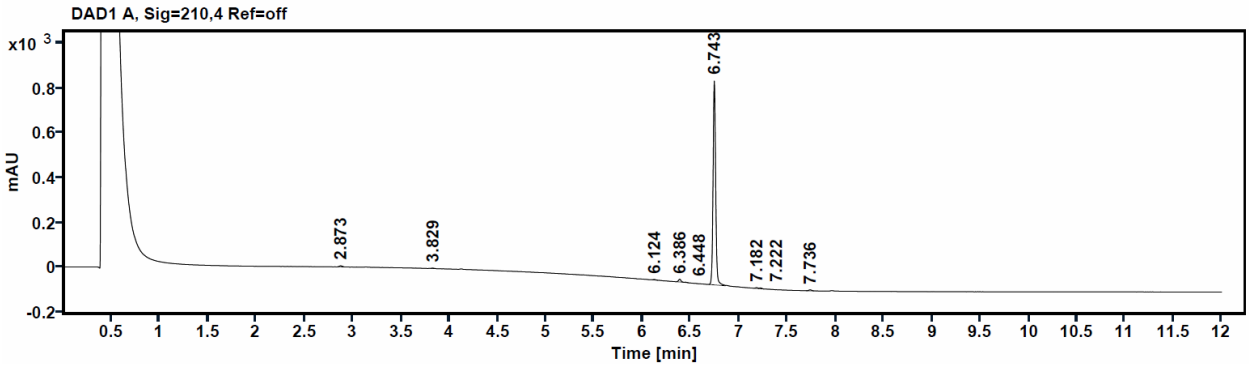
Compound ID	Time	Area	Height	Area%
S110	4.924	8.2721	4.4	0.6
***epi*-S111**	5.862	8.4912	4.2	0.6
***epi*-S110-INT**	5.908	-	-	0.0
S111	6.047	1478.6241	840.2	98.5
S111-over-addition	7.104	5.2326	3.1	0.3
Total	-	-	-	100.0

HPLC of **16** formation (reaction mixture)



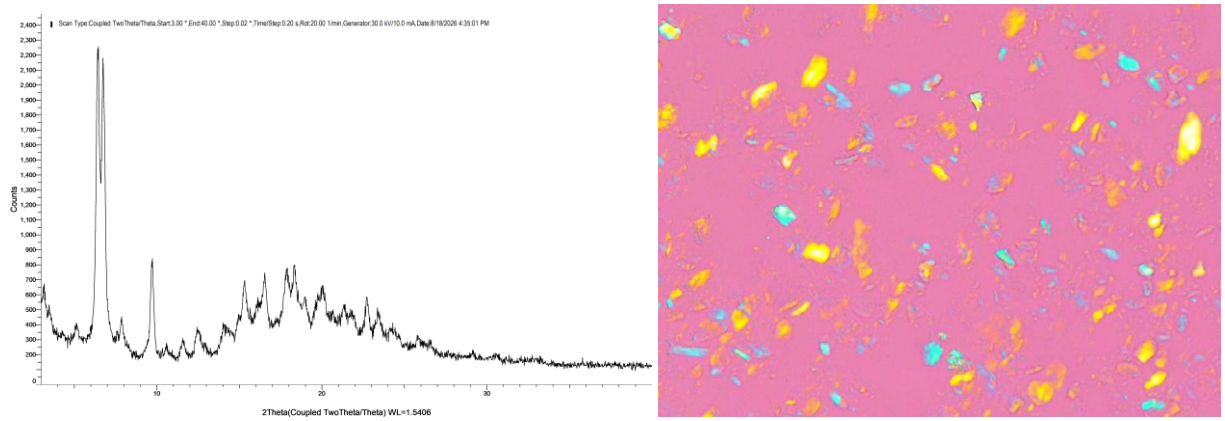
Compound ID	Time	Area	Height	Area% ratio
S111	6.044	24.0771	12.6	0.9
16	6.378	2546.4148	1419.5	98.5
***epi*-16**	6.499	8.1057	4.2	0.3
16-over-addition	6.831	6.4889	4.5	0.3
***epi*-S111-INT**	7.025	-	-	0.0
Total	-	-	-	100.0

HPLC of isolated **16**

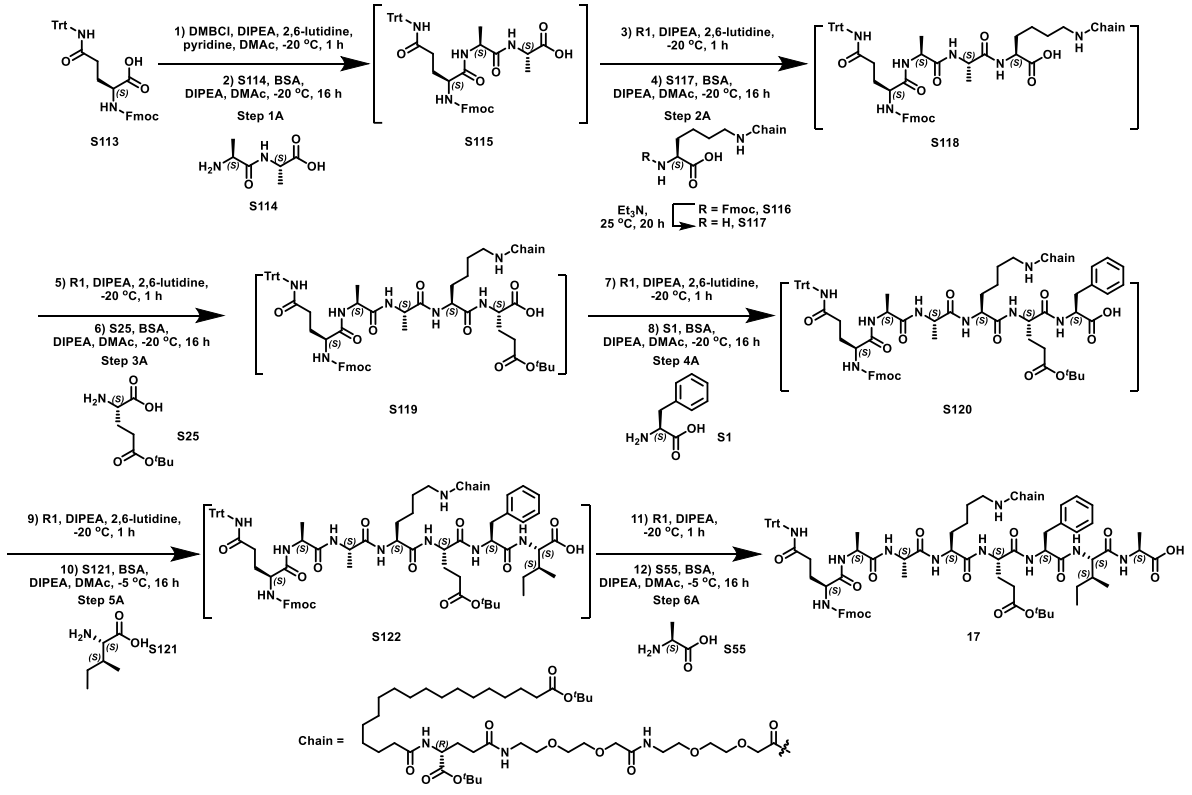


Compound ID	Time	Area	Height	Area%
	2.873	9.6311	4.7	0.5
	3.829	2.6912	1.6	0.2
	6.124	4.1051	2.2	0.2
	6.386	21.8823	12.2	1.2
	6.448	2.0211	1.3	0.1
16	6.743	1728.6075	914.9	96.5
	7.182	6.1590	3.5	0.3
	7.222	6.4501	3.7	0.4
	7.736	9.5586	4.4	0.5

XRPD and PLM of isolated **16**



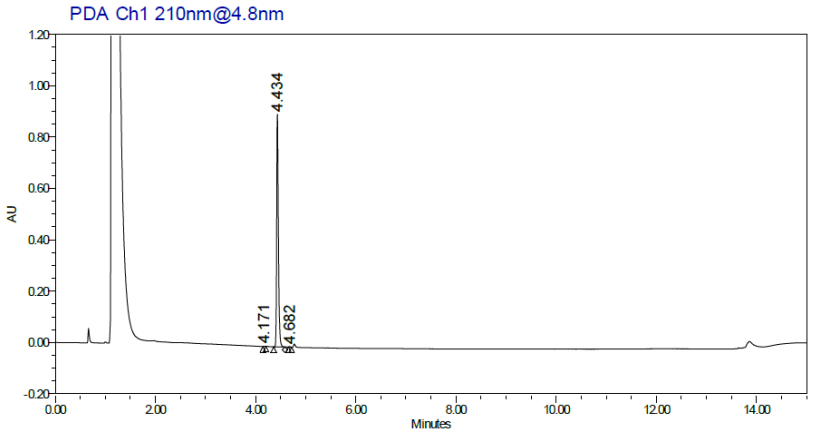
Fragment 17



Synthesis of H-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct(O*t*Bu)]-OH (S117). To a 5000 mL single-necked jacketed vessel was added **S116** (80.00 g, 66.86 mmol) and MeCN (3000 mL). Et_3N (47.36 g, 468.02 mmol) was added and the resulting reaction mixture was stirred at 25 °C for 20 h. Upon completion, the mixture was filtered. The collected wet cake was washed sequentially with MeCN (240 mL) and *n*-heptane (240 mL). The wet cake was dried with N_2 stream for 24 h to obtain 65.14 g of **S117** as a white solid in 100% isolated yield, which was used in the next reaction directly.

Synthesis of Fmoc-Gln(Trt)-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct(O*t*Bu)]-Glu(O*t*Bu)-Phe-Ile-Ala-OH (17). To a 500 mL single-necked jacketed vessel was added **S113** (30.00 g, 49.12 mmol) and DMAc (120 mL). After the solution was cooled to -20 °C, 2,6-lutidine (5.26 g, 49.12 mmol), DIPEA (6.35 g, 49.12 mmol), pyridine (0.78 g, 9.82 mmol) and DMBCl (6.94 g, 51.58 mmol) were added in sequence. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S113**:**S113-INT** was 0.76:99.24. The pre-cooled (0 °C) solution of **S114** (8.58 g, 53.54 mmol), BSA (19.99 g, 98.25 mmol), and DIPEA (3.17 g, 24.56 mmol) in DMAc (120 mL), which was prepared by stirring **S114**, BSA, and DIPEA in DMAc at 50 °C for 1 h and then cooled to 0 °C, was added to the reaction over 10 min. The reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the ratio of **S113**:**S115**:**S115-over-addition** was 0.12:99.79:0.09 and the reaction assay yield was 99%. Then *i*-PrOH (11.81 g, 196.49 mmol) was added to the reaction and stirred for 2 h at 25 °C. The resulting solution was directly used for next stage.

HPLC of **S115** formation

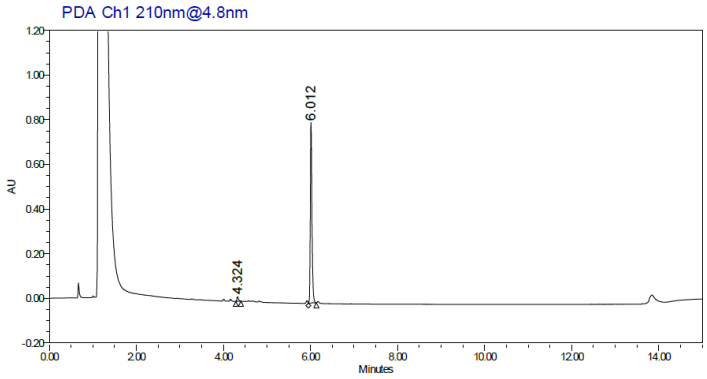


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S115-over-addition	4.171	1845	944	0.09
S115	4.434	2163904	905866	99.79
S113	4.682	2631	1402	0.12
Total	-	-	-	100.00

In a 1000 mL single-necked jacketed vessel, **S115** solution was cooled to -20 °C. DIPEA (3.81 g, 29.47 mmol), 2,6-lutidine (1.05 g, 9.82 mmol) and **R1** (9.49 g, 63.86 mmol) were added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S115**:**S115-INT** was 0.47:99.53. The pre-cooled (0 °C) solution of **S117** (56.48 g, 57.97 mmol), BSA (19.99 g, 98.25 mmol) and DIPEA (3.17 g, 24.56 mmol) in DMAc (300 mL), which was prepared by stirring **S117,** BSA and DIPEA in DMAc at 50 °C for 1 h and then cooled to 0 °C, was added to the reaction over 10 min. The

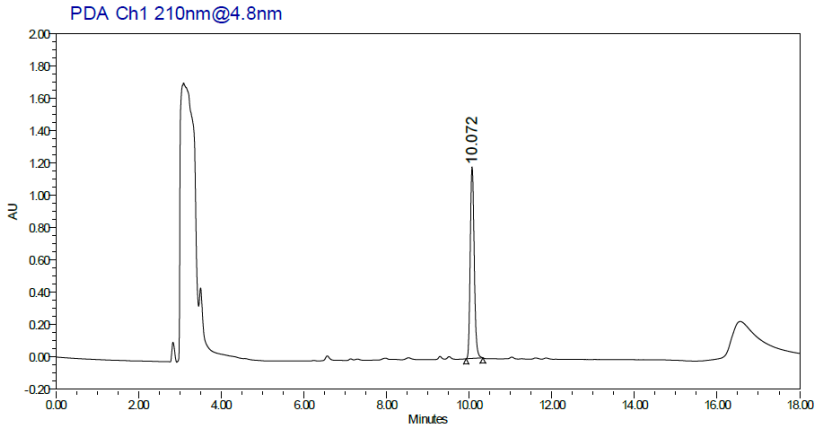
reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the ratio of **S115**:**S118**:***epi*-S118**:***epi*-S115-INT**:**S118-over-addition** was 2.4:97.6:0.0:0.0:0.0 and the reaction assay yield was 97%. Then *i*-PrOH (11.81 g, 196.49 mmol) was added to the reaction and stirred for 2 h at 25 °C. The resulting solution was directly used for next stage as **S118** solution.

HPLC of **S118** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S118-over-addition	7.025	-	-	-
S118	6.012	2099857	807706	97.60
S115	4.324	51670	20484	2.40
***epi*-S115-INT**	5.254	-	-	-
Total	-	-	-	100.0

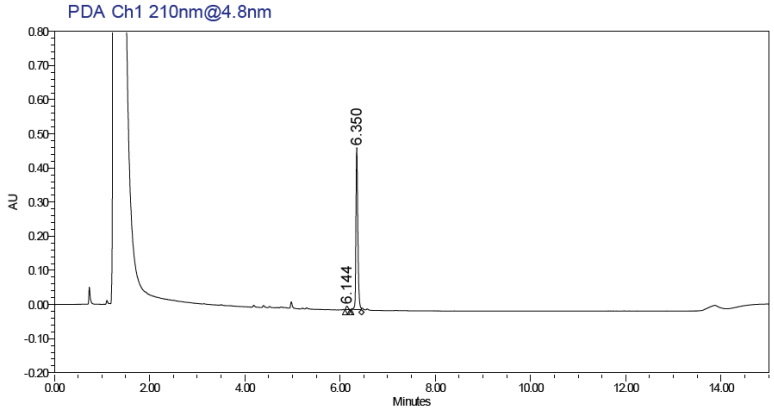
HPLC of **S118** chiral purity (**Method 2**)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S118	10.072	7891651	1184907	100.0
epi-**S118**	10.341	-	-	-
Total	-	-	-	100.0

The above solution of **S118** in 2000 mL single-necked jacketed vessel was cooled to -20 °C, DIPEA (5.08 g, 39.30 mmol), 2,6-lutidine (1.05 g, 9.82 mmol) and **R1** (9.86 g, 66.32 mmol) were added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S118**:**S118-INT** was 0.93:99.07. The pre-cooled (0 °C) solution of **S25** (11.98 g, 58.95 mmol), BSA (49.97 g, 245.62 mmol) and DIPEA (3.17 g, 24.56 mmol) in DMAc (120 mL), which was prepared by stirring **S25**, BSA and DIPEA in DMAc at 50 °C for 1 h and then cooled to 0 °C, was added to the reaction over 10 min. The reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the ratio of **S118**:**S119**:***epi*-S119**:***epi*-S118-INT**:**S119-over-addition** was 2.2:96.8:1.0:0.0:0.0 and the reaction assay yield was 96%. Then *i*-PrOH (29.52 g, 491.23 mmol) was added to the reaction and stirred for 2 h at 25 °C. The resulting solution was directly used for next stage as **S119** solution.

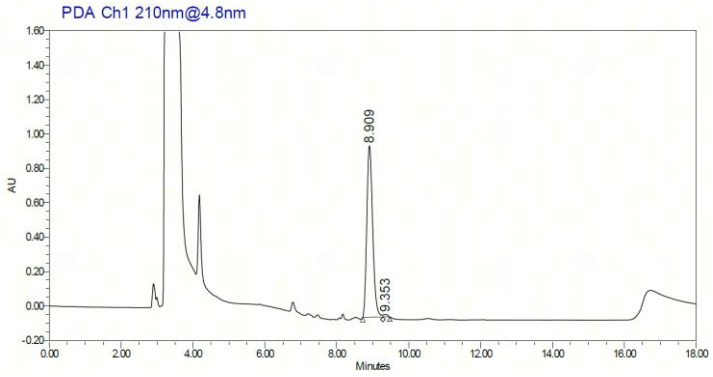
HPLC of **S119** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S119-over-addition	6.548	-	-	-
S119	6.350	1300719	474411	97.81*
S118	6.144	29090	9535	2.19
epi-**S118**-INT	6.706	-	-	-
Total	-	-	-	100.0

*Note: Contains 1.04% *epi*-**S119**

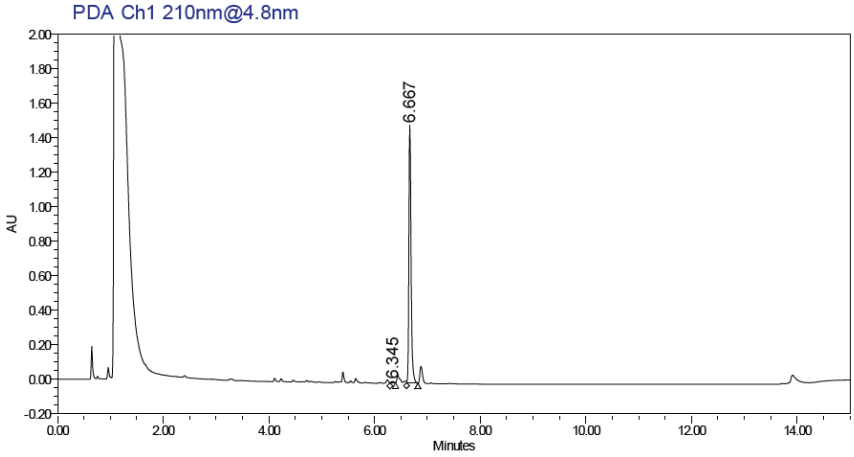
HPLC of **S119** chiral purity (**Method 3**)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S119	8.909	11253060	995756	98.96
epi-**S119**	9.353	118335	14721	1.04
Total	-	-	-	100.0

The above solution of **S119** in 2000 mL single-neck jacketed vessel was cooled to -20 °C, DIPEA (6.03 g, 46.67 mmol), 2,6-lutidine (1.05 g, 9.82 mmol) and **R1** (10.81 g, 72.70 mmol) were added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S119**:**S119-INT** (mixed anhydride intermediate quenched by 1-phenylethanamine) was 0.87:99.13. The pre-cooled (0 °C) solution of **S1** (9.74 g, 58.95 mmol), BSA (69.95 g, 343.86 mmol) and DIPEA (3.17 g, 24.56 mmol) in DMAc (120 mL), which was prepared by stirring **S1**, BSA and DIPEA in DMAc at 50 °C for 1 h and then cooled to 0 °C, was added to the reaction over 10 min. The reaction mixture was stirred at -20 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 95%, and the ratio of **S119**:**S120**:***epi*-S120**:***epi*-S119-INT**:**S120-over-addition** was 0.63:98.55:0.82:0:0. Then *i*-PrOH (41.33 g, 687.72 mmol) was added to the reaction and stirred for 2 h at 25 °C. The resulting solution was directly used for next stage as **S120** solution.

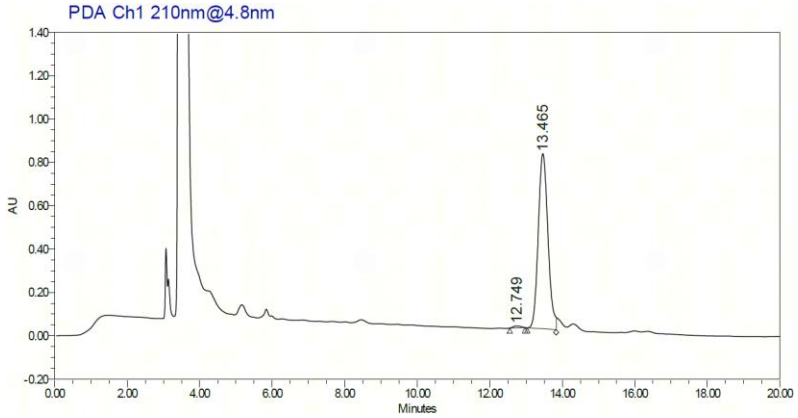
HPLC of **S120** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S120-over-addition	7.195	-	-	-
S120	6.667	4232894	1492377	99.37*
S119	6.345	26661	7220	0.63
epi-**S119**-INT	6.504	-	-	-
Total	-	-	-	100.0

*Note: Contains 0.83% *epi*-**S120**

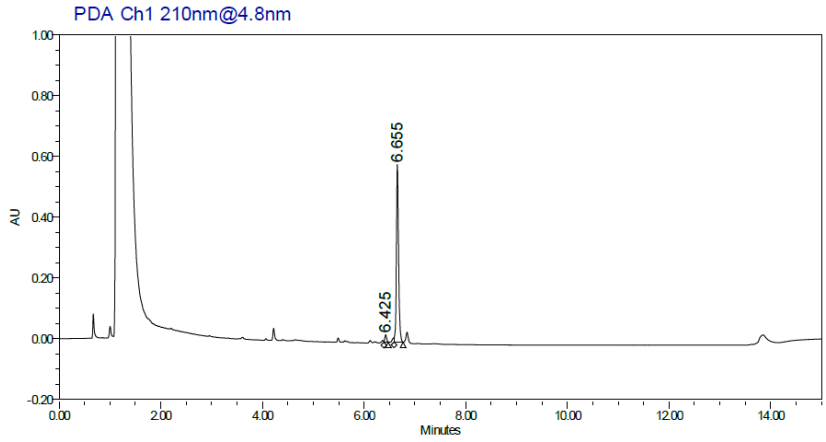
HPLC of **S120** chiral purity (**Method 4**)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S120	13.465	14894727	807751	99.17
epi-**S120**	12.749	123957	8688	0.83
Total	-	-	-	100.0

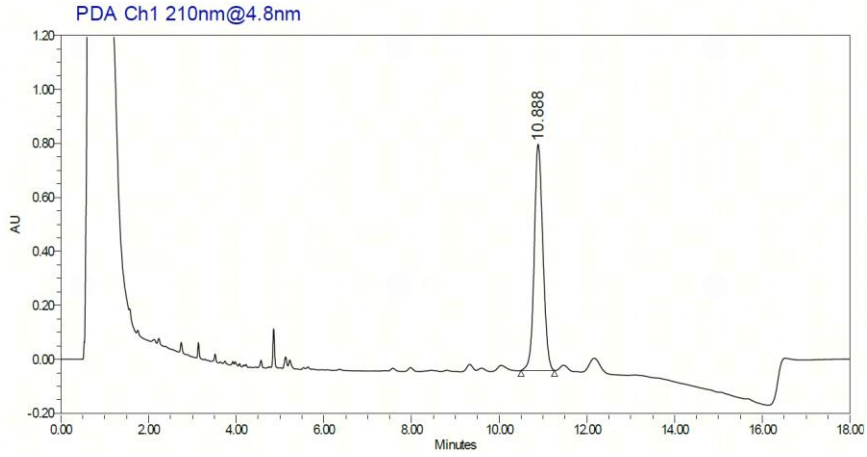
The above solution of **S120** in 2000 mL single neck jacketed vessel was cooled to -20 °C, DIPEA (6.03 g, 46.67 mmol), 2,6-lutidine (1.05 g, 9.82 mmol) and **R1** (11.68 g, 78.60 mmol) were added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S120**:**S120-INT** (mixed anhydride intermediate quenched by 1-phenylethanamine) was 0.99:99.01. Then the resulting solution was added into a pre-cooled solution of **S121** over 10 min, which was prepared by stirring **S121** (12.89 g, 98.25 mmol), BSA (99.93 g, 491.23 mmol) and DIPEA (3.17 g, 24.56 mmol) in DMAc (120 mL) at 50 °C for 1 h then cooling down to -5 °C. The reaction mixture was stirred at -5 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the ratio of **S120**:**S122**:***epi*-S122**:***epi*-S120-INT**:**S122-over-addition** was 3.43:96.57:0.0:0.0:0.0 and the reaction assay yield was 95%. Then *i*-PrOH (59.05 g, 982.46 mmol) was added to the reaction and stirred for 2 h at 25 °C. The resulting solution was directly used for next stage as **S122** solution.

HPLC of **S122** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S122-over-addition	7.106	-	-	-
S122	6.655	1713124	584202	96.57
S120	6.425	60910	24057	3.43
epi-**S120**-INT	6.934	-	-	-
Total	-	-	-	100.0

HPLC of **S122** chiral purity (**Method 5**)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S122	10.888	11498419	838377	100.0
***epi*-S122**	11.212	-	-	-
Total	-	-	-	100.0

The above solution of **S122** in 3000 mL single neck jacketed vessel was cooled to -20 °C. DIPEA (17.46 g, 135.09 mmol) and **R1** (24.09 g, 162.11 mmol) were added. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S122**:**S122-INT** (mixed anhydride intermediate quenched by 1-phenylethanamine) was 3.1:96.9. Then the resulting solution was added into a pre-cooled solution of **S55** over 10 min, which was prepared by stirring **S55** (17.51 g, 196.49 mmol), BSA (119.92 g, 589.48 mmol) and DIPEA (3.17 g, 24.56 mmol) in DMAc (120 mL) at 50 °C for 1 h then cooling down to -5 °C. The reaction mixture was stirred at -5 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 94%, and the ratio of **S122**:**17**:***epi*-17**:***epi*-S122-INT**:**17-over-addition** was 4.34:94.68:0.98:0.0:0.0. Then 25 wt% $NH_3 \cdot H_2O$ (6.68 g, 98.25 mmol) was added and the reaction mixture was stirred at 25 °C for 2 h. The reaction was diluted with H_2O (200 mL) and EtOAc (300 mL) and transferred to a separatory funnel. The aqueous phase was extracted with EtOAc (300 mL x 2). The combined organic layers were washed with brine, dried with Na_2SO_4, and concentrated by rotary evaporation. The residue was added into MeOH/MeCN/H_2O (10:10:6, 780 mL) over 10 mins and stirred for 12 h at 10 °C to allow crystallization. The weakly crystalline solid was filtered, washed with MeCN/H_2O (1:1, 100 mL), and dried under an N_2 stream to provide 87.47 g (90 wt%, 78.72 g by assay) of **17** as a white solid in 72% isolated yield and 92.7% HPLC purity over 6 steps.

Starting from **S113**, 3541 g of reagents/solvents were used to synthesize 78.72 g of **17**, giving a PMI of 45.

mp 215 °C. ^{1}H NMR (600 MHz, 3:1 CD_3CN/D_2O, 298 K) δ 7.79 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.5 Hz, 2H), 7.37 (t, *J* = 7.5 Hz, 2H), 7.28 (td, *J* = 7.5, 1.1 Hz, 2H), 7.26 – 7.08 (m, 20H), 4.50 (dd, *J* = 9.9, 4.9 Hz, 1H), 4.36 (dd, *J* = 10.6, 7.0 Hz, 1H), 4.27 (dd, *J* = 10.6, 7.3 Hz, 1H), 4.23 – 3.98 (m, 8H), 3.92 (dd, *J* = 9.0, 5.4 Hz, 1H), 3.89 (s, 2H), 3.79 (s, 2H), 3.60 – 3.49 (m, 8H), 3.47 (t, *J* = 5.6 Hz, 2H), 3.45 (t, *J* = 5.6 Hz, 2H), 3.32 (t, *J* = 5.6 Hz, 2H), 3.28 – 3.23 (m, 2H), 3.16 (dd, *J* = 14.0, 5.0 Hz, 1H), 3.12 – 3.00 (m, 2H), 2.86 (dd, *J* = 14.0, 9.9 Hz, 1H), 2.51 – 2.36 (m, 2H), 2.17 (t, *J* = 7.7 Hz, 2H), 2.15 – 2.04 (m, 6H), 2.01 – 1.95 (m, 2H), 1.87 – 1.62 (m, 7H), 1.54 – 1.38 (m, 8H), 1.36 (s, 9H), 1.36 (s, 9H), 1.32 (s, 9H), 1.31 – 1.23 (m, 11H), 1.23 – 1.15 (m, 22H), 1.13 – 1.03 (m, 2H), 0.84 (d, *J* = 6.8 Hz, 3H), 0.79 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, 3:1 CD_3CN/D_2O, 298 K) δ 174.9, 174.7, 174.6, 173.8, 173.8, 173.6, 173.5, 172.68 – 172.65 (3C), 172.2, 171.7, 171.5, 171.3 (2C), 171.0, 157.2, 144.5, 143.9, 143.8, 141.0, 137.2, 129.3, 128.5, 128.4, 127.9, 127.9, 127.7, 127.3, 126.8, 126.6, 125.3, 125.2, 120.1, 81.9, 80.7, 80.1, 70.4, 70.4, 69.9, 69.8, 69.6, 69.5, 69.1, 69.0, 66.8, 58.0, 55.4, 54.8, 54.5, 53.5, 52.7, 50.8, 50.6, 48.9, 46.9, 38.9, 38.3, 38.2, 36.9, 36.4, 35.5, 35.2, 32.6, 31.9, 31.3, 30.2, 29.3, 29.3, 29.2, 29.2, 29.1, 29.0, 29.0, 28.8, 28.8, 28.6, 28.5, 27.3, 27.3, 27.1, 27.0, 26.9, 26.0, 25.5, 24.8, 24.4, 23.1, 17.0, 16.1, 16.1, 14.7, 10.5. HRMS (ESI): m/z calcd for $C_{121}H_{174}O_{26}N_{13}$ $[M+H]^+$: 2225.2687, found: 2225.2661. FTIR (neat): 3282, 2926, 1727, 1628, 1529, 1448, 1151, 848, 698, 623 cm^{-1}.

Method 1: HPLC condition: Acquity UPLC CSH Fluoro-Phenyl, 2.1 × 150 mm, 1.7 μm column; 0.05% FA (v/v) in H_2O (solvent A), 0.05% FA (v/v) in MeCN (solvent B); gradient 40%-82% (solvent B) in 6.0 min, gradient 82%-95% (solvent B) in 4.0 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength 210 nm/254nm.

Method 2: HPLC condition: CHIRALPAK IG, 4.6 × 250 mm, 5.0 μm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN:THF=8:2 (solvent B); gradient 50%-95% (solvent B) in 11.0 min, hold 1 min; flow rate = 1.0 mL/min; detection wavelength = 210 nm.

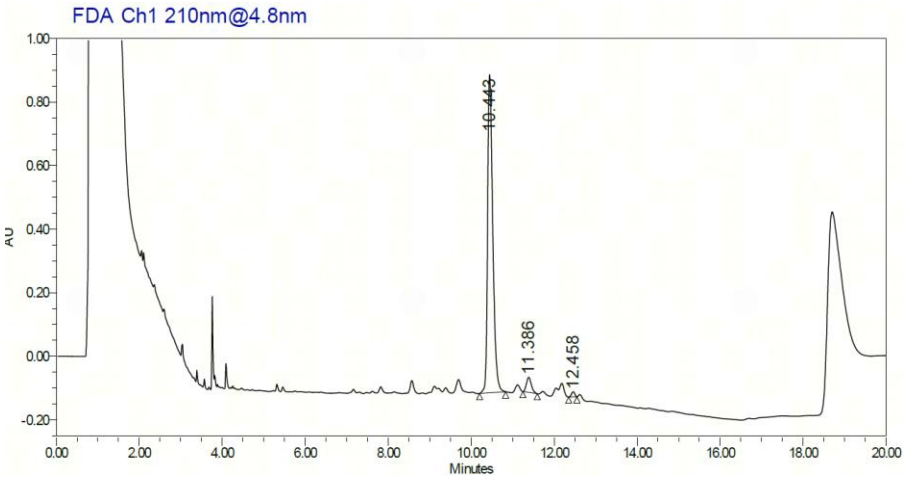
Method 3: HPLC condition: CHIRALPAK IG, 4.6 × 250 mm, 5.0 μm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN:MeOH=8:2 (solvent B); gradient 50%-90% (solvent B) in 3.0 min, gradient 90%-95% (solvent B) in 8.0 min, hold 1 min; flow rate = 1.0 mL/min; detection wavelength = 210 nm.

Method 4: HPLC condition: Daicel IC, 4.6 × 250 mm, 5.0 μm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN:MeOH=8:2 (solvent B); gradient 70%-95% (solvent B) in 15.0 min, hold 2 min; flow rate = 1.0 mL/min; detection wavelength = 210 nm.

Method 5: HPLC condition: ACQUITY UPLC Peptide CSH C18, 2.1 × 150 mm, 1.7 μm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN (solvent B); gradient 50%-70% (solvent B) in 3 min, gradient 70%-75% (solvent B) in 9 min, gradient 75%-95% (solvent B) in 3 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

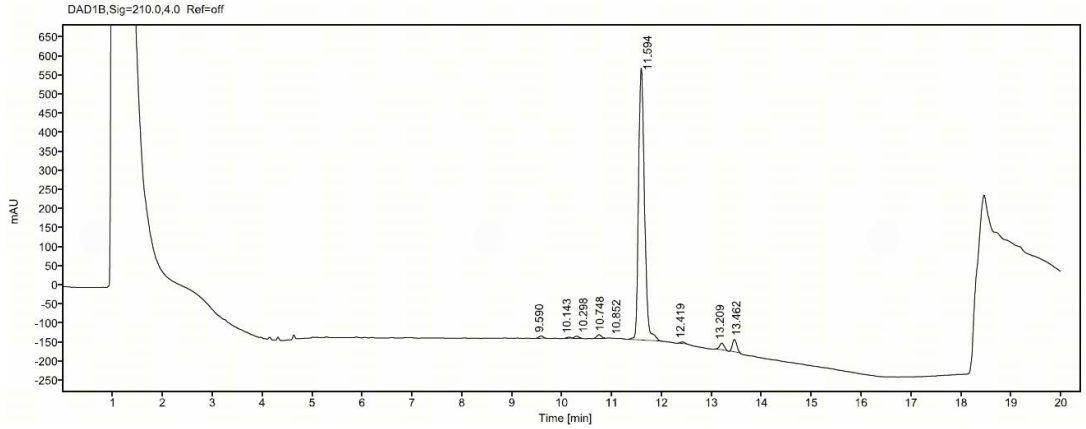
Method 6: HPLC condition: ACQUITY UPLC Peptide CSH C18, 2.1 × 150 mm, 1.7 μm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN (solvent B); gradient 40%-85% (solvent B) in 2 min, hold 8 min, gradient 85%-95% (solvent B) in 5 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

HPLC of **17** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
17-over-addition	11.643	-	-	-
17	10.443	8586153	1000749	94.68
***epi*-17**	12.458	89055	16164	0.98
S122	11.386	393328	47750	4.34
***epi*-S122-INT**	10.815	-	-	-
Total	-	-	-	100.0

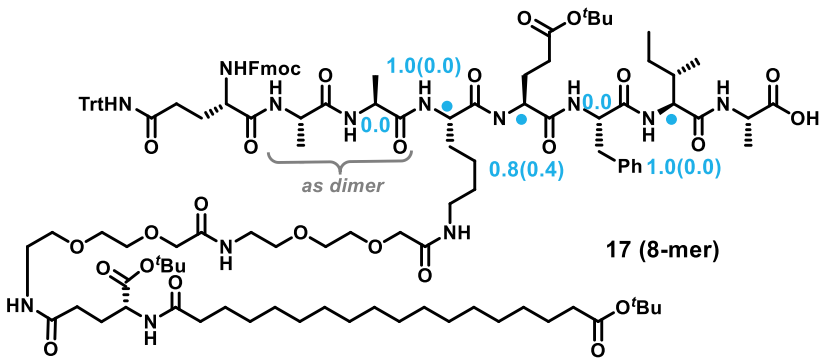
HPLC of isolated **17** (**Method 6**)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	%Area
17-over-addition	12.695	-	-	-
17	11.594	5912	713	92.7
***epi*-17**	12.935	-	-	0.0*
S122	12.419	24	4	0.4
***epi*-S122-INT**	12.065	-	-	-
Total	-	-	-	93.1

*Overlapped with impurities. DCl degradation confirmed 0.0% epimer of isoleucine.

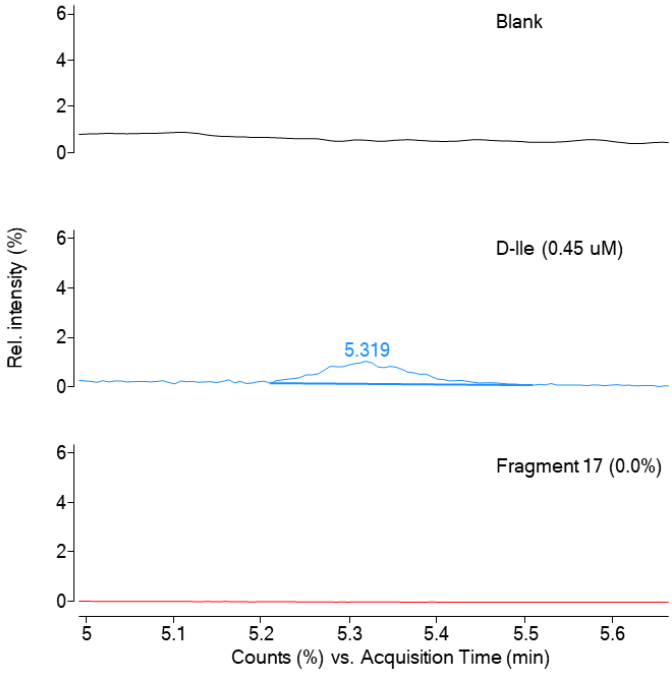
DCl degradation to determine Amino Acid chiral purity in isolated 17



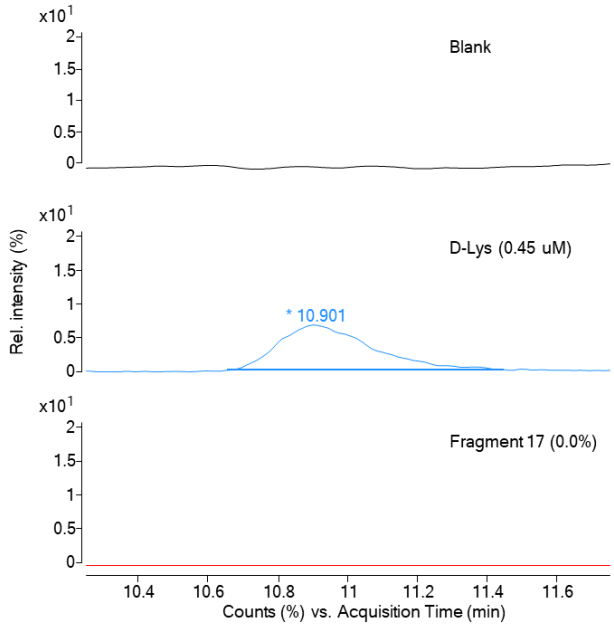
epimer% in reaction (epimer% in isolated 17)

Degradation procedure: for peptide hydrolysis, 1.5 mg of peptide sample was weighed into a 10 mL glass microwave hydrolysis tube. A hydrolysis solution consisting of 3 mL of 6 M DCl in D_2O and 30 µL of phenol was added, and the headspace was purged with nitrogen. The tube was then placed in a microwave synthesis reactor and heated at 150 °C for 15 min under microwave irradiation. After hydrolysis, the solvent was removed by lyophilization, and the residue was re-dissolved in MeCN/H_2O (1/1, v/v).

Representative EICs of blank, D-Ile (0.45 µM), and Fragment **17**.
Calibration curve: $R^2 = 1$, calibration range 0.45-225 µM (0.2%-100%).

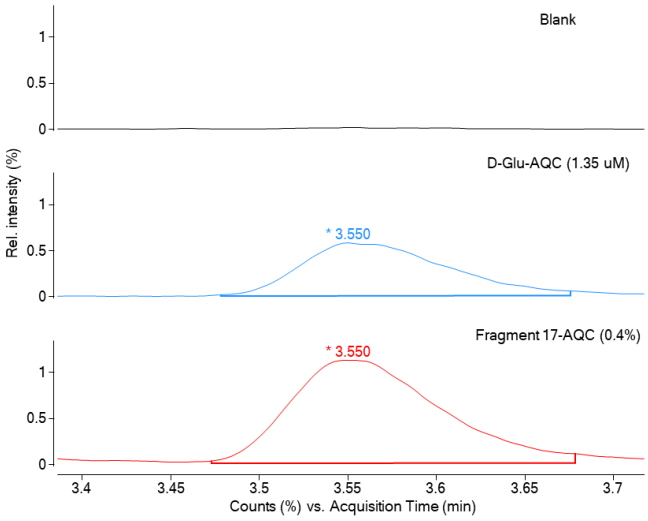


Representative EICs of blank, D-Lys (0.45 μM), and Fragment 17.
Calibration curve: $R^2 = 0.9999$, calibration range 0.45-225 μM (0.2%-100%).

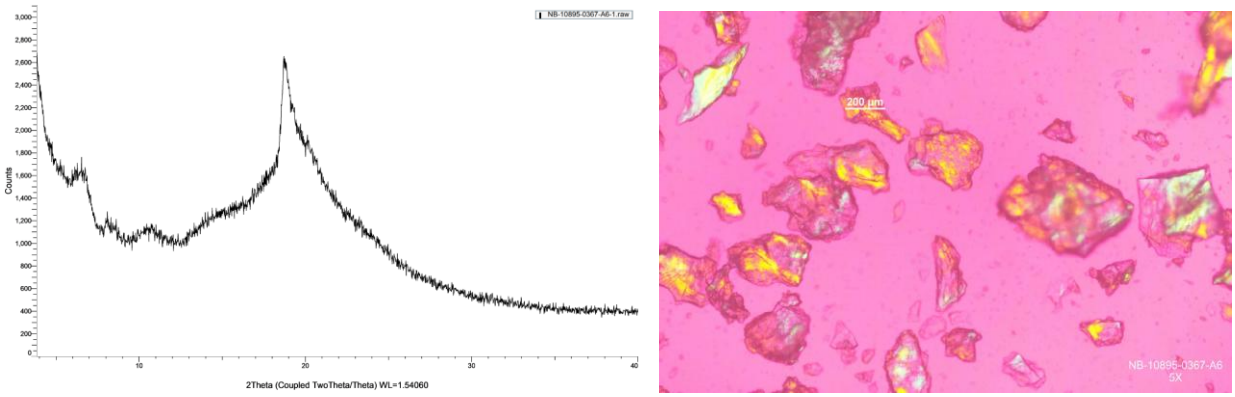


For amino acid derivatization, the derivatization reagent 6-Aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC) was prepared at a concentration of 5 mM in anhydrous acetonitrile and stored at -20°C. A 10 μL of sample solution was mixed with 170 μL of 0.4 M sodium borate buffer (pH 8.8), followed by the addition of 20 μL of AQC reagent solution. The reaction mixture was immediately heated at 55°C for 10 min with shaking at 1,000 rpm.

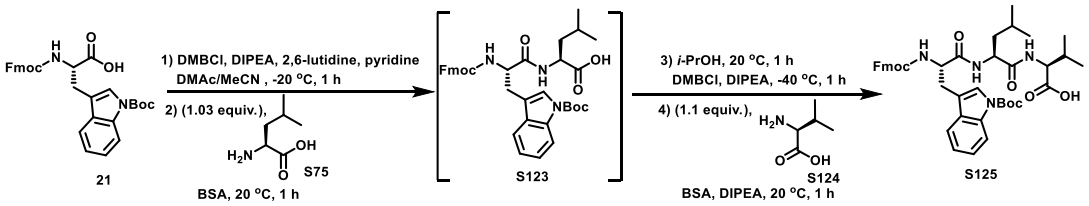
Representative EICs of blank, D-Glu-AQC (1.35 μM), and Fragment 17-AQC.
Calibration curve: $R^2 = 0.9999$, calibration range 1.35-33.75 μM (0.2%-5%).



XRPD and PLM of isolated **17**



Fragment 18



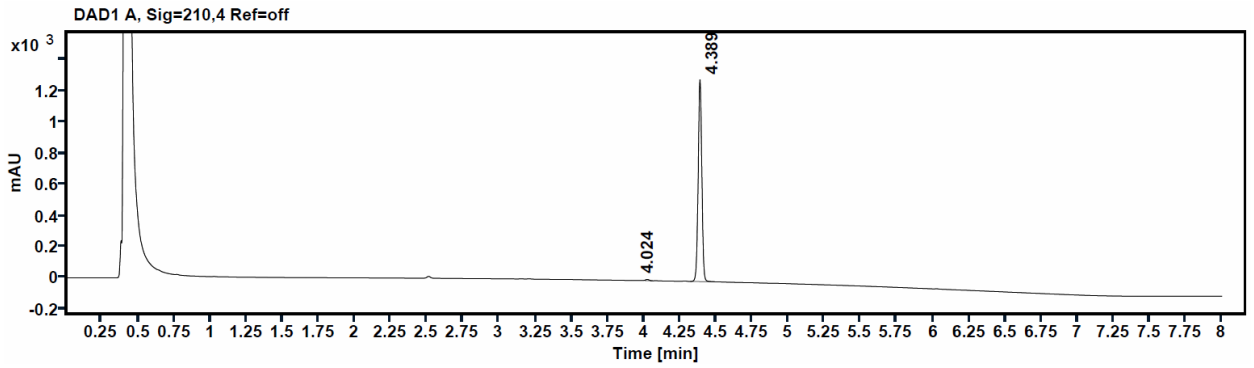
Synthesis of Fmoc-L-Trp(Boc)-L-Leu-L-Val-OH (**S125**). In a 500 mL 3-neck round bottom flask, **Fmoc-L-Trp(Boc)-OH (21)** (50.00 g, 94.95 mmol), pyridine (1.50 g, 18.99 mmol) and 2,6-lutidine (10.17 g, 94.95 mmol) were dissolved in DMAc/MeCN (1:1, 250 mL). The solution was cooled to -20 °C, DMBCl (13.16 g, 97.80 mmol) and DIPEA (12.27 g, 94.95 mmol) were added into the reaction mixture. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **21:21-INT** was 0.5:99.5. The reaction mixture was added slowly over 1h to a DMAc/MeCN solution of **L-Leu-OH (S75)** and BSA at 20 °C. The solution was prepared by mixing **S75** (12.83 g, 97.80 mmol) and BSA (38.63 g, 189.90 mmol) in DMAc/MeCN (1:1, 250 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **21:S123:S123-over-addition** was 0.6:99.4:0.0. Then *i*-PrOH (22.83 g, 379.79 mmol) was added to the reaction mixture. The reaction mixture was stirred at 20 °C and stirred for 1 h. The reaction was cooled to -40 °C, DMBCl (16.61 g, 123.43 mmol) and DIPEA (12.27 g, 94.95 mmol) were added to the reaction mixture. The reaction mixture was stirred at -40 °C for 1 h, then monitored by HPLC (**Method 1**). HPLC showed the ratio of **S123:S123-INT:*epi*-S123-INT** was 0.7:99.3:0.0. The reaction mixture was added slowly over 1 h to a DMAc/MeCN solution of **L-Val-OH (S124)**, BSA and DIPEA at 20 °C. The solution was prepared by mixing **S124** (12.23 g, 104.44 mmol) and BSA (57.95 g, 284.85 mmol) in DMAc/MeCN (1:1, 250 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C, then adding DIPEA (3.68 g, 28.48 mmol) before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC

(**Method 1**), which showed the ratio of **S123:S125:*epi*-S125:*epi*-S123-INT:S125-over-addition** was 0.7:99.1:0.0:0.0:0.2. The assay yield of the reaction was 96% over two steps. The reaction solution was slowly added dropwise into 6.5% KH_2PO_4 solution (1000 mL) at 20 °C. Then extracted with MTBE (750 mL). Upon phase separation, the organic phase was washed with 5% NaCl solution (250 mL), dried with anhydrous Na_2SO_4, filtered and concentrated to ~100 mL, then added dropwise into n-heptane (500 mL) at 0 °C over 1 h and stirred at 0 °C for 1 h. The solid was collected by filtration and dried under N_2 for overnight to provide 69.4 g of **S125** as a white solid (98.1% LCAP, 92.0 wt%) in 91% isolated yield (**Method 1**).

mp 140-145°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.66 (br s, 1H), 8.41 - 8.21(m, 1H), 8.02 (d, J = 8.4 Hz, 1H), 7.88 - 7.82 (m, 3H), 7.78 (d, J = 7.6 Hz, 1H), 7.73 (d, J = 8.8 Hz, 1H), 7.66 - 7.57 (m, 3H), 7.40 - 7.35 (m, 2H), 7.32 (t, J = 7.6 Hz, 1H), 7.27 - 7.19 (m, 3H), 4.54 - 4.39 (m, 2H), 4.20 - 3.95 (m, 4H), 3.10 - 3.06 (m, 1H), 2.95 (dd, J = 15.2, 10.8 Hz, 1H), 2.10 - 2.01 (m, 1H), 1.72 - 1.56 (m, 10H), 1.50 (t, J = 7.2 Hz, 2H), 0.90 - 0.84 (m, 12H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm) 172.83, 172.02, 171.30, 155.85, 149.04, 143.71, 143.66, 140.65, 140.62, 134.66, 130.30, 127.58, 127.00, 126.97, 125.28, 125.20, 124.26, 124.11, 122.38, 120.07, 119.59, 116.84, 114.63, 83.41, 65.76, 57.11, 54.44, 51.11, 46.54, 40.73, 31.26, 29.96, 27.64, 27.31, 26.82, 24.13, 23.08, 21.70, 19.05, 17.98. HRMS (ESI): m/z calcd. for $C_{42}H_{50}N_4O_8$ $[M+H]^+$: 739.3701, found: 739.3680. FTIR (neat): 3286, 3066, 2960, 2933, 2872, 1720, 1702, 1648, 1541, 1525, 1450, 1368, 1252, 1226, 1154, 1087, 1044, 1018, 855, 757, 740 cm^{-1}.

Method 1: Express C18 3.0 × 100 mm, particle size 2.7 μm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 50-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

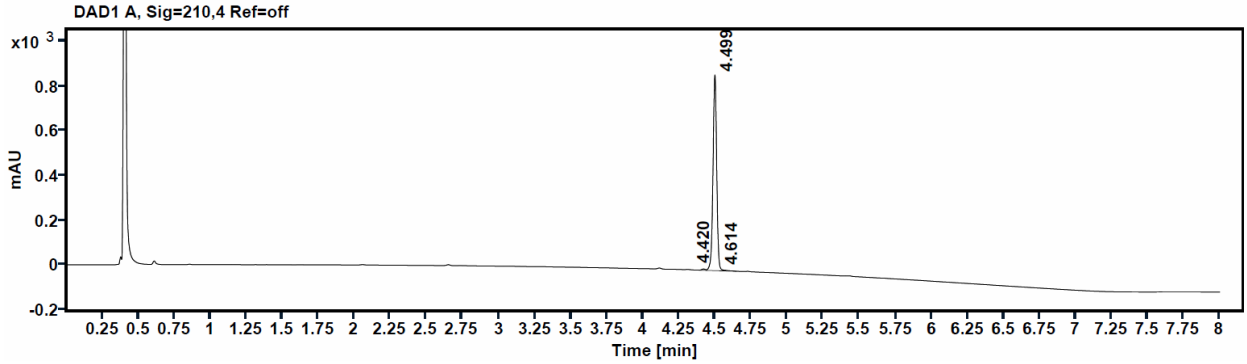
HPLC of **S123** formation (reaction mixture)



Compound ID	Time	Area	Height	Area%
21	4.024	14.1287	8.6	0.6
S123	4.389	2287.2495	1305.7	99.4

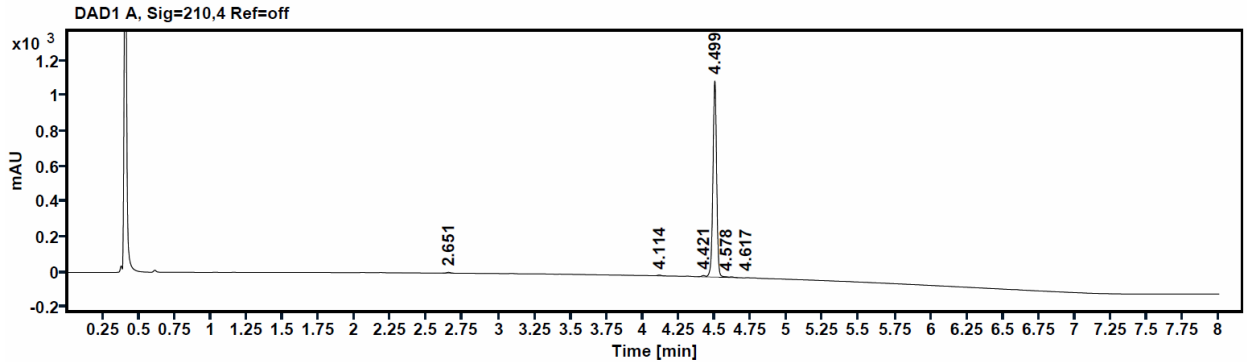
S123-over-addition	4.662	0.0	0.0	0.0
Total	-	-	-	100.0

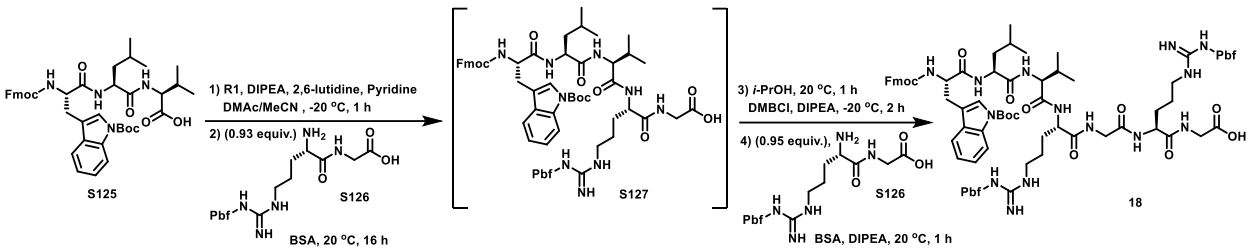
HPLC of **S125** formation (reaction mixture)



Compound ID	Time	Area	Height	Area%
S123	4.42	10.9853	6.1	0.7
S125	4.499	1568.9612	880.8	99.1
***epi*-S125**	4.560	0.0	0.0	0.0
S125-over-addition	4.614	3.0681	1.6	0.2
***epi*-S123-INT**	5.233	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of isolated **S125**





Synthesis of Fmoc-L-Trp(Boc)-L-Leu-L-Val-L-Arg(Pbf)-Gly-L-Arg(Pbf)-Gly-OH (**18**). In a 500 mL 3-neck round bottom flask, **Fmoc-L-Trp(Boc)-L-Leu-L-Val (S125)** 32.61 g (98.1% LCAP, 92.0 wt%, 40.60 mmol), pyridine (0.64 g, 8.12 mmol) and 2,6-lutidine (4.35 g, 40.60 mmol) were dissolved in DMAc/MeCN (1:1, 150 mL). The solution was cooled to -20 °C. **R1** (6.03 g, 40.60 mmol) and DIPEA (5.25 g, 40.60 mmol) were added into the reaction mixture. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC, which showed the ratio of **S125:S125-INT:*epi*-S125-INT** was 0.8:99.1:0.1. The reaction mixture was added slowly over 1 h to a DMAc/MeCN solution of **L-Arg (Pbf)-Gly (S126)** and BSA at 20 °C. The solution was prepared by mixing **S126** (18.26 g, 37.76 mmol) and BSA (24.78 g, 121.80 mmol) in DMAc/MeCN (1:1, 150 mL), stirring at 20 °C for 1 h before use. The reaction mixture was stirred at 20 °C for 16 h and monitored by HPLC, which showed the ratio of **S125:S127:*epi*-S127:*epi*-S125-INT:S127-over-addition** was 1.8:93.8:0.3:3.2:0.9. Then *i*-PrOH (12.20 g, 203.00 mmol) was added to the reaction mixture. The reaction mixture was stirred at 20 °C for 1 h. The reaction was cooled to -20 °C, DMBCl (6.01 g, 44.66 mmol) and DIPEA (4.46 g, 34.51 mmol) were added to the reaction mixture. The reaction mixture was stirred at -20 °C for 2 h, and monitored by HPLC. HPLC showed the ratio of **S127:S127-INT** was 1.2:98.8. The reaction mixture was added slowly over 1 h to a DMAc/MeCN solution of **L-Arg (Pbf)-Gly (S126)**, BSA and DIPEA at 20 °C. The solution was prepared by mixing **S126** (18.65 g, 38.57 mmol) and BSA (33.04 g, 162.40 mmol) in DMAc/MeCN (1:1, 150 mL), stirring at 40 °C for 1 h, cooling to 20 °C, then adding DIPEA (2.62 g, 20.30 mmol) before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC, which showed the ratio of **S127:18:18-over-addition** was 2.9:96.3:0.8. The assay yield of the reaction was 85% over two steps. The reaction solution was slowly added dropwise into 0.05M HCl aq. solution (1200 mL) at 10 °C over 2 h and stirred at 10 °C for 2 h. The solid was collected by filtration and dried under N_2. The crude solid (KF: 6%) was slurried with MeCN (1500 mL) at 50 °C for 16 h to allow crystallization. The weakly crystalline solid was collected by filtration and dried under N_2 for overnight to provide 54.1 g of **18** as a white solid (94.8% LCAP, 94.0 wt%) in 75% isolated yield.

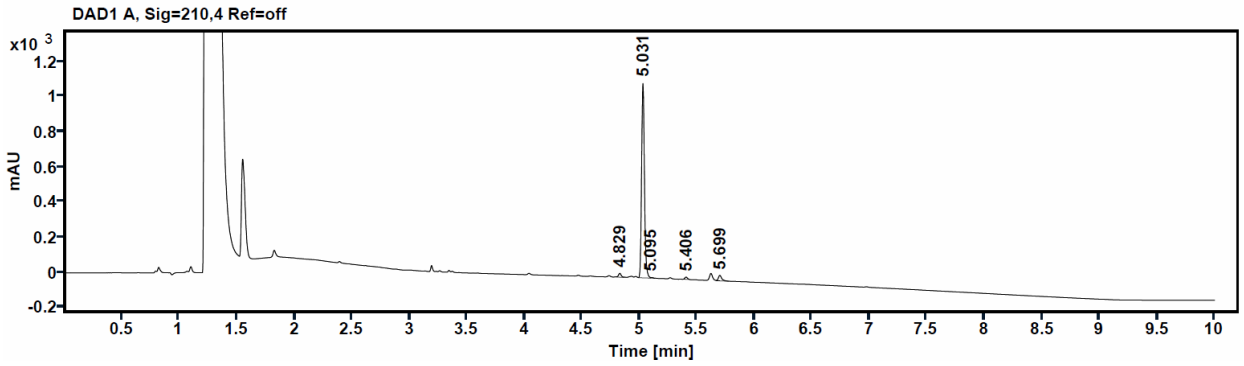
Starting from **21**, 4378 grams of reagents/solvents were used to synthesize 54.1 g (94.0 wt%) of **18**, giving a PMI of 86.

mp 180-186°C. ^{1}H-NMR (400 MHz, DMSO-d6) δ (ppm) 12.48 (br s, 1H), 8.33 (d, J = 8.0 Hz, 1H), 8.09-8.00 (m, 4H), 7.86 (d, J = 7.6 Hz, 4H), 7.78 (t, J = 7.6 Hz, 2H), 7.60 - 7.56 (m, 3H), 7.40 - 7.18 (m, 7H),

6.87 (br s, 3H), 6.46 (br s, 2H), 4.44 - 4.39 (m, 2H), 4.26 - 4.08 (m, 6H), 3.73 (d, J = 4.0 Hz, 2H), 3.57 (br s, 2H), 3.05 - 2.98 (m, 5H), 2.95 - 2.93 (m, 5H), 2.47 (s, 5H), 2.42 (s, 6H), 2.00 - 1.95 (m, 7H), 1.70 (br s, 2H), 1.55 (s, 10H), 1.49 (t, J = 7.2 Hz, 4H), 1.39 (d, J = 2.4 Hz, 16H), 0.86 - 0.81 (m, 13H). ^{13}C-NMR (101 MHz, DMSO-d6) δ (ppm) 172.24, 171.99, 171.83, 171.43, 171.35, 169.09, 157.91, 157.87, 156.83, 156.61, 156.30, 149.51, 144.17, 144.13, 141.11, 141.08, 137.73, 137.70, 135.12, 134.76, 134.65, 131.90, 131.87, 130.77, 128.05, 127.47, 127.44, 125.76, 125.67, 124.78, 124.74, 124.57, 122.88, 120.53, 120.10, 117.35, 116.71, 115.07, 86.74, 86.70, 83.88, 66.22, 58.05, 54.91, 52.68, 51.74, 47.00, 42.94, 42.55, 41.07, 40.65, 40.45, 31.03, 28.77, 28.11, 27.81, 25.86, 25.57, 24.62, 23.48, 22.20, 19.69, 19.43, 18.58, 18.07, 12.74. HRMS (ESI): m/z calcd. for $C_{84}H_{112}N_{14}O_{18}S_2$ $[M+H]^+$: 1669.7793, found: 1669.7733. FTIR (neat): 3293, 2967, 2930, 1731, 1638, 1543, 1452, 1368, 1251, 1156, 1090, 759, 740 cm^{-1}.

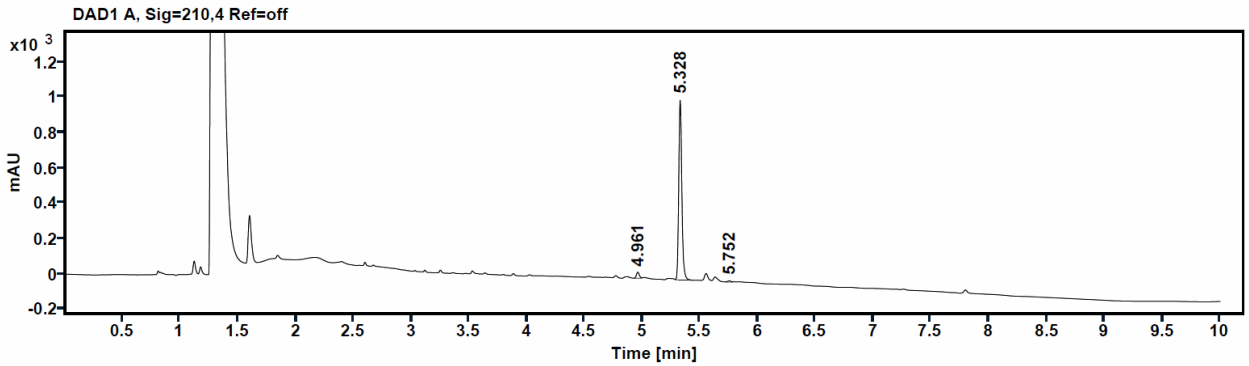
HPLC conditions: SB-Aq 4.6 × 100 mm, particle size 1.8 μm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN (solvent B); gradient 10-65% (solvent B) in 2 min, 65-95% (solvent B) in 8 min, hold 2 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

HPLC of **S127** formation (reaction mixture)



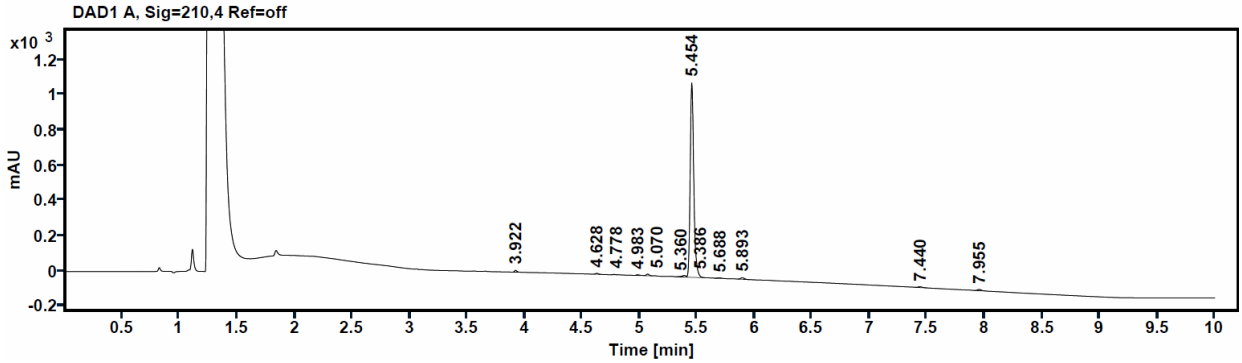
Compound ID	Time	Area	Height	Area% ratio
S125	4.829	36.9172	22.2	1.8
S127	5.031	1899.5353	1111.2	93.8
***epi*-S127**	5.095	5.6927	3.5	0.3
S127-over-addition	5.406	18.8372	12.4	0.9
***epi*-S125-INT**	5.699	64.2543	31.5	3.2
Total	-	-	-	100.0

HPLC of **18** formation (reaction mixture)



Compound ID	Time	Area	Height	Area%
S127	4.961	57.362	33.6	2.9
18	5.328	1886.2157	1019.4	96.3
18-over-addition	5.752	15.0363	7.2	0.8
Total	-	-	-	100.0

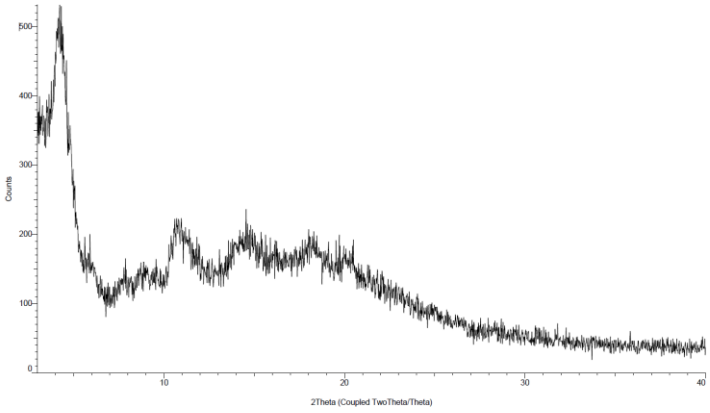
HPLC of isolated **18**

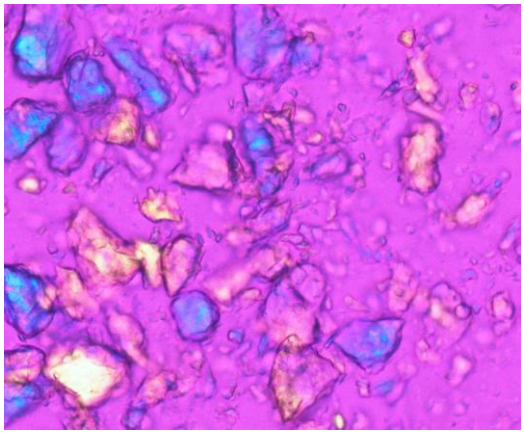


Compound ID	Time	Area	Height	Area%
S127	5.070	16.0146	9.2	0.6
18	5.454	2393.7822	1107.3	94.8
18-over-addition	5.893	19.8891	9.0	0.8
others	-	-	-	3.8
Total	-	-	-	100.0

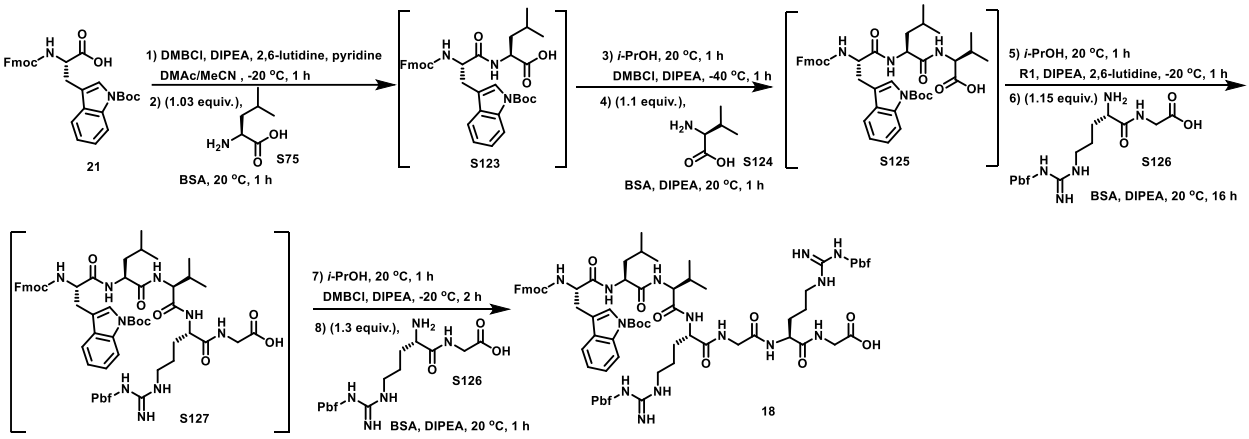
The compound **18** was slurry with MIBK (Methyl Isobutyl Ketone)/MeCN (1:1, 20 V) at 20 °C for 16 h, the solid was collected by filtration and dried under vacuum to provide **18** as a white solid (Crystal).

XRPD and PLM of isolated **18**





Synthesis of fragment 18 by continuous elongation.



Synthesis of Fmoc-L-Trp(Boc)-L-Leu-L-Val-L-Arg(Pbf)-Gly-L-Arg(Pbf)-Gly-OH (**18**). In a 500 mL 3-neck round bottom flask, **Fmoc-L-Trp(Boc)-OH (21)** (20.00 g, 37.98 mmol), pyridine (0.60 g, 7.60 mmol) and 2,6-lutidine (4.07 g, 37.98 mmol) were dissolved in DMAc/MeCN (1:1, 100 mL). The solution was cooled to -20 °C, DMBCl (5.27 g, 39.12 mmol) and DIPEA (4.91 g, 37.98 mmol) were added into the reaction mixture. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **21**:**21-INT** was 0.5:99.5. The reaction mixture was added slowly over 1 h to a DMAc/MeCN solution of **L-Leu-OH (S75)** and BSA at 20 °C, which was prepared by mixing **S75** (5.13 g, 39.12 mmol) and BSA (15.45 g, 75.96 mmol) in DMAc/MeCN (1:1, 100 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by

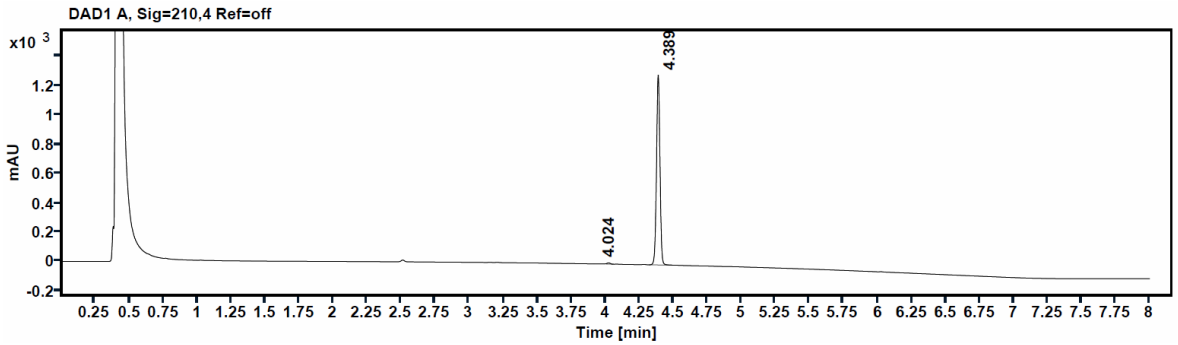
HPLC (**Method 1**), which showed the ratio of **21:S123:S123-over-addition** was 0.6:99.4:0.0. Then *i*-PrOH (9.13 g, 151.92 mmol) was added into the reaction mixture. The reaction mixture was stirred at 20 °C for 1 h. The reaction was cooled to -40 ºC, DMBCl (6.65 g, 49.37 mmol) and DIPEA (4.91 g, 37.98 mmol) were added into the reaction mixture. The reaction mixture was stirred at -40 °C for 1 h and monitored by HPLC (**Method 1**). HPLC showed the ratio of **S123:S123-INT**:***epi*-S123-INT** was 0.7:99.3:0.0. The reaction mixture was added slowly over 1 h to a DMAc/MeCN solution of **L-Val-OH (S124),** BSA and DIPEA at 20 °C. The solution was prepared by mixing **S124** (4.80 g, 41.02 mmol) and BSA (23.18 g, 113.94 mmol) in DMAc/MeCN (1:1, 100 mL), stirring at 40 °C for 1 h, and then cooling to 20 °C, then adding DIPEA (1.47 g, 11.38 mmol) before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S123:S125:*epi*-S125:*epi*-S123-INT:S125-over-addition** was 0.7:99.1:0.0:0.0:0.2. Then *i*-PrOH (13.70 g, 227.88 mmol) was added into the reaction mixture. The reaction mixture was stirred at 20 °C for 1 h. The reaction was cooled to -20 ºC, **R1** (9.04 g, 60.77 mmol), 2,6-lutidine (2.03 g, 18.99 mmol) and DIPEA (4.91 g, 37.98 mmol) were added into the reaction mixture. The reaction mixture was stirred at -20 °C for 1 h and monitored by HPLC (**Method 2**). HPLC showed the ratio of **S125:S125-INT:*epi*-S125-INT** was 1.4:98.5:0.1. The reaction mixture was added slowly over 1 h to a DMAc/MeCN solution of **L-Arg (Pbf)-Gly-OH (S126),** BSA and DIPEA at 20 °C. The solution was prepared by mixing **S126** (21.12 g, 43.68 mmol) and BSA (38.63 g, 189.90 mmol) in DMAc/MeCN (1:1, 100 mL), stirring at 20 °C for 1 h, and then adding DIPEA (1.47 g, 11.38 mmol) before use. The reaction mixture was stirred at 20 °C for 16 h and monitored by HPLC (**Method 2**), which showed the ratio of **S125:S127:*epi*-S127:*epi*-S125-INT:S127-over-addition** was 2.1:93.1:0.2:2.6:2.0. Then *i*-PrOH (18.26 g, 303.84 mmol) was added into the reaction mixture. The reaction mixture was stirred at 20 °C for 1 h. The reaction was cooled to -20 ºC, DMBCl (9.71 g, 72.16 mmol) and DIPEA (7.85 g, 60.77 mmol) were added into the reaction mixture. The reaction mixture was stirred at -20 °C for 2 h, then monitored by HPLC (**Method 2**). HPLC showed the ratio of **S127:S127-INT** was 1.5:98.5. The reaction mixture was added slowly over 1 h to a DMAc/MeCN solution of **L-Arg (Pbf)-Gly-OH (S126)** and BSA at 20 °C. The solution was prepared by mixing **S126** (23.88 g, 49.37 mmol) and BSA (61.81 g, 303.84 mmol) in DMAc/MeCN (1:1, 100 mL), stirring at 20 °C for 1 h, and then adding DIPEA (2.45 g, 18.99 mmol) before use. The reaction mixture was stirred at 20 °C for 1 h and monitored by HPLC (**Method 2**), which showed the ratio of **S127:18:18-over-addition** was 2.8:95.6:1.6. The assay yield was 80% over four steps. The reaction solution was slowly added dropwise into 0.05M aq. HCl solution (800 mL) at 10 ºC over 1 h and stirred at 10 ºC for 2 h. Then extracted with EtOAc (400 mL), upon phase separation, the organic phase was washed with 5% NaCl solution (200 mL), dried with anhydrous Na_2SO_4, filtered and concentrated. The crude product was purified by medium-pressure flash chromatography (C18 column, mobile phase, MeCN in Water (0.05% HCl), 50-90% gradient in 50 min; detection wavelength: 210 nm) to provide 40.9 g **18** as a white solid (94.1% LCAP, 93.0 wt%) in 60%

isolated yield (**Method 2**). This gives slightly lower IY and purity than the above method with the isolation of intermediate **S125**.

Method 1: Express C18 3.0 × 100 mm, particle size 2.7 µm column; 0.05% TFA (v/v) in H_2O (solvent A), 0.05% TFA (v/v) in MeCN (solvent B); gradient 50-95% (solvent B) in 6 min, hold 2 min; flow rate: 1.0 mL/min; detection wavelength: 210 nm.

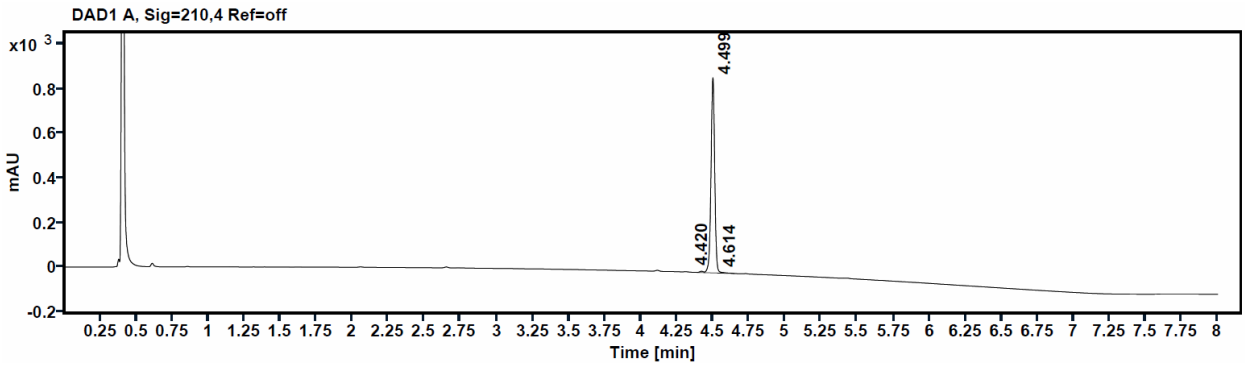
Method 2: SB-Aq 4.6 × 100 mm, particle size 1.8 µm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in MeCN (solvent B); gradient 10-65% (solvent B) in 2 min, 65-95% (solvent B) in 8 min, hold 2 min; flow rate: 1.5 mL/min; detection wavelength: 210 nm.

HPLC of **S123** formation (reaction mixture)



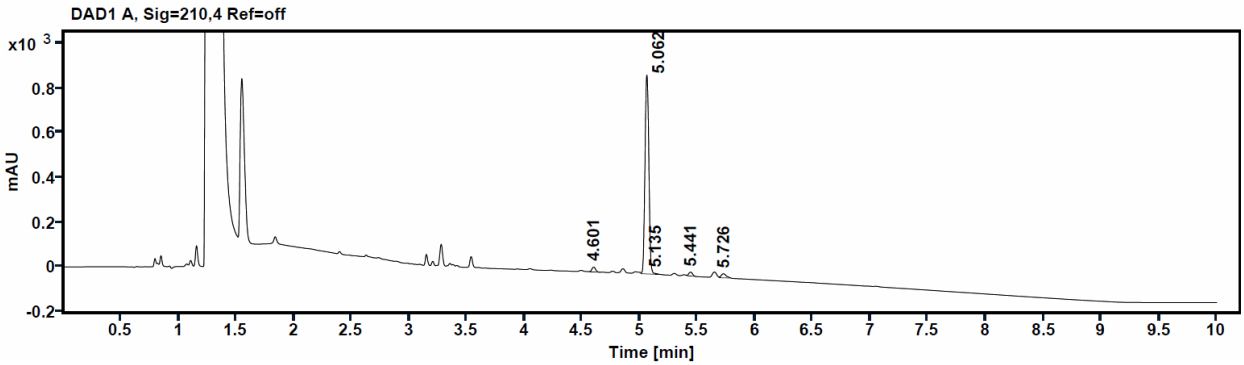
Compound ID	Time	Area	Height	Area%
21	4.024	14.1287	8.6	0.6
S123	4.389	2287.2495	1305.7	99.4
S123-over-addition	4.662	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of **S125** formation (reaction mixture)



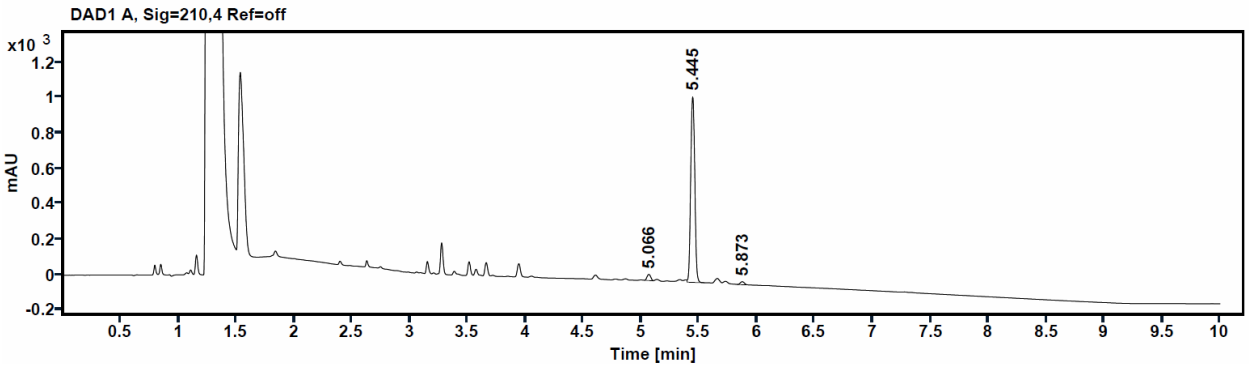
Compound ID	Time	Area	Height	Area%
S123	4.42	10.9853	6.1	0.7
S125	4.499	1568.9612	880.8	99.1
***epi*-S125**	4.560	0.0	0.0	0.0
S125-over-addition	4.614	3.0681	1.6	0.2
***epi*-S123-INT**	5.233	0.0	0.0	0.0
Total	-	-	-	100.0

HPLC of **S127** formation (reaction mixture)



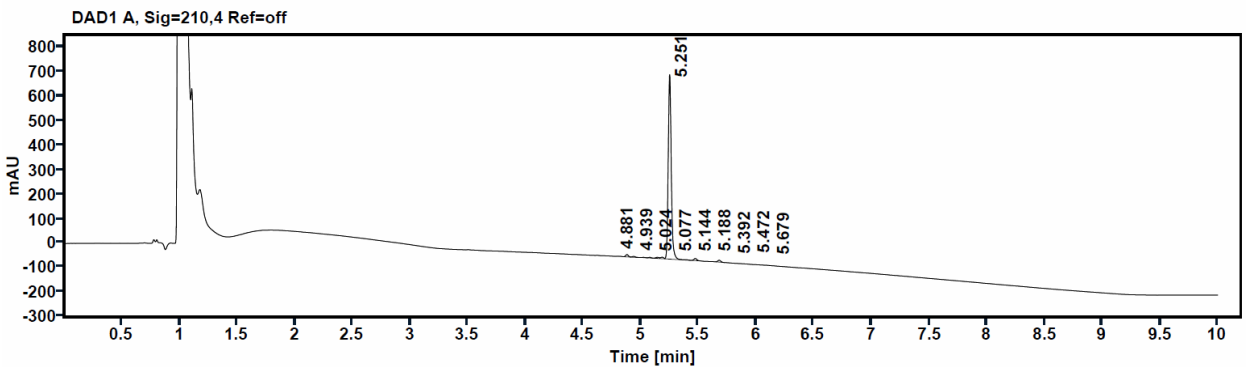
Compound ID	Time	Area	Height	Area% ratio
S125	4.601	46.0056	21.3	2.1
S127	5.062	2067.1443	895.1	93.1
***epi*-S127**	5.125	5.2921	3.6	0.2
S127-over-addition	5.441	44.4615	18.4	2.0
***epi* -S125-INT**	5.726	57.582	18.2	2.6
Total	-	-	-	100.0

HPLC of **18** formation (reaction mixture)

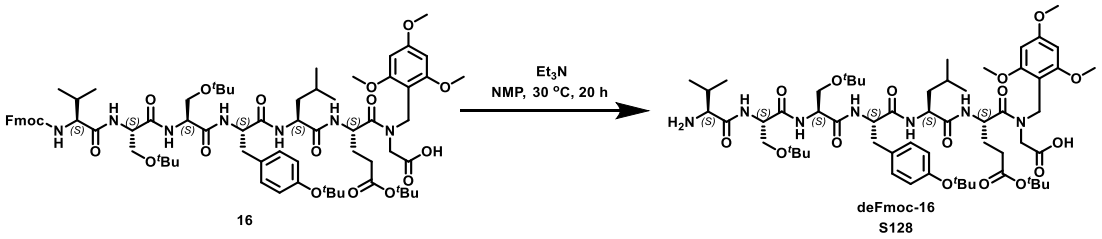


Compound ID	Time	Area	Height	Area% ratio
S127	5.066	74.3537	34	2.8
18	5.445	2535.675	1045.8	95.6
18-over-addition	5.872	41.6918	16.9	1.6
Total	-	-	-	100.0

HPLC of isolated **18**



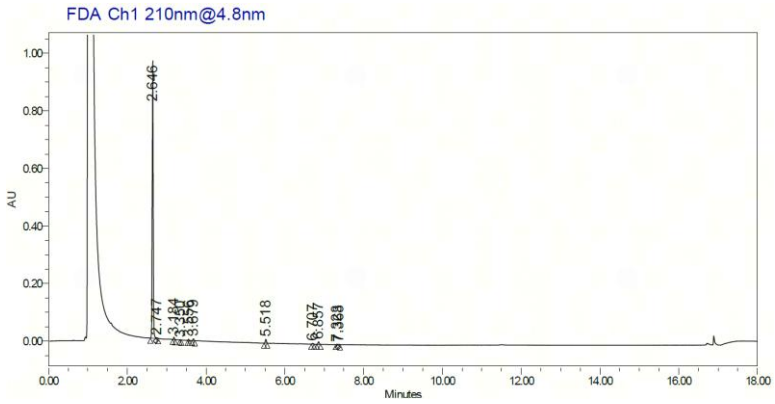
Compound ID	Time	Area	Height	Area% ratio
S127	4.881	15.4000	9.6	1.1
18	5.251	1317.3966	757.1	94.1
18-over-addition	5.679	13.6157	7.6	0.9
others	-	-	-	3.9
Total	-	-	-	100.0



Synthesis of H-Val-Ser(O*t*Bu)-Ser(O*t*Bu)-Tyr(O*t*Bu)-Leu-Glu(O*t*Bu)-Gly(Tmb)-OH (deFmoc-16, S128). To a 50 mL single neck jacketed vessel was added 1-methylpyrrolidin-2-one (NMP) (25 mL), **16** (5.00 g, 3.62 mmol) and Et_3N (1.83 g, 18.11 mmol). The reaction mixture was stirred at 30 °C for 20 h and monitored by HPLC (**Method 1**), which showed the ratio of **16**:**S128** was 0.29:96.18. The reaction was added into MTBE (150 mL) and stirred for 2 h at 20 °C. The solid was collected by filtration and washed with MTBE (25 mL), dried under N_2 to provide 4.25 g of **S128** (94.72% HPLC purity and 95.7wt%) as a white solid with 97% isolated yield. mp 200.5 °C. HRMS (ESI): m/z calcd for $C_{59}H_{96}O_{16}N_7$ $[M+H]^+$: 1158.6908, found: 1158.6908. FTIR (neat): 3281, 2972, 1727, 1672, 1628, 1504, 1364, 1150, 1129, 689, 470 cm^{-1}.

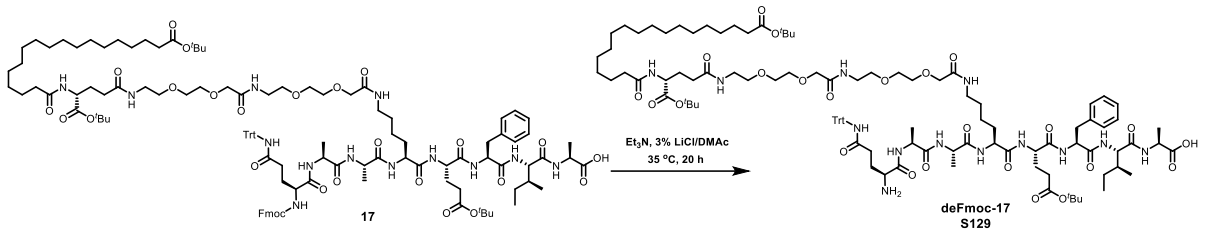
Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 40%-85% (solvent B) in 8.5 min, gradient 85%-90% (solvent B) in 3.5 min, gradient 90%-95% (solvent B) in 1 min; flow rate 0.4 mL/min; detection wavelength = 210 nm.

HPLC of isolated **S128**



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
9-Methylene-9H-fluorene	5.518	26612	13463	1.52
16	8.030	-	-	-
S128	2.646	1661910	964273	94.72
Others	-	-	-	3.76
Total	-	-	-	100.00

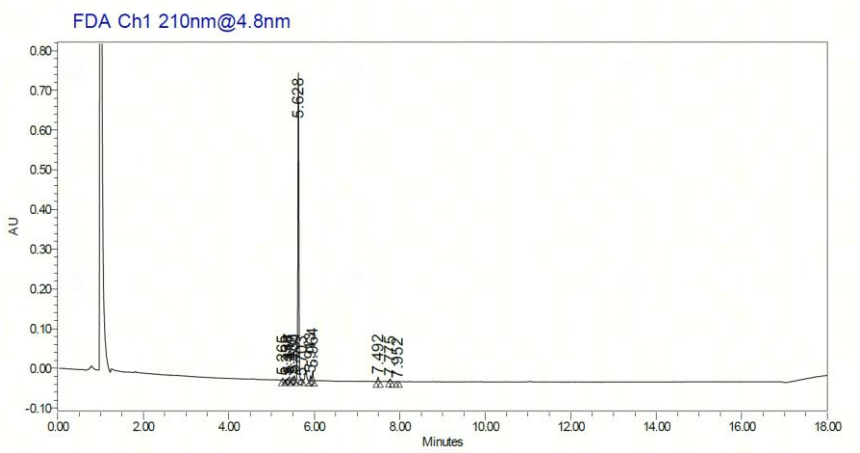
Synthesis of H-Gln(Trt)-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct(O*t*Bu)]-Glu(O*t*Bu)-Phe-Ile-Ala-OH (S129)



Synthesis of H-Gln(Trt)-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct(O*t*Bu)]-Glu(O*t*Bu)-Phe-Ile-Ala-OH (deFmoc-17, S129). To a 100 mL single neck jacketed vessel was added 3% LiCl/ DMAc solution (80 mL), **17** (5.00 g, 2.25 mmol) and Et_3N (1.82 g, 17.97 mmol). The reaction mixture was stirred at 35 °C for 20 h and monitored by HPLC (**Method 1**), which showed the ratio of **17**:**S129** was 0.71:85.85. The reaction was added into MTBE/H_2O:1/1 (480 mL) and stirred for 1 h at 20 °C. The solid was collected by filtration and washed with MTBE (25 mL), dried under N_2 to provide 4.89 g of **S129** as a white solid (88.8% HPLC purity and 88.3 wt%) with 96% isolated yield. mp: 226.5 °C. ^{1}H NMR (600 MHz, DMSO-d_6, 298 K) δ 8.63 (s, 1H), 8.18 (d, J = 7.7 Hz, 2H), 8.08 (d, J = 6.8 Hz, 1H), 8.05 (d, J = 7.5 Hz, 1H), 8.01 – 7.93 (m, 2H), 7.93 – 7.86 (m, 3H), 7.72 – 7.62 (m, 2H), 7.30 – 7.23 (m, 6H), 7.23 – 7.11 (m, 14H), 4.57 (td, J = 8.4, 4.7 Hz, 1H), 4.35 – 4.29 (m, 1H), 4.29 – 4.23 (m, 1H), 4.23 – 4.13 (m, 3H), 4.11 – 4.06 (m, 1H), 4.04 (ddd, J = 9.1, 7.5, 5.3 Hz, 1H), 3.87 (s, 2H), 3.84 (s, 2H), 3.59 – 3.50 (m, 8H), 3.44 (t, J = 6.0 Hz, 2H), 3.40 (t, J = 6.0 Hz, 2H), 3.27 (q, J = 6.0 Hz, 2H), 3.24 – 3.21 (m, 1H), 3.19 (q, J = 5.9 Hz, 2H), 3.03 (td, J = 14.2, 5.7 Hz, 3H), 2.79 (dd, J = 14.1, 9.1 Hz, 1H), 2.35 (t, J = 7.8 Hz, 2H), 2.20 – 2.04 (m, 8H), 1.92 – 1.84 (m, 1H), 1.84 – 1.76 (m, 2H), 1.76 – 1.69 (m, 2H), 1.68 – 1.63 (m, 1H), 1.63 – 1.54 (m, 2H), 1.52 – 1.42 (m, 6H), 1.41 – 1.35 (m, 3H), 1.38 (s, 9H), 1.38 (s, 9H), 1.37 (s, 9H), 1.25 (d, J = 7.8 Hz, 3H), 1.23 (s, 25H), 1.21 – 1.16 (m, 6H), 1.08 (dt, J = 13.4, 7.8 Hz, 1H), 0.84 (d, J = 6.8 Hz, 3H), 0.80 (t, J = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6, 298 K) δ 174.4, 173.4, 172.8, 172.8, 172.5, 172.3, 172.2, 172.1, 171.8, 171.8, 171.7, 171.1, 170.9, 170.6, 169.7, 169.4, 145.4, 138.0, 129.6, 129.0, 128.4, 127.9, 126.8, 126.6, 80.7, 80.0, 79.8, 70.6, 70.5, 70.4, 69.8, 69.7, 69.6, 69.6, 69.3, 57.1, 54.1, 54.0, 52.9, 52.8, 52.0, 48.6, 48.5, 48.2, 39.0, 38.5, 38.4, 37.7, 37.3, 35.5, 35.2, 33.0, 32.0, 32.0, 31.7, 31.5, 30.5, 29.5, 29.5, 29.5, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 29.3, 29.1, 29.0, 28.8, 28.2, 28.2, 28.1, 27.4, 25.7, 25.1, 24.6, 23.2, 22.6, 19.1, 18.5, 17.9, 15.7, 14.4, 11.5. HRMS (ESI): m/z calcd for $C_{106}H_{165}O_{24}N_{13}$ $[M+2H]^{2+}$: 1002.1040, found: 1002.1039. FTIR (neat): 3281, 2927, 1727, 1625, 1518, 1448, 1366, 1150, 847, 699, 572 cm^{-1}.

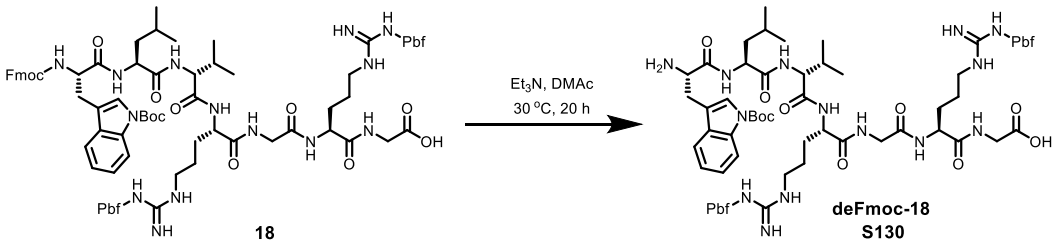
Method 1: HPLC condition: Acquity UPLC CSH Fluoro-Phenyl, 2.1 × 150 mm, 1.7 μm column; 0.05% FA (v/v) in H_2O (solvent A), 0.05% FA (v/v) in MeCN (solvent B); gradient 40%-82% (solvent B) in 6.0 min, gradient 82%-95% (solvent B) in 4.0 min, hold 2 min; flow rate 0.4 mL/min; detection wavelength = 210 nm/254nm.

HPLC of isolated **S129**



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
9-Methylene-9H-fluorene	5.762	-	-	-
17	10.845	-	-	-
S129	5.628	1228253	773196	88.79
Total	-	-	-	88.79

Synthesis of H-Trp(Boc)-Leu-Val-Arg(Pbf)-Gly-Arg(Pbf)-Gly-OH (S130)

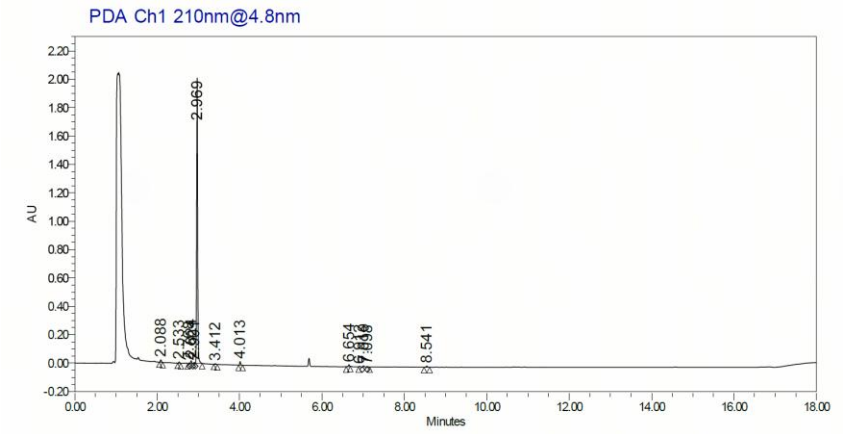


Synthesis of H-Trp(Boc)-Leu-Val-Arg(Pbf)-Gly-Arg(Pbf)-Gly-OH (deFmoc-18, S130). To a 50 mL single neck jacketed vessel was added DMAc (25 mL), **18** (5.00 g, 2.99 mmol) and Et_3N (1.51 g, 14.97 mmol). The reaction mixture was stirred at 30 °C for 20 h and monitored by HPLC (**Method 1**), which showed the ratio of **18**:**S130** was 0:91.7. The reaction was added into MTBE (125 mL) and stirred for 2 h at 20 °C. The solid was collected by filtration and washed with MTBE (25 mL), dried under N_2 to provide 4.56 g of **S130** as a white solid with quantitative yield and 94.0% HPLC purity. mp 167.8 °C. HRMS (ESI): m/z calcd for $C_{69}H_{103}O_{16}N_{14}S_2$ $[M+H]^+$: 1447.7112, found: 1447.7120. FTIR (neat): 3280, 2931, 1730, 1622, 1546, 1452, 1369, 1254, 1155, 1088, 660, 698, 567 cm^{-1}.

Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 40%-85% (solvent B) in 8.5 min, gradient

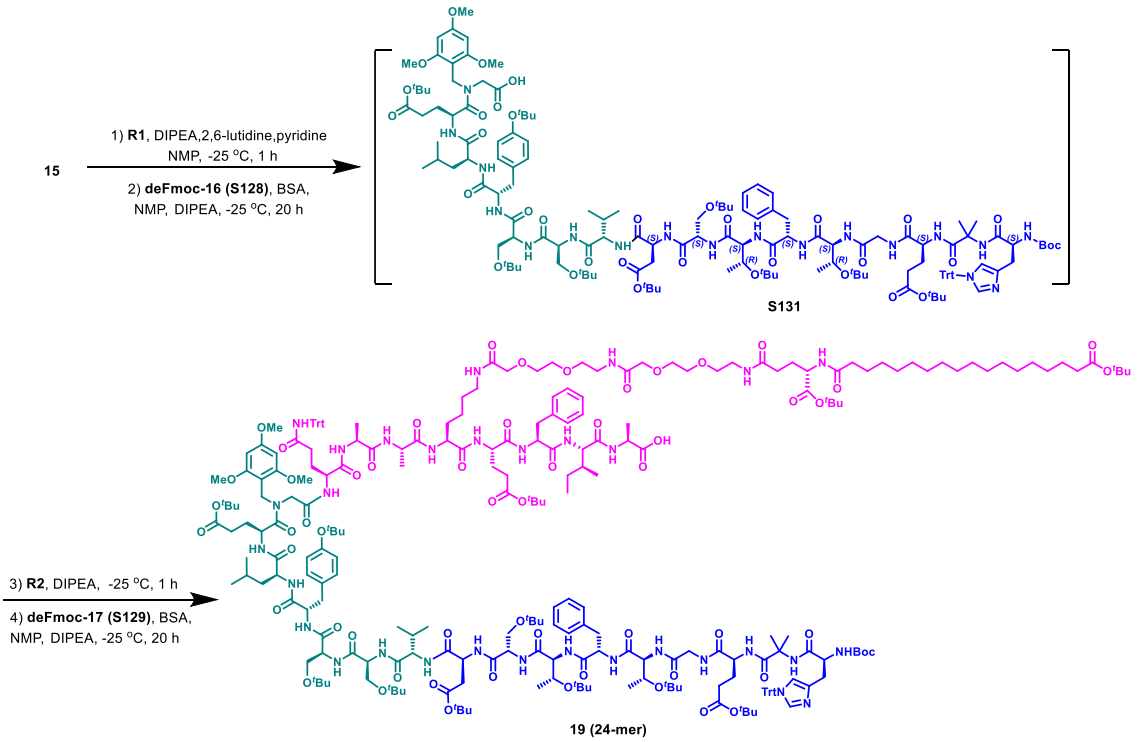
85%-90% (solvent B) in 3.5 min, gradient 90%-95% (solvent B) in 1 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

HPLC of isolated **S130**



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
9-Methylene-9H-fluorene	5.750	-	-	-
18	9.500	-	-	-
S130	2.969	3417574	2011772	94.01
Total	-	-	-	94.01

Fragment couplings:

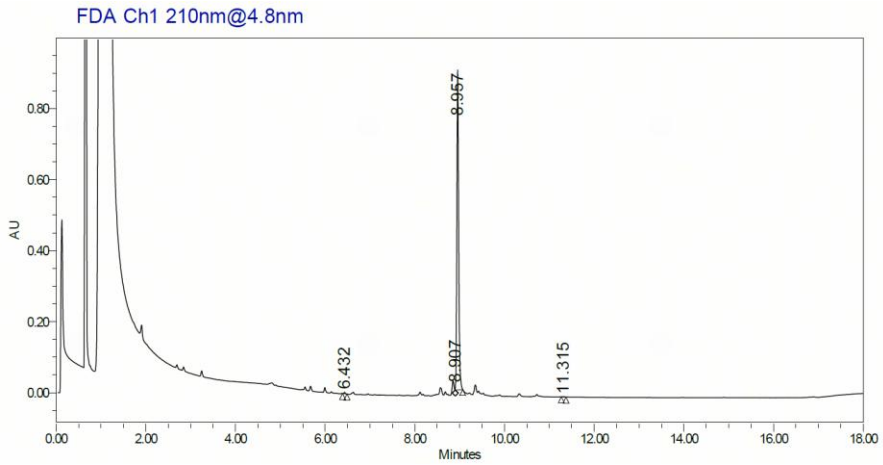


Synthesis of Boc-His(Trt)-Aib-Glu(O*t*Bu)-Gly-Thr(*t*Bu)-Phe-Thr(*t*Bu)-Ser(*t*Bu)-Ser(*t*Bu)-Val-Ser(O*t*Bu)-Ser(O*t*Bu)-Tyr(O*t*Bu)-Leu-Glu(O*t*Bu)-Gly(Tmb)-Gln(Trt)-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct(O*t*Bu)]-Glu(O*t*Bu)-Phe-Ile-Ala-OH (19). To a 250 mL three-neck jacketed vessel were added **15** (5.00 g, 3.12 mmol), NMP (75 mL). After the solution was cooled to -25 °C, 2,6-lutidine (334.66 mg, 3.12 mmol), DIPEA (464.20 mg, 3.59 mmol), pyridine (49.41 mg, 624.62 μmol) and 2-ethyl-3-methylbutanoyl chloride (**R1**) (580.24 mg, 3.90 mmol) were added. The reaction mixture was stirred at -25 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **15:15-INT** was 0.57:99.43. The solution of **S128**, which was prepared by stirring **S128** (4.34 g, 3.75 mmol), DIPEA (201.83 mg, 1.56 mmol), BSA (1.27 g, 6.25 mmol) in NMP (75 mL) at 25 °C was added to the above solution over 1 h. The reaction mixture was stirred at -25°C for 20 h and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 95%, and the ratio of **15:S131**:***epi*-S131**:***epi*-15-INT:S131-over-addition** was: 0.28:99.51:0.11:0:0.11. Then *i*-PrOH (750.75 mg, 12.49 mmol) was added to the reaction and stirred at 35 °C for 5 h. The above reaction solution was cooled to -25 °C. DIPEA (645.85 mg, 5.00 mmol) and **R2** (863.72 mg, 5.31 mmol) were added. The reaction mixture was stirred at -25 °C for 1 h and monitored by HPLC (**Method 1**), which showed the ratio of **S131**:**S131-INT** was 0.88:99.12. The solution of **S129** (7.19 g, 3.59 mmol), DIPEA (807.31 mg, 6.25 mmol), BSA (2.54 g, 12.49 mmol) in NMP (75 mL) was added to above solution over 1 h. The reaction mixture was stirred at -25 °C for 20 h and monitored by HPLC **(Method 1**). The HPLC showed the ratio of **S131:19**:**19-over-addition** was: 1.29:98.31:0.39 and the reaction assay yield was 90%. The reaction was added into MeCN/H_2O (1:5, 450 mL) and stirred for 16 h

at 20 ºC. The solid was collected by filtration and washed with THF/H_2O (1:1, 20 mL), dried under N_2 to provide 19.2 g (60 wt%, 11.5 g after assay) of **19** as a white solid in 78% isolated yield and 86.9% HPLC purity. mp 223.6 °C. HRMS (ESI): m/z calcd for $C_{250}H_{378}O_{57}N_{31}$ $[M+3H]^{3+}$: 1575.5872, found: 1575.5907. FTIR (neat): 3281, 2930, 1727, 1627, 1518, 1451, 1365, 1151, 1100, 747, 638 cm^{-1}.

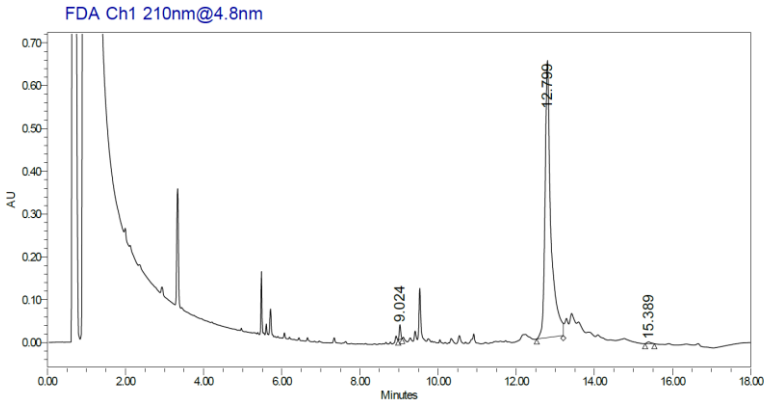
Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 40%-85% (solvent B) in 8.5 min, gradient 85%-90% (solvent B) in 3.5 min, gradient 90%-95% (solvent B) in 1 min; flow rate 0.4 mL/min; detection wavelength = 210 nm.

HPLC of **S131** formation



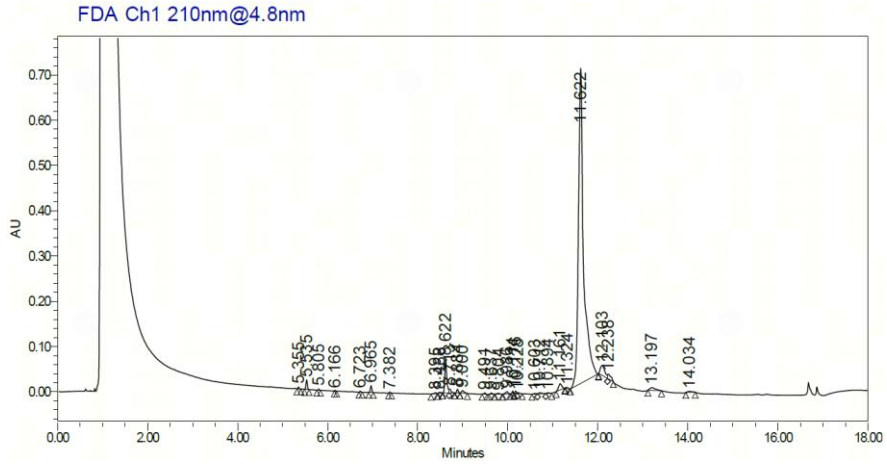
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S131-over-addition	11.315	2679	884	0.11
S131	8.957	2510672	900689	99.51
***epi*-S131**	8.910	-	-	0.11
***epi*-15-INT**	6.605	-	-	-
15	6.432	7045	4016	0.28
Total	-	-	-	100.00

HPLC of **19** formation (Reaction)

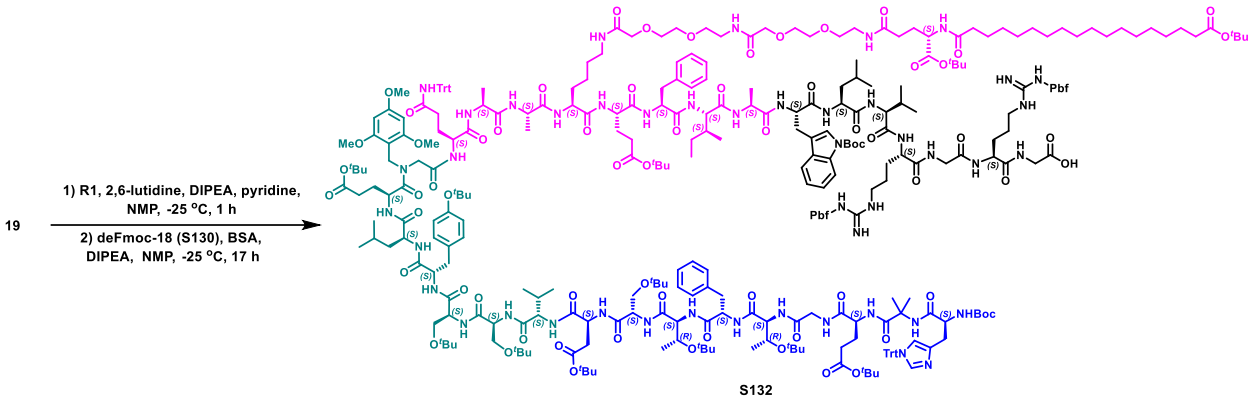


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
19-over-addition	15.389	26901	3989	0.39
19	12.799	6709349	647457	98.31
S131	9.024	88136	34172	1.29
Total	-	-	-	99.99

HPLC of isolated **19**



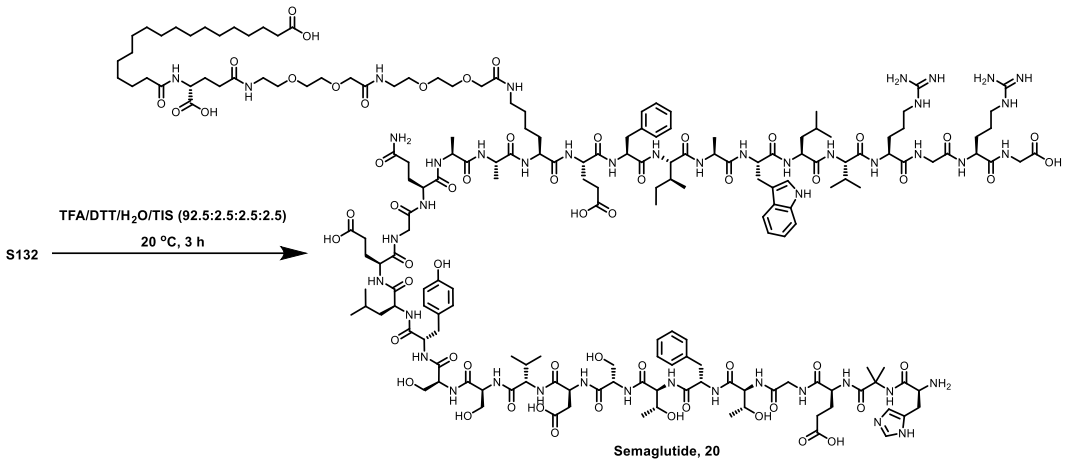
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
19-over-addition	14.034	15346	2393	0.2
19	11.622	5533792	695765	86.9
S131	8.395	6584	1959	0.1
Others	-	-	-	12.8
Total	-	-	-	100.0



Synthesis of Boc-His(Trt)-Aib-Glu(O*t*Bu)-Gly-Thr(*t*Bu)-Phe-Thr(*t*Bu)-Ser(*t*Bu)-Ser(*t*Bu)-Val-Ser(O*t*Bu)-Ser(O*t*Bu)-Tyr(O*t*Bu)-Leu-Glu(O*t*Bu)-Gly(Tmb)-Gln(Trt)-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct(O*t*Bu)]-Glu(O*t*Bu)-Phe-Ile-Ala-Trp(Boc)-Leu-Val-Arg(Pbf)-Gly-Arg(Pbf)-Gly-OH (S132). To a 500 mL three-neck jacketed vessel was added NMP (150 mL) and **19** (10.00 g, 2.12 mmol). After the solution was cooled to -25 °C, DIPEA (0.33 g, 2.54 mmol), 2,6-lutidine (0.23 g, 2.12 mmol), pyridine (0.033 g, 0.42 mmol) and **R1** (0.39 g, 2.65 mmol) were added in sequence. The reaction mixture was stirred at -25 °C for 1 h and monitored by UPLC (**Method 1**), which showed the ratio of **19:19-INT** was 2.90:97.10. The pre-cooled (0 °C) solution of **S130** and BSA and DIPEA in NMP was added to the reaction over 10 min. The **S130** solution was prepared by stirring **S130** (4.29 g, 2.97 mmol), BSA (0.86 g, 4.24 mmol) and DIPEA (0.14 g, 1.06 mmol) in NMP (50 mL) at 50 °C for 1 h and then cooled to 0 °C. The reaction mixture was stirred at -25 °C for 17 h and monitored by UPLC (**Method 2**). The UPLC showed the ratio of **19:S132** was: 2.2:97.8. The reaction mixture of **S132** was charged into MeCN/H_2O (v:v=1:5, 700 mL) at 20 °C. The solid was filtered, then slurried with MeCN/H_2O (v:v=1:1, 150 mL). The solid was filtered and dried for 18 h under N_2 sweep to provide 12.00 g of **S132** as a white solid. HRMS (ESI): m/z calcd for $C_{319}H_{477}O_{72}N_{45}S_2$ $[M+2H]^{2+}$: 3077.2239, found: 3077.2194.

Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 40%-85% (solvent B) in 8.5 min, gradient 85%-90% (solvent B) in 3.5 min, gradient 90%-95% (solvent B) in 1 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

Method 2: HPLC condition: ACQUITY UPLC® Protein BEH C4, 2.1 × 50 mm, 1.7 µm column; 0.1% TFA (v/v) in H_2O (solvent A), 0.1% TFA (v/v) in THF (solvent B); gradient 40%-85% (solvent B) in 8.5 min, gradient 20%-90% (solvent B) in 10 min, hold 2 min; flow rate = 0.5 mL/min; detection wavelength = 210 nm.



Synthesis of H-His-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Ser-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH (Semaglutide, 20). To a 250 mL three-neck jacketed vessel was added TFA/dithiothreitol (DTT)/H_2O/ triisopropylsilane (TIS) (92.5:2.5:2.5:2.5, 120 mL). After the solution was cooled to 5 °C, **S132** (12.00 g, 2.12 mmol) was added. The reaction mixture was stirred at 20 °C for 3 h and monitored by UPLC (**Method 1**). The UPLC showed the reaction assay yield was 78%, and the ratio of **S132:Semaglutide** was 0.0:100.0. The reaction mixture was charged into pre-cooled (0 °C) MTBE (720 mL) over 10 min. The reaction mixture was stirred at 0 °C for 0.5 h. The solid was filtered and slurried with MTBE (60 mL) for 1 h at 20 °C. The solid was filtered and dried for 18 h under N_2 stream to provide 14.6 g (43 wt%, 6.27 g by assay) of crude **Semaglutide 20** as a white solid in 72% isolated yield from **19** over 2 steps and 73.0% UPLC purity.

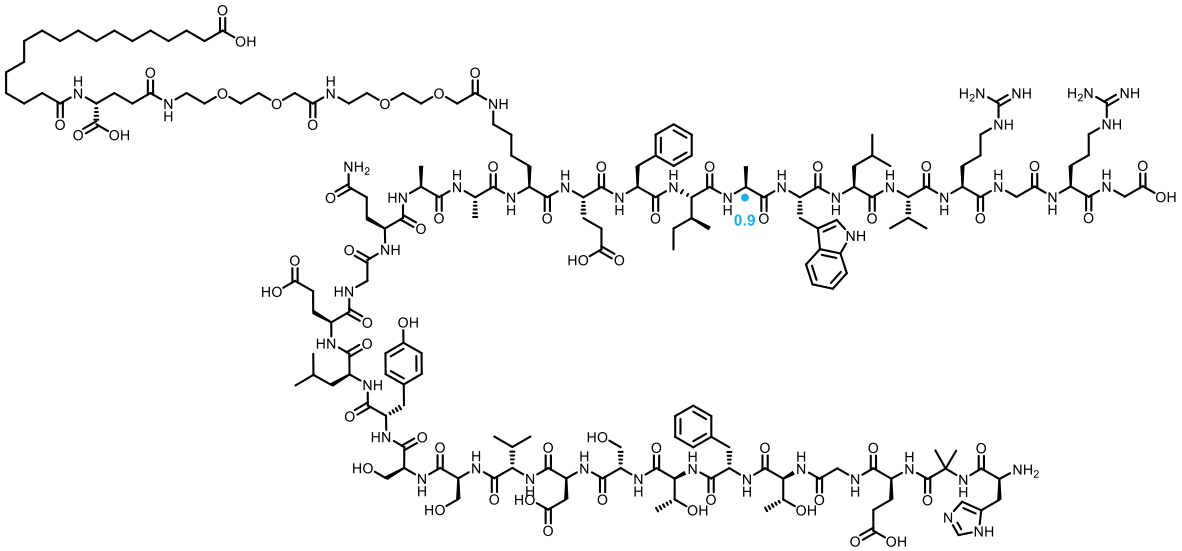
Starting from **S93**, **S106**, **S113**, **21**, 5756 g of reagents/solvents were used as described above to synthesize 6.27 g of semaglutide **20**, giving a synthesis PMI of 918.

The crude solid was purified by prep-HPLC (Prep-HPLC condition (RP): Kromasil C18, 20*250 mm, 10 μm column; 50 mM NH_4HCO_3 (solvent A), MeCN (solvent B); gradient 26%-41% (solvent B) in 60 min, flow rate = 10 mL/min; detection wavelength = 220 nm, 280 nm) to give pure **Semaglutide 20** as a white solid with 68% recovery. ^{1}H NMR (500 MHz, 1:1 CD_3CN/D_2O/NaOH, 298 K) δ 7.67 (d, *J* = 1.2 Hz, 1H), 7.34 (d, *J* = 8.2 Hz, 1H), 7.29 (d, *J* = 7.9 Hz, 1H), 7.27 – 7.10 (m, 11H), 7.07 (t, *J* = 7.6 Hz, 1H), 6.98 (d, *J* = 8.2 Hz, 2H), 6.96 – 6.90 (m, 2H), 6.67 (d, *J* = 8.4 Hz, 2H), 4.60 (dd, *J* = 9.0, 5.8 Hz, 1H), 4.54 (t, *J* = 6.8 Hz, 1H), 4.37 – 3.63 (m, 41H), 3.63 – 3.49 (m, 14H), 3.47 (t, *J* = 5.6 Hz, 2H), 3.44 (dd, *J* = 13.9, 9.4 Hz, 1H), 3.34 (t, *J* = 5.6 Hz, 2H), 3.25 (td, *J* = 5.5, 1.5 Hz, 2H), 3.24 – 2.85 (m, 15H), 2.63 (dd, *J* = 15.9, 7.0 Hz, 1H), 2.55 (dd, *J* = 15.9, 6.6 Hz, 1H), 2.40 – 1.55 (m, 38H), 1.55 – 1.27 (m, 24H), 1.26 (s, 3H), 1.24 (s, 3H), 1.22 – 1.13 (m, 24H), 1.06 (d, *J* = 6.4 Hz, 3H), 0.95 (d, *J* = 6.4 Hz, 3H), 0.91 (d, *J* = 6.6 Hz, 3H), 0.88 – 0.74 (m, 21H), 0.68 (d, *J* = 6.4 Hz, 3H), 0.68 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (126 MHz, 1:1

CD_3CN/D_2O/NaOH, 298 K) δ 181.58, 181.13, 179.87, 179.51, 177.36, 176.93, 176.72, 176.68, 176.41, 176.39, 176.34, 176.31, 176.14, 175.63, 175.52, 175.49, 175.39, 175.24, 175.08, 175.01, 174.68, 174.61, 174.54, 174.49, 174.45, 174.27, 174.01, 173.95, 173.84, 172.84, 172.75, 172.67, 172.52, 172.48, 171.87, 171.50, 171.34, 171.26 (2C), 171.11, 171.00, 169.35, 156.63, 156.61, 155.23, 136.54, 136.20, 136.04, 135.91, 131.73, 130.38, 129.24, 129.15, 128.57, 128.50, 127.46, 127.27, 126.91, 124.21, 121.34, 116.74, 115.34, 111.55, 108.94, 70.39, 69.66, 69.48, 69.45, 68.94, 68.87, 67.14, 66.92, 61.56, 61.43, 61.09, 60.80, 60.40, 60.31, 59.11, 58.74, 58.39, 57.99, 57.81, 57.43, 57.04, 56.73, 56.33, 55.83, 55.43, 55.27, 54.99, 54.72, 54.51, 54.33, 53.76, 53.34, 53.11, 52.44, 52.05, 51.78, 43.29, 42.86, 42.52, 40.64, 40.57, 39.58, 39.26, 38.88, 38.48, 38.31, 36.86, 36.80, 36.56, 35.95, 35.60, 34.66, 34.27, 33.53, 32.29, 30.83, 29.83, 29.39, 29.28, 29.25, 29.20, 29.11, 28.98, 28.87, 28.44, 27.83, 27.65, 27.25, 26.61, 26.39, 26.01, 25.85, 25.79, 25.74, 25.50, 24.68, 24.18, 23.63, 23.11, 22.70, 22.51, 22.48, 20.99, 20.21, 18.75, 18.67, 18.65, 18.48, 18.39, 18.03, 15.50, 15.45, 15.28, 14.87, 9.69.

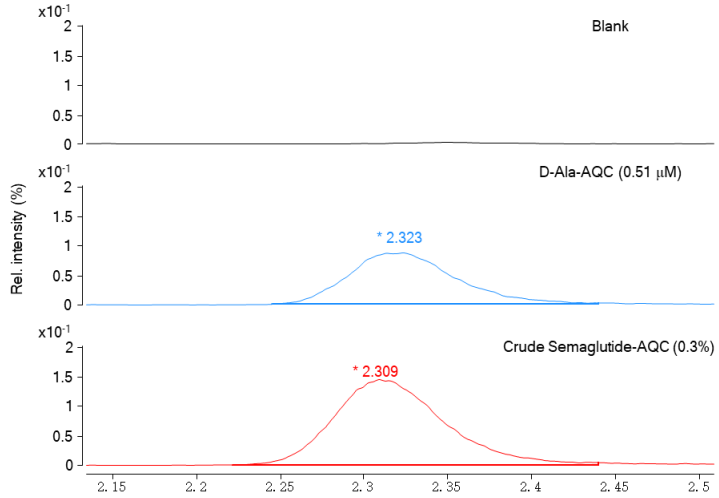
DCl degradation of crude Semaglutide to determine the epimerization of coupling 19 + deFmoc-18

Semaglutide contains three alanine residues and exhibits an overall epimer level of 0.3% by EIC analysis. Given that the first two alanine residues showed no detectable epimerization (0.0%), the epimerization is attributed to the final alanine coupling, corresponding to an estimated 0.9% epimer level at that position.

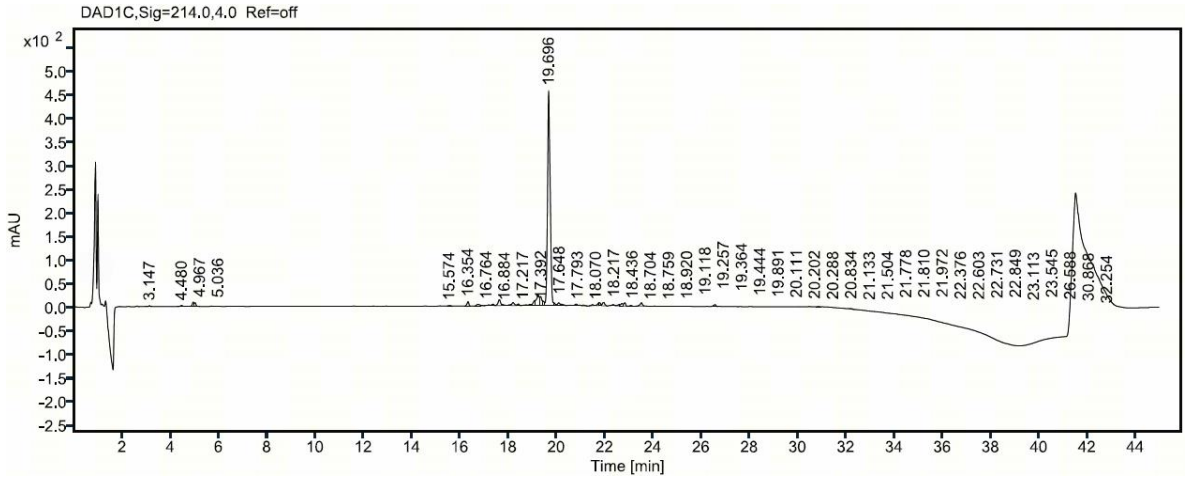


crude Semaglutide, 20

Representative EICs of blank, D-Ala-AQC (0.51 μM), and Crude Semaglutide.
Calibration curve: $R^2 = 1$, calibration range 0.51-253 μM (0.2%-100%).

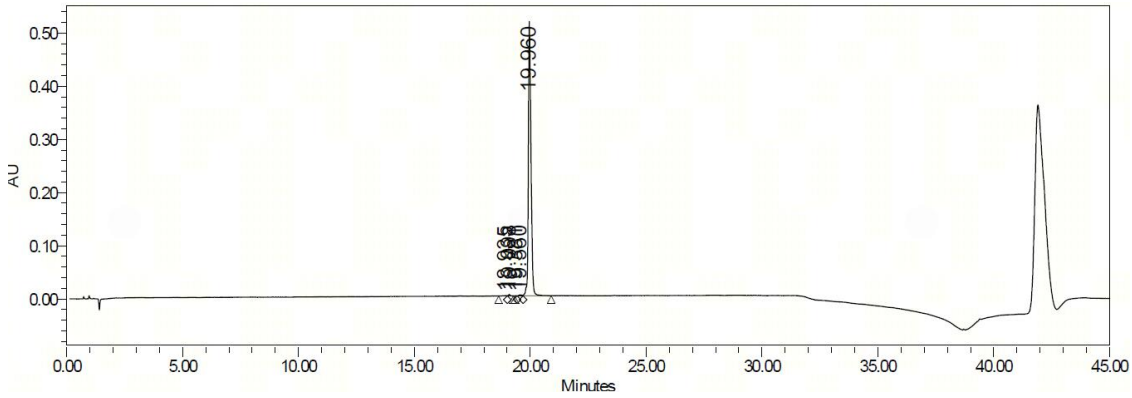


UPLC of crude **Semaglutide 20**



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S132	38.114	-	-	-
20 (Semaglutide)	19.696	3397.11	455.91	73.01
Others	-	-	-	26.99
Total	-	-	-	100.00

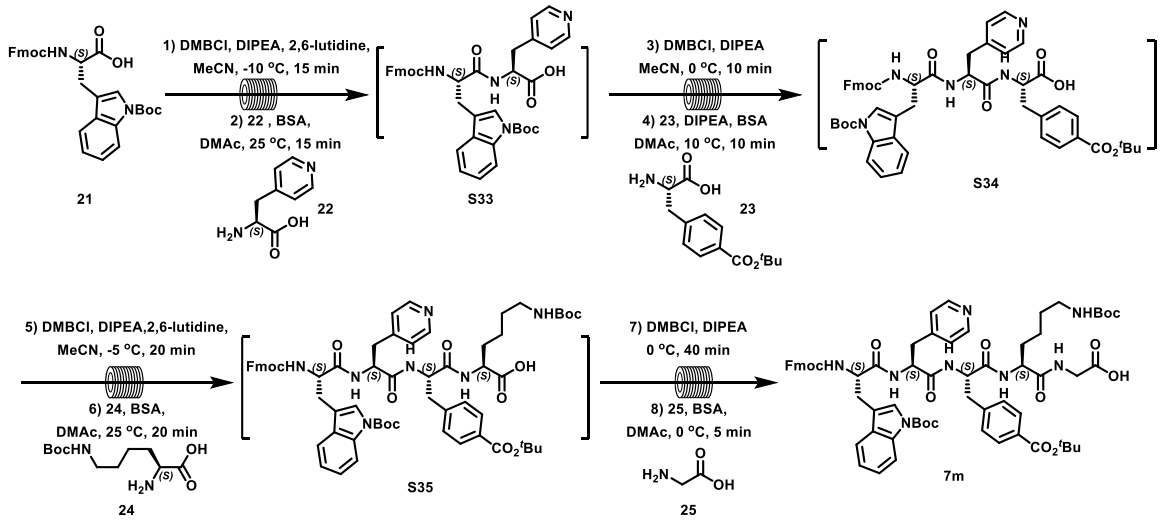
UPLC of purified **Semaglutide 20**

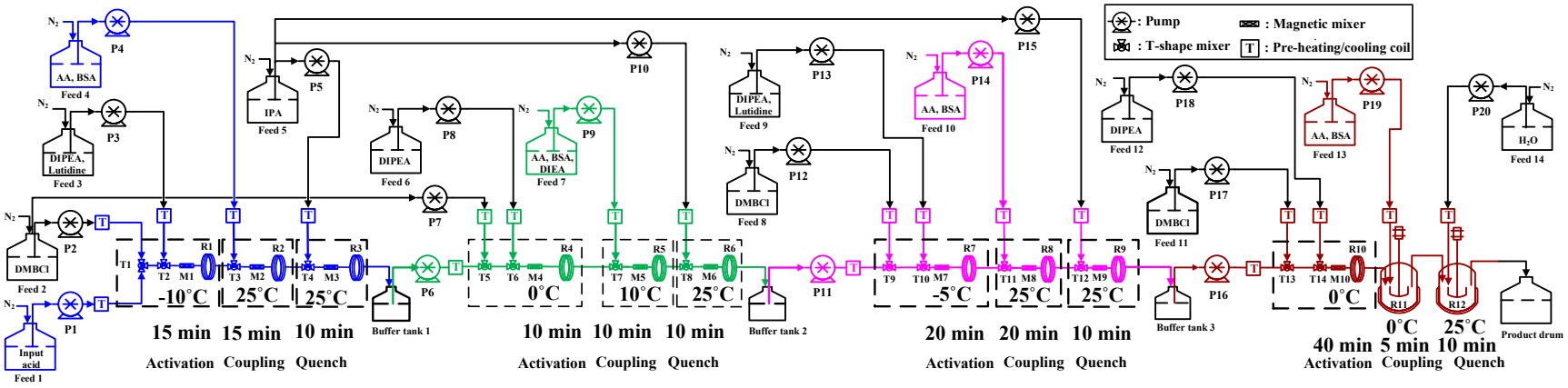


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
20 (Semaglutide)	19.960	4043235	515600	99.05
Others	-	-	-	0.95
Total	-	-	-	100.00

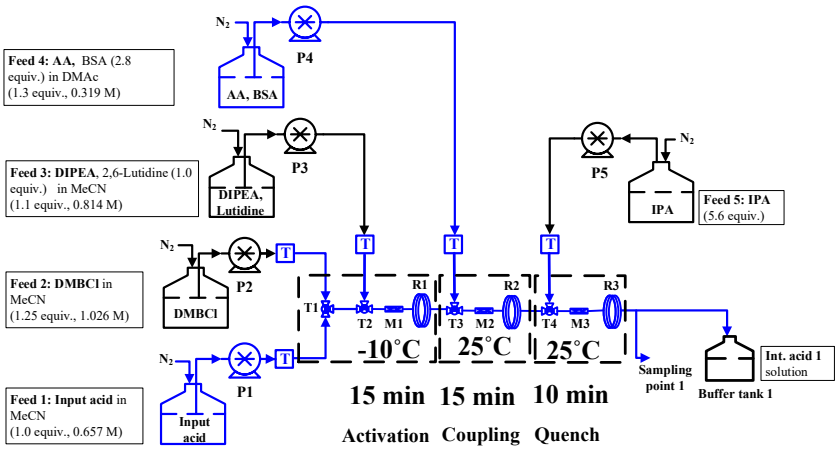
Experimental procedures and characterizations for Figure 6

Fig. 6a Synthesis of Fmoc-Trp(Boc)-Pal-pCO$_2$*t*BuPhe-Lys(Boc)-Gly-OH (7m) under continuous flow conditions.





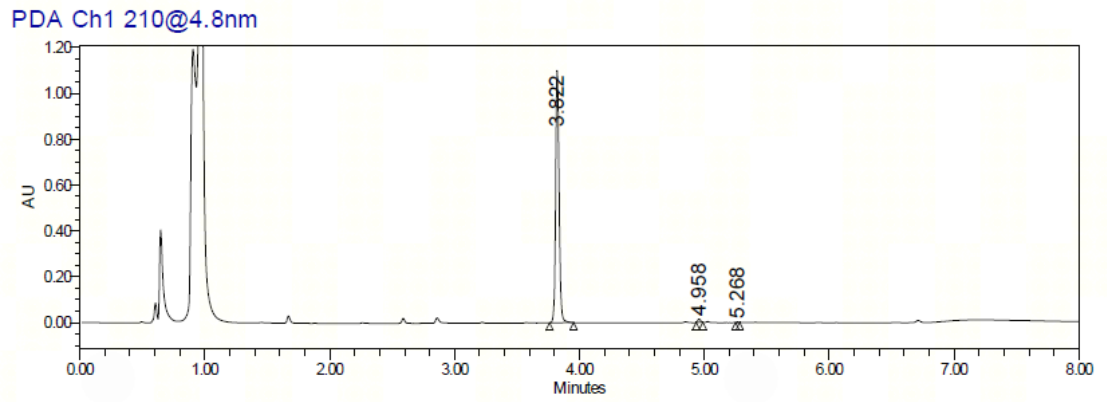
Coupling 1:



A solution of compound **21** (500.00 g, 949.52 mmol, Input acid) in MeCN (1000 mL) (**Feed 1**, flow rate of pump **P1**: 4.106 mL/min) and a solution of DMBCl (159.76 g, 1186.90 mmol) in MeCN (1000 mL) (**Feed 2**, flow rate of pump **P2**: 3.289 mL/min) were introduced to the T-shape mixer **T1** at -10 °C with the syringe pumps. Then, the resultant mixture and a solution of DIPEA (134.99 g, 1044.48 mmol) and 2,6-lutidine (101.74 g, 949.52 mol) in MeCN (1000 mL) (**Feed 3**, flow rate of pump **P3**: 3.645 mL/min) were introduced to the T-shape mixer **T2** at -10 °C with the syringe pumps. The resultant mixture was passed through the magnetic mixer **M1** and reaction tube **R1** (1/4 PFA tubing, length: 13 m, volume: 160.2 mL, residence time: 15 min) at the same temperature. Then, the resultant mixture and a solution of compound **22** (**Feed 4**, flow rate of pump **P4**: 11.004 mL/min), which was prepared by stirring compound **22** (205.13 g, 1234.38 mmol) and BSA (540.85 g, 2658.67 mmol) in DMAc (3000 mL) at 50 °C for 1 h and then cooled to 25 °C, were introduced to the T-shape mixer **T3** at 25 °C with the syringe pumps. The resultant mixture was passed through the magnetic mixer **M2** and reaction tube **R2** (1/4 PFA tubing, length: 27.0 m, volume: 332.8 mL, residence time: 15 min) at the same temperature. Then, the resultant mixture and 2-propanol (*i*-PrOH) (319.57 g, 5317.33 mmol) (**Feed 5**, flow rate of pump **P5**: 1.157 mL/min) were introduced to the T-shape mixer **T4** at 25 °C with the syringe pumps to quench the reaction. The resultant mixture was passed through the magnetic mixer **M3** and reaction tube **R3** (1/4 PFA tubing, length: 19.0 m, volume: 234.2 mL, residence time: 10 min) at 25 °C. IPC sample taken from sampling point 1 was analyzed by HPLC (**Method 1**). The HPLC showed the ratio of **21**:**S33**:**21-INT**:**S33-over-addition** was 1.01:98.95:0.04:0:0.

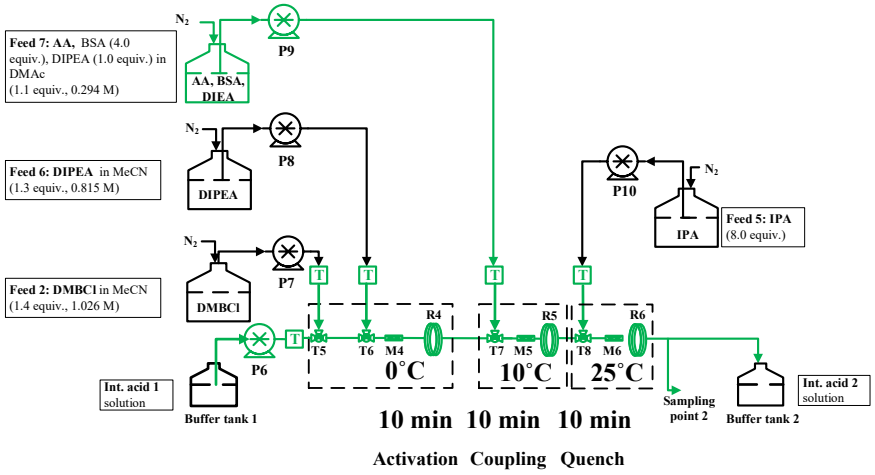
Method 1: HPLC condition: Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5-95% (solvent B) in 5.5 min, hold 2.5 min; flow rate 0.5 mL/min; detection wavelength 210 nm.

UPLC of **S33** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
21	4.958	21671	14105	1.01
S33	3.822	2124126	1100711	98.95
21-INT	5.269	892	754	0.04
S33-over-addition	3.234	-	-	-
Total	-	-	-	100.0

Coupling 2:

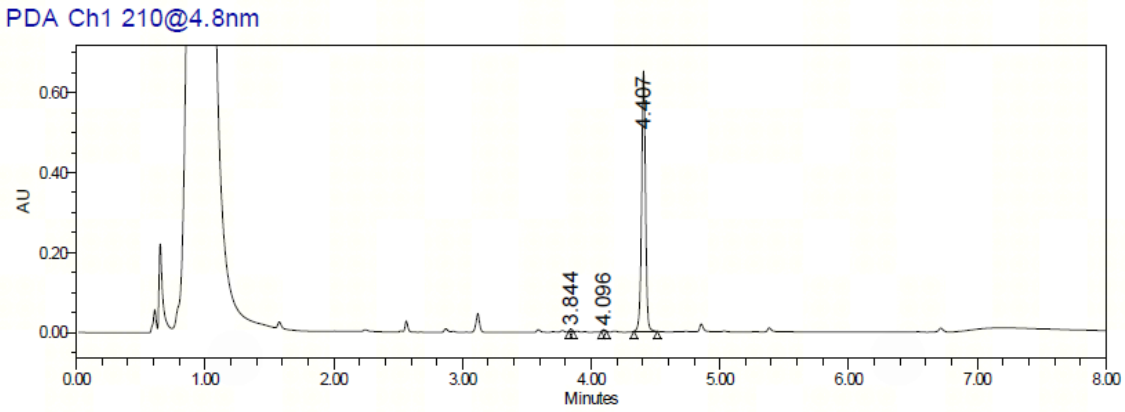


The resultant mixture (**S33 solution in buffer tank 1**, flow rate of pump **P6**: 23.201 mL/min) and a solution of DMBCl (178.93 g, 1329.33 mmol) in MeCN (1120 mL) (**Feed 2**, flow rate of pump **P7**: 3.684 mL/min) were introduced to the T-shape mixer **T5** at 0 °C with the syringe pumps. Then, the resultant mixture and a solution of DIPEA (159.54 g, 1234.38 mmol) in MeCN (1300 mL) (**Feed 6**, flow rate of pump **P8**: 4.304 mL/min) were introduced to the T-shape mixer **T6** at 0 °C with the syringe pumps. The resultant mixture was passed through the magnetic mixer **M4** and reaction tube **R4** (1/4 PFA tubing, length: 25 m, volume:

308.1 mL, residence time: 10 min) at the same temperature. Then, the resultant mixture and a solution of compound **23** (277.10 g, 1044.48 mmol), DIPEA (122.73 g, 949.52 mmol) and BSA (772.65 g, 3798.09 mmol) in DMAc (2000 mL) (Feed 7, flow rate of pump P9: 10.109 mL/min) were introduced into the T-shape mixer T7 at 10 °C using the syringe pumps. The resultant mixture was passed through the magnetic mixer **M5** and reaction tube **R5** (1/4 PFA tubing, length: 33 m, volume: 406.7 mL, residence time: 10 min) at 10 °C. Then, the resultant mixture and 2-propanol (*i*-PrOH) (456.53 g, 7596.19 mmol) (**Feed 5**, flow rate of pump **P10**: 1.653 mL/min) were introduced to the T-shape mixer **T8** at 25 °C with the syringe pumps to quench the reaction. The resultant mixture was passed through the magnetic mixer **M6** and reaction tube **R6** (1/4 PFA tubing, length: 35 m, volume: 431.4 mL, residence time: 10 min) at the same temperature. The IPC sample taken from sampling point 2 was analyzed by HPLC (**Method 1**). The HPLC showed the ratio of **S33**:**S34**:**S33-INT**:***epi*-S34**:**S34-over-addition** was 0.45:99.14:0.40:0.0:0.0.

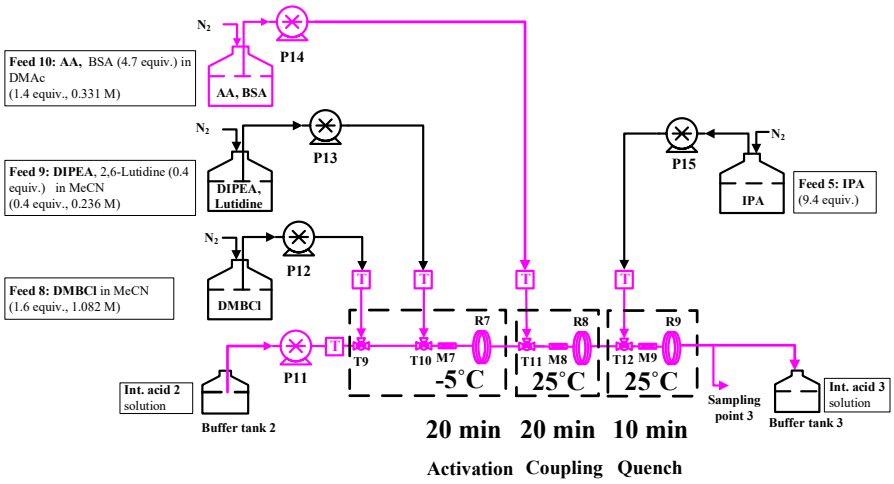
Method 1: HPLC condition: Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-95% (solvent B) in 5.5 min, hold 2.5 min; flow rate 0.5 mL/min; detection wavelength = 210 nm.

UPLC of **S34** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S33	3.844	6302	5422	0.45
S34	4.407	1374741	651341	99.14
S33-INT	4.096	5589	4085	0.40
***epi*-S34**	4.335	-	-	-
S34-over-addition	4.768	-	-	-
Total	-	-	-	100.0

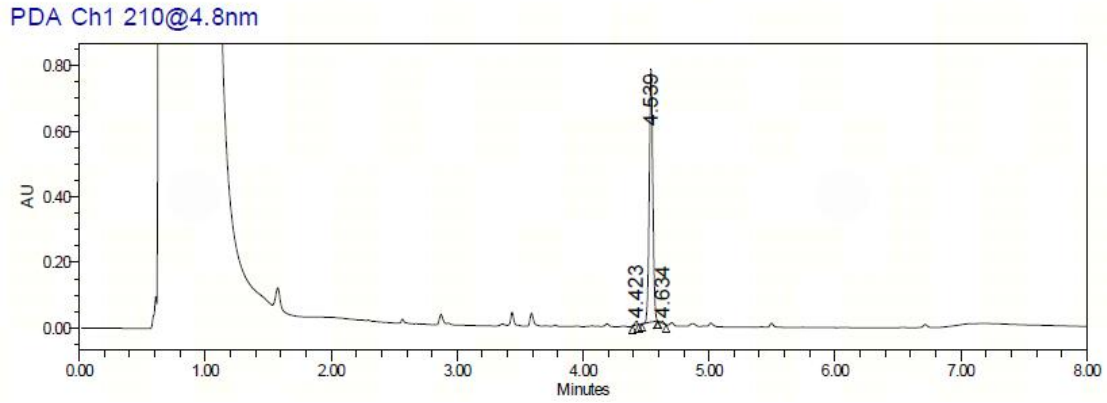
Coupling 3:



The resultant mixture (**S34 solution in buffer tank 2**, flow rate of pump **P11**: 42.950 mL/min) and a solution of DMBCl (204.49 g, 1519.24 mmol) in MeCN (1200 mL) (**Feed 8**, flow rate of pump **P12**: 3.992 mL/min) were introduced to the T-shape mixer **T9** at -5 °C with the syringe pumps. Then, the resultant mixture and a solution of DIPEA (49.09 g, 379.81 mmol) and 2,6-lutidine (40.70 g, 379.81 mmol) in MeCN (1500 mL) (**Feed 9**, flow rate of pump **P13**: 4.564 mL/min) were introduced to the T-shape mixer **T10** at -5 °C with the syringe pumps. The resultant mixture was passed through the magnetic mixer **M7** and reaction tube **R7** (3/8 PFA tubing, length: 31 m, volume: 989.1 mL, residence time: 20 min) at the same temperature. Then, the resultant mixture and a solution of compound **23** (**Feed 10**, flow rate of pump **P14**: 11.403 mL/min), which was prepared by stirring compound **24** (327.43 g, 1329.33 mmol) and BSA (907.86 g, 4462.76 mmol) in DMAc (2350 mL) at 50 °C for 1 h and then cooled to 25 °C, were introduced to the T-shape mixer **T11** at 25 °C with the syringe pumps. The resultant mixture was passed through the magnetic mixer **M8** and reaction tube **R8** (3/8 PFA tubing, length: 38 m, volume: 1212.5 mL, residence time: 20 min) at 25 °C. Then, the resultant mixture and *i*-PrOH (536.42 g, 8925.52 mmol) (flow rate: 1.942 mL/min) were introduced to the T-shape mixer **T12** at 25 °C with the syringe pumps to quench the reaction. The resultant mixture was passed through the magnetic mixer **M9** and reaction tube **R9** (3/8 PFA tubing, length: 20 m, volume: 638.1 mL, residence time: 10 min) at the same temperature. The IPC sample taken from sampling point 3 was analyzed by HPLC (**Method 1**). The HPLC showed the ratio of **S34**:**S35**:**S34-INT**:***epi*-S35**:**S35-over-addition** was 1.04:98.30:0.66:0:0.

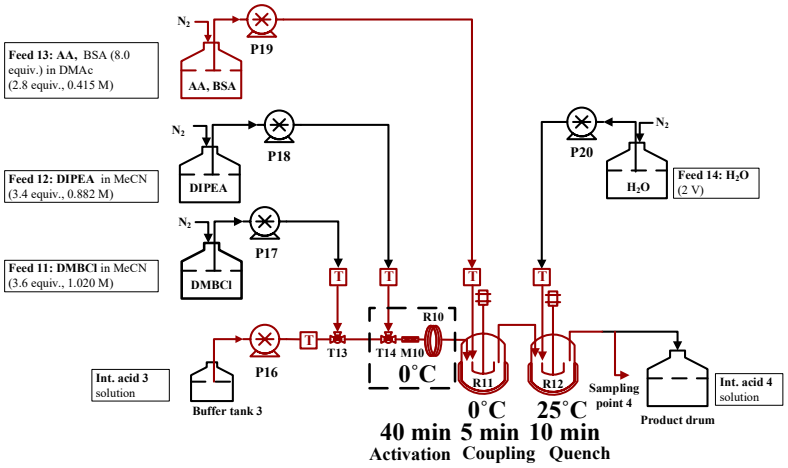
Method 1: HPLC condition: Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-95% (solvent B) in 5.5 min, hold 2.5 min; flow rate 0.5 mL/min; detection wavelength = 210 nm.

UPLC of **S35** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S34	4.423	16082	10631	1.04
S35	4.539	1519401	772196	98.30
S34-INT	4.634	10175	5260	0.66
***epi*-S35**	4.486	-	-	-
S35-over-addition	4.910	-	-	-
Total	-	-	-	100.0

Coupling 4:

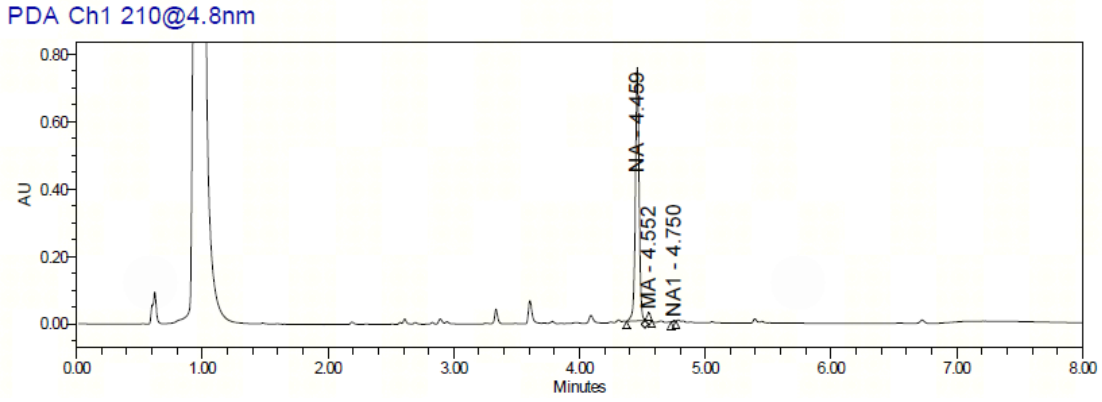


The resultant mixture (**S35 solution in buffer tank 3**, flow rate of pump **P16**: 64.850 mL/min) and a solution of DMBCl (460.10 g, 3418.28 mmol) in MeCN (2900 mL) (**Feed 11**, flow rate of pump **P17**: 9.526 mL/min) were introduced to the T-shape mixer **T13** at 0 °C with the syringe pumps. Then, the resultant mixture and a solution of DIPEA (417.27 g, 3228.38 mmol) in MeCN (3100 mL) (**Feed 12**, flow rate of

pump **P18**: 10.397 mL/min) were introduced to the T-shape mixer **T14** at 0 °C using the syringe pumps. The resultant mixture was passed through the magnetic mixer **M10** and reaction tube **R10** (3/8 PFA tubing, length: 95 m, volume: 3031.2 mL, residence time: 40 min) at 0 °C. Then, the resultant mixture and a solution of compound **24** (**Feed 13**, flow rate of pump **P19**:18.211 mL/min), which was prepared by stirring compound **25** (199.59 g, 2658.67 mmol) and BSA (1545.29 g, 7596.19 mmol) in DMAc (4000 mL) at 50 °C for 1 h and then cooled to 25 °C, were introduced to the **R11** (Continuous Stirred Tank Reactor, retention volume: 456.0 mL, residence time: 5 min) at 0°C with the syringe pump and piston pump. Then, the resultant mixture and water (H_2O) (1000.00 g) (**Feed 14**, flow rate of pump **P20**: 2.844 mL/min) were introduced to the **R12** (retention volume: 936.7 mL, residence time: 10 min) to quench the reaction at 25 °C. IPC sample taken from sampling point 4 was analyzed by HPLC (**Method 1**). The HPLC showed the ratio of **S35**:**7m**:**S35-INT**:***epi*-7m**:**7m-over-addition** was 2.69:97.00:0.31:0.0:0.0. The resulting mixture was added into a mixed solvent of MeCN (60 L) and H_2O (22 L) at 20 °C (Note: after adding 1/3 reaction mixture, seed was added) and stirred for 16 h. The solid was collected by filtration and washed with MeCN/H_2O (3:1, 4000 mL), dried under vacuum at 45 °C to provide 1078.2 g of **7m** (Assay is 84 wt% by QNMR: containing 6.5% DMAc and 3.5% MeCN) as a white solid in 79% isolated yield and 96.5% HPLC purity over 4 steps.

Method 1: HPLC condition: Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-95% (solvent B) in 5.5 min, hold 2.5 min; flow rate 0.5 mL/min; detection wavelength = 210 nm.

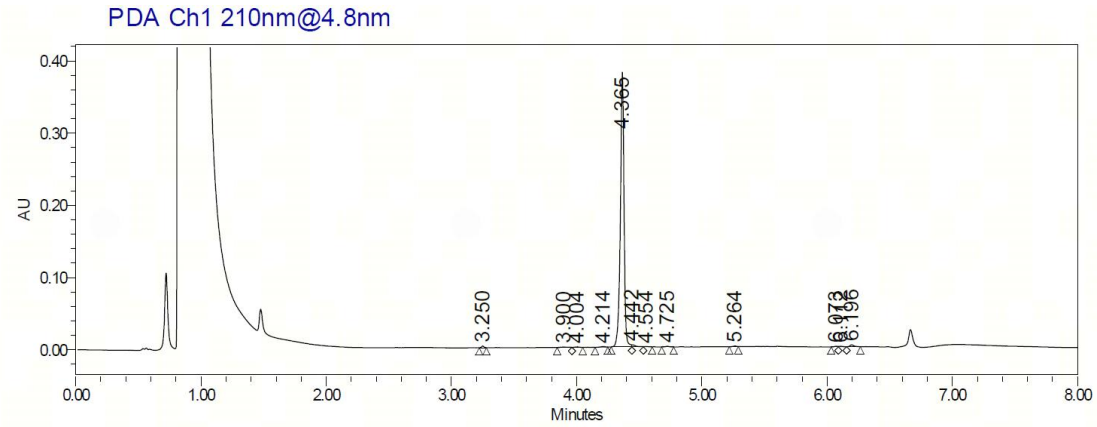
UPLC of **7m** formation (Reaction)



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
S35	4.552	42966	23348	2.69
7m	4.459	1548381	752910	97.00
S35-INT	4.752	4881	3285	0.31
***epi*-7m**	4.450	-	-	-

7m-over-addition	4.359	-	-	-
Total	-	-	-	100.00

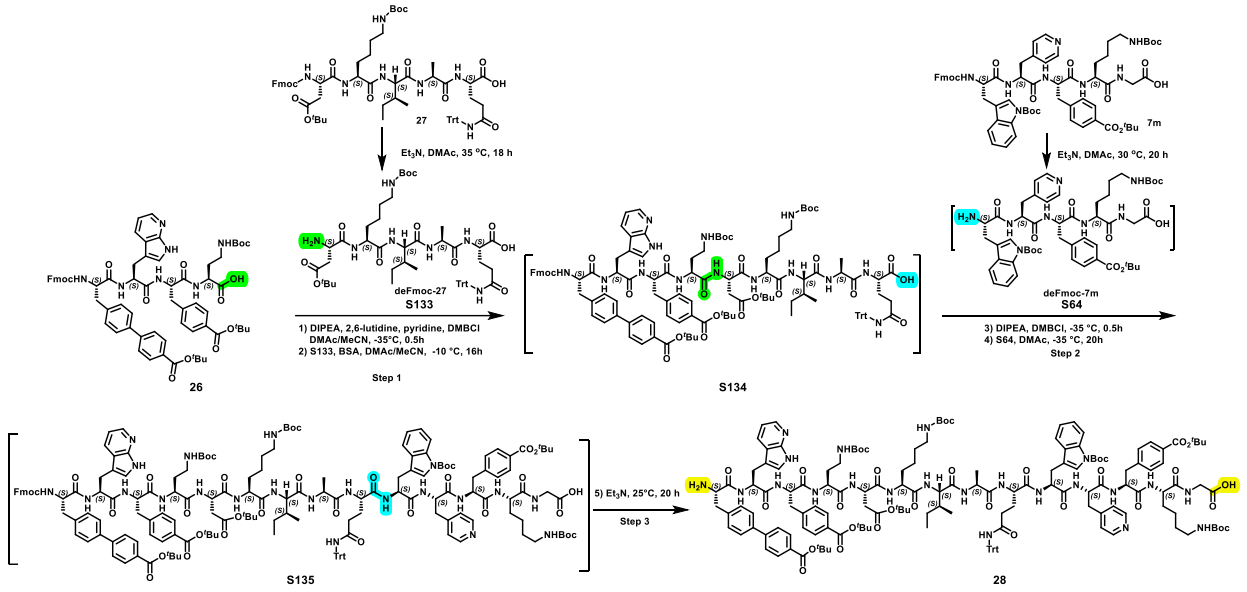
UPLC of isolated **7m**



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S35	4.442	42966	23348	0.77
7m	4.365	1548381	752910	96.51
S35-INT	4.652	-	-	-
***epi*-7m**	4.356	-	-	-
7m-over-addition	4.267	-	-	-
Others	-	-	-	2.72
Total	-	-	-	100.00

2) Experimental Procedure for Fig. 6b

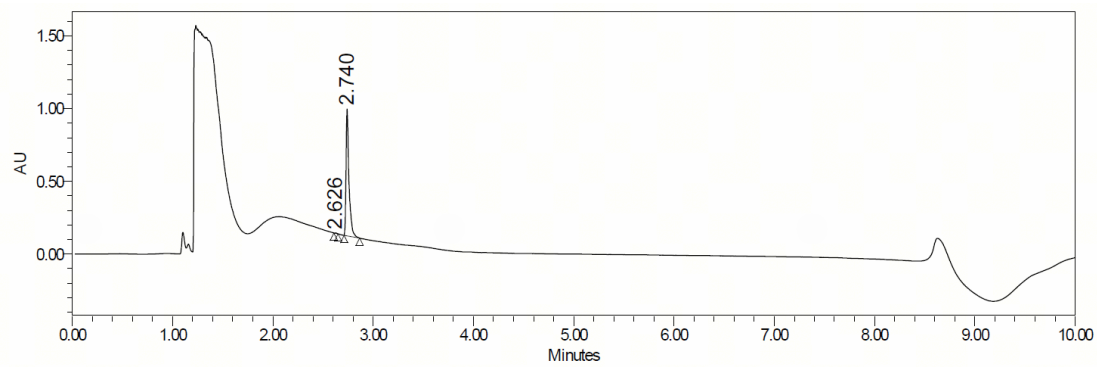
Continuous fragment couplings enabled by batch mode



Synthesis of H-Asp(O*t*Bu)-Lys(Boc)-Ile-Ala-Gln(Trt)-OH (deFmoc-27, S133). To a 1000 mL three-neck jacketed vessel were added **27** (66.00 g, 55.26 mmol) and DMAc (330 mL) and Et_3N (27.96 g, 276.30 mmol). The reaction mixture was stirred at 35 °C for 18 h and monitored by HPLC (**Method 1**), which showed the ratio of **27:S133** was 0.00:99.79. The reaction solution was added into 1500 mL MTBE. The slurry was stirred for 2 h at 15 °C. The solid was collected by filtration. Then wet solid was washed with 300 mL MTBE, dried under N_2 stream for 24 h to give 53.72 g white solid with quantitative yield and 99.81% purity. mp 202.0 °C, HRMS (ESI): m/z calcd for $C_{52}H_{74}O_{11}N_7$ $[M+H]^+$: 972.5441, found: 972.5409. FTIR (neat): 3273, 2933, 1689, 1623, 1514, 1447, 1160, 751, 698, 624 cm^{-1}.

Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-70% (solvent B) in 4 min, gradient 70%-95% (solvent B) in 6 min, hold 2 min; flow rate 0.4 mL/min; detection wavelength = 210 nm.

HPLC of isolated **S133**

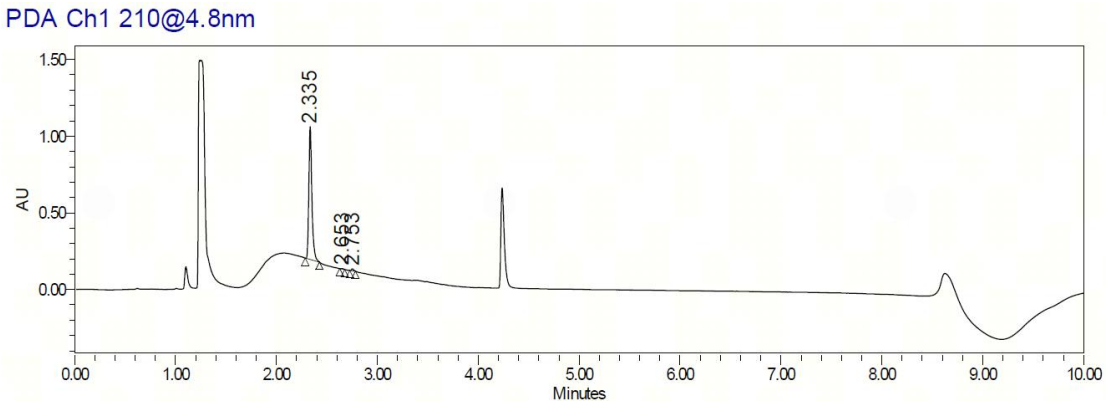


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
Unknown peak	2.626	3356	2723	0.19
S133	2.738	1748798	872182	99.81
Total	-	-	-	100.00

Synthesis of H-Trp(Boc)-Pal(4)-Phe(4-Boc)-Lys(Boc)-Gly-OH (deFmoc-7m, S64). To a 500 mL three-neck jacketed vessel were added **7m** (50.00 g, 41.41 mmol), DMAc (250 mL) and Et_3N (20.95 g, 207.06 mmol). The reaction mixture was stirred at 30 °C for 20 h and monitored by HPLC (**Method 1**), which showed the ratio of **7m**:**S64** was 0.00:98.85. The reaction solution was directly used in the next step.

Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-70% (solvent B) in 4 min, gradient 70%-95% (solvent B) in 6 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

HPLC of **S64** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
9-Methylene-9H-fluorene	4.231	-	-	-
7m	3.830	0	0	0.00
S64	2.335	1807780	866418	98.85
Total	-	-	-	98.85

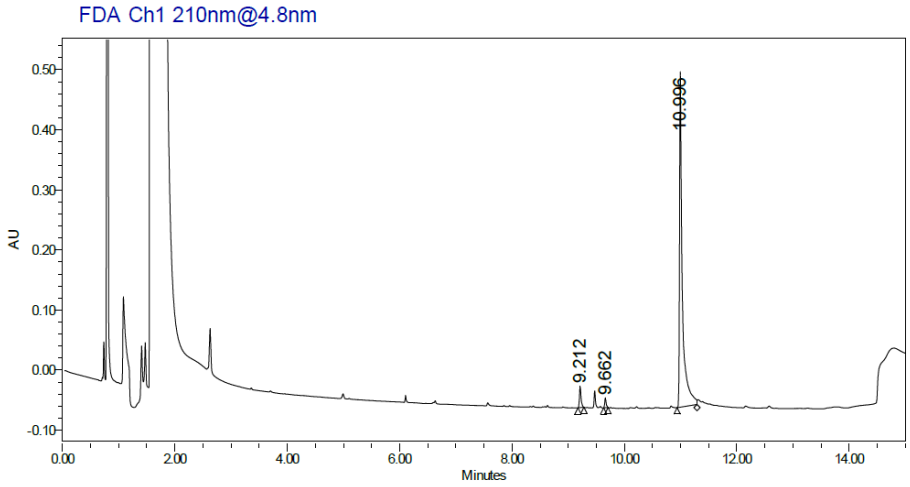
Continuous fragment Couplings and Fmoc deprotection in batch mode

Synthesis of Fmoc-Bip(4'-Boc)-Pyr(3)-Phe(4-Boc)-Dab(Boc)-Asp(O*t*Bu)-Lys(Boc)-Ile-Ala-Gln(Trt)-OH (S134). To a 250 mL three-necked jacket was added **26** (5.00 g, 4.18 mmol) and DMAc/MeCN (50 mL, v/v=4/1). After the solution was cooled to -35 °C, 2,6-lutidine (0.45 g, 4.18 mmol), DIPEA (0.54 g, 4.18 mmol), pyridine (66 mg, 0.84 mmol) and DMBCl (0.59 g, 4.39 mmol) were added in sequence. The

reaction mixture was stirred at -35 °C for 0.5 h and monitored by HPLC (**Method 1**), which showed the ratio of **26**:**26-INT** was 1.96:98.04. The pre-cooled (0 °C) solution of **S133** in DMAc/MeCN, which was prepared by stirring **S133** (4.51 g, 4.64 mmol), BSA (1.70 g, 8.36 mmol) in DMAc/MeCN (50 mL, v/v=4/1) at 50 °C for 1 h and cooled to 0 °C, was added to the reaction over 5 min. The reaction mixture was stirred at -35 °C for 3 h followed at -10 °C for 16 h and monitored by HPLC (**Method 1**). The HPLC showed the ratio of **26**:**26-INT**:**S134**:***epi*-26-INT**:***epi*-S134**:**S134-over-addition** was 3.5:1.4:95.1:0.0:0.0:0.0. Then *i*-PrOH (1.00 g, 16.69 mmol) was added to the reaction and stirred for 16 h at -10 °C. The resulting solution was directly used for the next step as **S134** solution.

Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-70% (solvent B) in 4 min, gradient 70%-95% (solvent B) in 6 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

HPLC of **S134** formation



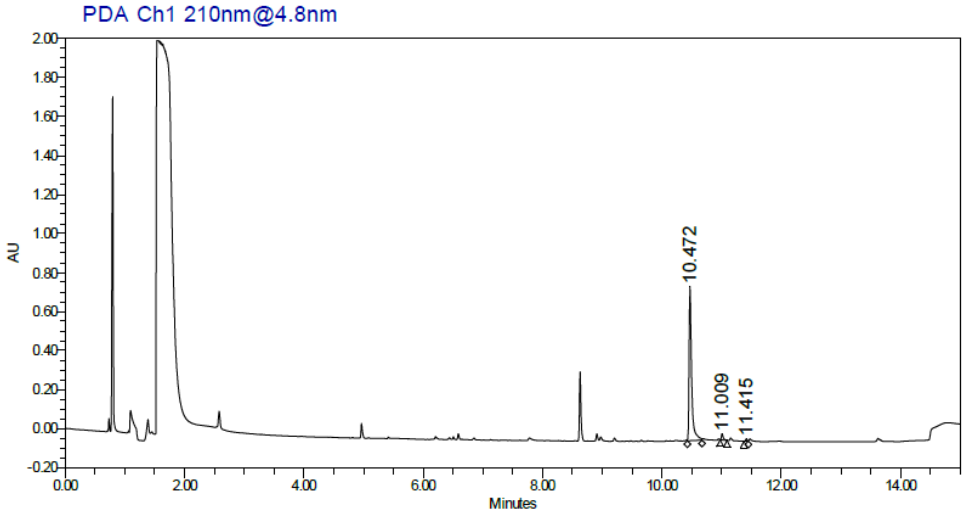
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S134-over-addition	5.960	-	-	-
26	9.212	70502	35112	3.50
26-INT	9.662	28893	16408	1.43
***epi*-26-INT**	9.599	-	-	-
S134	10.996	1915246	558242	95.07
***epi*-S134**	10.846	-	-	-

Synthesis of Fmoc-Bip(4'-Boc)-Pyr(3)-Phe(4-Boc)-Dab(Boc)-Asp(O*t*Bu)-Lys(Boc)-Ile-Ala-Gln(Trt)-Trp(Boc)-Pal(4)-Phe(4-Boc)-Lys(Boc)-Gly-OH (S135). The above solution of **S134** was cooled to -35 °C, DIPEA (0.566 g, 4.38 mmol) and DMBCl (0.67 g, 5.00 mmol) were added. The reaction mixture was

stirred at -35 °C for 0.5 h and monitored by HPLC (**Method 1**), which showed the ratio of **S134**:**S134-INT** was 1.0:99.0. The above pre-cooled (0 °C) solution of **S64** reaction solution in DMAc (5.22 mmol), was added to the reaction over 10 min. The reaction mixture was stirred at -35 °C for 20 h and monitored by HPLC (**Method 1**). The HPLC showed the ratio of **S134**:**S134-INT**:**S135**:***epi*-S135**:***epi*-S134-INT**:**S135-over-addition** was 2.8:0.9:96.3:0:0:0. Then $NH_3 \cdot H_2O$ (0.73 g, 25wt%, 10.74 mmol) was added to the reaction and stirred for 2 h at 25 °C. The resulting solution was directly used for the next step as **S135** solution.

Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-70% (solvent B) in 4 min, gradient 70%-95% (solvent B) in 6 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

HPLC of **S135** formation

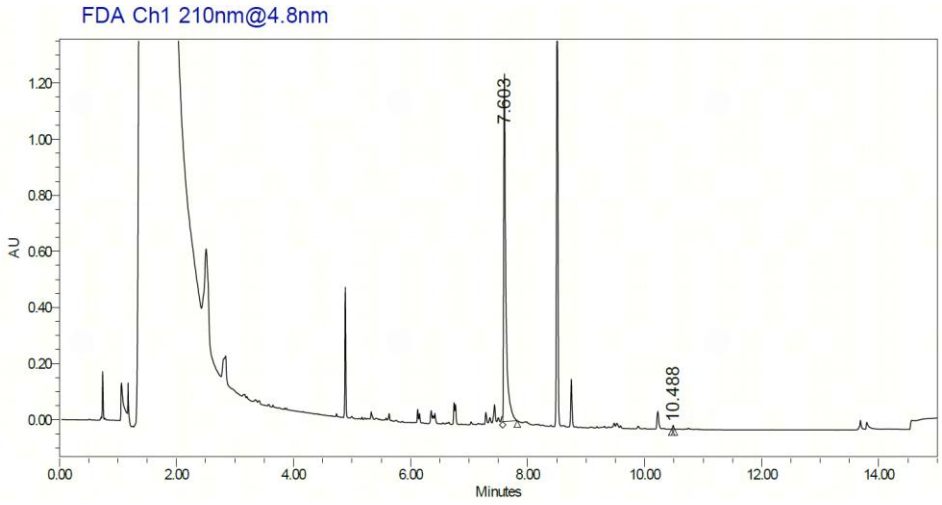


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
$BnNH_2$	4.879	-	-	-
9-Methylene-9H-fluorene	8.727	-	-	-
S134	11.009	67611	31738	2.8
S134-INT	11.415	21369	31738	0.9
***epi*-S134-INT**	11.361	-	-	-
S135	10.472	2302399	790905	96.3
S135-over-addition	7.640	-	-	-
***epi*-S135**	10.436	-	-	-

Synthesis of H-Bip(4'-Boc)-Pyr(3)-Phe(4-Boc)-Dab(Boc)-Asp(O*t*Bu)-Lys(Boc)-Ile-Ala-Gln(Trt)-Trp(Boc)-Pal(4)-Phe(4-Boc)-Lys(Boc)-Gly-OH (28). To the above **S135** solution (4.18 mmol), Et_3N (2.11 g, 20.9 mmol) was added. The reaction mixture was stirred at 25 °C for 20 h and monitored by HPLC (**Method 1**), which showed the ratio of **S135:28** was 0.55:99.45. The reaction solution was added into MTBE/H_2O (1/1, 150 mL) and stirred for 1 h at 25 °C. The solids were collected by filtration and dried under vacuum at 35 °C for 24 h to obtain 12.24 g (82 wt%, 10.04 g by assay) of crude **28** as a white solid in 82.3% purity and 83% isolated yield over 3 steps, which was used in the next reaction directly.

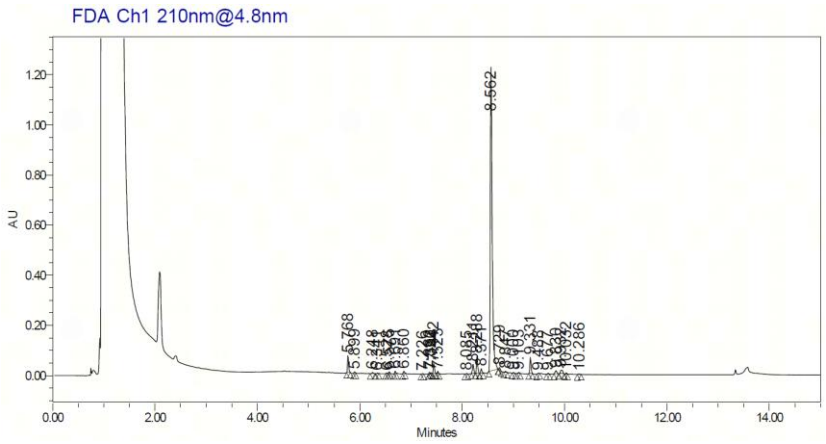
Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-70% (solvent B) in 4 min, gradient 70%-95% (solvent B) in 6 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

HPLC of **28** formation



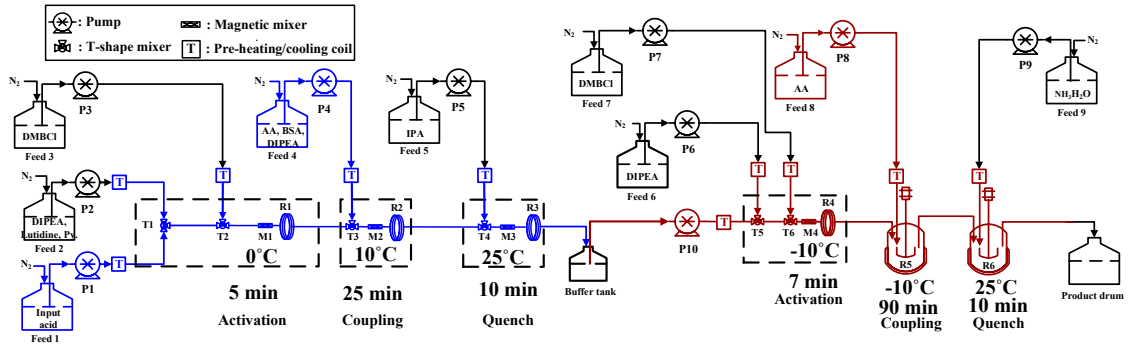
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
9-Methylene-9H-fluorene	8.526	-	-	-
S135	10.488	17570	13537	0.55
28	7.603	3156377	1239166	99.45
Total	-	-	-	100.0

HPLC of isolated 28

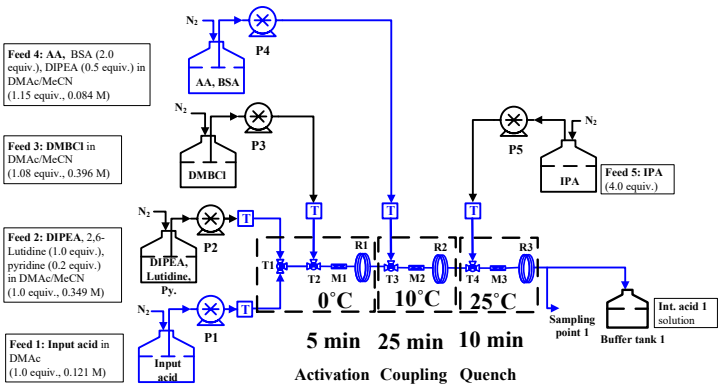


Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S135	12.013	-	-	-
28	8.562	3200289	1211059	82.34
Others	-	-	-	17.66
Total	-	-	-	100.00

Fragment Couplings Enabled by Continuous Flow



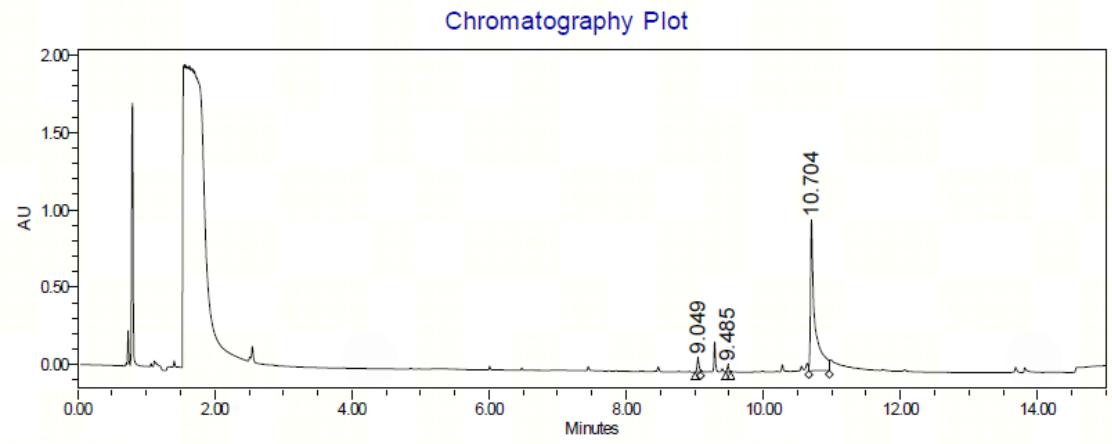
Coupling 1:



Synthesis of Fmoc-Bip(4'-Boc)-Pyr(3)-Phe(4-Boc)-Dab(Boc)-Asp(O*t*Bu)-Lys(Boc)-Ile-Ala-Gln(Trt)-OH (S134). A solution of compound **26** (10.00 g, 8.345 mmol, Input acid) in DMAc (60 mL, 6 V) (**Feed 1**, flow rate of pump **P1**: 0.323 mL/min) and a solution of DIPEA (1.08 g, 8.345 mmol), 2,6-lutidine (0.89 g, 8.345 mmol) and pyridine (0.13 g, 1.669 mmol) in DMAc (10 mL, 1 V) and MeCN (10 mL, 1 V) (**Feed 2**, flow rate of pump **P2**: 0.112 mL/min) were introduced to the T-shape mixer **T1** at 0 °C with the syringe pumps. Then, the resultant mixture and a solution of DMBCl (1.21 g, 9.012 mmol) in DMAc (10 mL, 1 V) and MeCN (10 mL, 1 V) (**Feed 3**, flow rate of pump **P3**: 0.107 mL/min) were introduced to the T-shape mixer **T2** at 0 °C with the syringe pumps. The resultant mixture was passed through the magnetic mixer **M1** and reaction tube **R1** (1/8 PFA tubing, length: 1.0 m, volume: 2.1 mL, residence time: 5 min) at the same temperature. Then, the resultant mixture and a solution of compound **S133** (9.33 g, 9.596 mmol), BSA (3.40 g, 16.689 mmol) and DIPEA (0.54 g, 4.172 mmol) in DMAc (80 mL, 8 V) and MeCN (20 mL, 2 V) (**Feed 4**, flow rate of pump **P4**: 0.535 mL/min) were introduced to the T-shape mixer **T3** at 10 °C with the syringe pumps. The resultant mixture was passed through the magnetic mixer **M2** and reaction tube **R2** (1/8 PFA tubing, length: 13.0 m, volume: 27.8 mL, residence time: 25 min) at the same temperature. Then, the resultant mixture and *i*-PrOH (2.01 g, 33.378 mmol) (**Feed 5**, flow rate of pump **P5**: 0.012 mL/min) were introduced to the T-shape mixer **T4** at 25 °C with the syringe pumps to quench the reaction. The resultant mixture was passed through the magnetic mixer **M3** and reaction tube **R3** (1/8 PFA tubing, length: 5.0 m, volume: 10.7 mL, residence time: 10 min) at 25 °C. IPC sample taken from sampling point 1 was analyzed by HPLC (**Method 1**). The HPLC showed the ratio of **26**:**26-INT**:**S134**:***epi*-S134**:***epi*-26-INT**:**S134-over-addition** was 3.7:1.5:94.8:0:0:0.

Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-70% (solvent B) in 4 min, gradient 70%-95% (solvent B) in 6 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

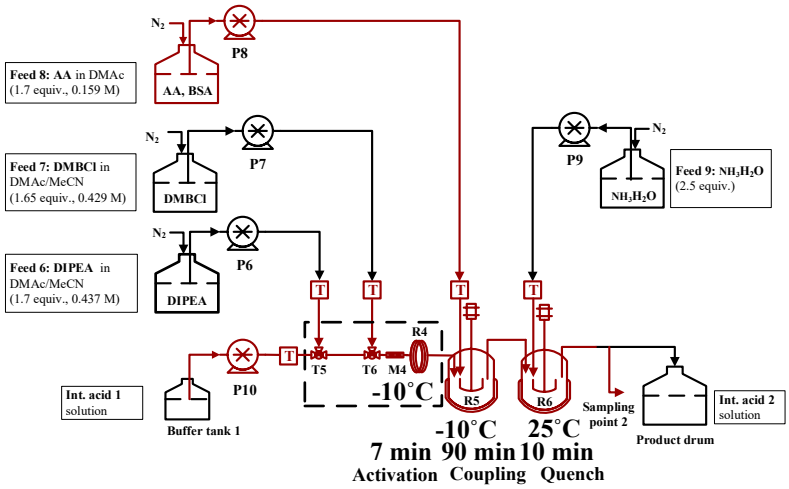
HPLC of **S134** formation



Compound Number	RT (min)	Area	Height	area% ratio

		(mAU*min)	(mAU)	
S134-over-addition	5.803	-	-	-
26	9.049	168166	91402	3.71
26-INT	9.485	67954	44952	1.50
***epi*-26-INT**	9.462	-	-	-
S134	10.704	4292700	971752	94.79
***epi*-S134**	10.692	-	-	-
Total	-	-	-	100.0

Coupling 2:

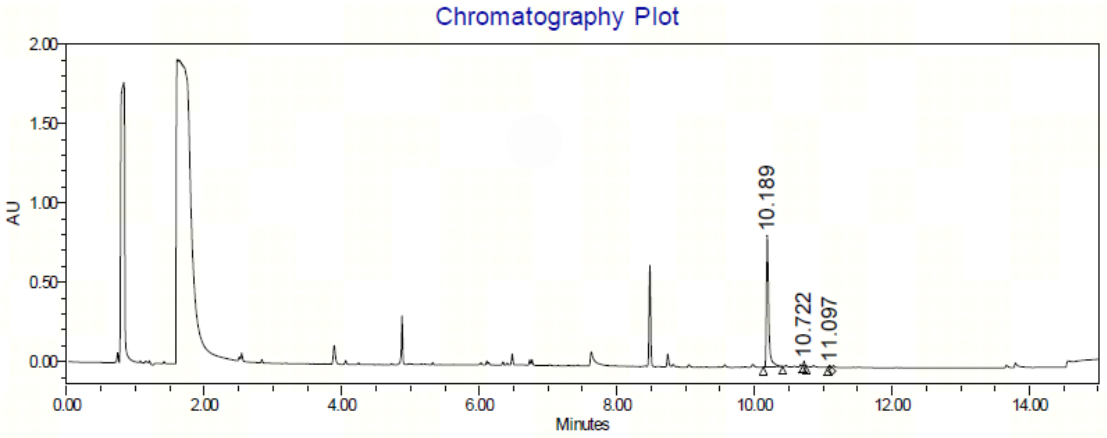


Synthesis of Fmoc-Bip(4'-Boc)-Pyr(3)-Phe(4-Boc)-Dab(Boc)-Asp(O*t*Bu)-Lys(Boc)-Ile-Ala-Gln(Trt)-Trp(Boc)-Pal(4)-Phe(4-Boc)-Lys(Boc)-Gly-OH (S135). The resultant mixture (**S134** solution in buffer tank 1, flow rate of pump **P10**: 1.088 mL/min) and a solution of DIPEA (1.83 g, 14.186 mmol) in DMAc (10 mL, 1 V) and MeCN (20 mL, 2 V) (**Feed 6**, flow rate of pump **P6**: 0.152 mL/min) were introduced to the T-shape mixer **T5** at -10 °C with the syringe pumps. Then, the resultant mixture and a solution of DMBCl (1.85 g, 13.768 mmol) in DMAc (10 mL, 1 V) and MeCN (20 mL, 2 V) (**Feed 7**, flow rate of pump **P7**: 0.150 mL/min) were introduced to the T-shape mixer **T6** at -10 °C with the syringe pumps. The resultant mixture was passed through the magnetic mixer **M4** and reaction tube **R4** (1/8 PFA tubing, length: 5.0 m, volume: 10.7 mL, residence time: 7 min) at the same temperature. Then, the resultant mixture and a solution of compound **S64** (85.58 g, wt% is 16.33%, 14.185 mmol) in DMAc (Feed 8, flow rate of pump **P8**: 0.418 mL/min) were introduced into the **R5** (Continuous Stirred Tank Reactor, retention volume: 162.6 mL, residence time: 90 min) at -10 °C with the syringe pump. Then, the resultant mixture and $NH_3 \cdot H_2O$ (1.42 g, 25wt%, 20.861 mmol) (**Feed 14**, flow rate of pump **P9**: 0.016 mL/min) were introduced to the **R6** (retention volume: 21.4 mL, residence time: 10 min) to quench the reaction at 25 °C. IPC sample taken

from sampling point 2 was analyzed by HPLC (**Method 1**). The HPLC showed the ratio of **S134**:**S134-INT:S135**:***epi*-S135**:***epi*-S134-INT**:**S135-over-addition** was 1.9:0.9:97.2:0.0:0.0:0.0. The resulting solution was stirred for 2 h at 25 °C and directly used for next stage as **S135** solution.

Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-70% (solvent B) in 4 min, gradient 70%-95% (solvent B) in 6 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

HPLC of **S135** formation



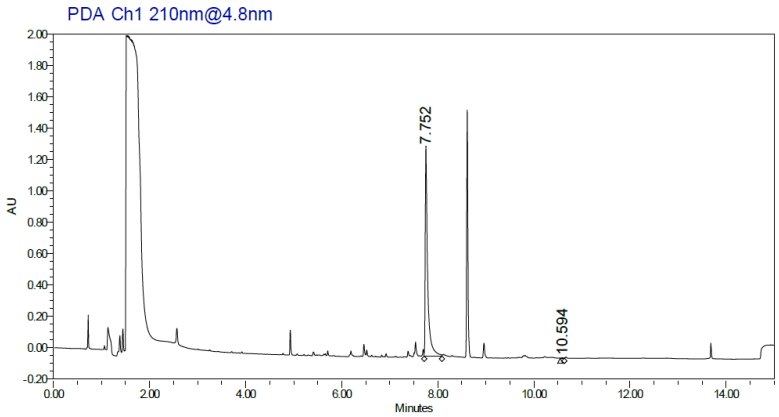
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
$BnNH_2$	4.768	-	-	-
DMB-NHBn	6.324	-	-	-
9-Methylene-9H-fluorene	8.491	-	-	-
S134	10.722	37141	25892	1.90
S134-INT	11.097	17044	10779	0.87
***epi*-S134-INT**	11.054	-	-	-
S135	10.189	1902799	829759	97.23
S135-over-addition	7.470	-	-	-
***epi*-S135**	10.154	-	-	-
Total	-	-	-	100.0

Synthesis of H-Bip(4'-Boc)-Pyr(3)-Phe(4-Boc)-Dab(Boc)-Asp(O*t*Bu)-Lys(Boc)-Ile-Ala-Gln(Trt)-Trp(Boc)-Pal(4)-Phe(4-Boc)-Lys(Boc)-Gly-OH (28). To a portion of solution of **S135** (15.60 g by assay, 5.00 mmol) was added Et_3N (2.53 g, 25.0 mmol) was added. The reaction mixture was stirred at 25 °C for 16 h and monitored by HPLC (**Method 1**), which showed the ratio of **S135:28** was 0.18:99.82. The reaction solution was added into MTBE/H_2O (1/1, 150 mL) and stirred for 1 h at 25 °C. The solids were collected by filtration and dried under vacuum at 35 °C for 24 h to obtain 17.03 g (80 wt%, 13.62 g solid by assay)

of crude **28** as a white solid in 80.2% purity and 80% isolated yield over 3 steps, which was used in the next reaction directly. HRMS (ESI): m/z calcd for $C_{156}H_{203}O_{32}N_{22}$ $[M+H]^+$: 2896.4928, found: 2896.4895. FTIR (neat): 3043, 2889, 1649, 1467, 1434, 1190, 1121, 839, 800, 723, 517 cm^{-1}.

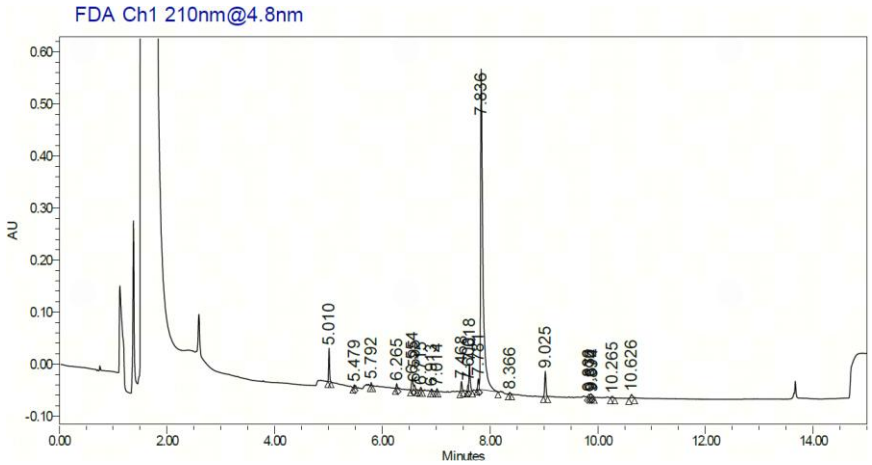
Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 µm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-70% (solvent B) in 4 min, gradient 70%-95% (solvent B) in 6 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

HPLC of **28** formation



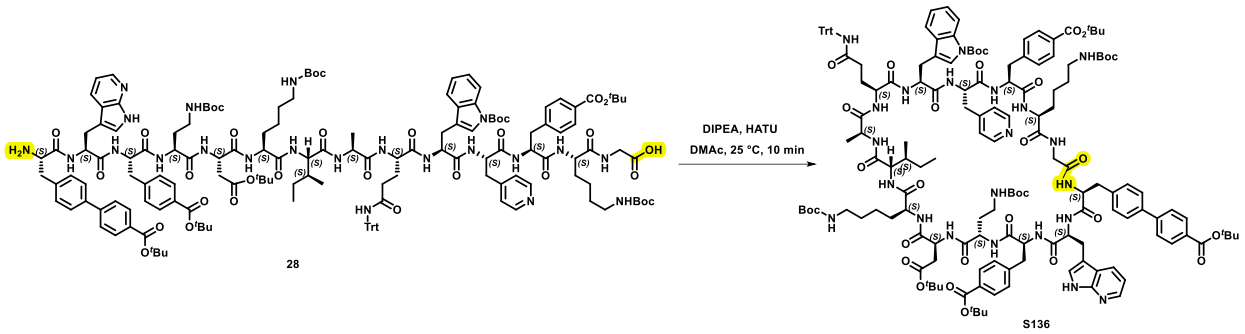
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
9-Methylene-9H-fluorene	8.612	-	-	-
S135	10.594	8260	3035	0.18
28	7.752	4618219	1340477	99.82
Total	-	-	-	100.0

HPLC of isolated 28



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%

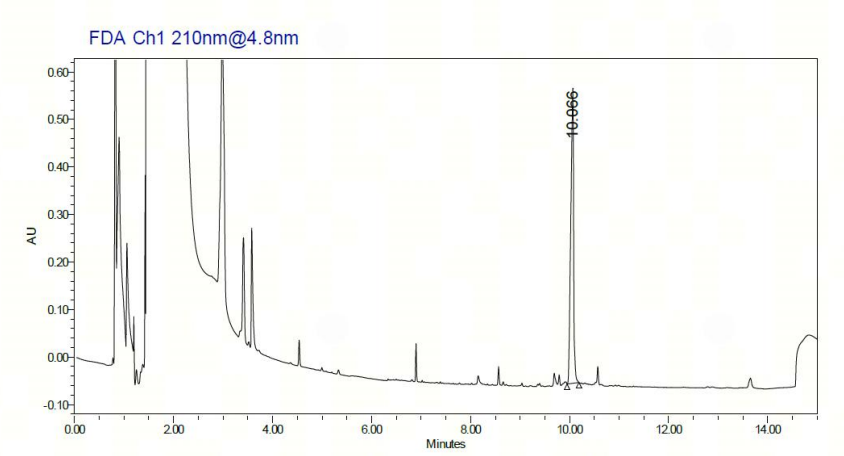
S135	10.994	-	-	-
28	7.836	1871513	616371	80.15
Others	-	-	-	19.85
Total	-	-	-	100.00



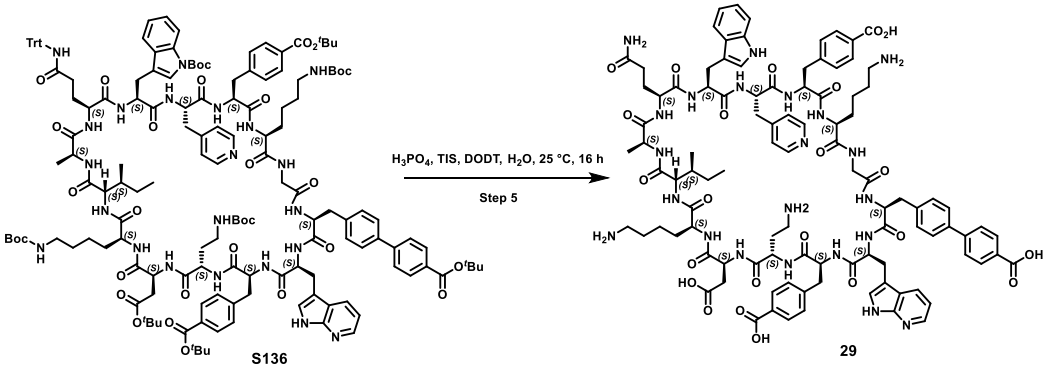
Synthesis of cyclo[Bip(4'-Boc)-Pyr(3)-Phe(4-Boc)-Dab(Boc)-Asp(O*t*Bu)-Lys(Boc)-Ile-Ala-Gln(Trt)-Trp(Boc)-Pal(4)-Phe(4-Boc)-Lys(Boc)-Gly] (S136). To a 250 mL three-neck jacketed vessel was added DIPEA (0.54 g, 4.18 mmol) and HATU (1.58 g, 4.15 mmol) and DMAc (40 mL). The pre-dissolved solution of **28** (4.00 g, 1.38 mmol) and DMAc (40 mL), which was prepared by stirring **28** in DMAc at 25 °C for 0.5 h, was added to the above HATU solution over 0.5 h at 25 °C. The reaction mixture was stirred at 25 °C for 10 min and monitored by HPLC (**Method 1**). The HPLC showed the reaction assay yield was 93% and the ratio of **28:S136** was 0.0:100.0. The product was precipitated by charging the reaction solution into H_2O (300 mL) and stirring for 1 h at 25 °C. The solid was collected by filtration and dried with N_2 stream for 24 h to obtain 6.2 g (~58 wt%) of **S136** as a white solid in 90% isolated yield, which was used in the next reaction directly. HRMS (ESI): m/z calcd for $C_{156}H_{202}O_{31}N_{22}$ $[M+2H]^{2+}$: 1439.7448, found: 1439.7476. FTIR (neat): 3282, 2932, 1708, 1627, 1514, 1449, 1366, 1252, 1160, 844, 700, 590 cm^{-1}.

Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-70% (solvent B) in 4 min, gradient 70%-95% (solvent B) in 6 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

HPLC of **S136** formation



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area% ratio
HOAt	3.220	-	-	-
TMU	3.459	-	-	-
28	7.976	-	-	-
S136	10.066	937555	340879	100.0
Total	-	-	-	100.0



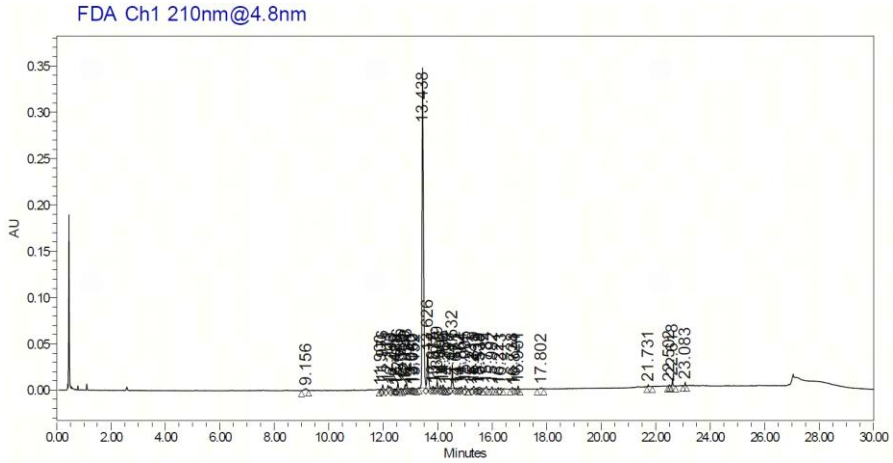
Synthesis of cyclo[Bip(4'-COOH)-Pyr(3)-Phe(4-COOH)-Dab-Asp-Lys-Ile-Ala-Gln-Trp-Pal(4)-Phe(4-COOH)-Lys-Gly] (29). To a 100 mL single-neck jacketed vessel was added a solution of 85 wt% H_3PO_4/TIS/ 3,6-Dioxa-1,8-octanedithiol (DODT)/H_2O (92.5/2.5/2.5/2.5, 37.5 mL) and MeCN (12.5 mL). **S136** (5 g by assay, 1.74 mmol) was added to the reaction over 5 min at 25 °C. The reaction mixture was stirred at 25 °C for 16 h and monitored by HPLC (**Method 1**) (Sample preparation method: 0.5 mL of reaction solution was added into 10 mL of MeOH, and 0.5 mL of 25 wt% aq. ammonia solution was added. The solid was filtered and dissolved with water). The HPLC showed the ratio of **S136:29** was 0.0:72.2. The reaction solution was added into MeOH (750 mL) and then 25 wt% aq. ammonia solution (50 mL) was added slowly at 5 °C and stirred for 30 min. The solid were filtered and washed with MeOH (50 mL). The

wet cake was dried at 35 °C under vacuum to give 154.7 g (1.9 wt%, 2.94 g by assay) crude product **29** (Note: contains ammonium phosphate) as a white solid with 80.2% HPLC purity and 84% isolated yield. Starting from single AAs, 1085 g of reagents/solvents were used in total to synthesize 2.94 g of **29**, giving a synthesis PMI of 369.

The crude product was purified by prep-HPLC (HPLC condition: first condition (RP): Kromasil C18, 30*250 mm, 10 um column; 50 mM $NH_4H_2PO_4$ (solvent A), MeCN (solvent B); gradient 16%-31% (solvent B) in 75 min, flow rate = 22.5 mL/min; detection wavelength = 225 nm, 280 nm; Second condition (Salt exchange): Kromasil C18, 30*250 mm, 10 μm column; 10 mM NH_4HCO_3 (solvent A), MeCN (solvent B); gradient 10%-30% (solvent B) in 100 min, flow rate = 22.5 mL/min; detection wavelength = 225 nm, 280 nm) to give pure product **29**. mp 211.67 °C. ^{1}H NMR (500 MHz, 1:1 D_2O/CD_3CN/NaOH, 323 K) δ 8.08–7.98 (m, 3H), 7.87–7.78 (m, 3H), 7.74 (d, *J* = 7.9 Hz, 2H), 7.70 (d, *J* = 8.0 Hz, 2H), 7.46–7.34 (m, 4H), 7.25–6.99 (m, 9H), 6.95 (dd, *J* = 7.9, 4.8 Hz, 1H), 6.83 (br, 1H), 6.73 (br, 2H), 6.57 (br, 2H), 4.97 (br, 1H), 4.75 (br, 1H), 4.59 (br, 1H), 4.55–4.22 (m, 7H), 4.10–3.72 (m, 4H), 3.61 (d, *J* = 16.0 Hz, 1H), 3.24–2.64 (m, 14H), 2.64–2.13 (m, 6H), 2.13–1.96 (m, 2H), 1.87–0.60 (m, 25H), 0.55 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, 1:1 D_2O/CD_3CN/NaOH, 323 K) δ 177.6, 176.7, 174.2, 174.0, 173.6, 173.0 (2C), 172.6, 172.5, 172.4, 172.3, 172.2, 172.1 (2C), 171.4, 171.35, 171.31, 171.2, 169.9, 148.3, 147.5, 147.2, 142.5, 142.0, 140.2, 139.3, 138.5, 136.7, 135.8, 135.4, 135.4, 130.1, 129.7, 129.3, 129.1, 129.1, 128.7, 128.1, 127.3, 126.8, 126.3, 124.7, 124.3, 123.4, 121.3, 120.2, 117.9, 115.4, 111.3, 108.8, 108.7, 57.0, 55.9, 54.4, 54.2, 54.2, 54.1, 54.0, 52.9, 52.7, 52.5, 51.3, 48.6, 42.7, 39.4, 39.3, 37.8, 37.0, 36.5, 34.9 (br), 34.5 (br), 31.1, 30.6, 29.1 (br), 28.6 (br), 28.1, 27.4, 26.4, 26.1, 24.6, 22.0, 21.8, 17.9, 14.1, 10.2. HRMS (ESI): m/z calcd for $C_{101}H_{123}O_{23}N_{22}$ $[M+H]^+$: 2011.9126, found: 2011.9117. FTIR (neat): 3280, 1658, 1630, 1514, 1426, 1199, 1133, 799, 507 cm^{-1}.

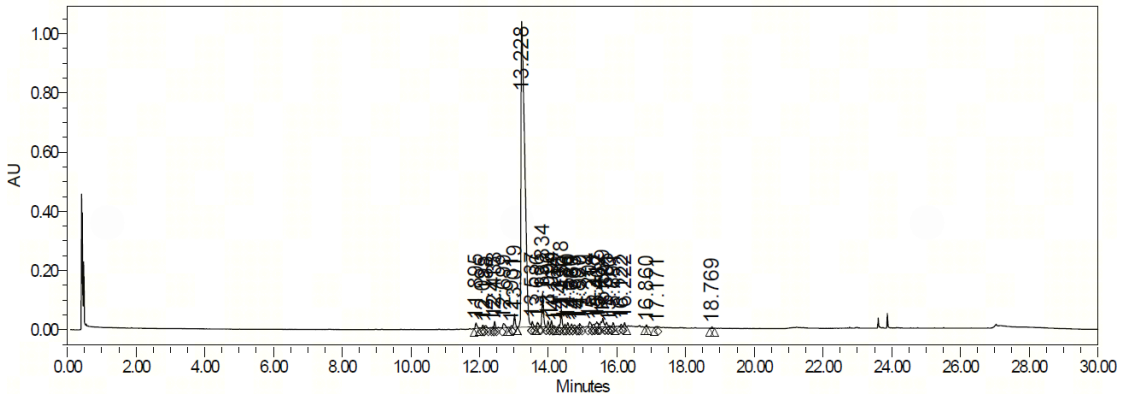
Method 1: HPLC condition: ACQUITY UPLC CSH Phenyl-Hexyl, 2.1 × 150 mm, 1.7 μm column; 0.1% H_3PO_4 (v/v) in H_2O (solvent A), MeCN (solvent B); gradient 5%-70% (solvent B) in 4 min, gradient 70%-95% (solvent B) in 6 min, hold 2 min; flow rate = 0.4 mL/min; detection wavelength = 210 nm.

HPLC of **29** formation



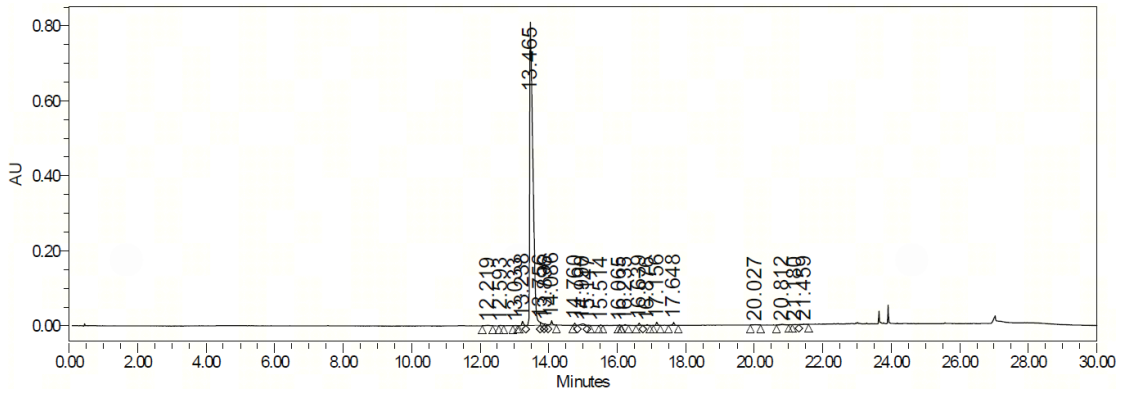
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S136	24.453	-	-	-
29	13.438	1247600	345600	72.16
Total	-	-	-	72.16

HPLC of crude **29**



Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S136	24.243	-	-	-
29	13.228	7673167	1032147	80.2
Others	-	-	-	19.8
Total	-	-	-	100.0

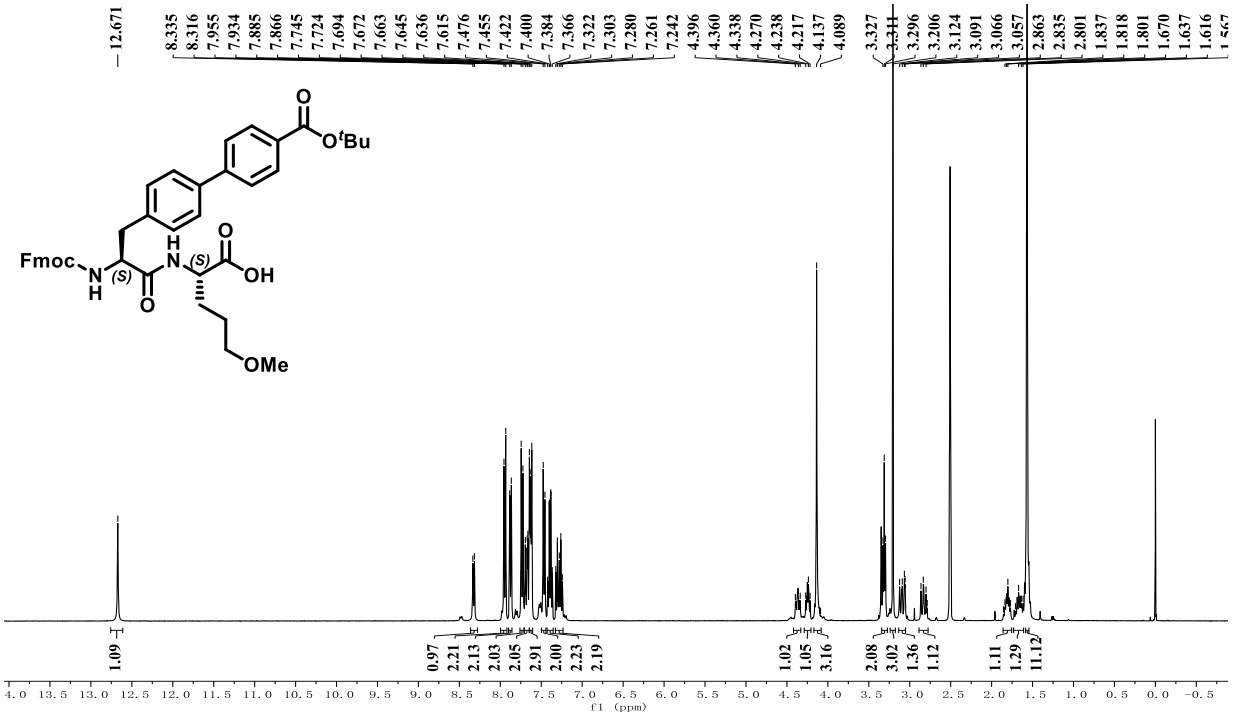
HPLC of purified **29**



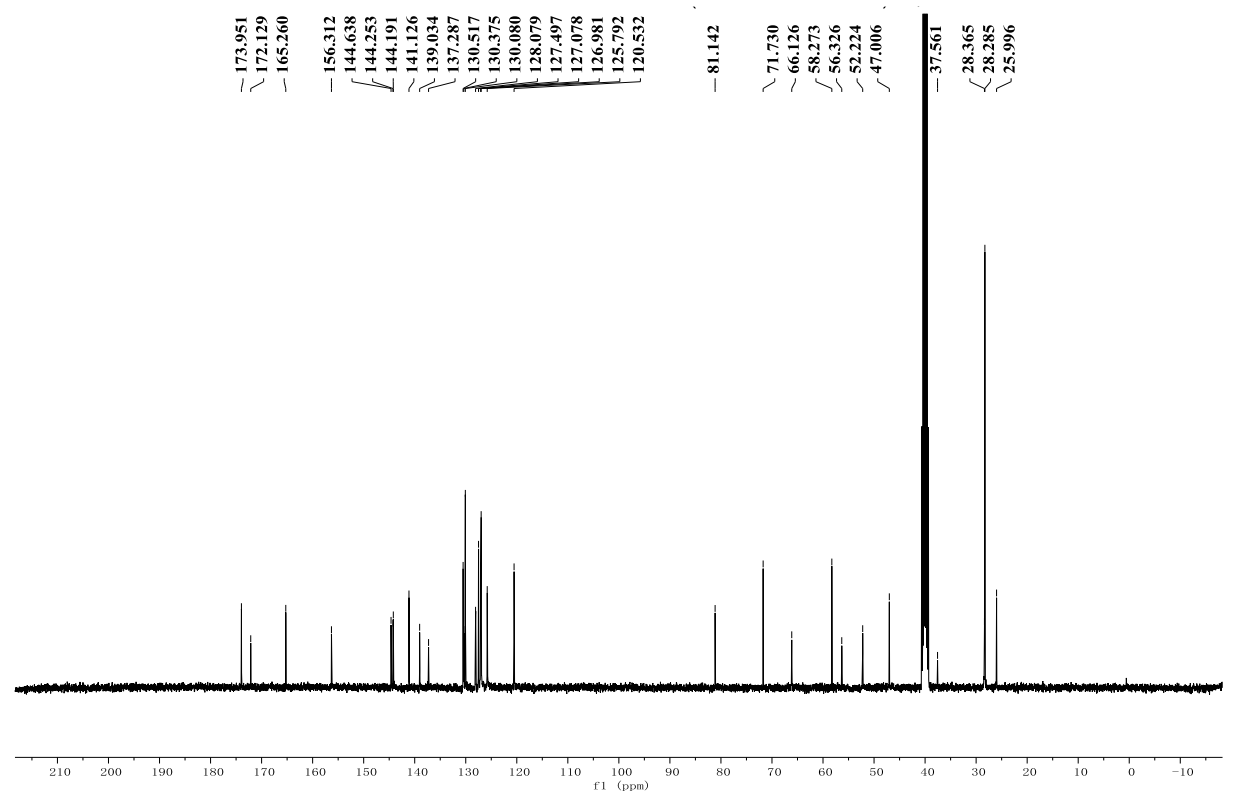
Compound Number	RT (min)	Area (mAU*min)	Height (mAU)	area%
S136	24.502	-	-	-
29	13.465	5162039	808877	94.48
Others	-	-	-	5.52
Total	-	-	-	100.00

Selective ^{1}H and ^{13}C NMR spectra

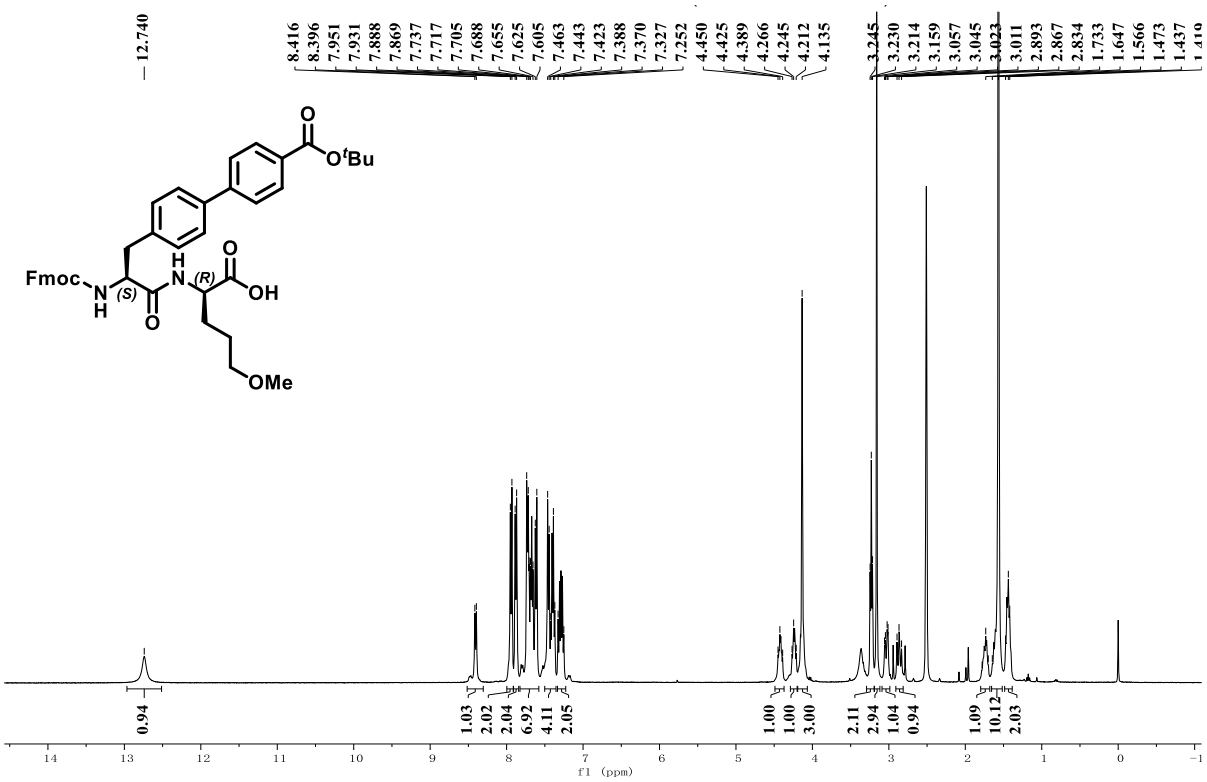
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-OH (3) (^{1}H NMR, 400 MHz, DMSO-d_6)



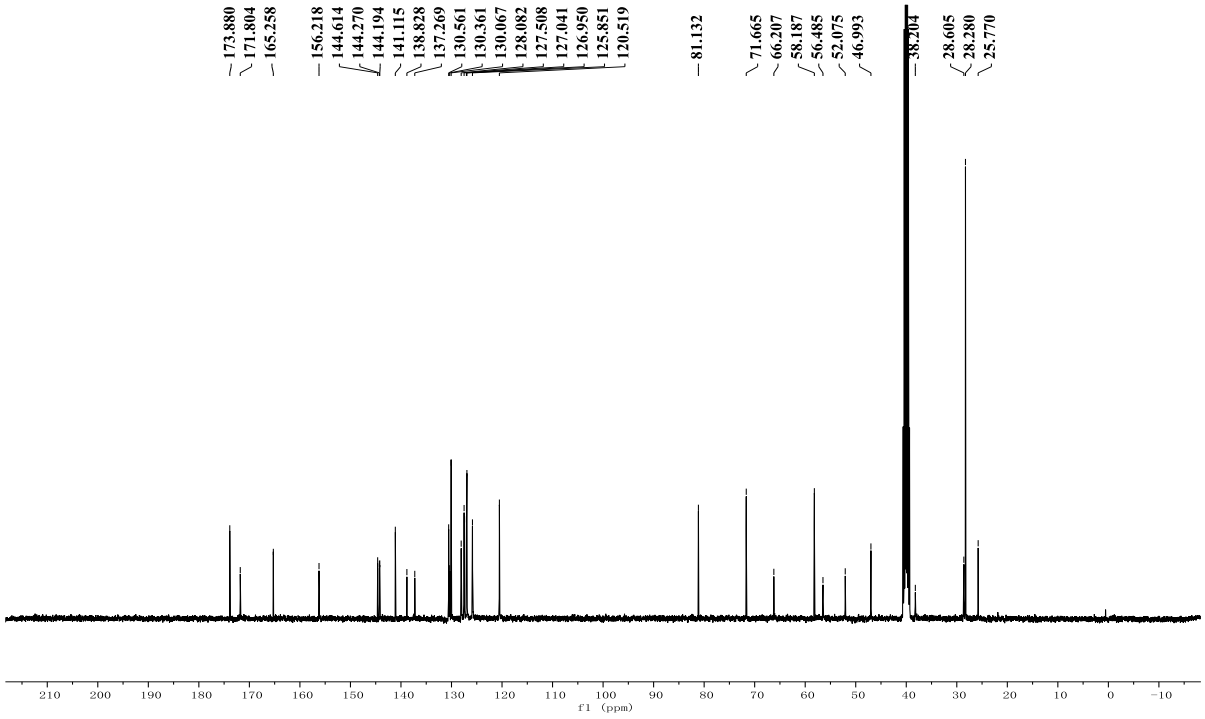
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-OH (3) (^{13}C NMR, 101 MHz, DMSO-d_6)



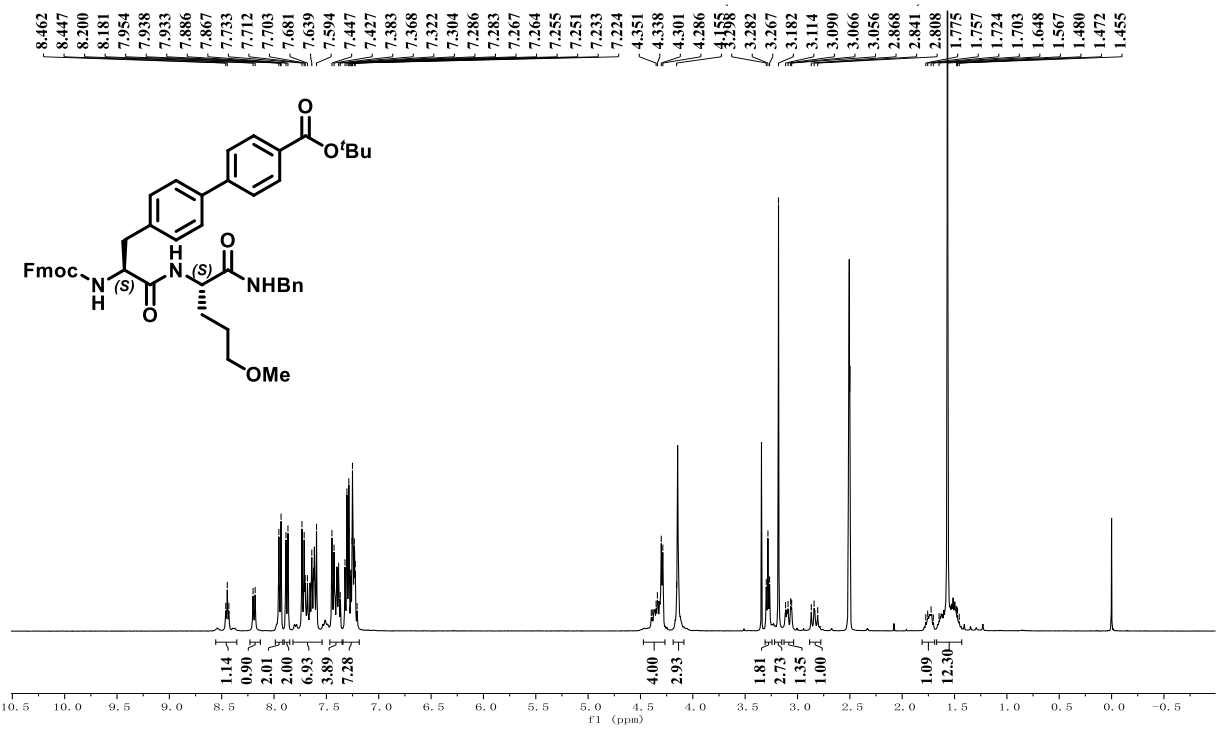
Fmoc-Bip-4-CO$_2$*t*Bu-D-5-OMe-NVA-OH (*epi*-3) (^{1}H NMR, 400 MHz, DMSO-d_6)



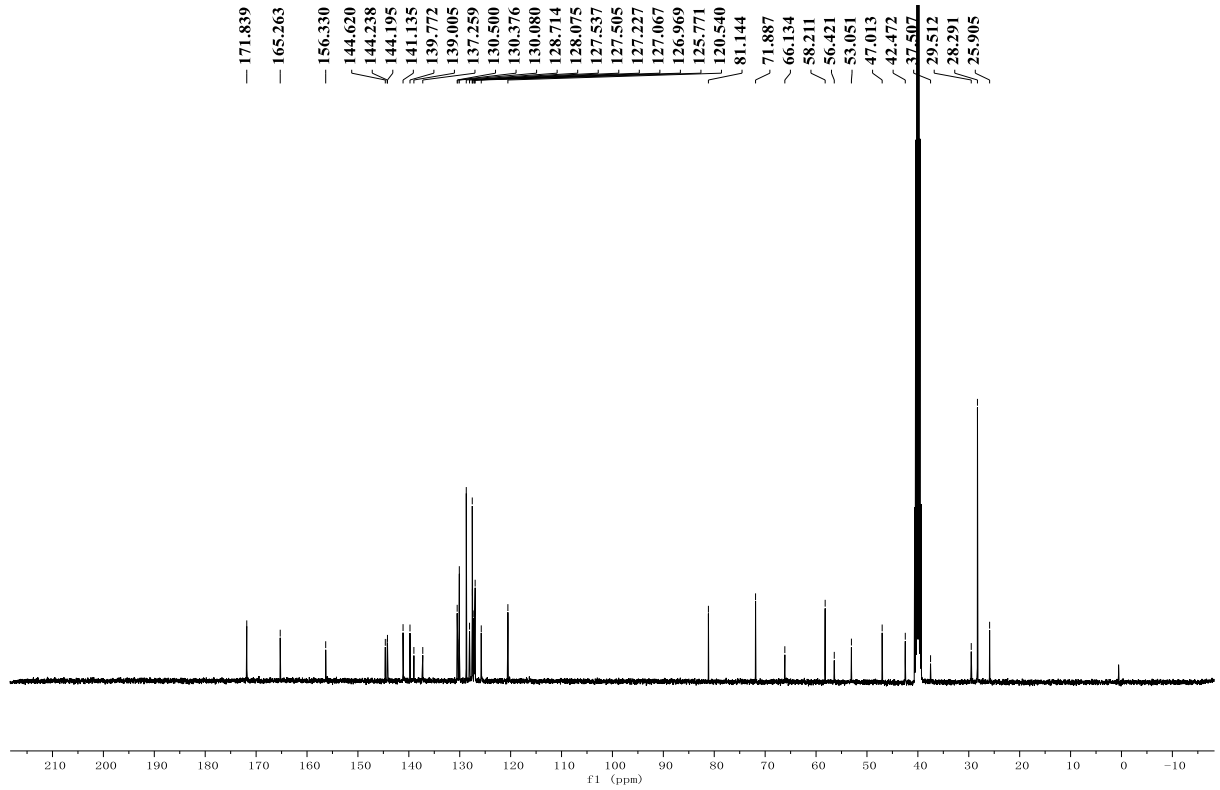
Fmoc-Bip-4-CO$_2$*t*Bu-D-5-OMe-NVA-OH (*epi*-3) (^{13}C NMR, 101 MHz, DMSO-d_6)



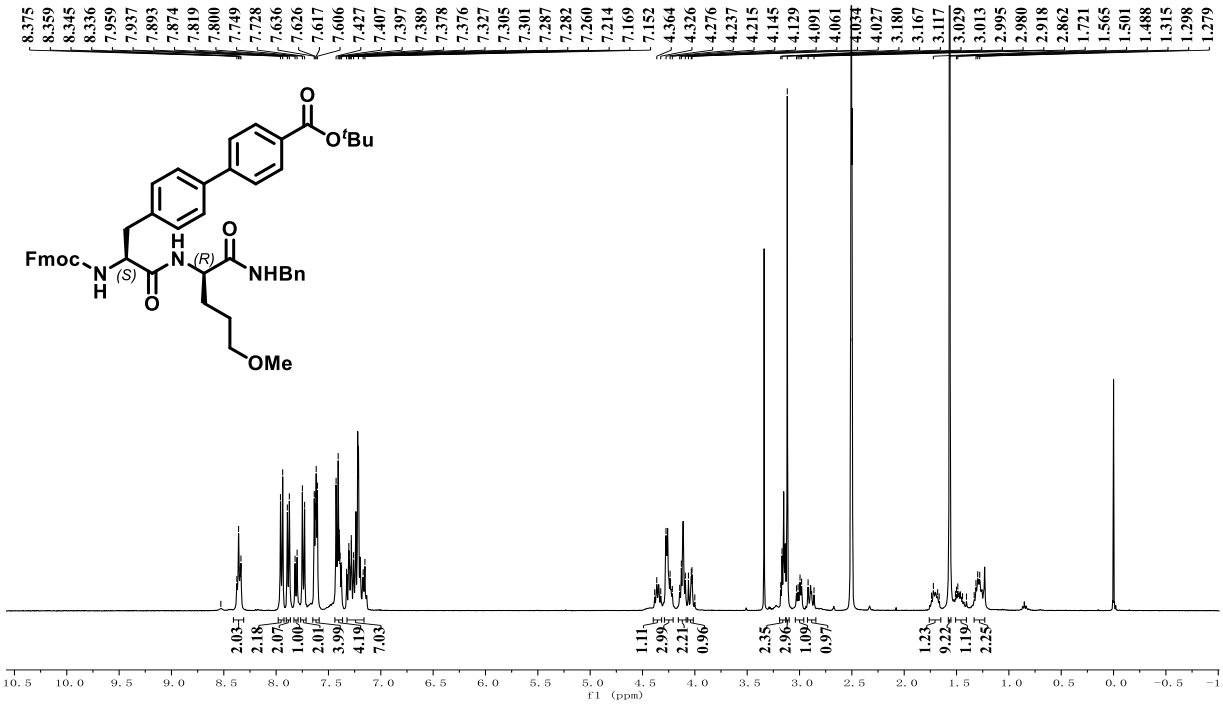
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-O-BnNH$_2$ (3-INT) (^{1}H NMR, 400 MHz, DMSO-d_6)



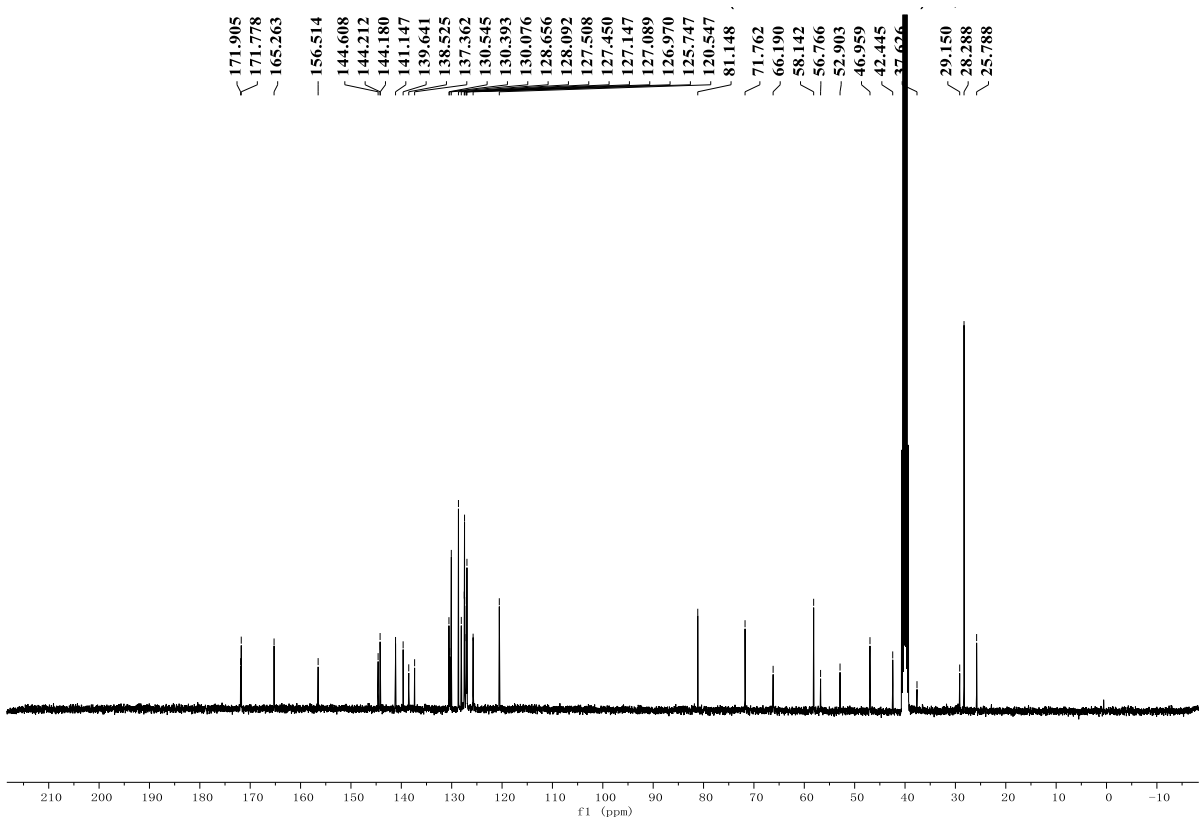
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-O-BnNH$_2$ (3-INT) (^{13}C NMR, 101 MHz, DMSO-d_6)



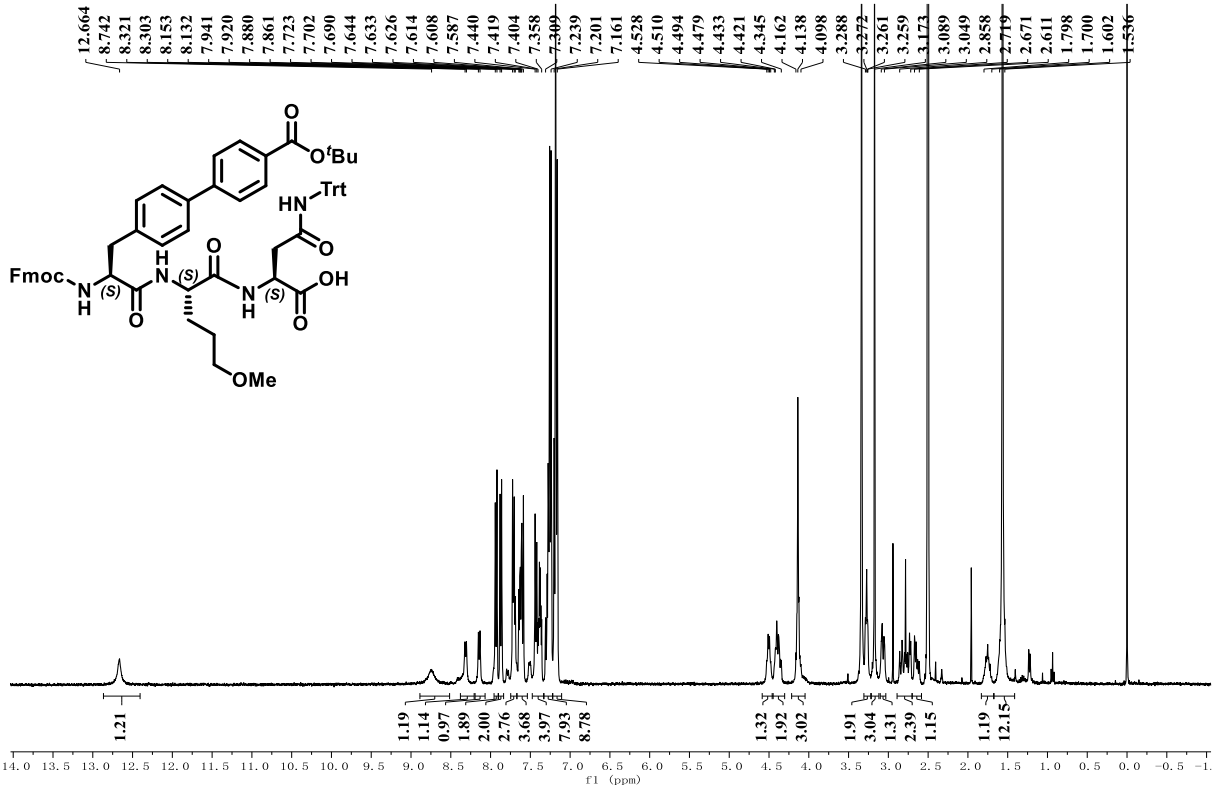
Fmoc-Bip-4-CO$_2$*t*Bu-D-5-OMe-NVA-O-BnNH$_2$ (*epi*-3-INT) (^{1}H NMR, 400 MHz, DMSO-d_6)



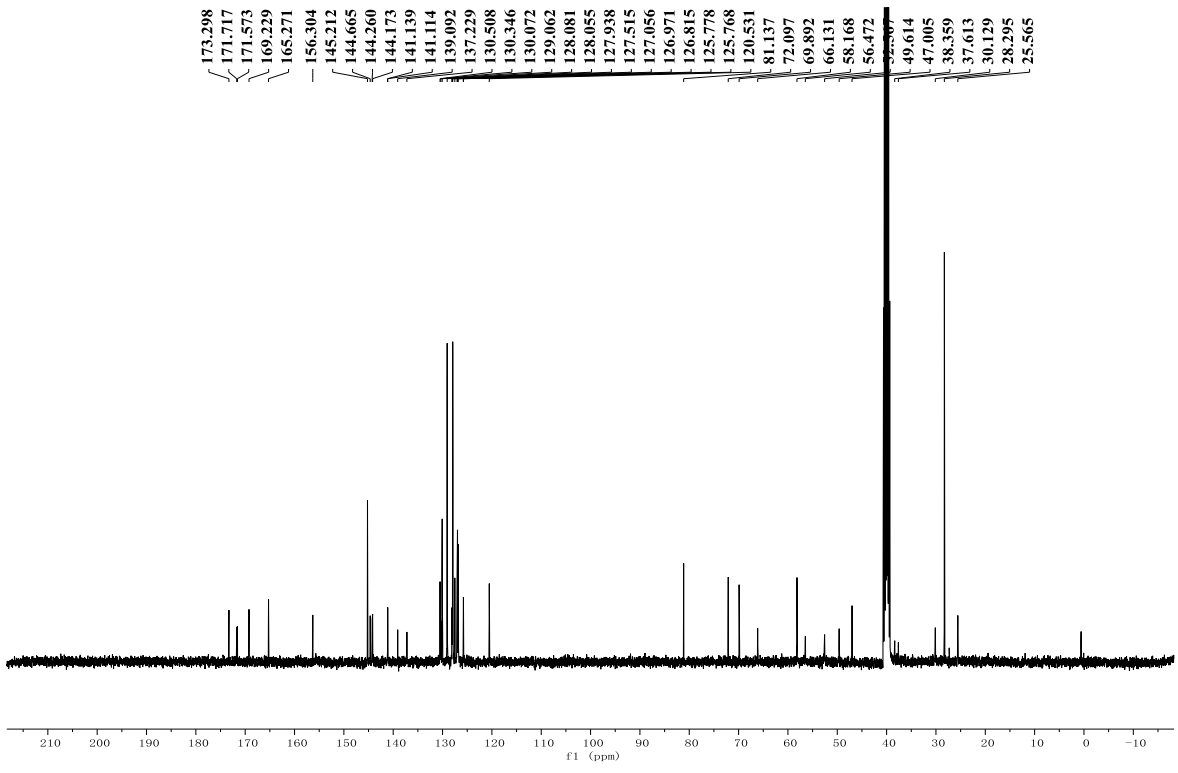
Fmoc-Bip-4-CO$_2$*t*Bu-D-5-OMe-NVA-O-BnNH$_2$ (*epi*-3-INT) (^{13}C NMR, 101 MHz, DMSO-d_6)



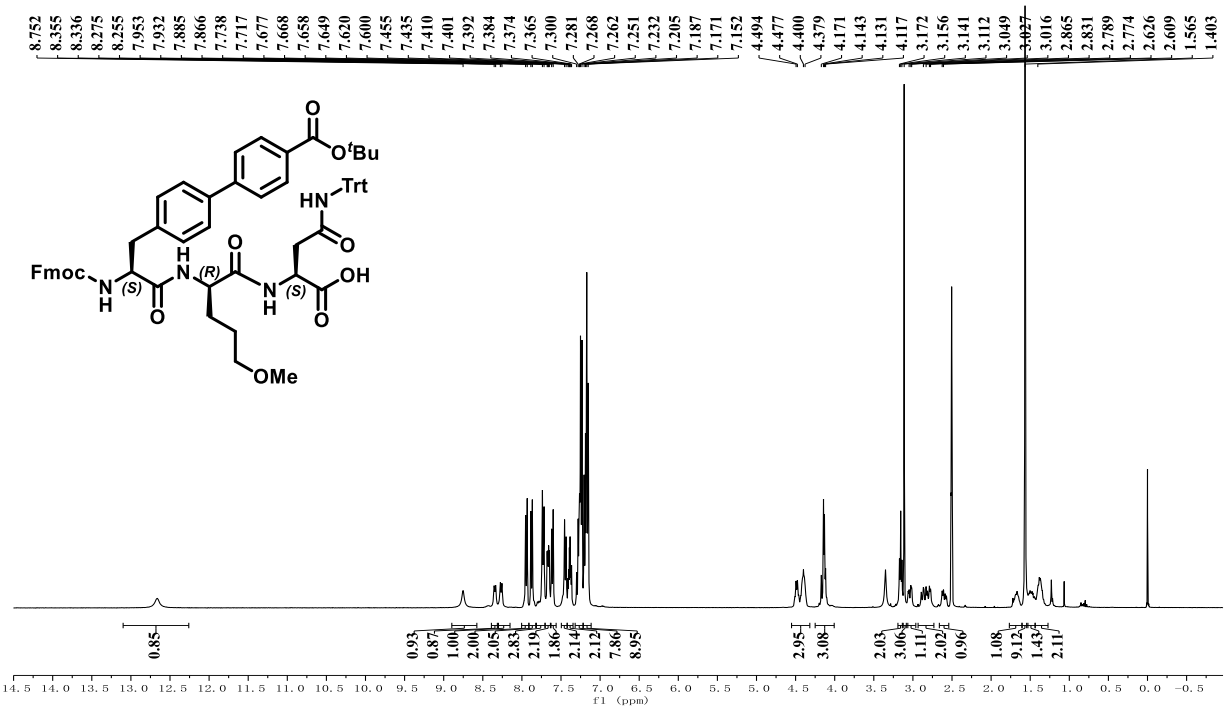
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-L-Gln(Trt)-OH (5) (^{1}H NMR, 400 MHz, DMSO-d_6)



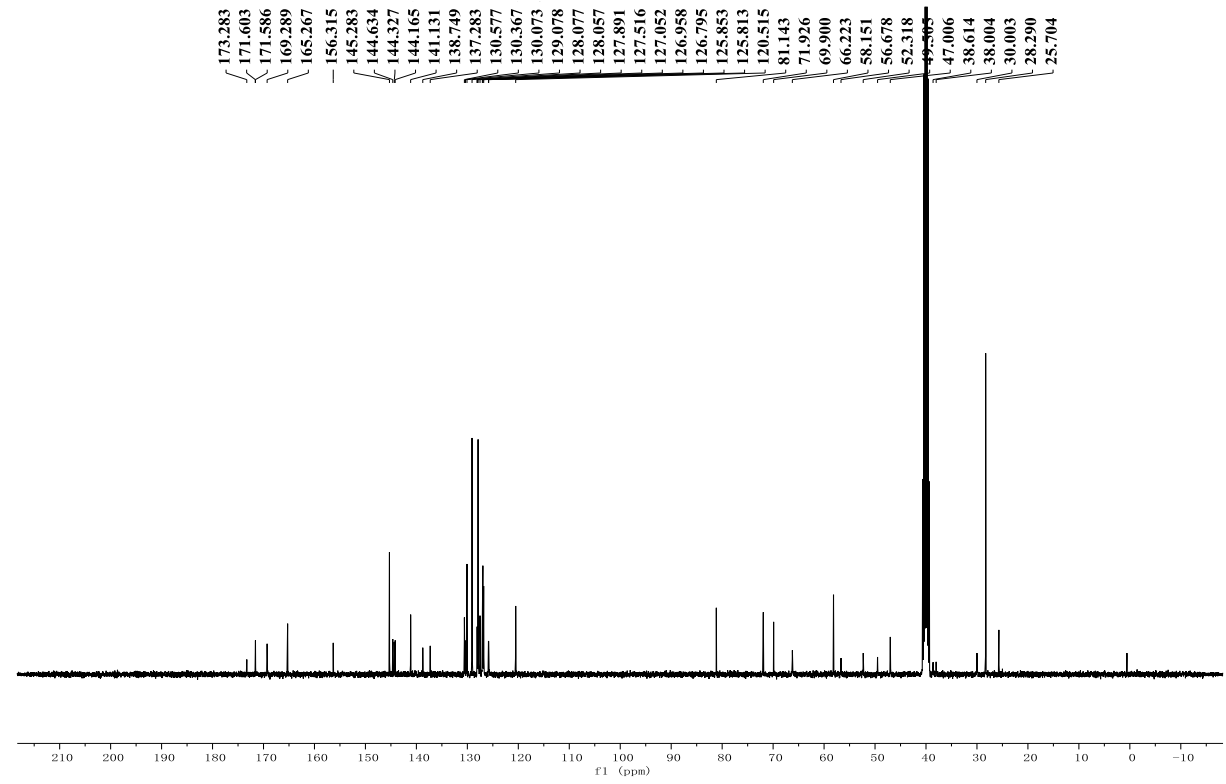
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-L-Gln(Trt)-OH (5) (^{13}C NMR, 101 MHz, DMSO-d_6)



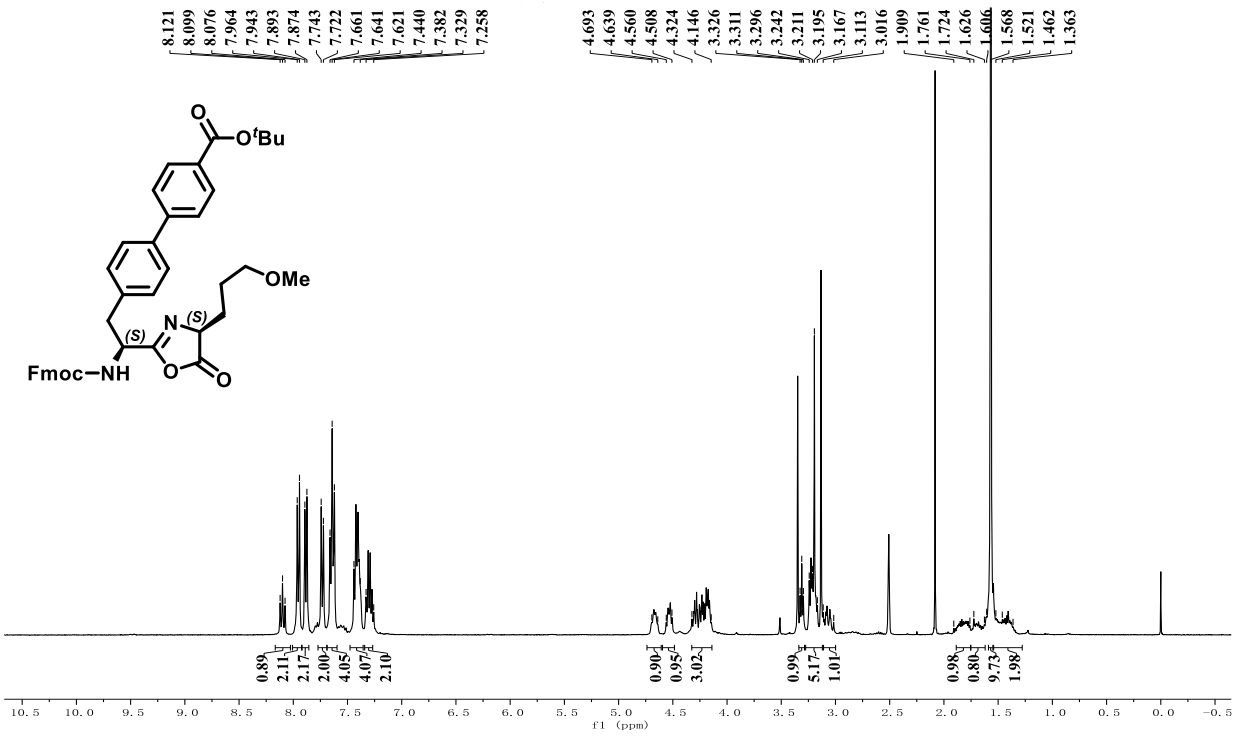
Fmoc-Bip-4-CO$_2$*t*Bu-D-5-OMe-NVA-L-Gln(Trt)-OH (*epi*-5) (^{1}H NMR, 400 MHz, DMSO-d_6)



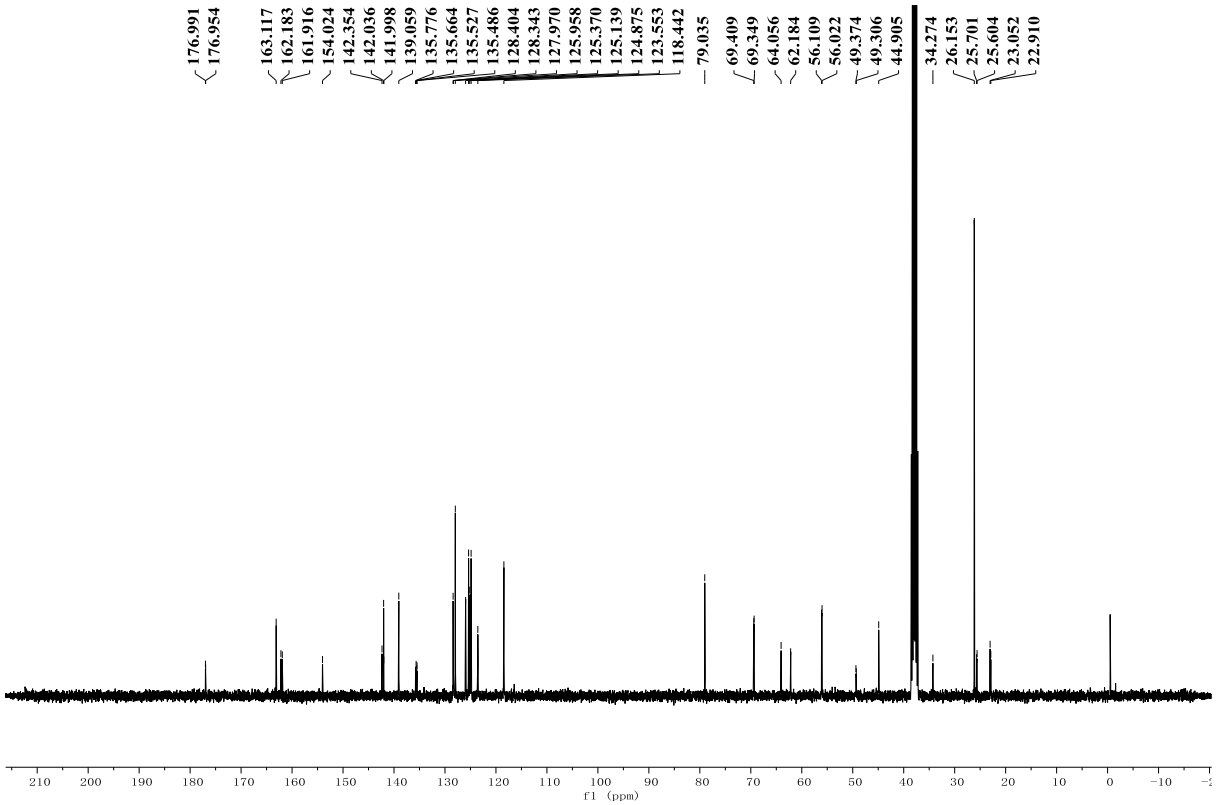
Fmoc-Bip-4-CO$_2$*t*Bu-D-5-OMe-NVA-L-Gln(Trt)-OH (*epi*-5) (^{13}C NMR, 101 MHz, DMSO-d_6)



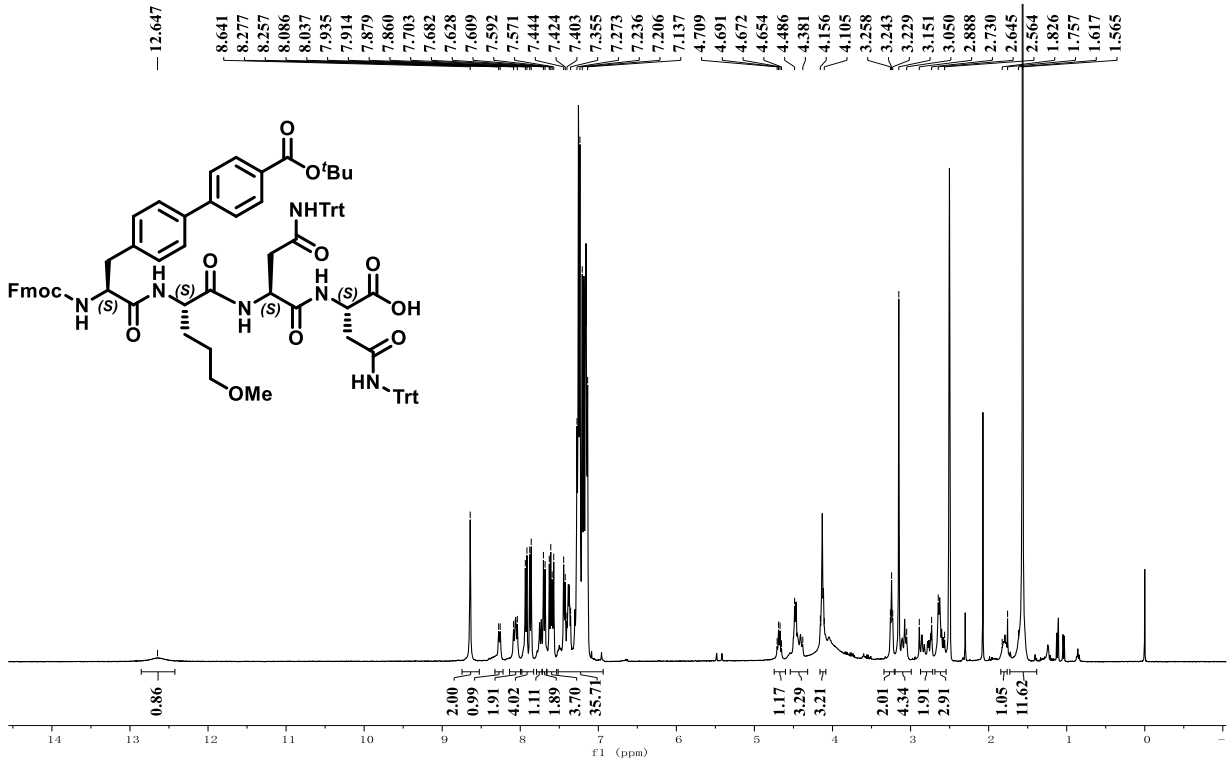
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-Oxazolone (3-Oxazolone) (^{1}H NMR, 400 MHz, DMSO-d_6)



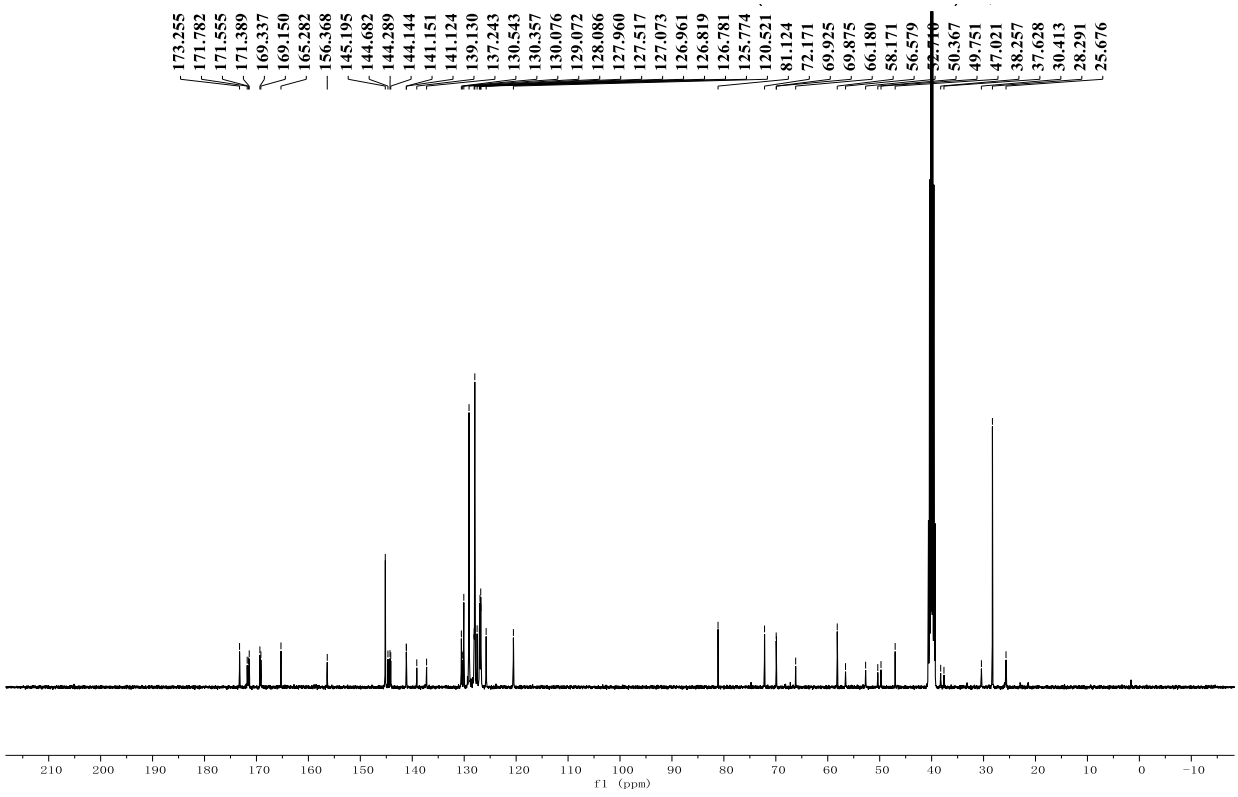
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-Oxazolone (3-Oxazolone) (^{13}C NMR, 101 MHz, DMSO-d_6)



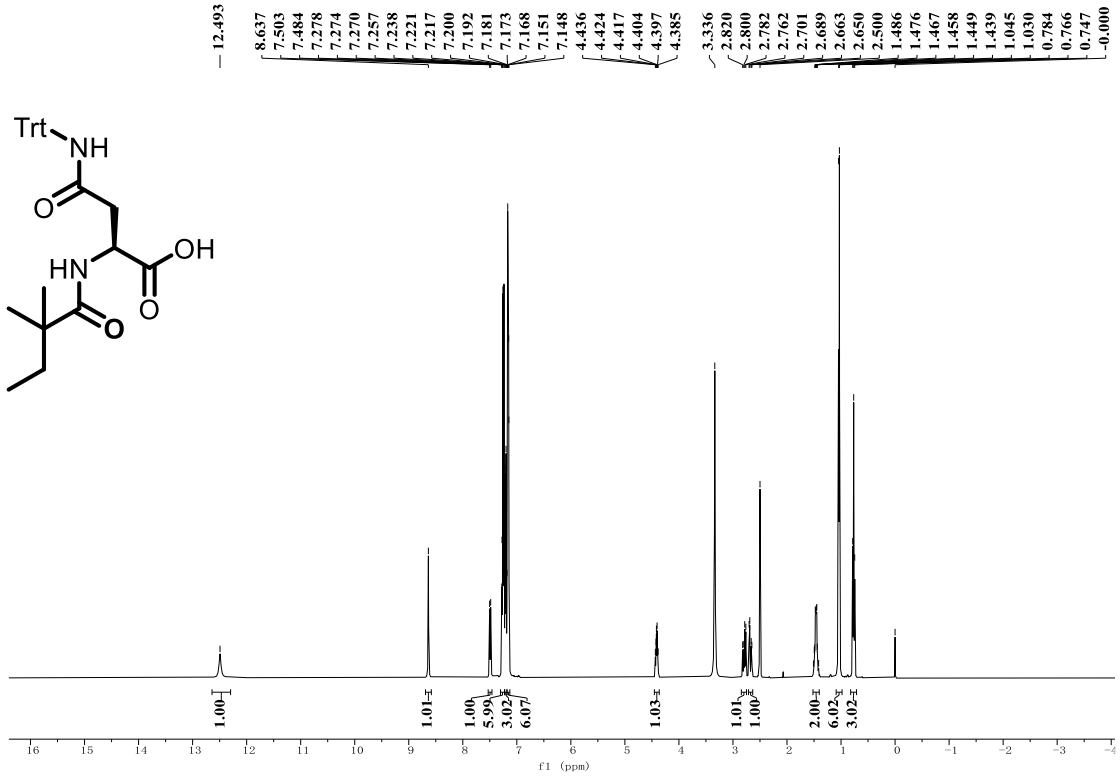
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-L-Gln(Trt)-L-Gln(Trt)-OH (6) (^{1}H NMR, 400 MHz, DMSO-d_6)



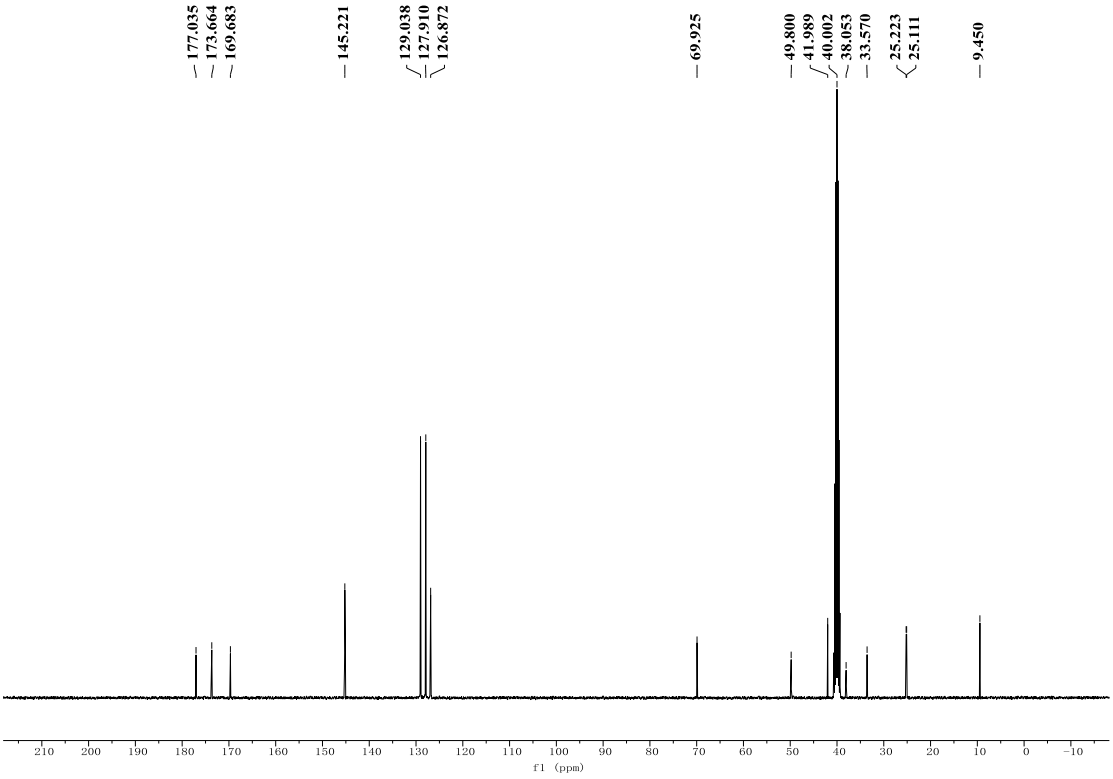
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-L-Gln(Trt)-L-Gln(Trt)-OH (6) (^{13}C NMR, 101 MHz, DMSO-d_6)



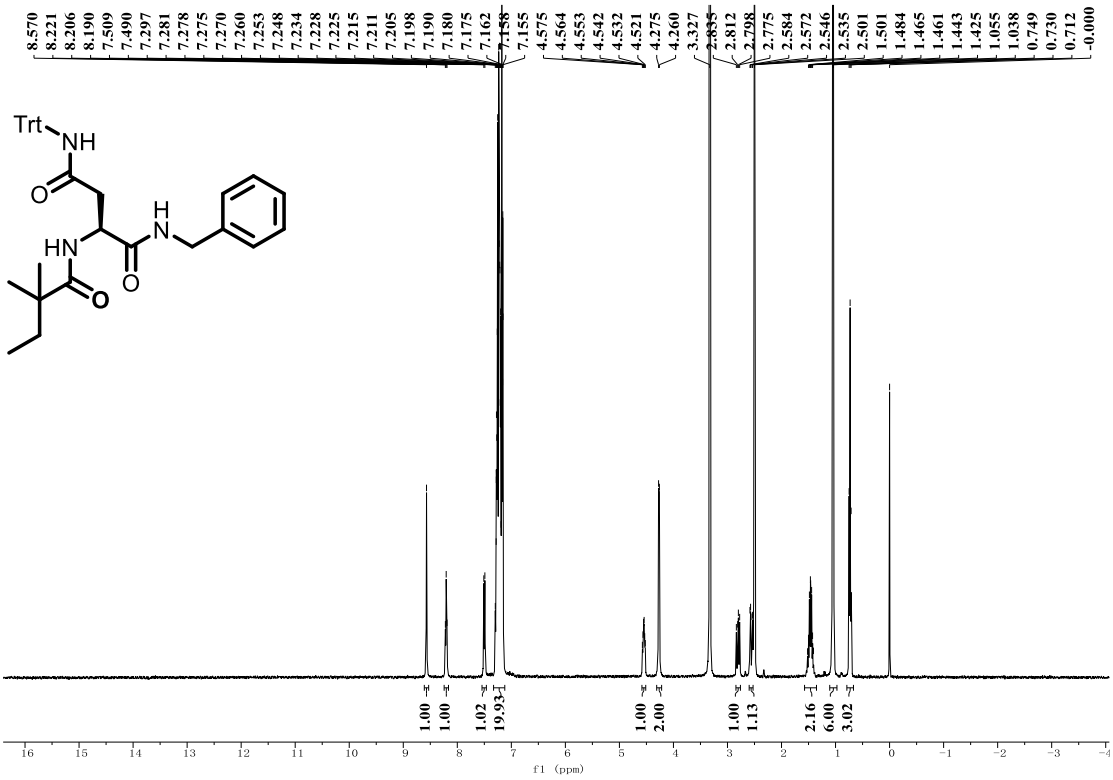
DMB-L-Gln(Trt)-OH (DMB-4) (^{1}H NMR, 400 MHz, DMSO-d_6)



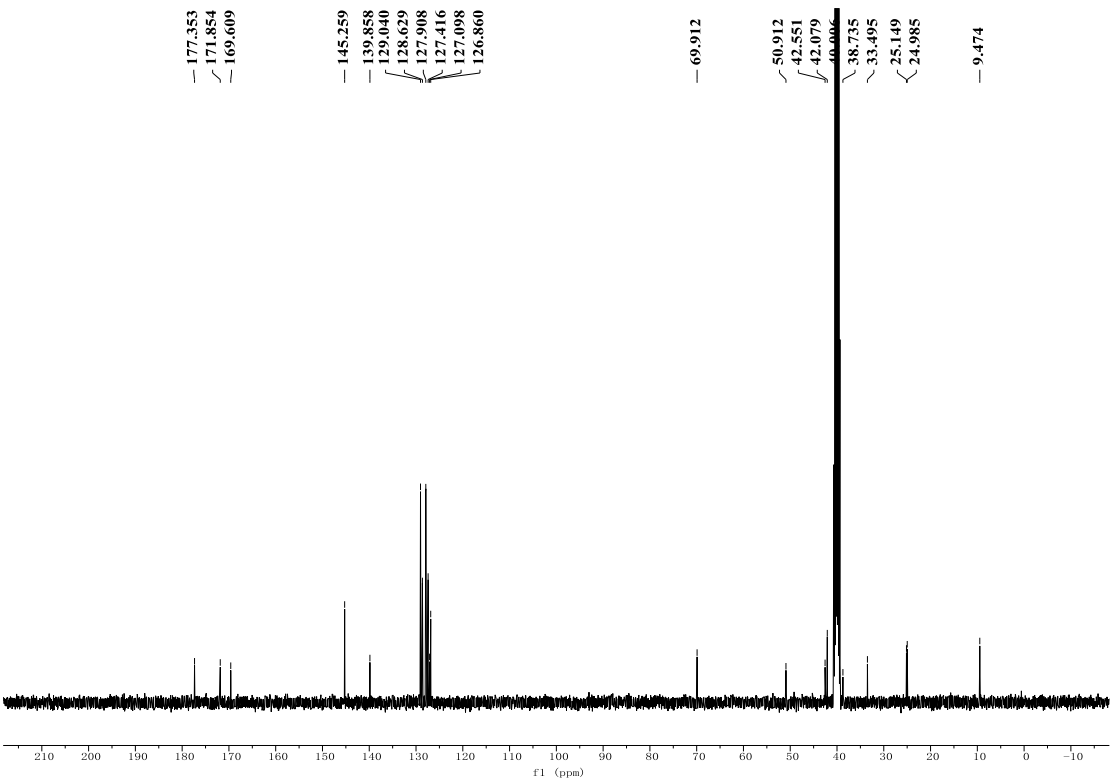
DMB-L-Gln(Trt)-OH (DMB-4) (^{13}C NMR, 101 MHz, DMSO-d_6)



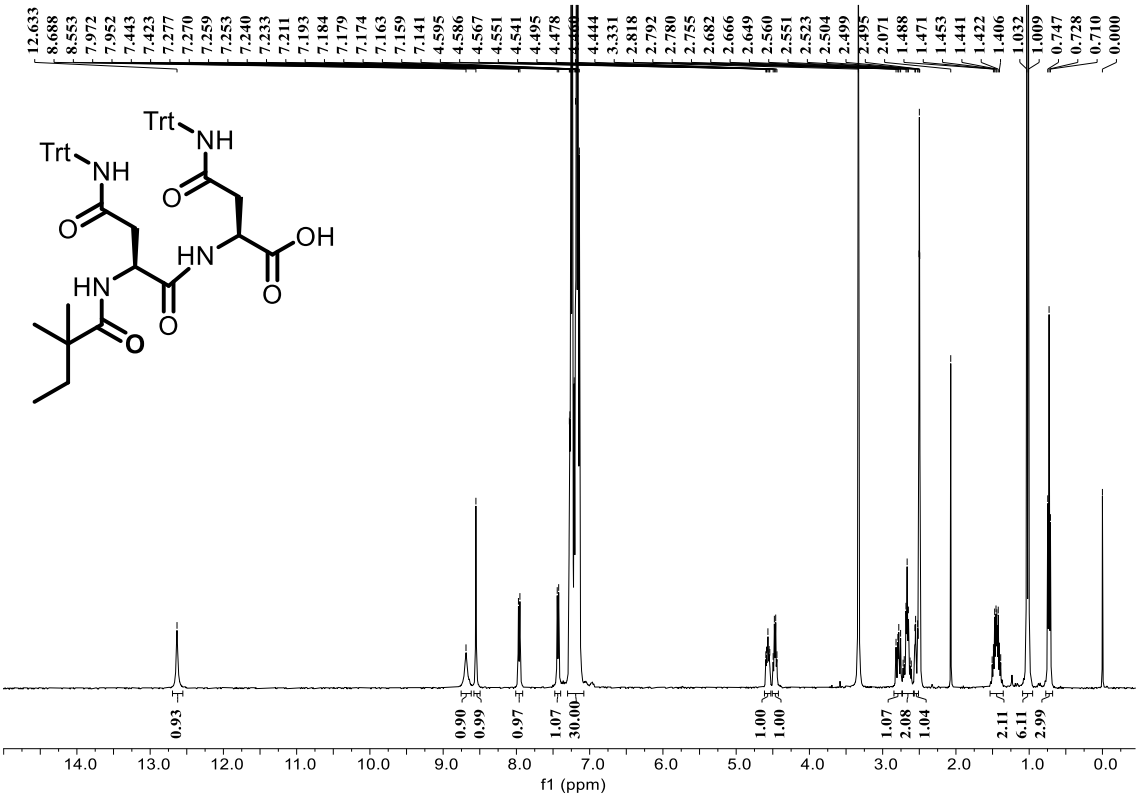
DMB-L-Gln(Trt)-BnNH$_2$ (DMB-4-Bn) (^{1}H NMR, 400 MHz, DMSO-d_6)



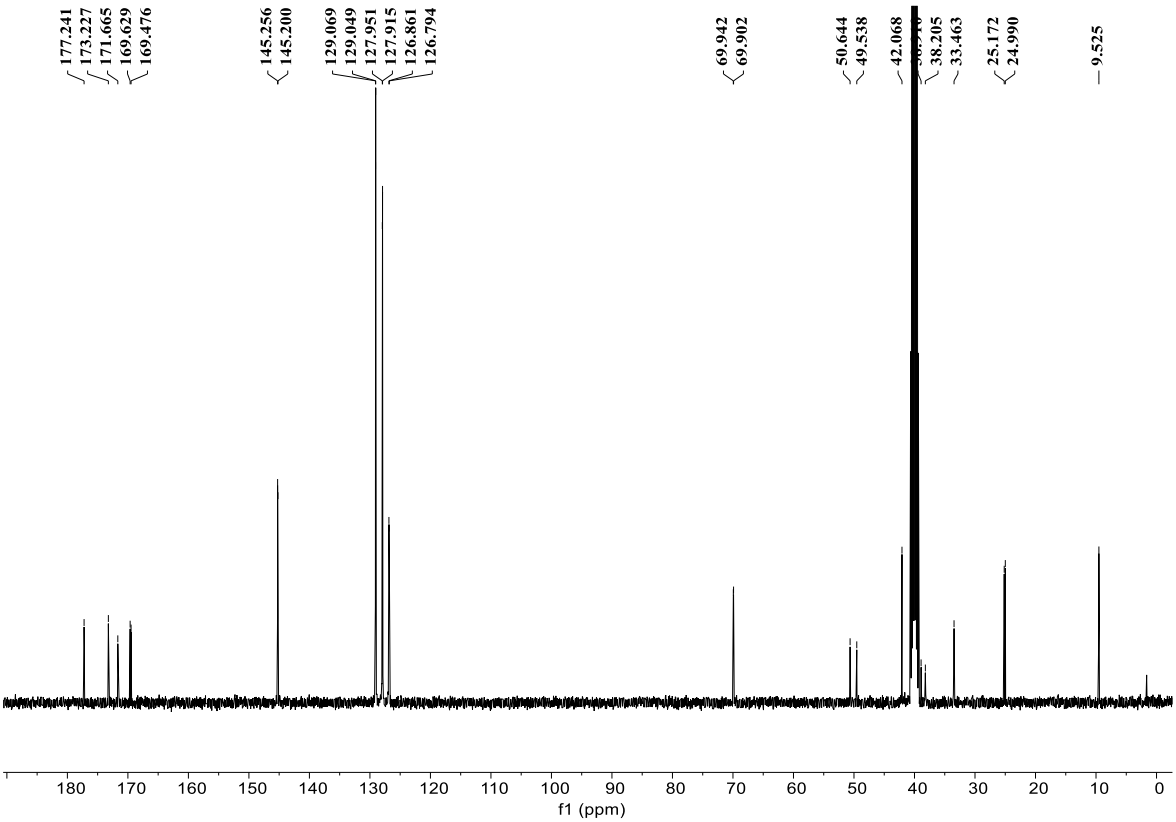
DMB-L-Gln(Trt)-BnNH$_2$ (DMB-4-Bn) (^{13}C NMR, 101 MHz, DMSO-d_6)



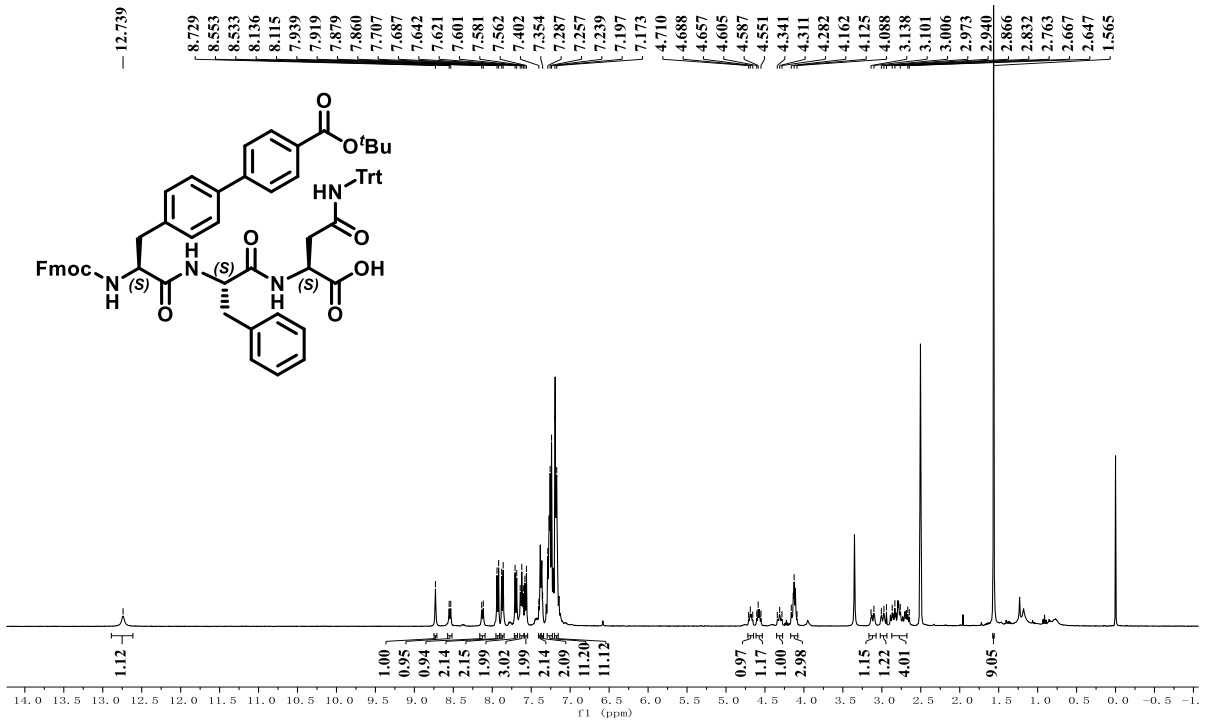
Synthesis of DMB-L-Gln(Trt)-L-Gln(Trt)-OH (DMB-4-4) (^{1}H NMR, 400 MHz, DMSO-d_6)



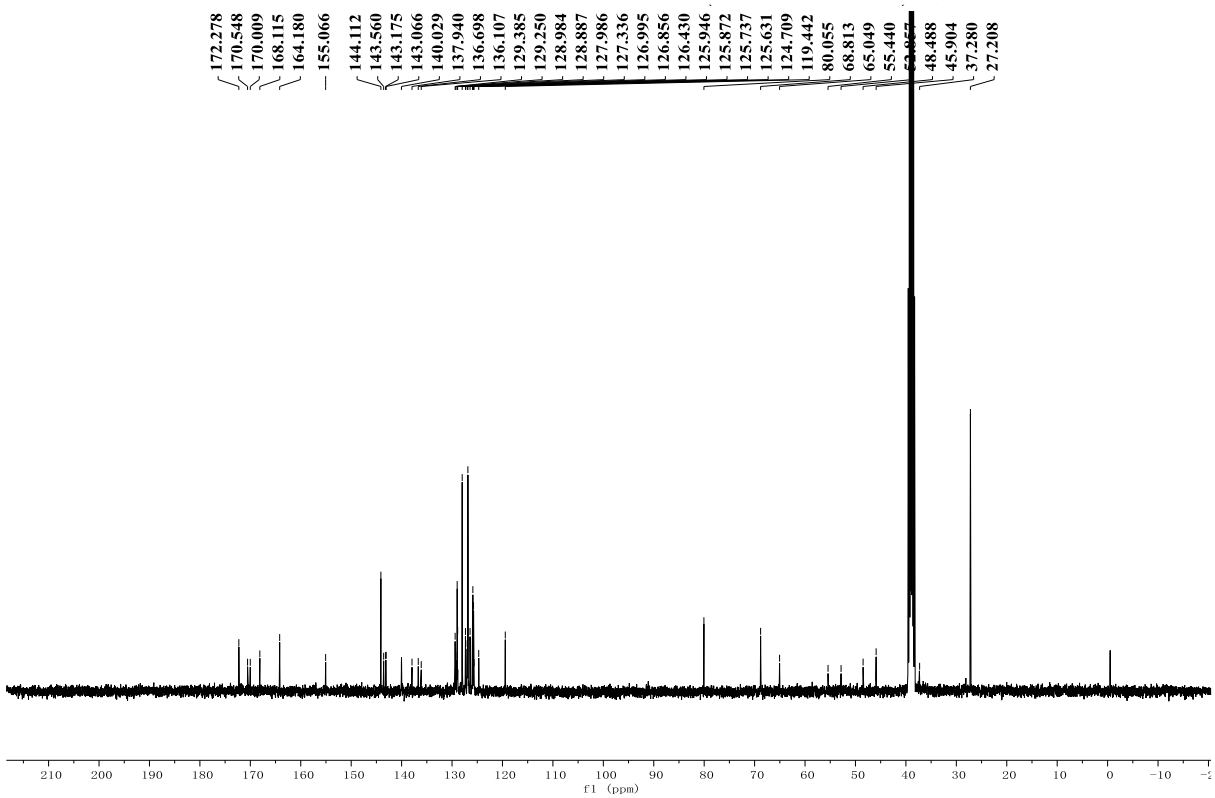
Synthesis of DMB-L-Gln(Trt)-L-Gln(Trt)-OH (DMB-4-4) (^{13}C NMR, 101 MHz, DMSO-d_6)



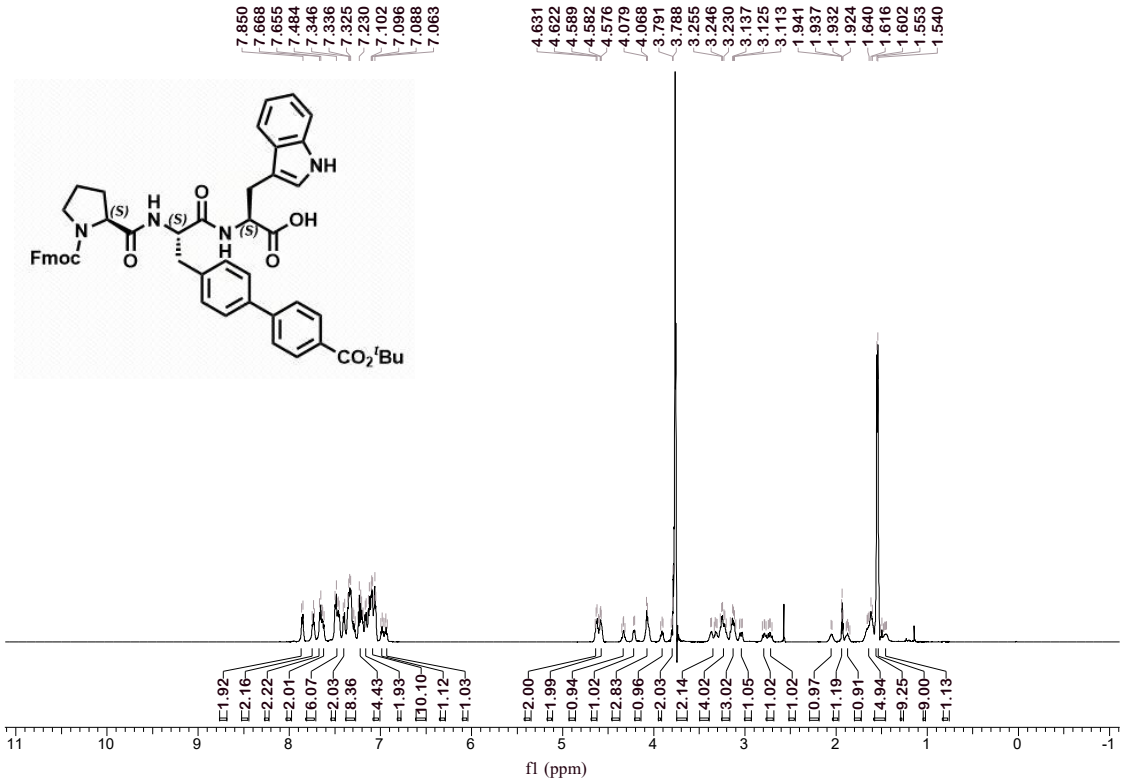
Fmoc-Bip-4-CO$_2$*t*Bu-L-Phe-L-Asn(Trt)-OH (7a) (^{1}H NMR, 400 MHz, DMSO-d_6)



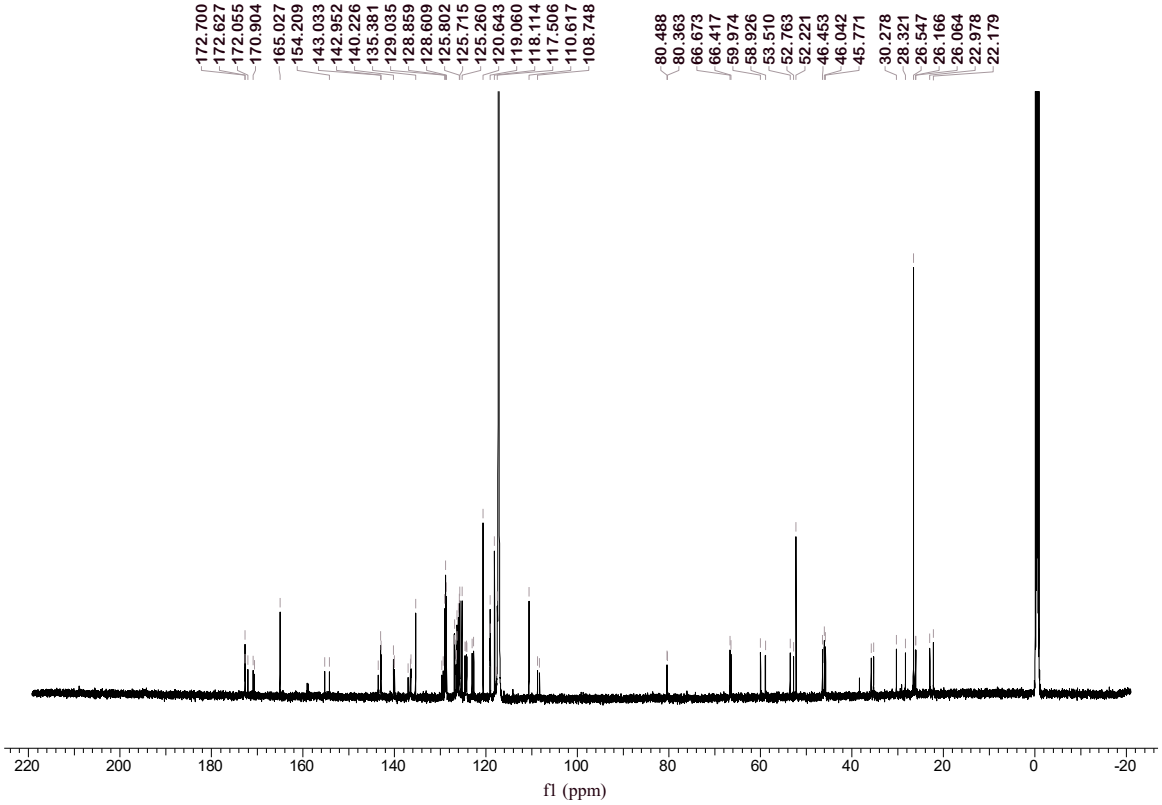
Fmoc-Bip-4-CO$_2$*t*Bu-L-Phe-L-Asn(Trt)-OH (7a) (^{13}C NMR, 101 MHz, DMSO-d_6)



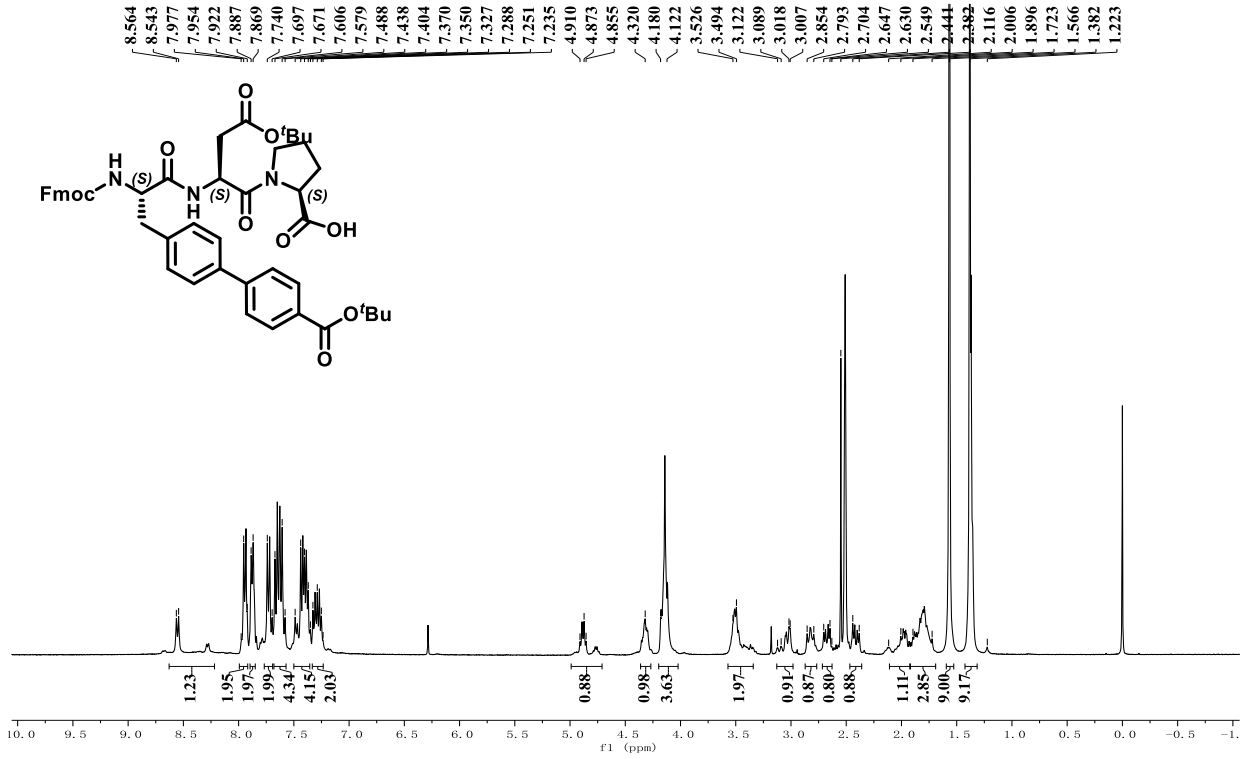
Fmoc-Pro-Bip-4-CO$_2$*t*Bu-Trp-OH (7b) (^{1}H NMR, 600 MHz, 3:1 CD_3CN/D_2O, 298 K)



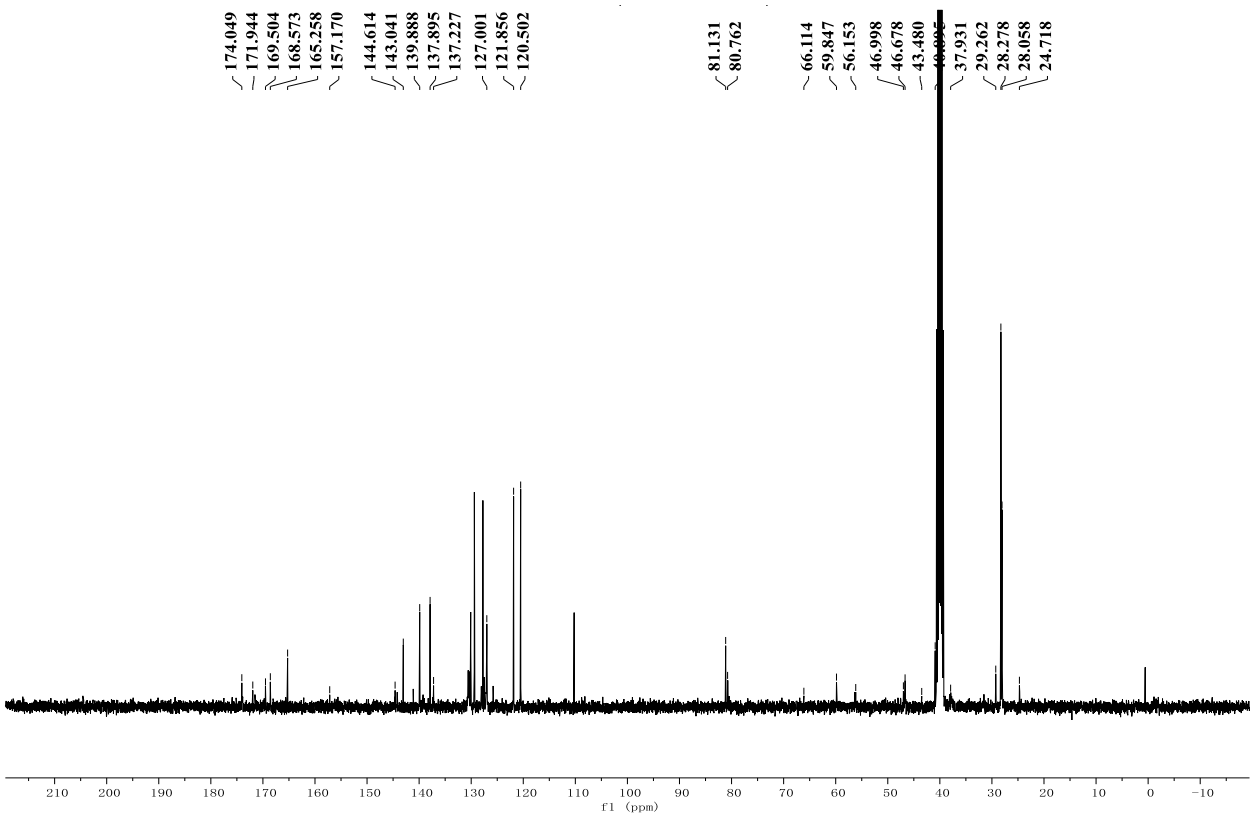
Fmoc-Pro-Bip-4-CO$_2$*t*Bu-Trp-OH (7b) (^{13}C NMR, 151 MHz, 3:1 CD_3CN/D_2O, 298 K)



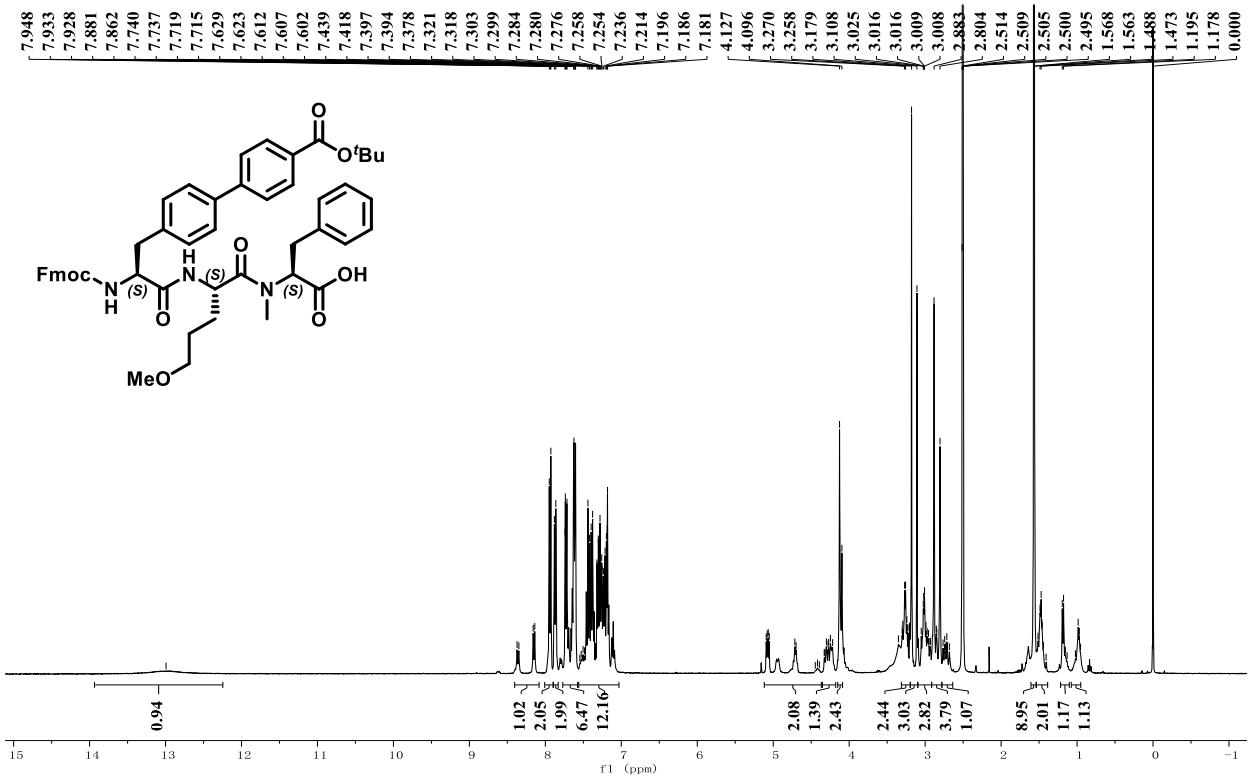
Fmoc-Bip-4-CO$_2$*t*Bu-L-Asp(O*t*Bu)-L-Pro-OH (7c) (^{1}H NMR, 400 MHz, DMSO-d_6)



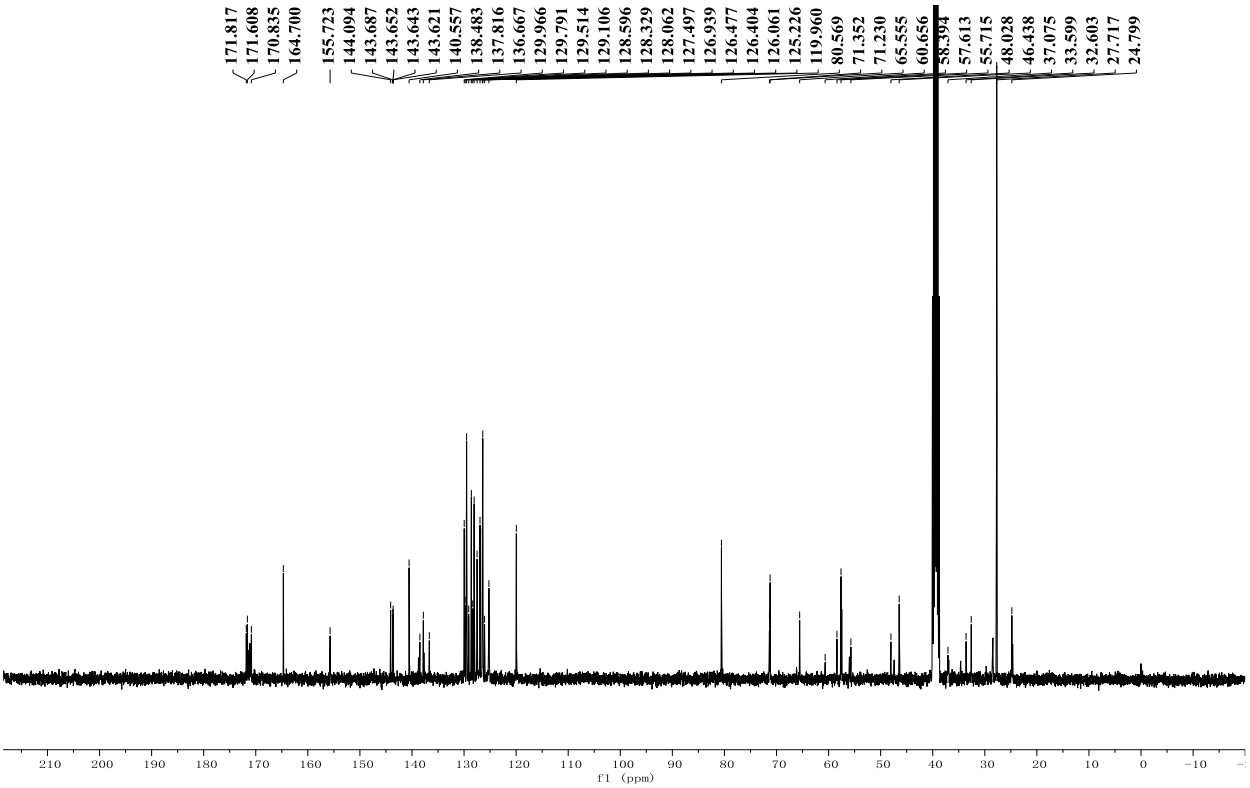
Fmoc-Bip-4-CO$_2$*t*Bu-L-Asp(O*t*Bu)-L-Pro-OH (7c) (^{13}C NMR, 101 MHz, DMSO-d_6)



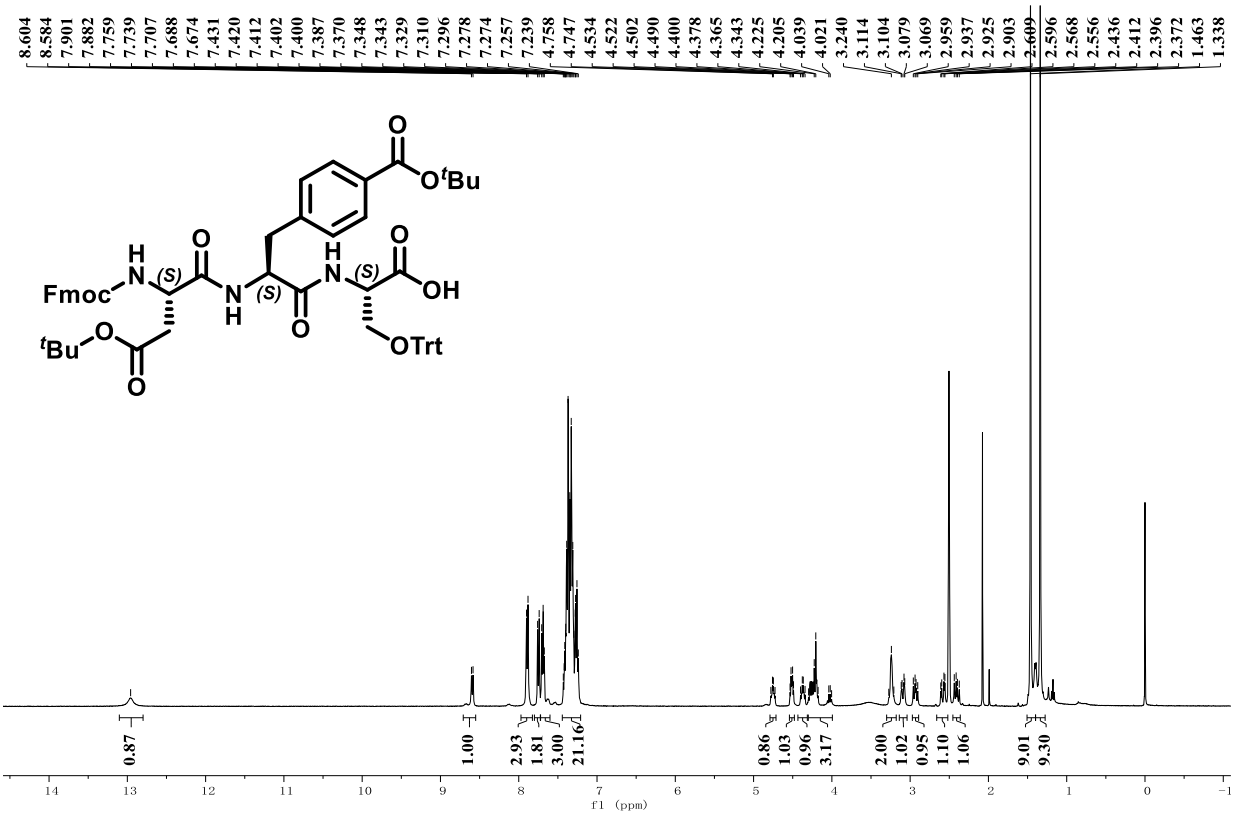
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-MePhe-OH (7d). (^{1}H NMR, 400 MHz, DMSO-d_6)



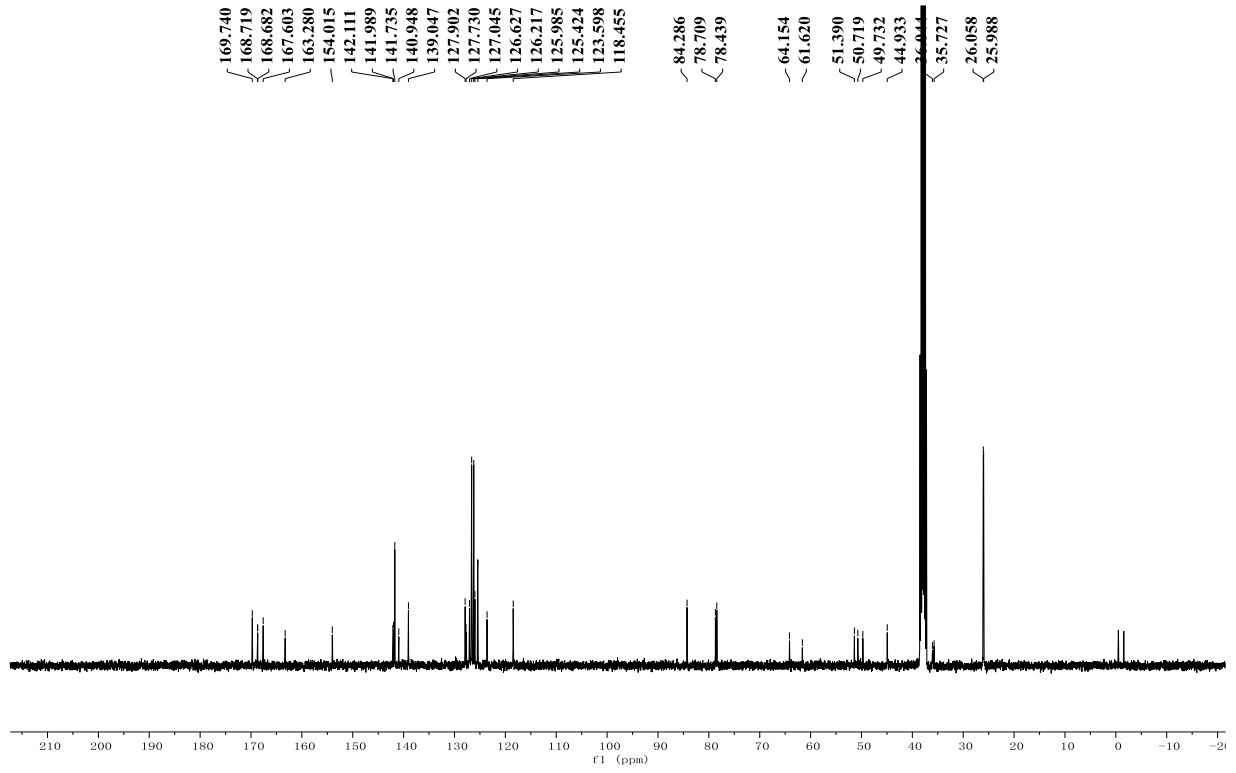
Fmoc-Bip-4-CO$_2$*t*Bu-5-OMe-NVA-MePhe-OH (7d) (^{13}C NMR, 101 MHz, DMSO-d_6)



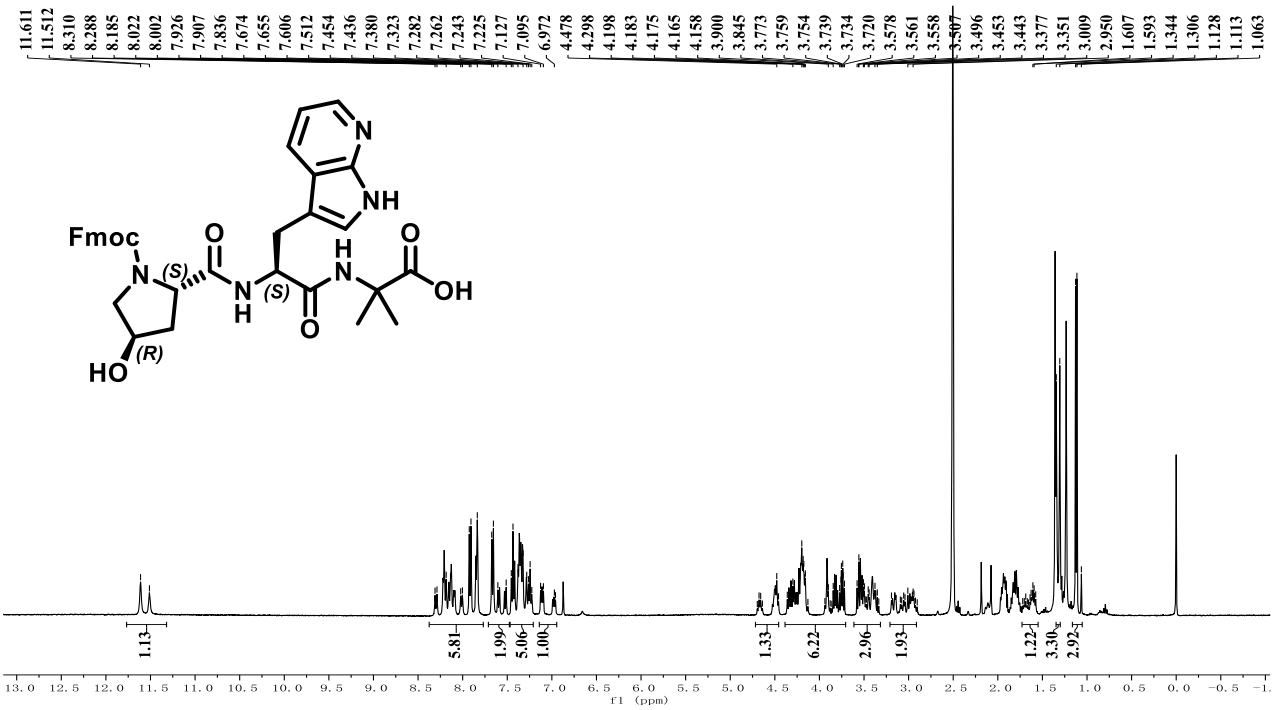
Fmoc-L-Asp(O*t*Bu)-L-Phe(4-CO_2*t*Bu)-L-Ser(Trt)-OH (**7e**) (^{1}H NMR, 400 MHz, DMSO-d_6)



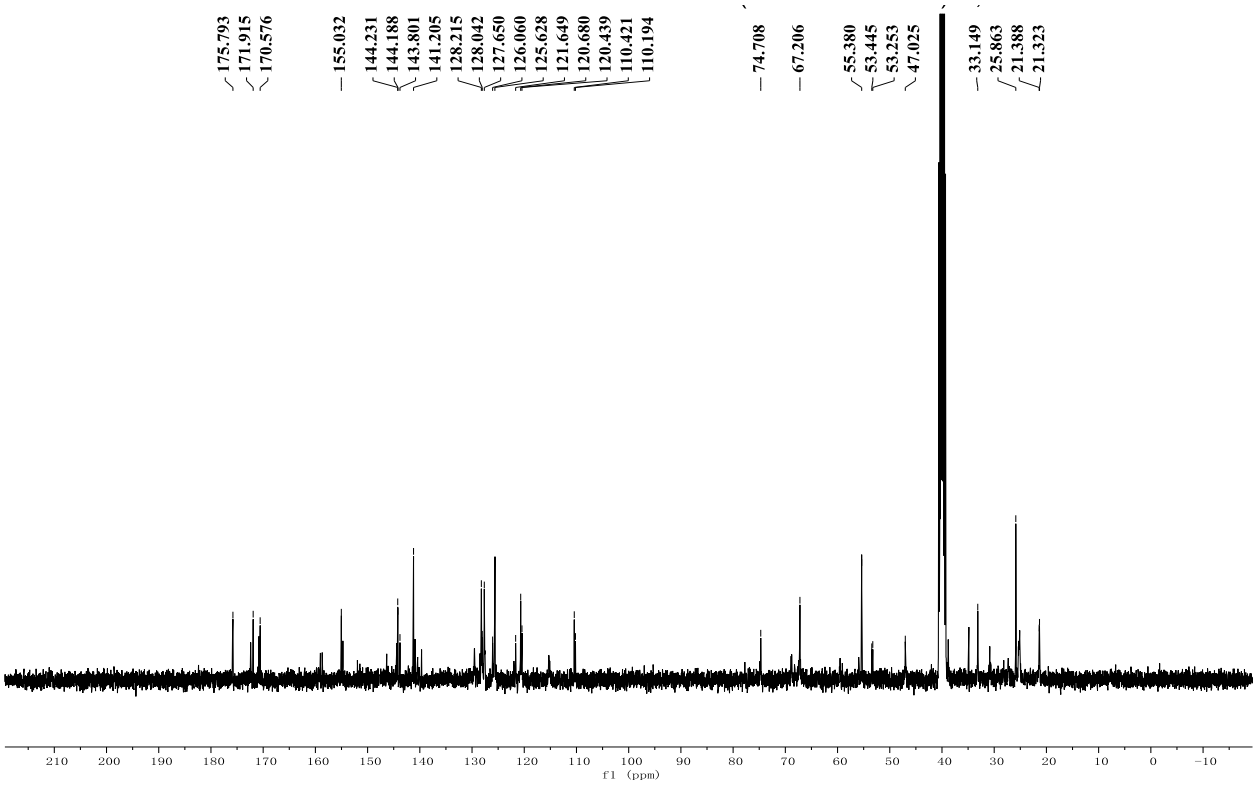
Fmoc-L-Asp(O*t*Bu)-L-Phe(4-CO_2*t*Bu)-L-Ser(Trt)-OH (**7e**) (^{13}C NMR, 101 MHz, DMSO-d_6)



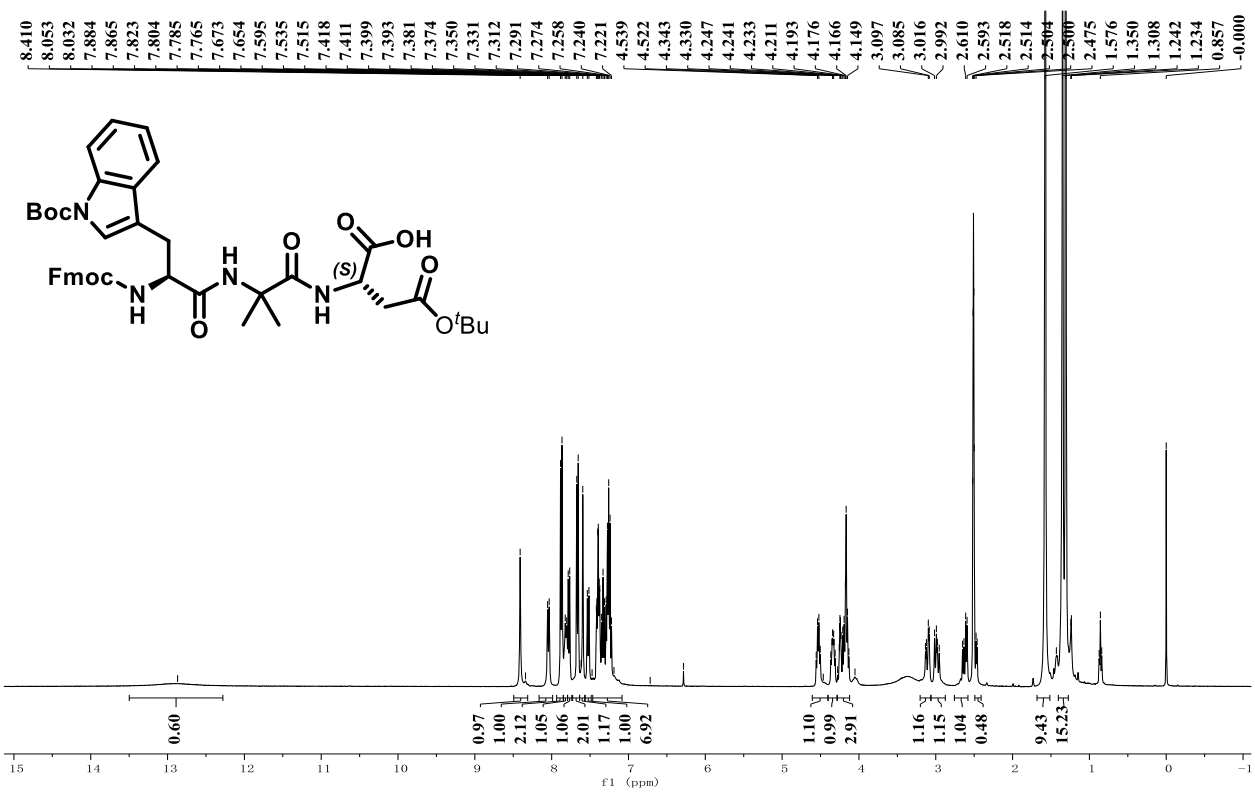
Fmoc-Pro(*t*4OH)-7-aza-Trp-Aib-OH (7f) (^{1}H NMR, 400 MHz, DMSO-d_6)



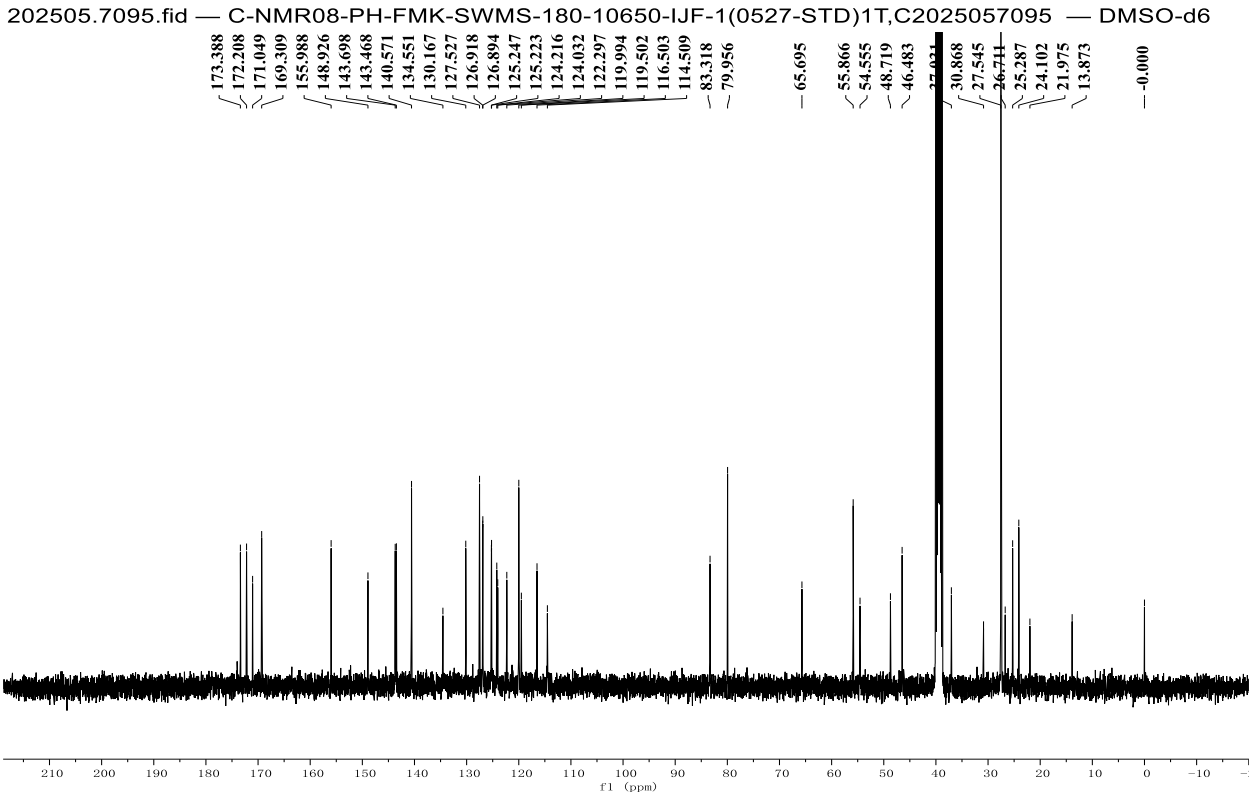
Fmoc-Pro(*t*4OH)-7-aza-Trp-Aib-OH (7f) (^{13}C NMR, 101 MHz, DMSO-d_6)



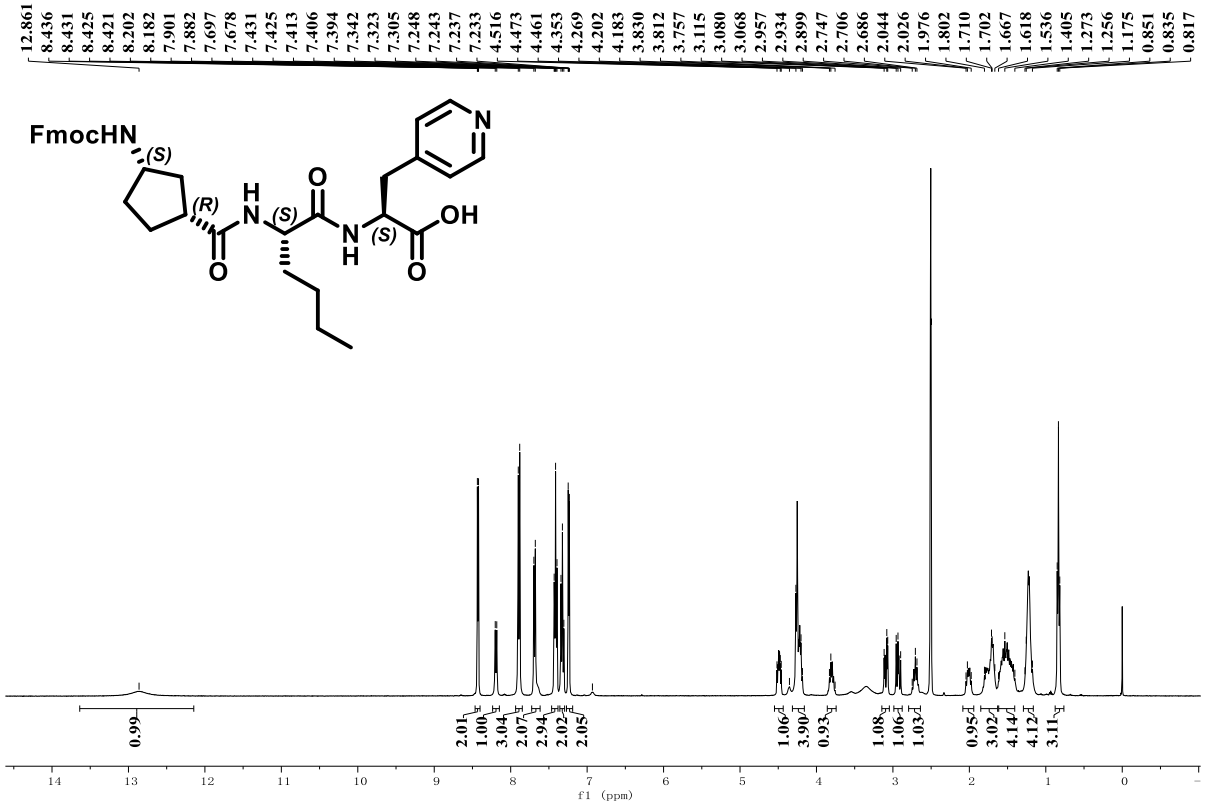
Fmoc-Trp(Boc)-Aib-L-Asp(O*t*Bu)-OH (7g). (^{1}H NMR, 400 MHz, DMSO-d_6)



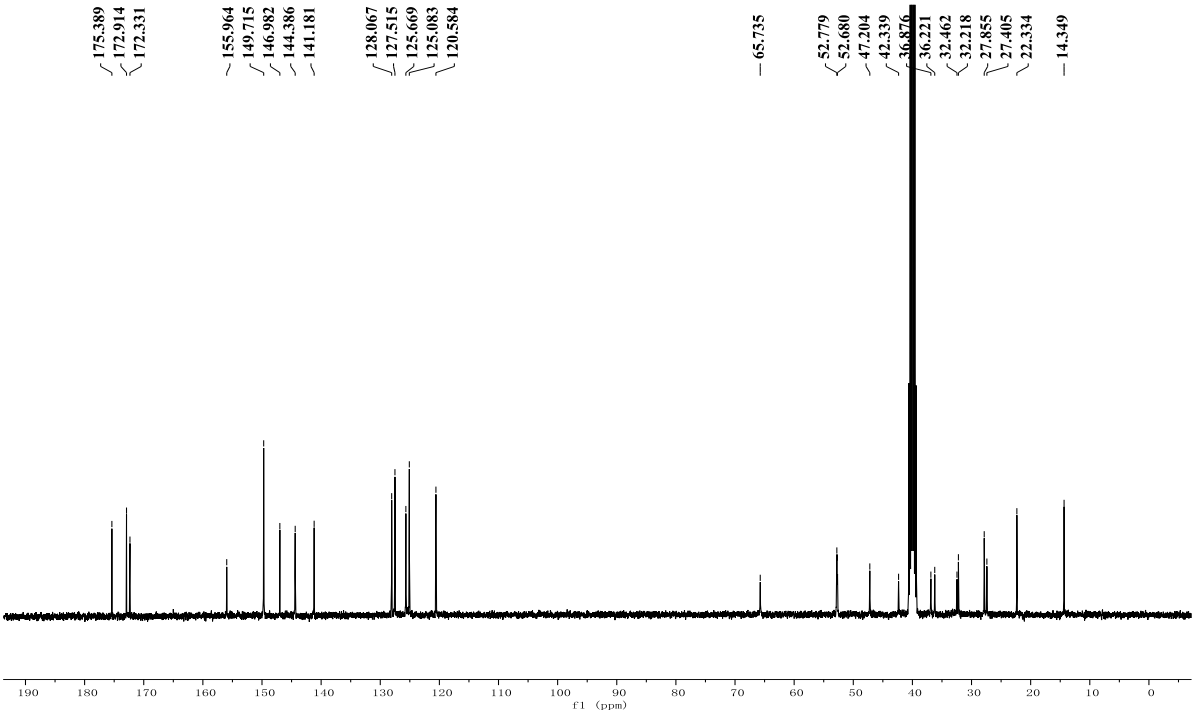
Fmoc-Trp(Boc)-Aib-L-Asp(O*t*Bu)-OH (7g) (^{13}C NMR, 101 MHz, DMSO-d_6)



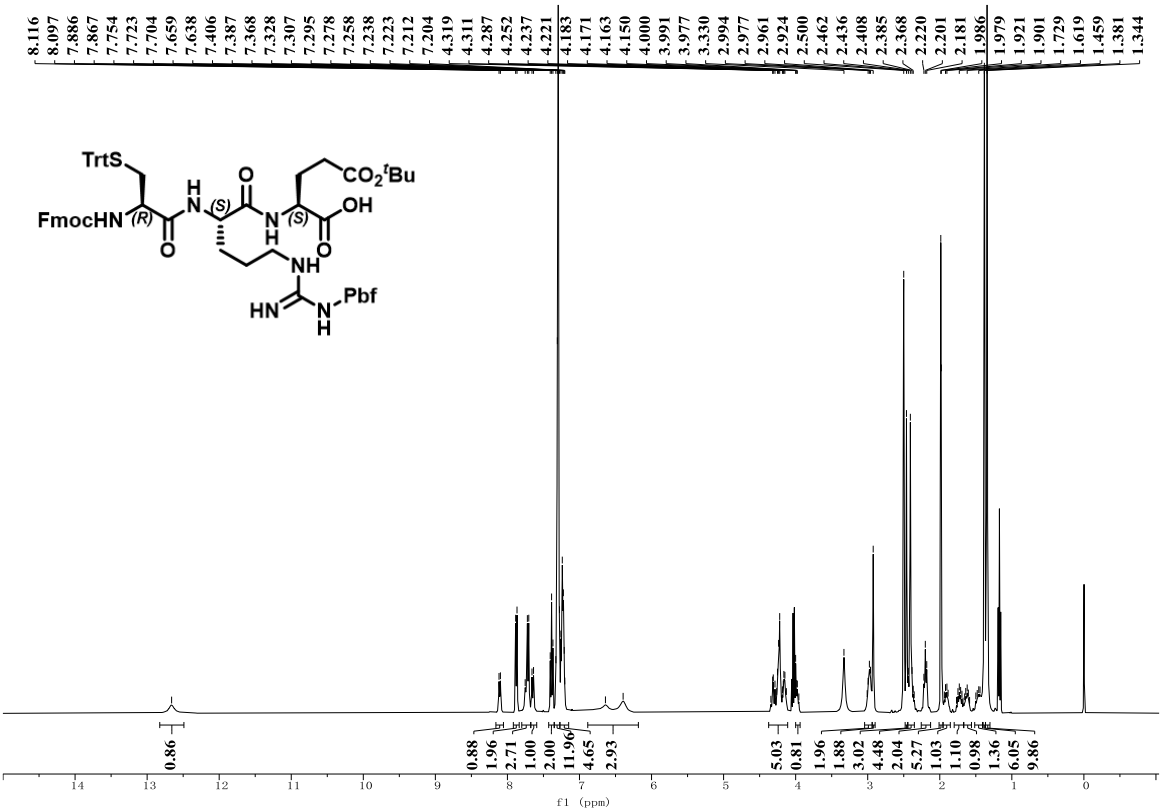
Fmoc-bhcLeu-L-Nle-L-Ala(4-PYRI)-OH (7h) (^{1}H NMR, 400 MHz, DMSO-d_6)



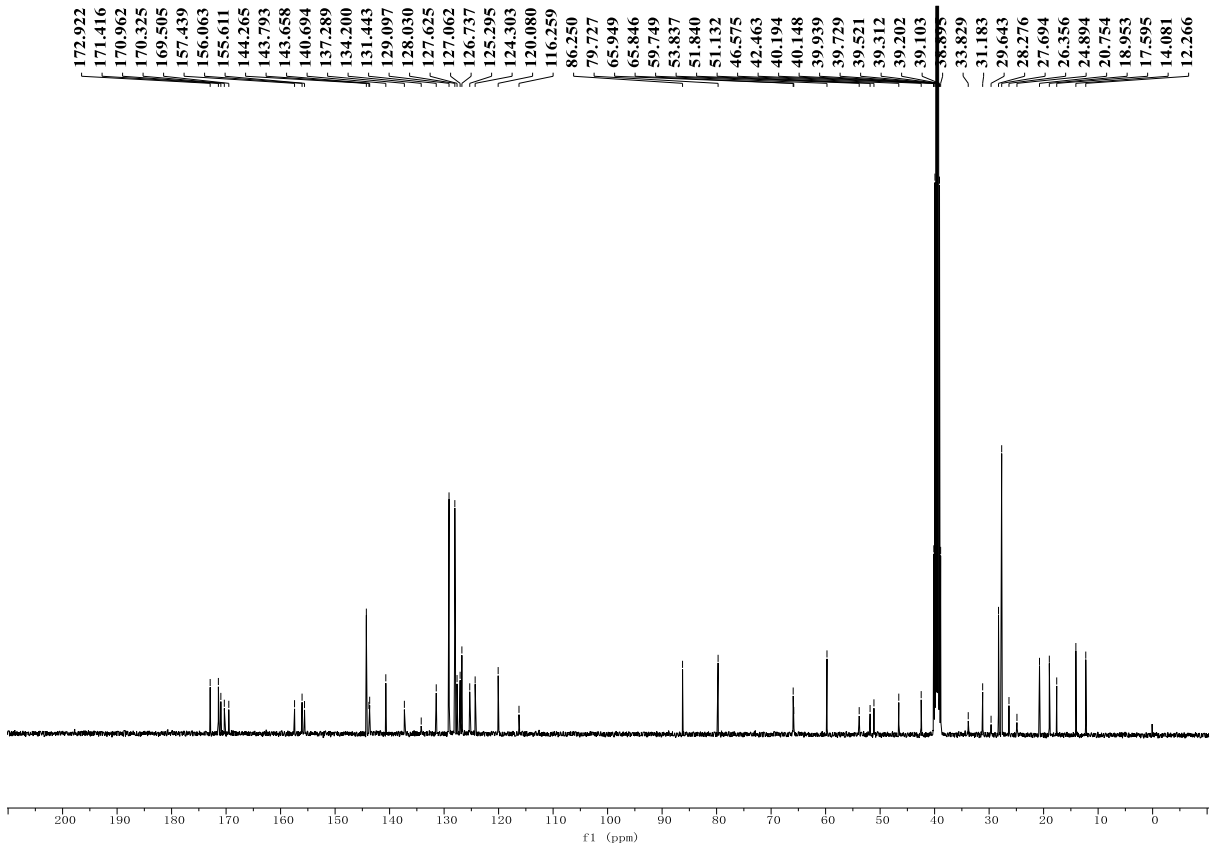
Fmoc-bhcLeu-L-Nle-L-Ala(4-PYRI)-OH (7h) (^{13}C NMR, 101 MHz, DMSO-d_6)



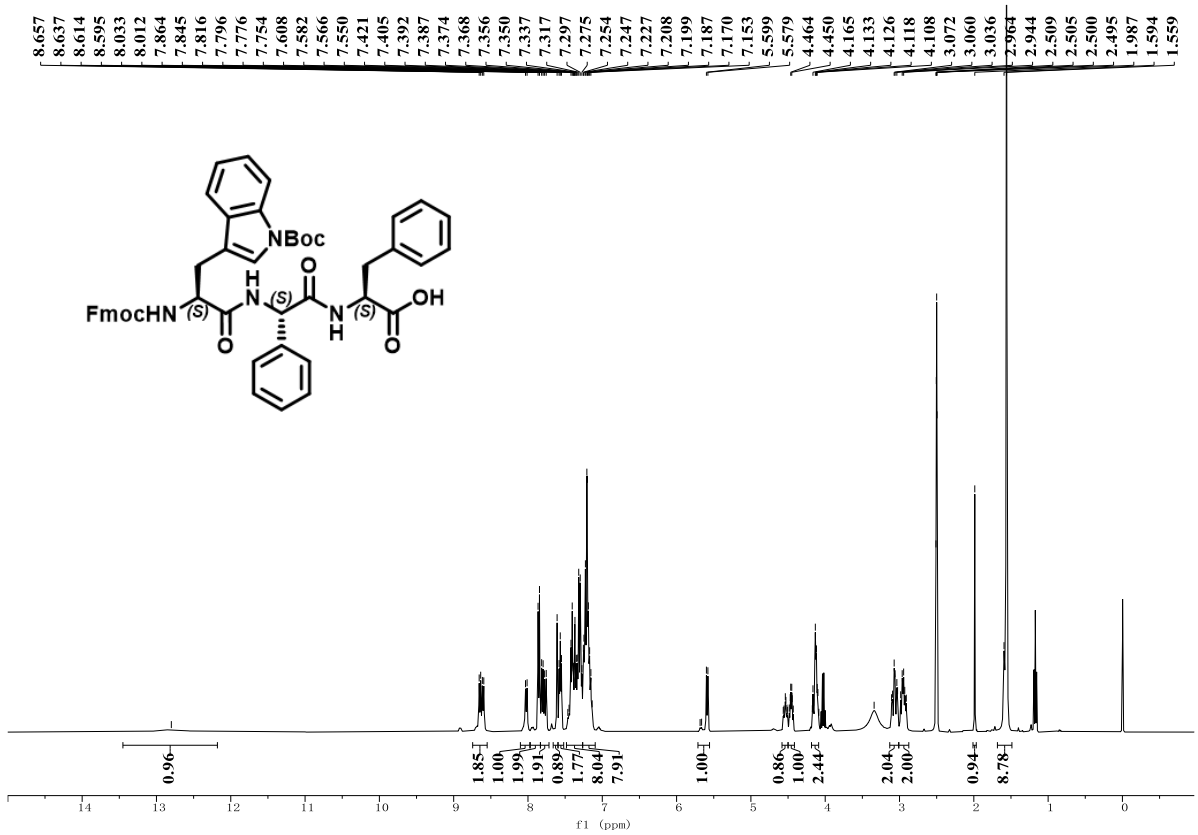
Fmoc-L-Cys(Trt)-L-Arg(Pbf)-L-Glu(5-CO_2*t*Bu)-OH (7i) (^{1}H NMR, 400 MHz, DMSO-d_6)



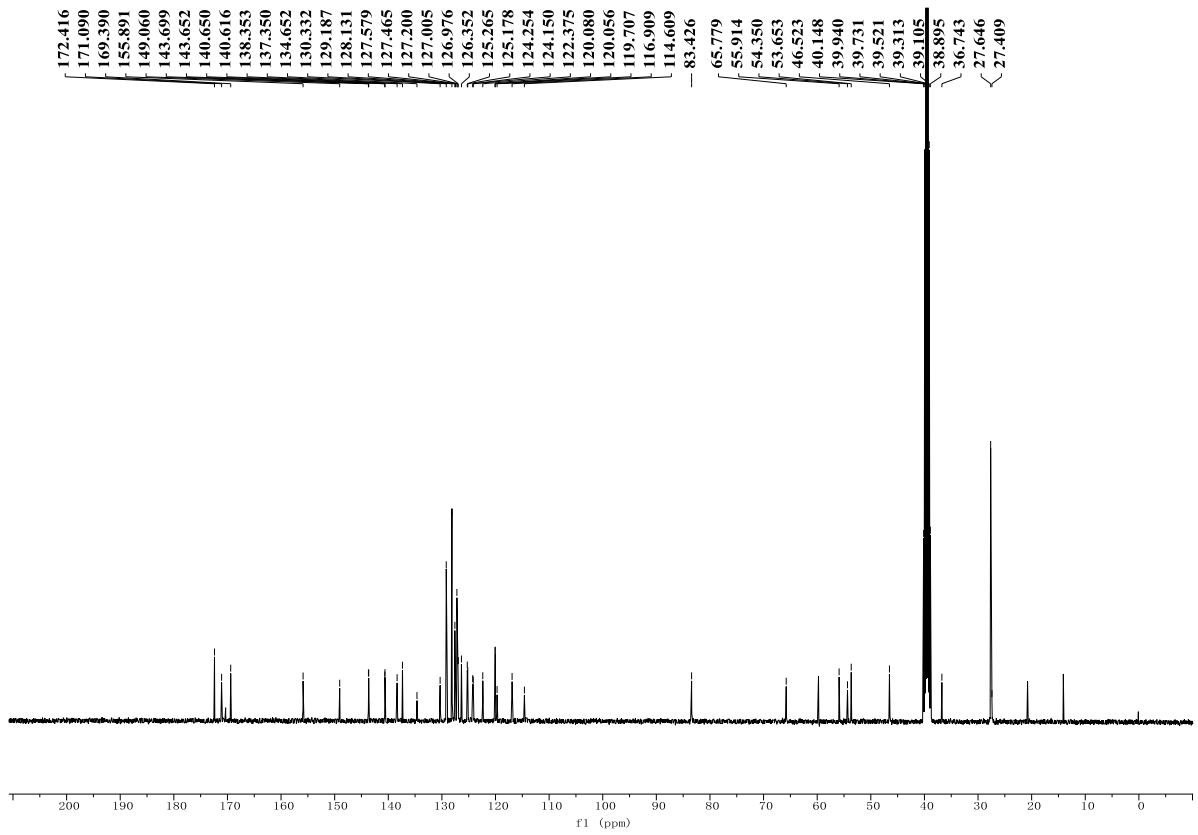
Fmoc-L-Cys(Trt)-L-Arg(Pbf)-L-Glu(5-CO_2*t*Bu)-OH (7i) (^{13}C NMR, 101 MHz, DMSO-d_6)



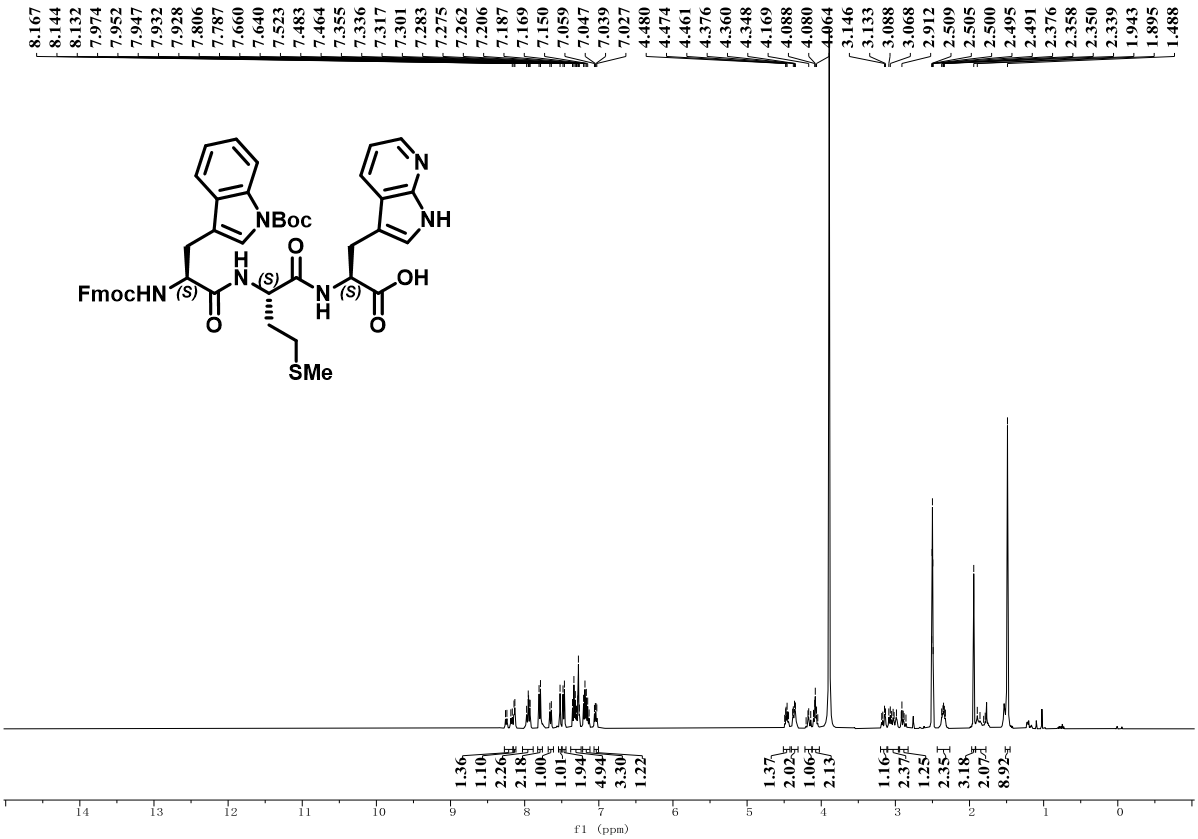
Fmoc-L-Trp(Boc)-L-Phg-L-Phe-OH (7j) (^{1}H NMR, 400 MHz, DMSO-d_6)



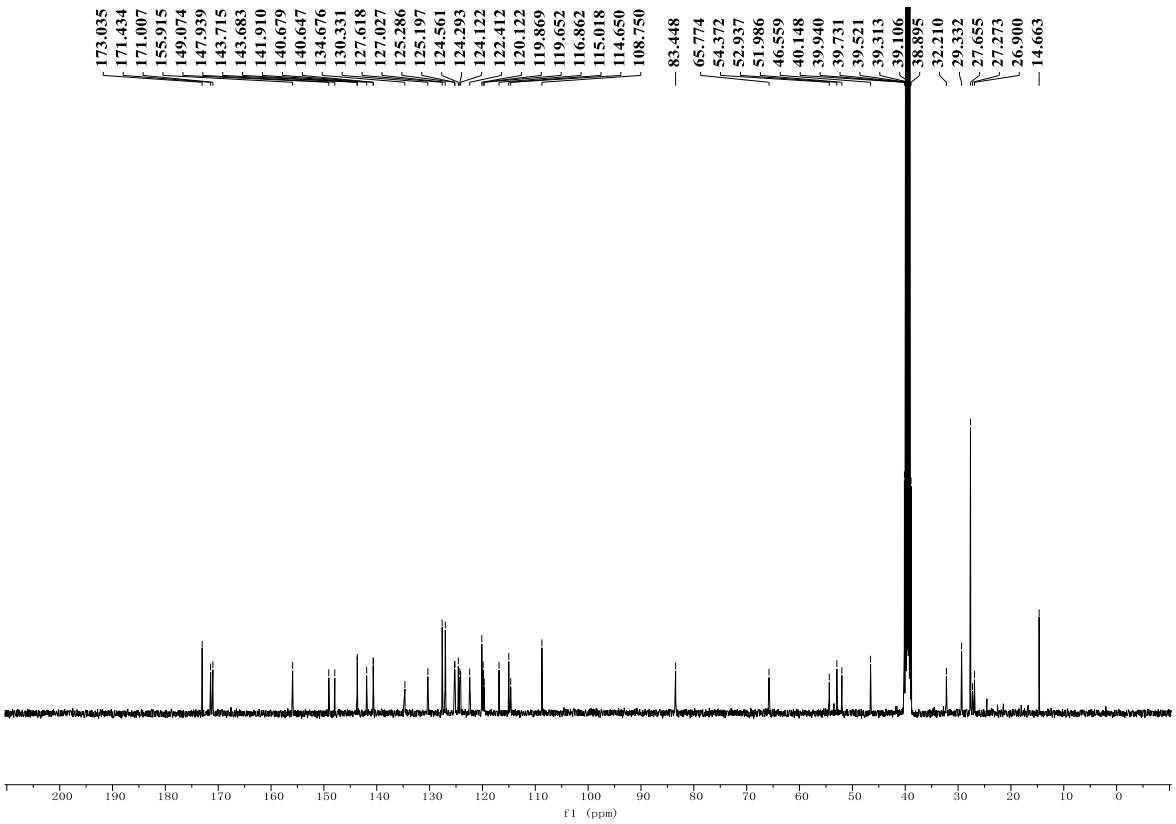
Fmoc-L-Trp(Boc)-L-Phg-L-Phe-OH (7j) (^{13}C NMR, 101 MHz, DMSO-d_6)



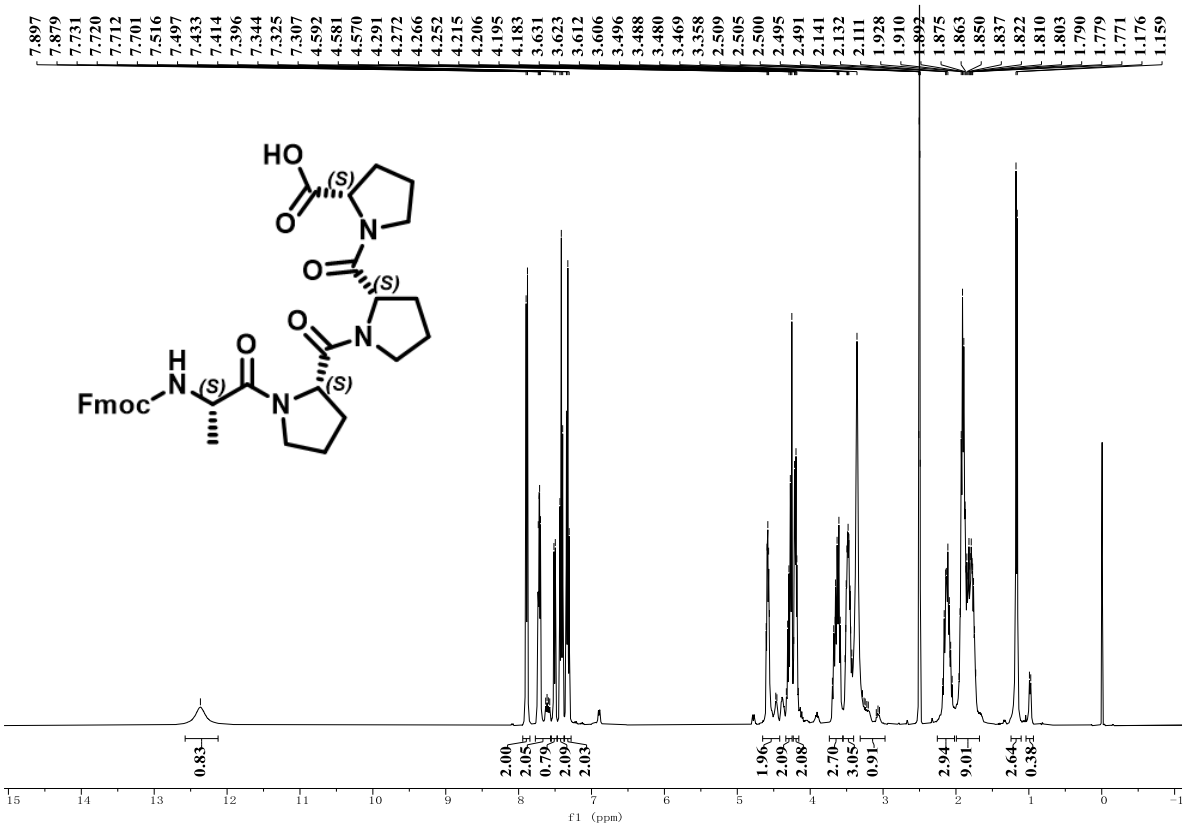
Fmoc-L-Trp(Boc)-L-Met-L-7-aza-Trp-OH (7k) (^{1}H NMR, 400 MHz, DMSO-d_6+D_2O)



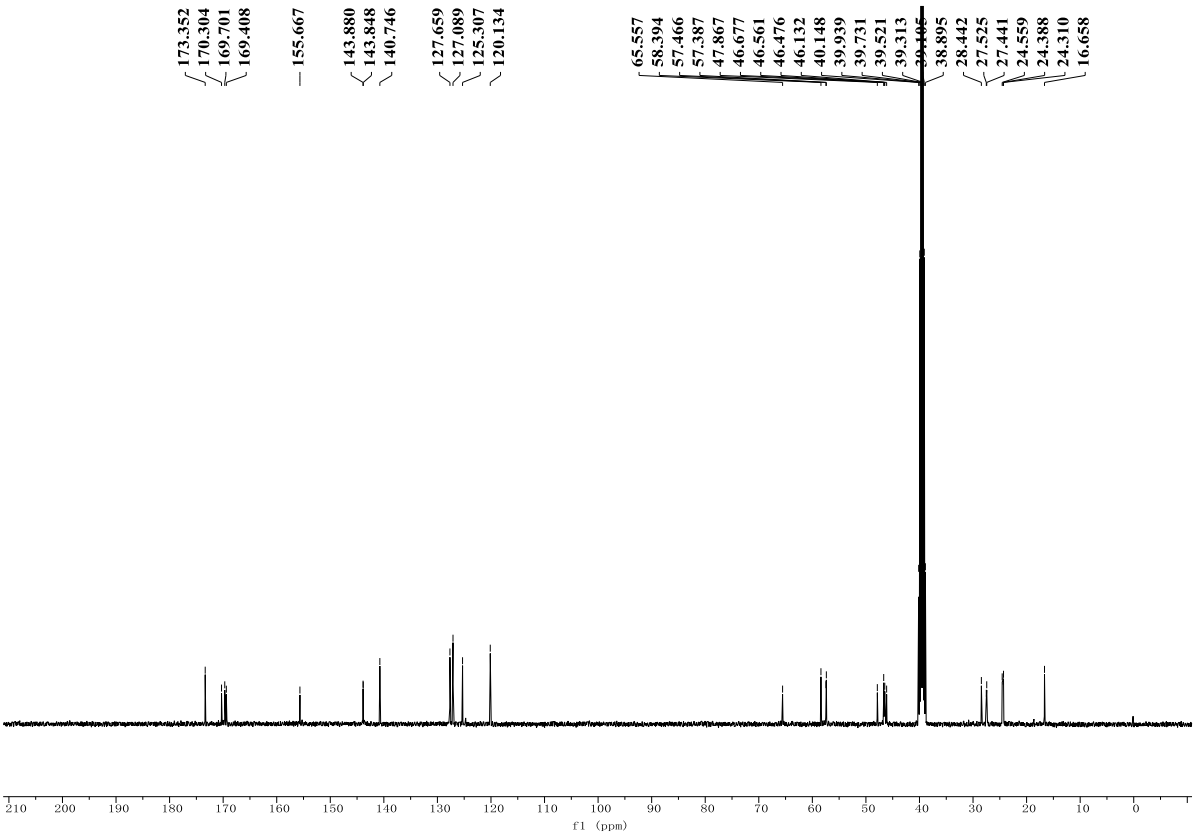
Fmoc-L-Trp(Boc)-L-Met-L-7-aza-Trp-OH (7k) (^{13}C NMR, 101 MHz, DMSO-d_6)



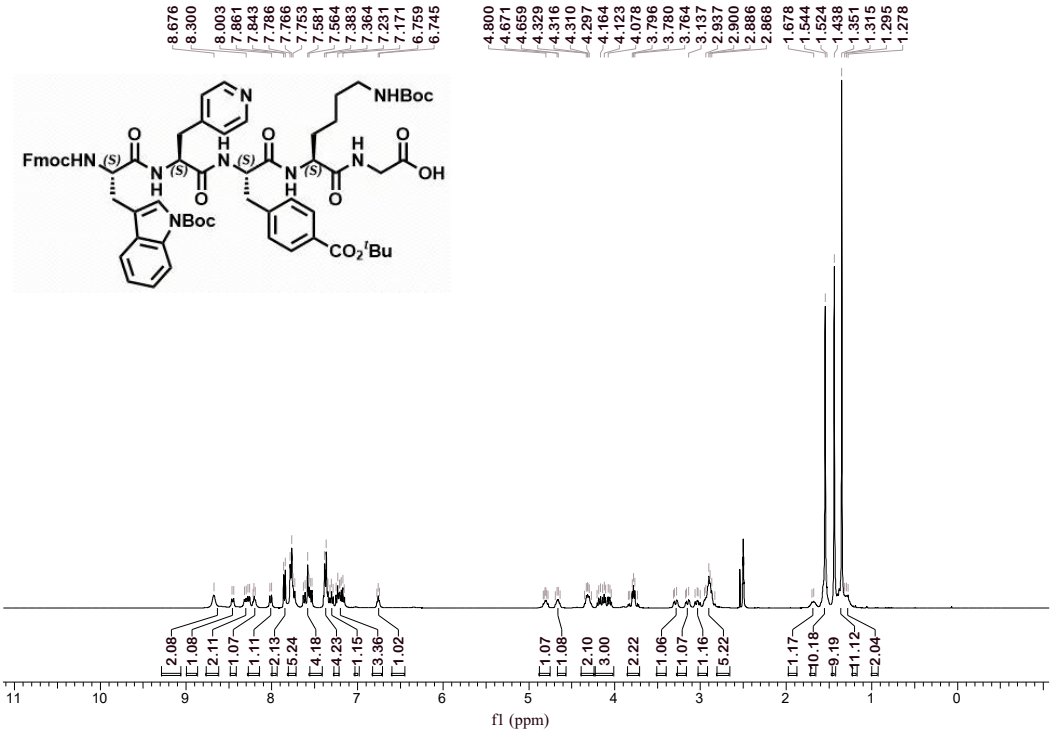
Fmoc-L-Ala-L-Pro-L-Pro-L-Pro-OH (7l) (^{1}H NMR, 400 MHz, DMSO-d_6)



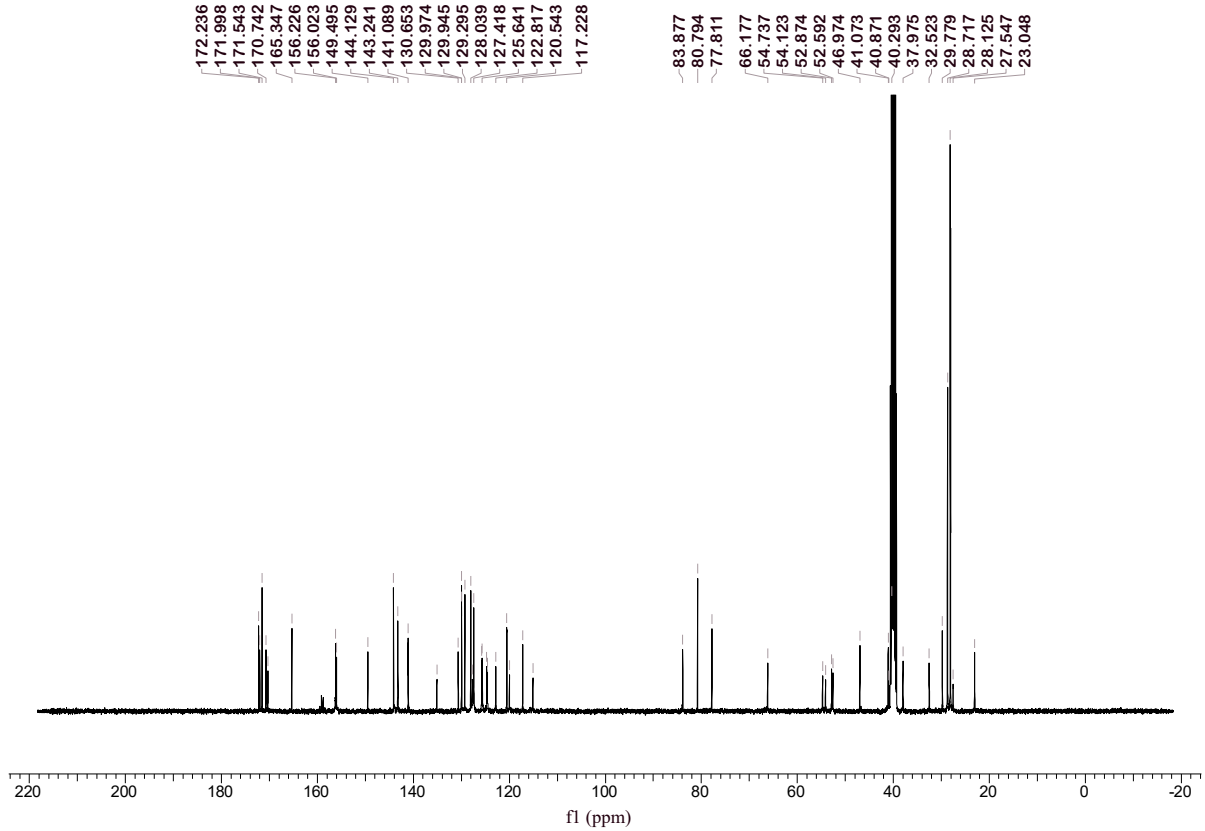
Fmoc-L-Ala-L-Pro-L-Pro-L-Pro-OH (7l) (^{13}C NMR, 101 MHz, DMSO-d_6)



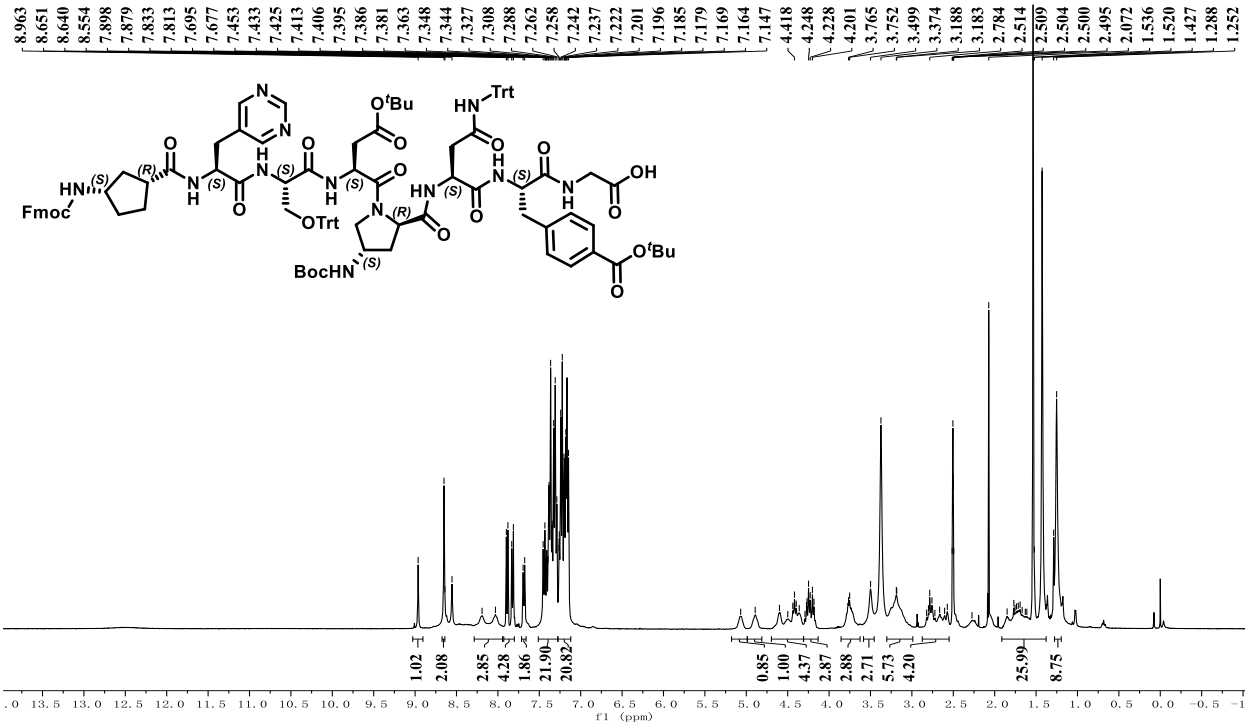
Fmoc-Trp(Boc)-Pal-pCO$_2$^{t}BuPhe-Lys(Boc)-Gly-OH (7m) (^{1}H NMR, 400 MHz, DMSO-d$_6$, 298 K)



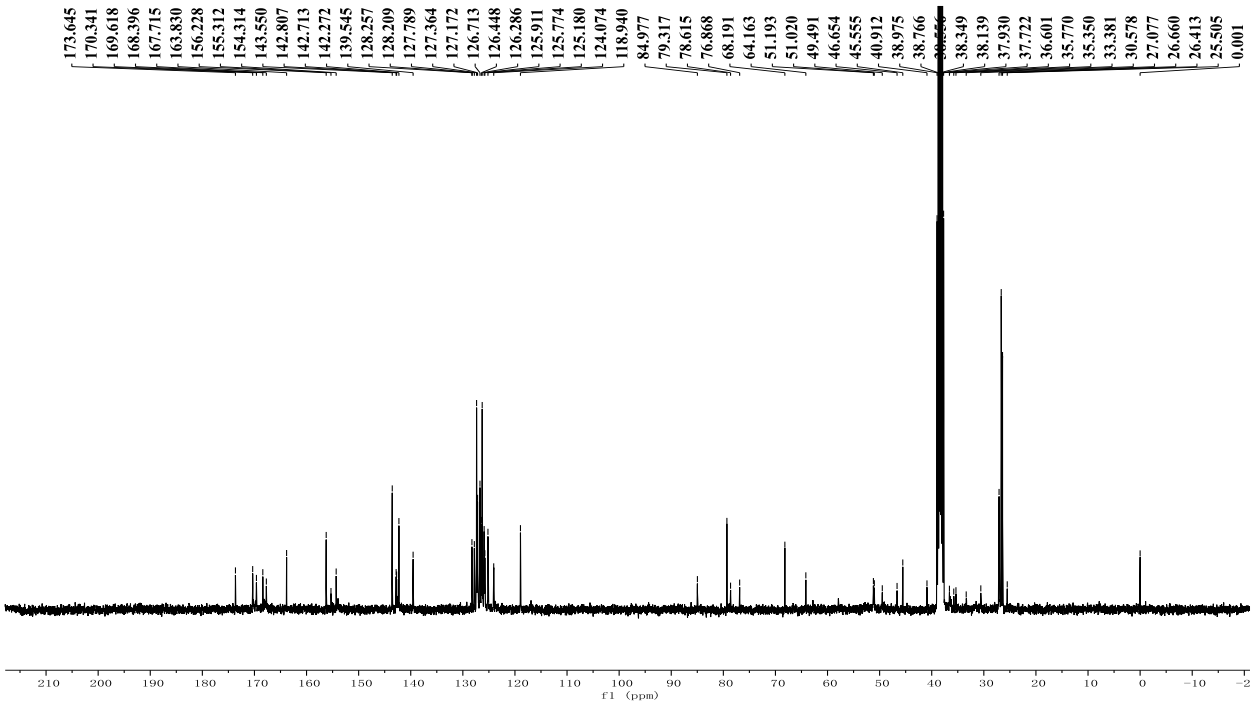
Fmoc-Trp(Boc)-Pal-pCO$_2$*t*BuPhe-Lys(Boc)-Gly-OH (7m) (^{13}C NMR, 101 MHz, DMSO-d$_6$, 298 K)



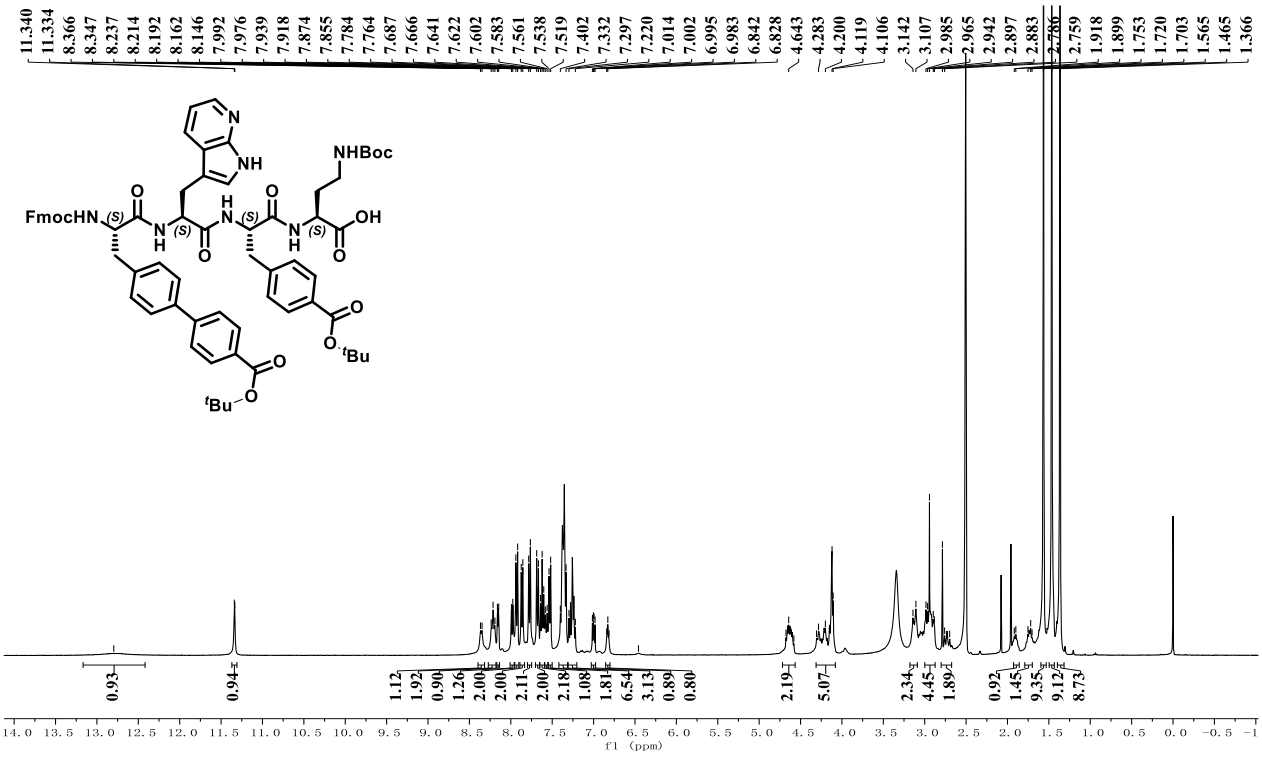
Fmoc-bhcLeu-3,5-pyrimi-Ala-L-Ser(Trt)-L-Asp(O*t*Bu)-D-Pro(*t*4NHBoc)-L-Asn(Trt)-L-Phe(O*t*Bu)-Gly-OH (7n) (^{1}H NMR, 400 MHz, DMSO-d_6)



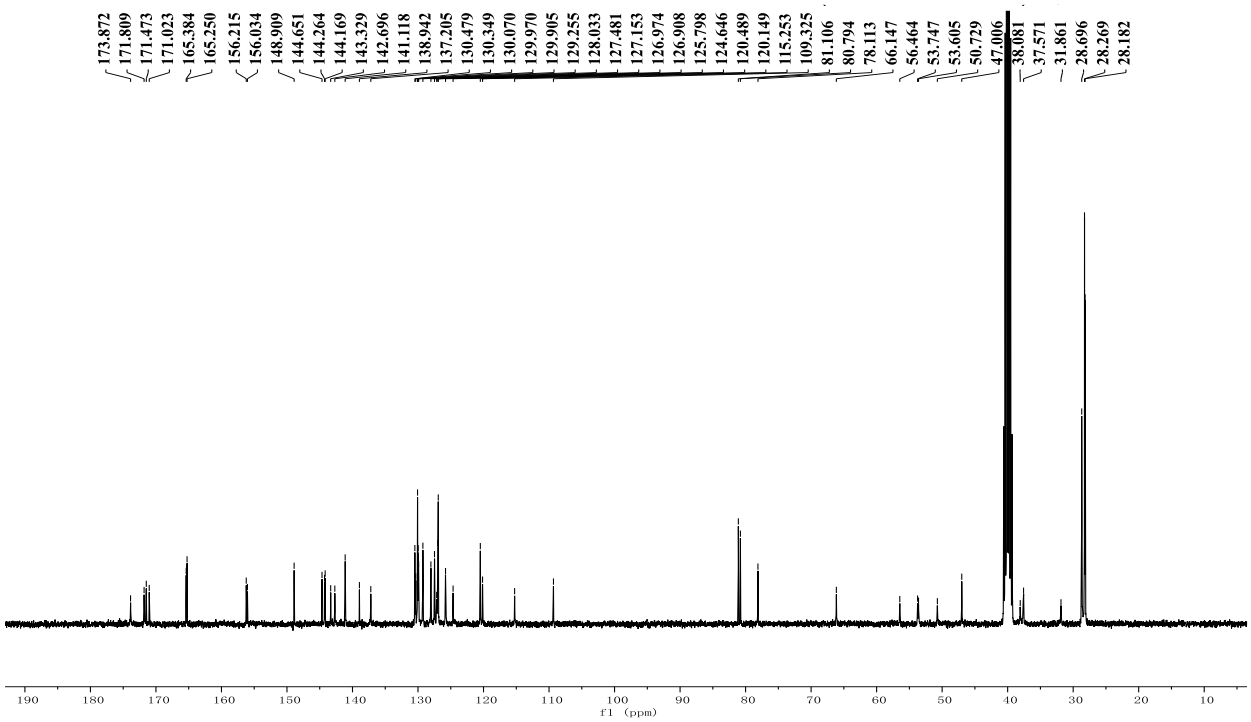
Fmoc-bhcLeu-3,5-pyrimi-Ala-L-Ser(Trt)-L-Asp(O*t*Bu)-D-Pro(*t*4NHBoc)-L-Asn(Trt)-L-Phe(O*t*Bu)-Gly-OH (7n) (^{13}C NMR, 101 MHz, DMSO-d_6)



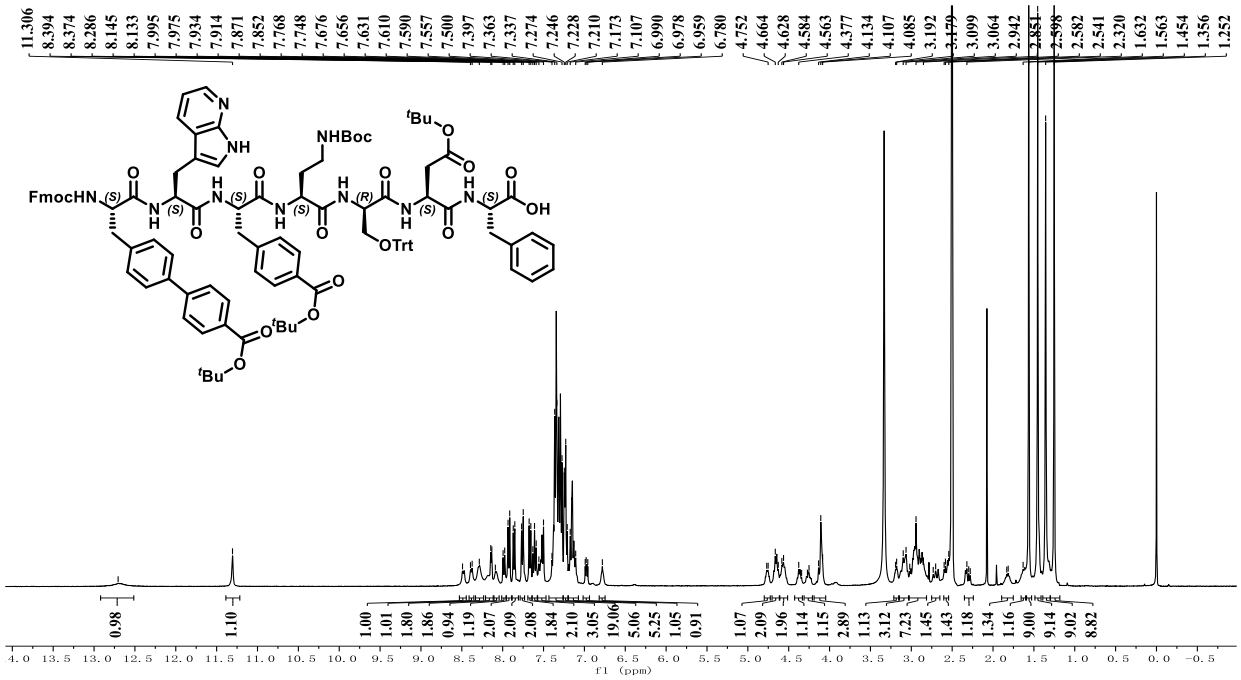
Fmoc-Bip-4-CO$_2$*t*Bu-7-aza-Trp-4-CO$_2$*t*BuPhe-L-Dab(Boc)-OH (26) (^{1}H NMR, 400 MHz, DMSO-d_6)



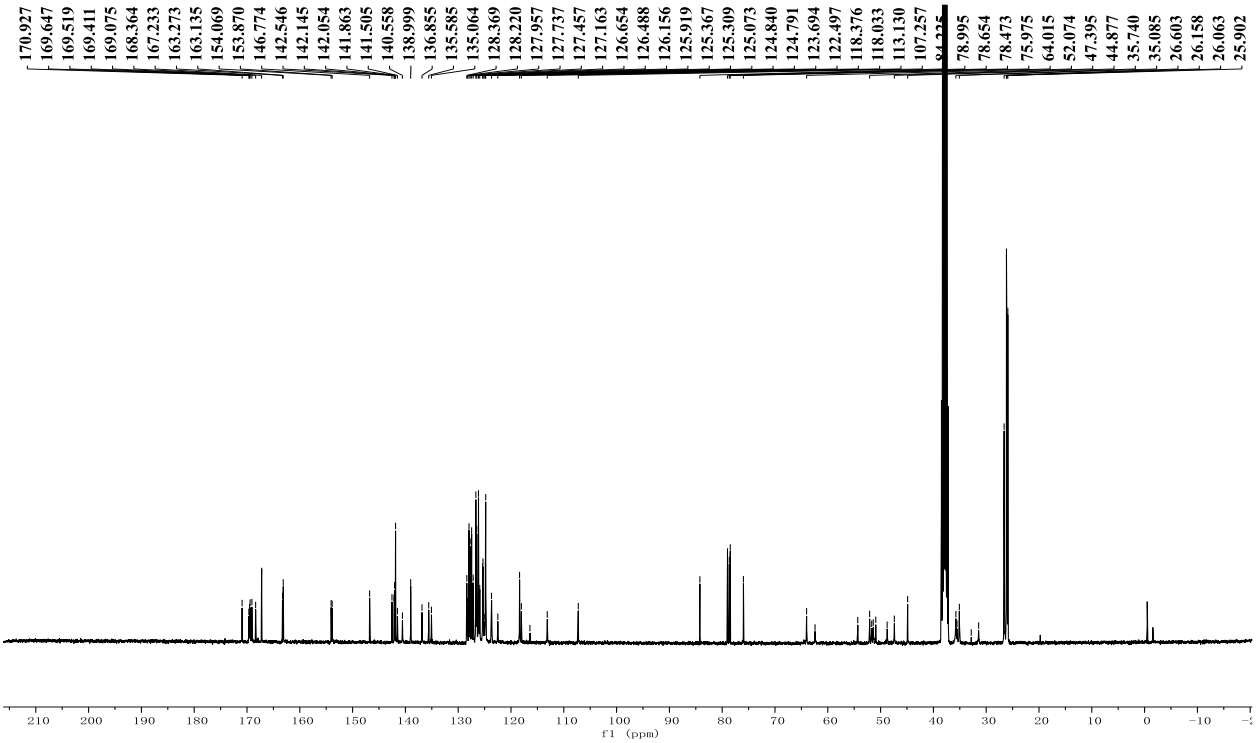
Fmoc-Bip-4-CO$_2$*t*Bu-7-aza-Trp-4-CO$_2$*t*BuPhe-L-Dab(Boc)-OH (26) (^{13}C NMR, 101 MHz, DMSO-d_6)



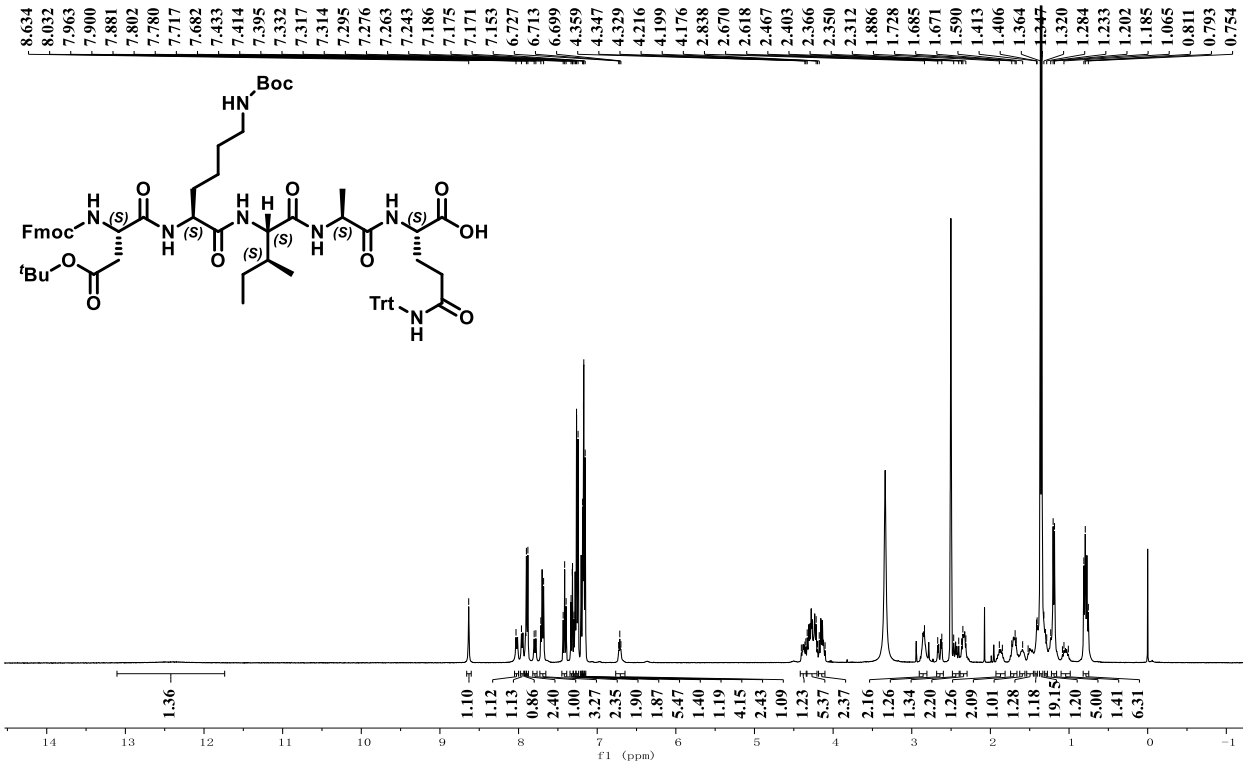
Fmoc-Bip-4-CO$_2$*t*Bu-7-aza-Trp-L-pCO$_2$*t*BuPhe-L-Dab(Boc)-D-Ser(Trt)-L-Asp(O*t*Bu)-L-Phe-OH (8) (^{1}H NMR, 400 MHz, DMSO-d_6)



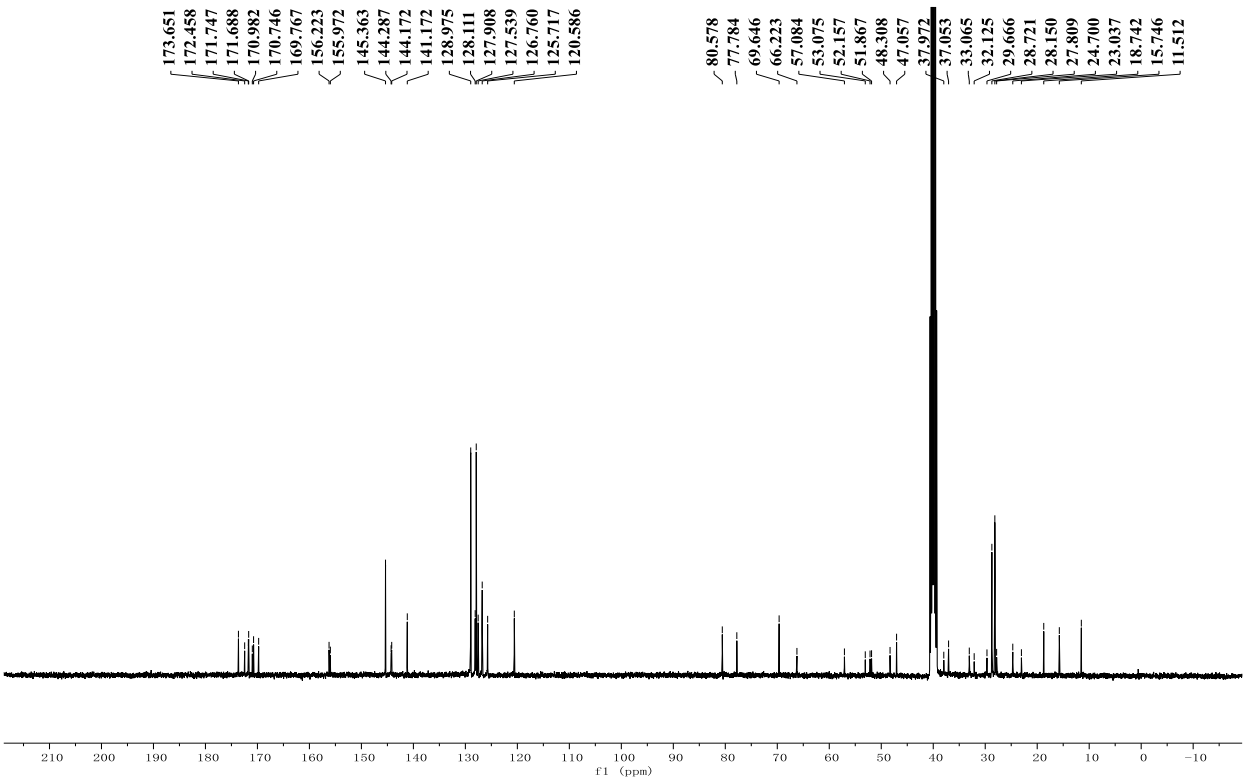
Fmoc-Bip-4-CO$_2$*t*Bu-7-aza-Trp-L-pCO$_2$*t*BuPhe-L-Dab(Boc)-D-Ser(Trt)-L-Asp(O*t*Bu)-L-Phe-OH (8) (^{13}C NMR, 101 MHz, DMSO-d_6)



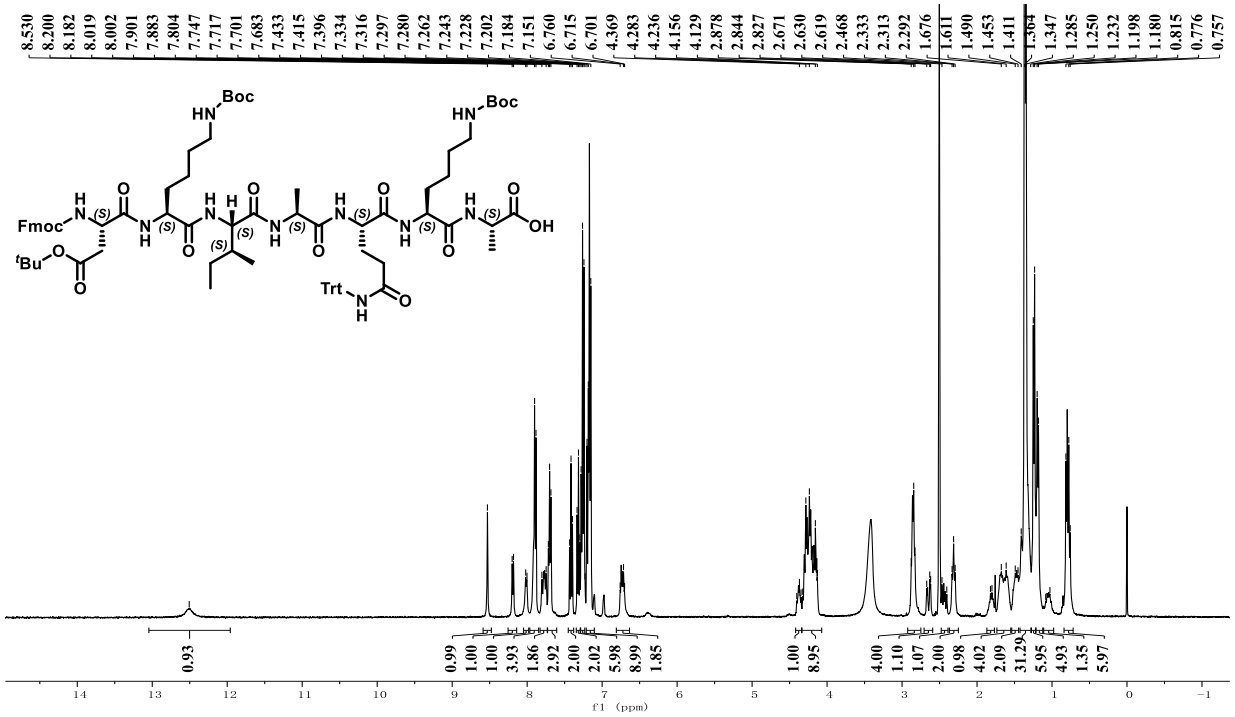
Fmoc-L-Asp(O*t*Bu)-L-Lys(Boc)-L-Lle-L-Ala-L-Gln(Trt)-OH (27) (^{1}H NMR, 400 MHz, DMSO-d_6)



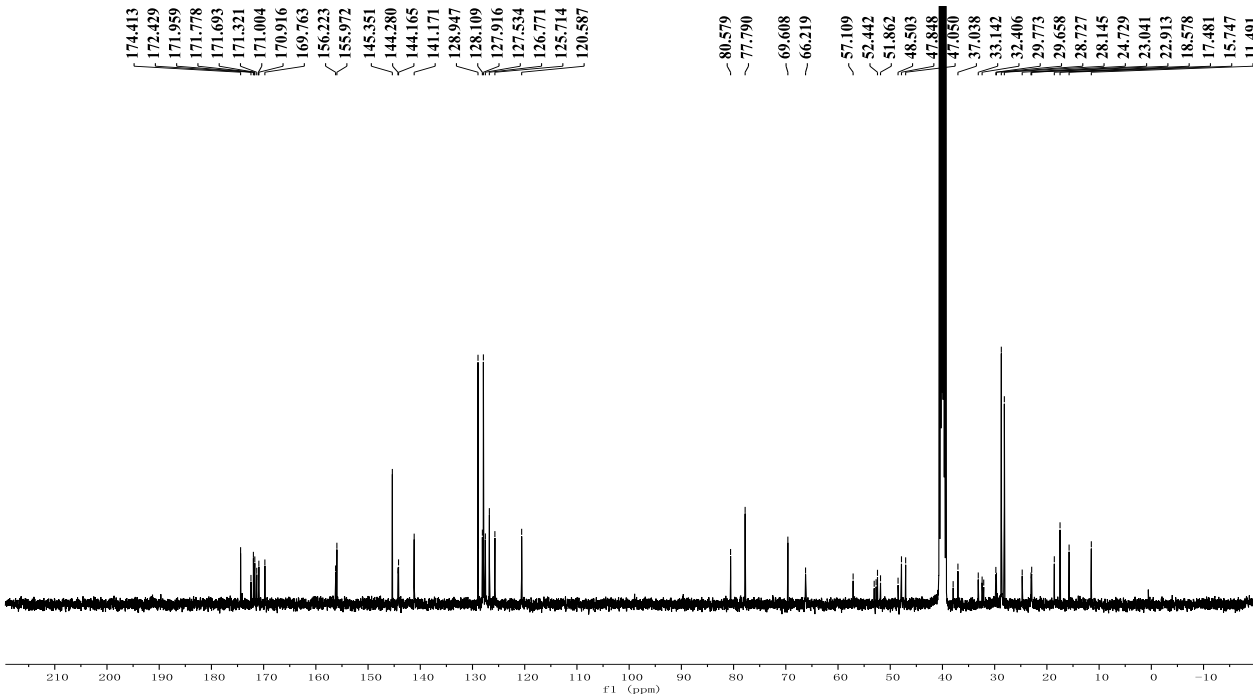
Fmoc-L-Asp(O*t*Bu)-L-Lys(Boc)-L-Lle-L-Ala-L-Gln(Trt)-OH (27) (^{13}C NMR, 101 MHz, DMSO-d_6)



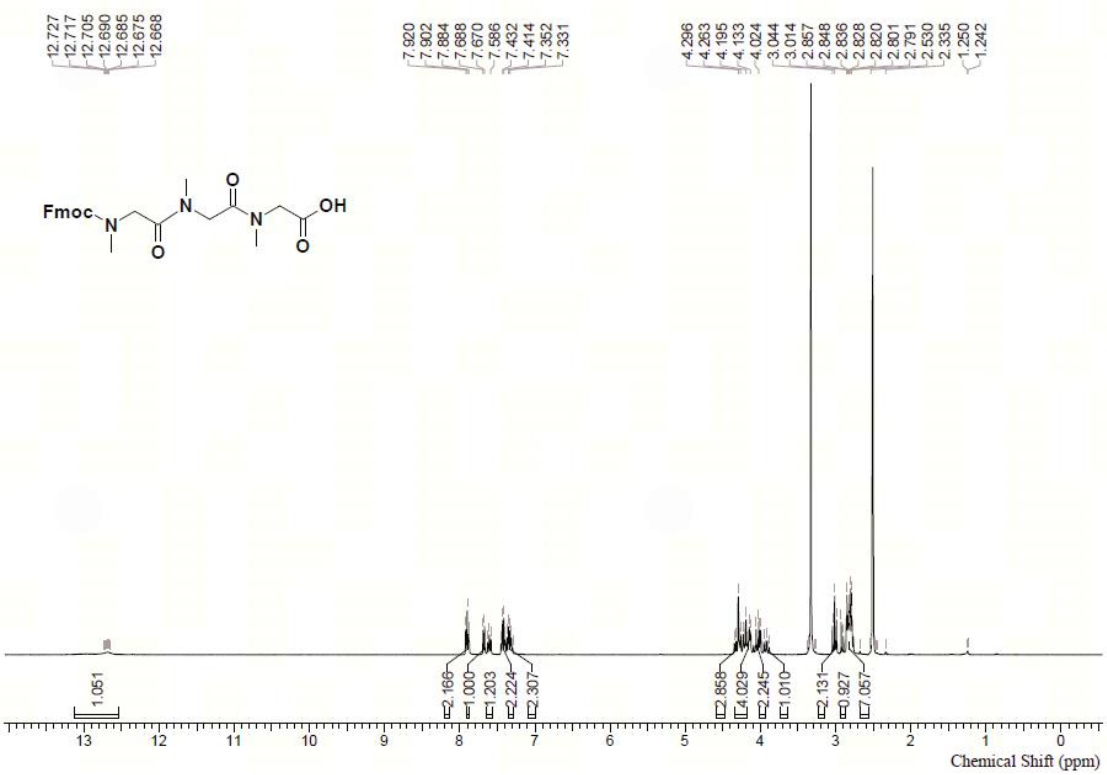
Fmoc-L-Asp(O*t*Bu)-L-Lys(Boc)-L-Lle-L-Ala-L-Gln(Trt)-L-Lys(Boc)-L-Ala-OH (9) (^{1}H NMR, 400 MHz, DMSO-d_6)



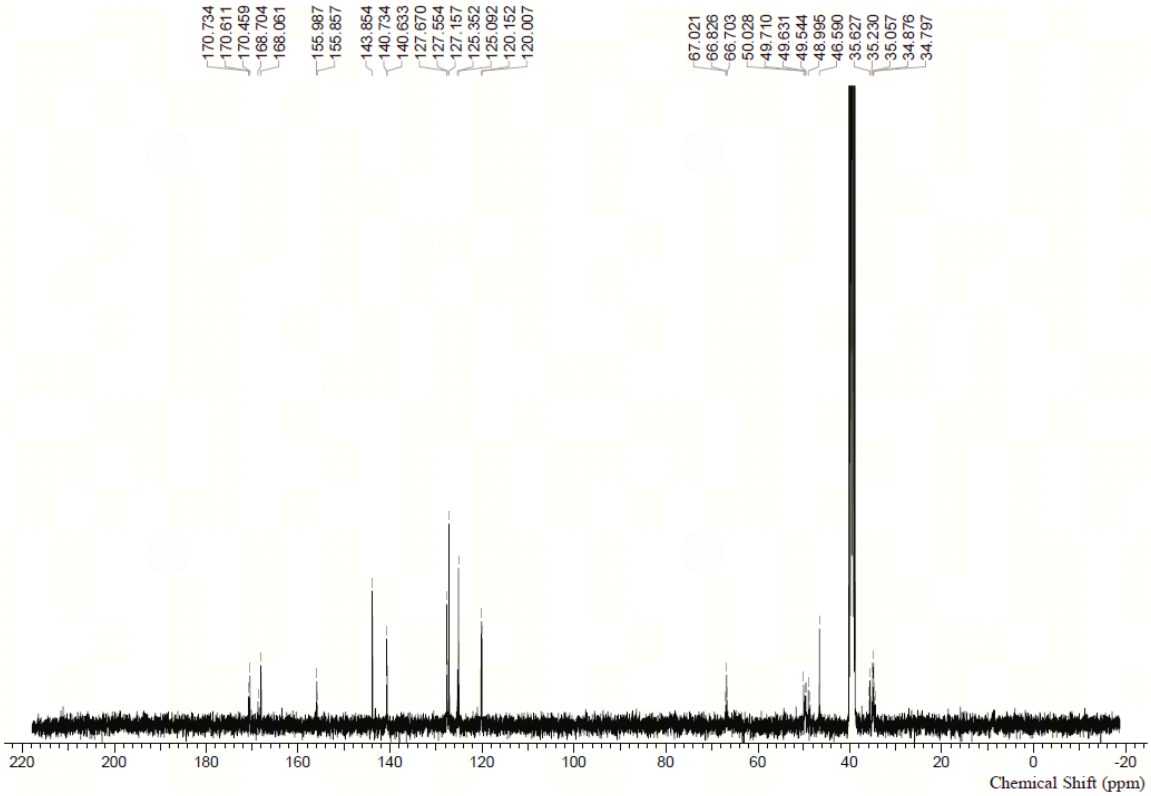
Fmoc-L-Asp(O*t*Bu)-L-Lys(Boc)-L-Lle-L-Ala-L-Gln(Trt)-L-Lys(Boc)-L-Ala-OH (9) (^{13}C NMR, 101 MHz, DMSO-d_6)



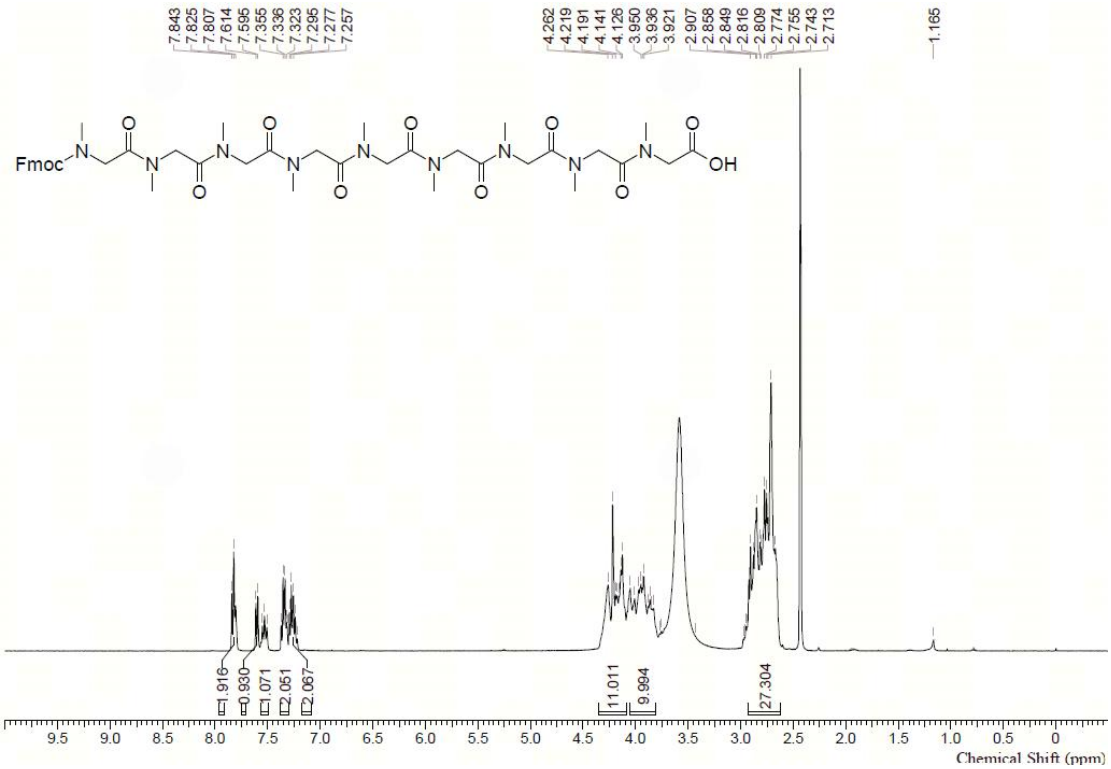
Fmoc-Sar-Sar-Sar-OH (S59) (^{1}H NMR, 400 MHz, DMSO-d6, 298 K)



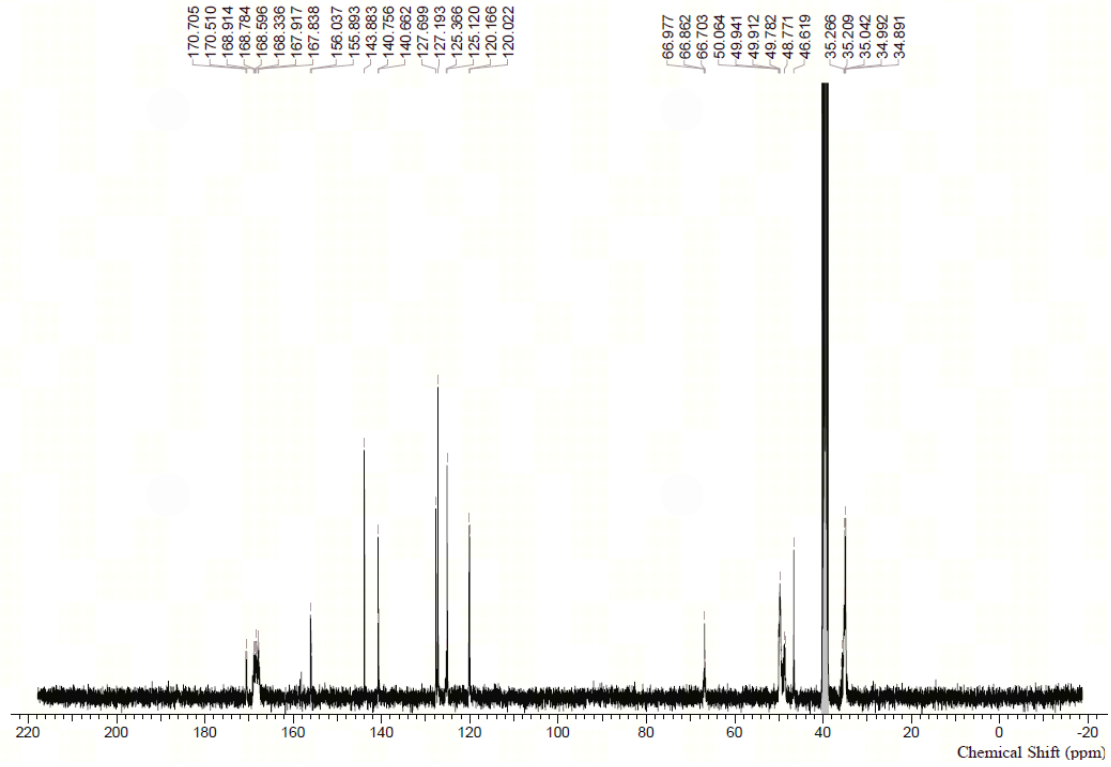
Fmoc-Sar-Sar-Sar-OH (S59) (^{13}C NMR, 101 MHz, DMSO-d6, 298 K)



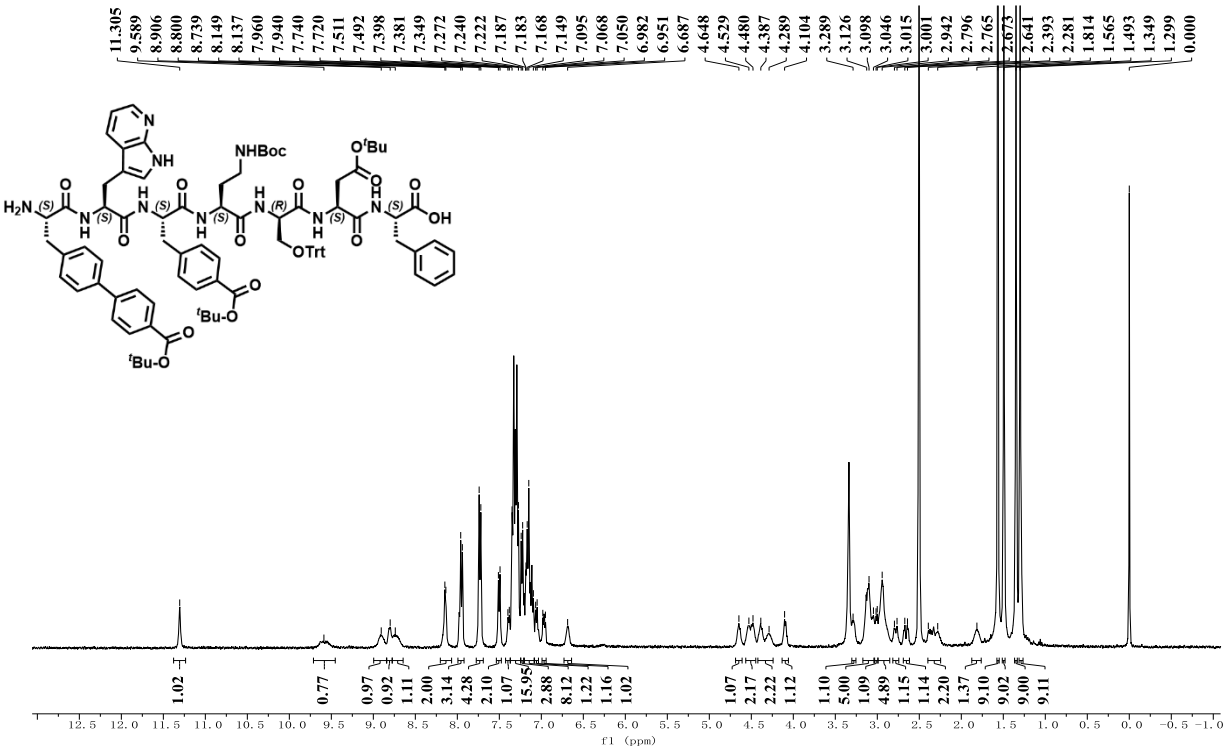
Fmoc-Sar-Sar-Sar-Sar-Sar-Sar-Sar-Sar-Sar-OH (10) (^{1}H NMR, 400 MHz, DMSO-d6, 298 K)



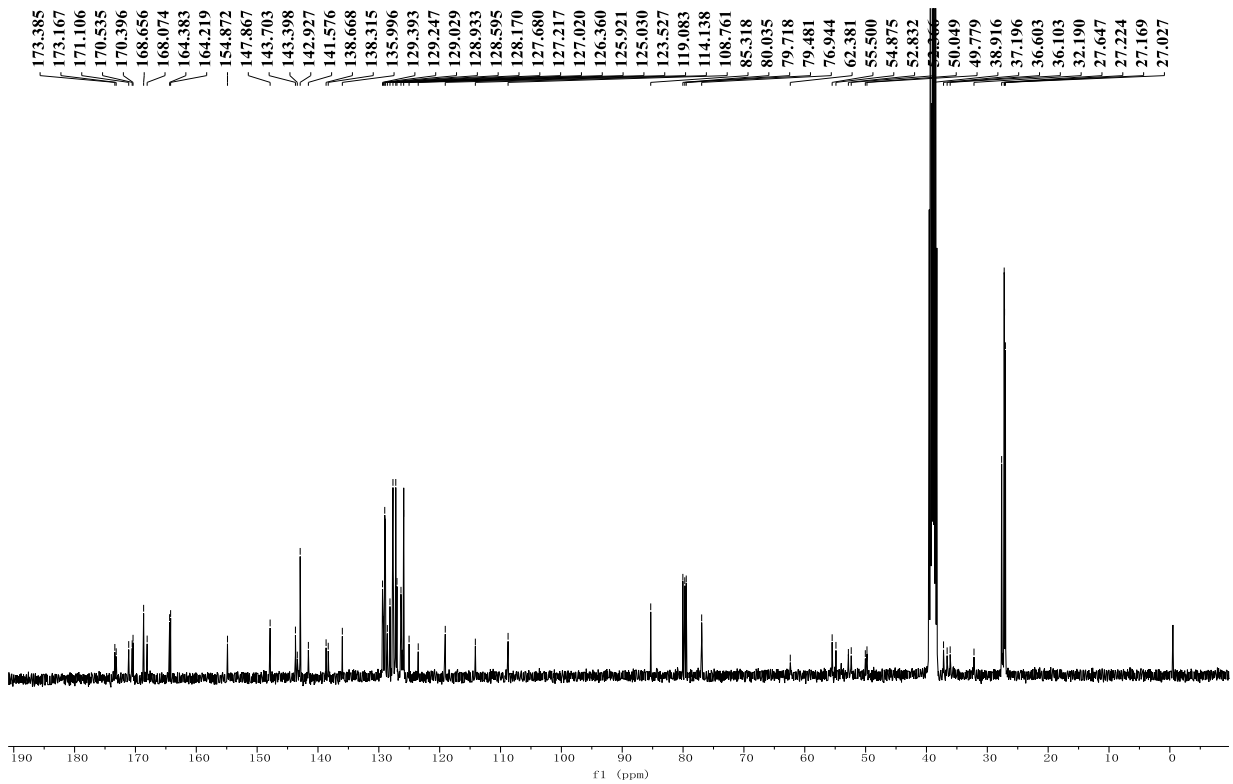
Fmoc-Sar-Sar-Sar-Sar-Sar-Sar-Sar-Sar-Sar-OH (10) (^{13}C NMR, 101 MHz, DMSO-d6, 298 K)



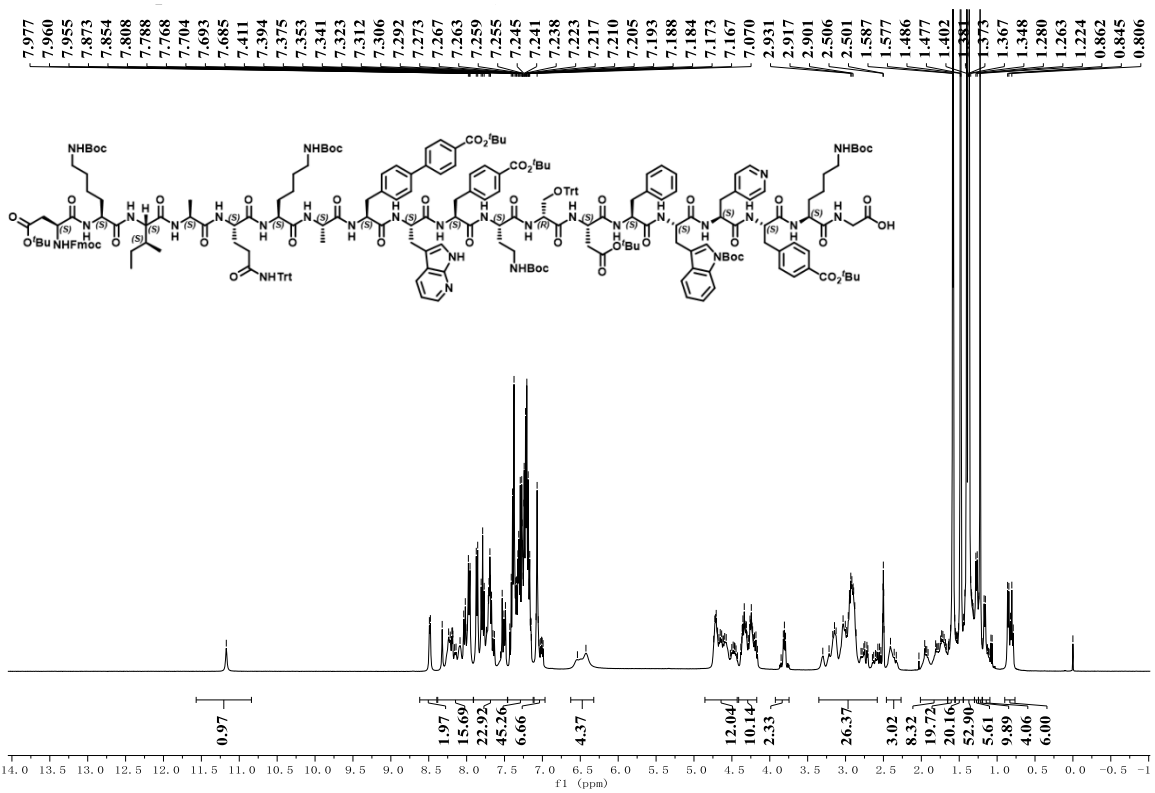
Bip-4-CO$_2$*t*Bu-7-aza-Trp-L-Phe(O*t*Bu)-L-Dab(Boc)-D-Ser(Trt)-L-Asp(O*t*Bu)-L-Phe (S62) (^{1}H NMR, 400 MHz, DMSO-d_6)



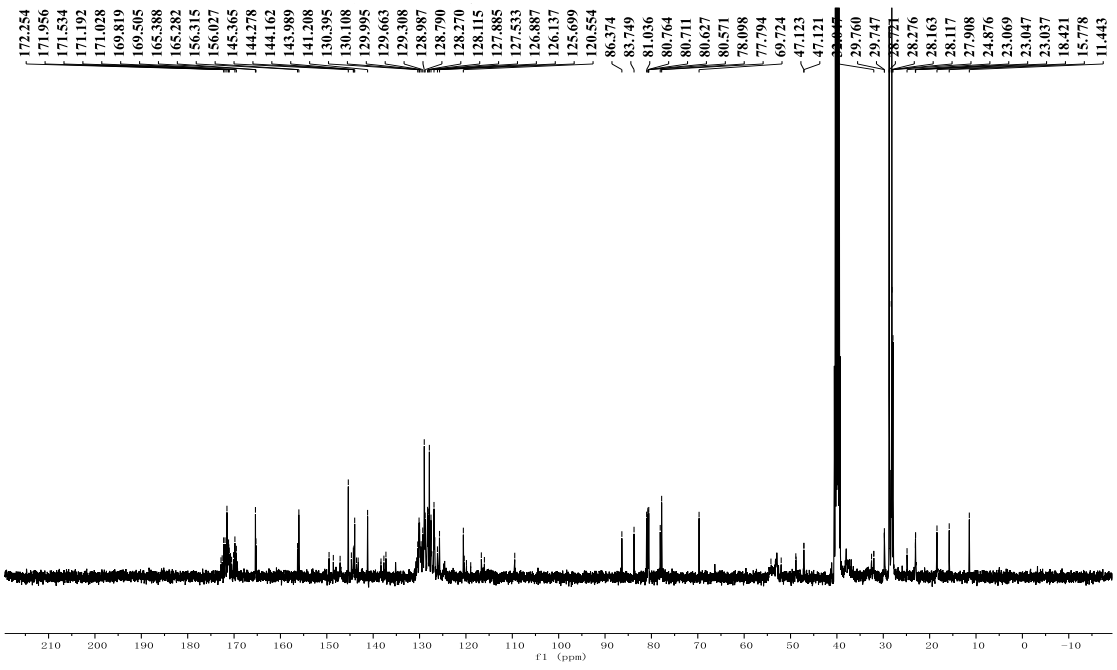
Synthesis of Bip-4-CO$_2$tBu-7-aza-Trp-L-Phe(OtBu)-L-Dab(Boc)-D-Ser(Trt)-L-Asp(OtBu)-L-Phe (9a, 7-mer) (^{13}C NMR, 101 MHz, DMSO-d_6)



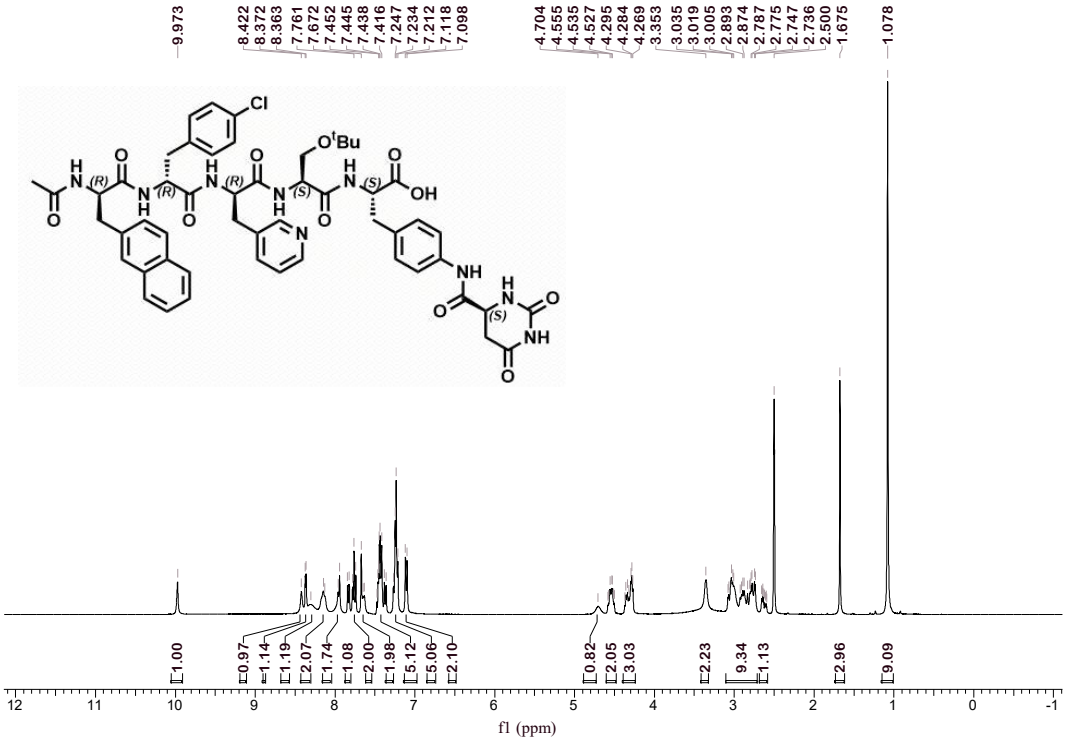
Fmoc-L-Asp(O*t*Bu)-L-Lys(Boc)-L-Lle-L-Ala-L-Gln(Trt)-L-Lys(Boc)-L-Ala-Bip-4-CO_2*t*Bu-7-aza-Trp-L-Phe(O*t*Bu)-L-Dab(Boc)-D-Ser(Trt)-L-Asp(O*t*Bu)-L-Phe-L-Trp(Boc)-L-Ala(4-PYRI)-L-Phe(O*t*Bu)-L-Lys(Boc)-Gly (S65, 19-mer) (^{1}H NMR, 400 MHz, DMSO-d_6)



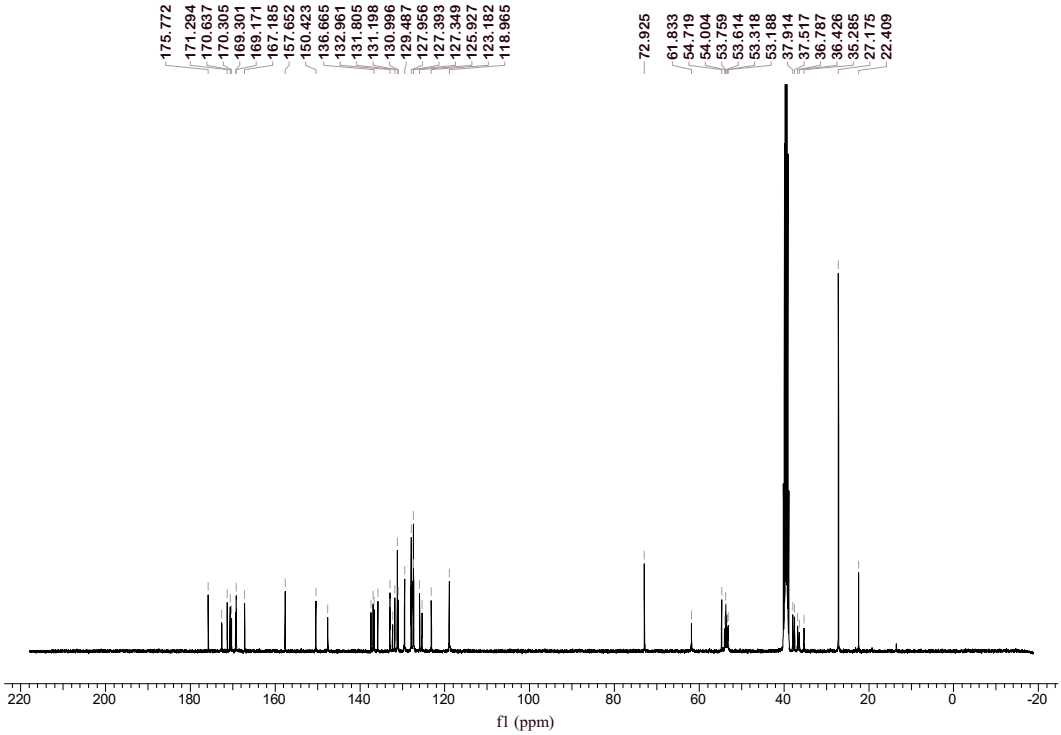
Fmoc-L-Asp(O*t*Bu)-L-Lys(Boc)-L-Lle-L-Ala-L-Gln(Trt)-L-Lys(Boc)-L-Ala-Bip-4-CO_2*t*Bu-7-aza-Trp-L-Phe(O*t*Bu)-L-Dab(Boc)-D-Ser(Trt)-L-Asp(O*t*Bu)-L-Phe-L-Trp(Boc)-L-Ala(4-PYRI)-L-Phe(O*t*Bu)-L-Lys(Boc)-Gly (S65, 19-mer) (^{13}C NMR, 101 MHz, DMSO-d_6)



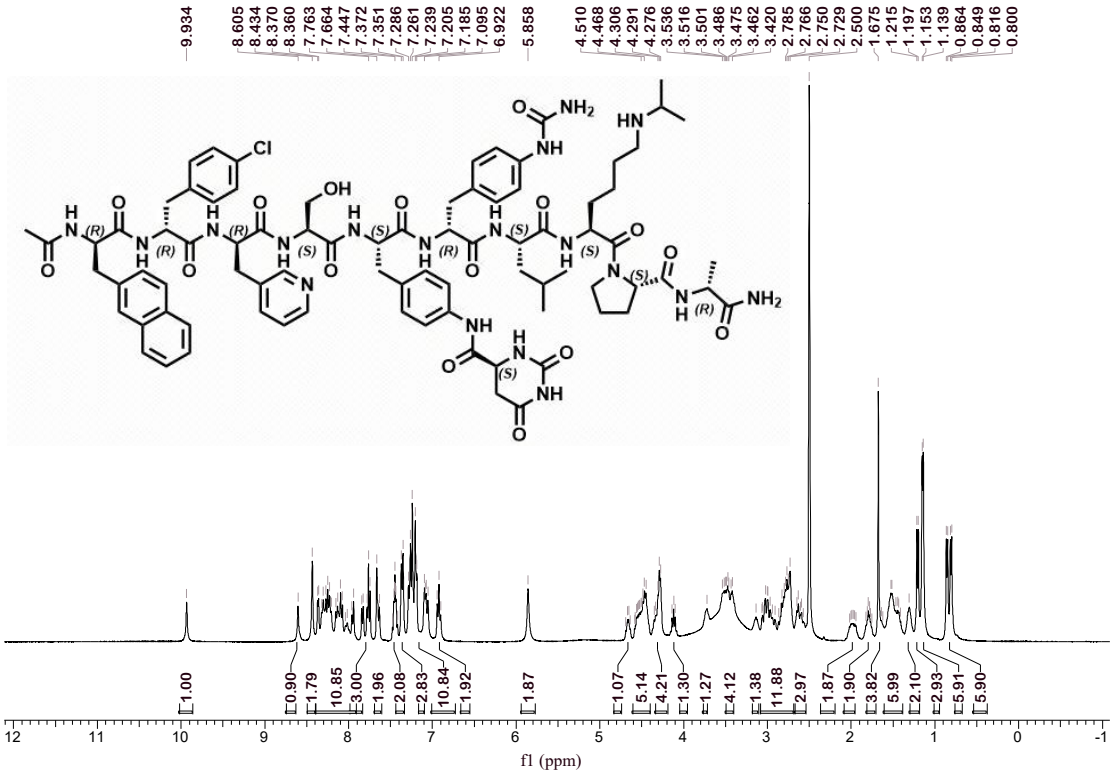
Ac-D-2-Nal-D-Phe(4-Cl)-3-D-Pal-Ser(*t*Bu)-Aph(Hor)-OH (**12**) (^{1}H NMR, 400 MHz, DMSO-d_6, 298 K)



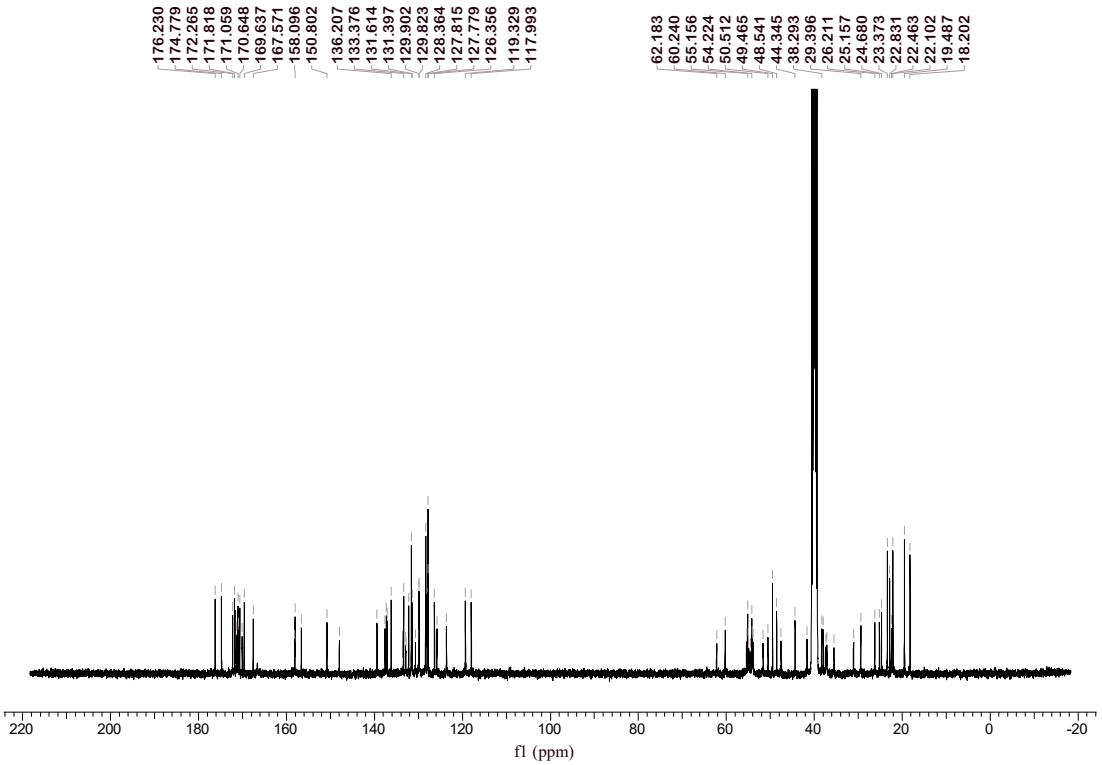
Ac-D-2-Nal-D-Phe(4-Cl)-3-D-Pal-Ser(*t*Bu)-Aph(Hor)-OH (**12**) (^{13}C NMR, 101 MHz, DMSO-d_6, 298 K)



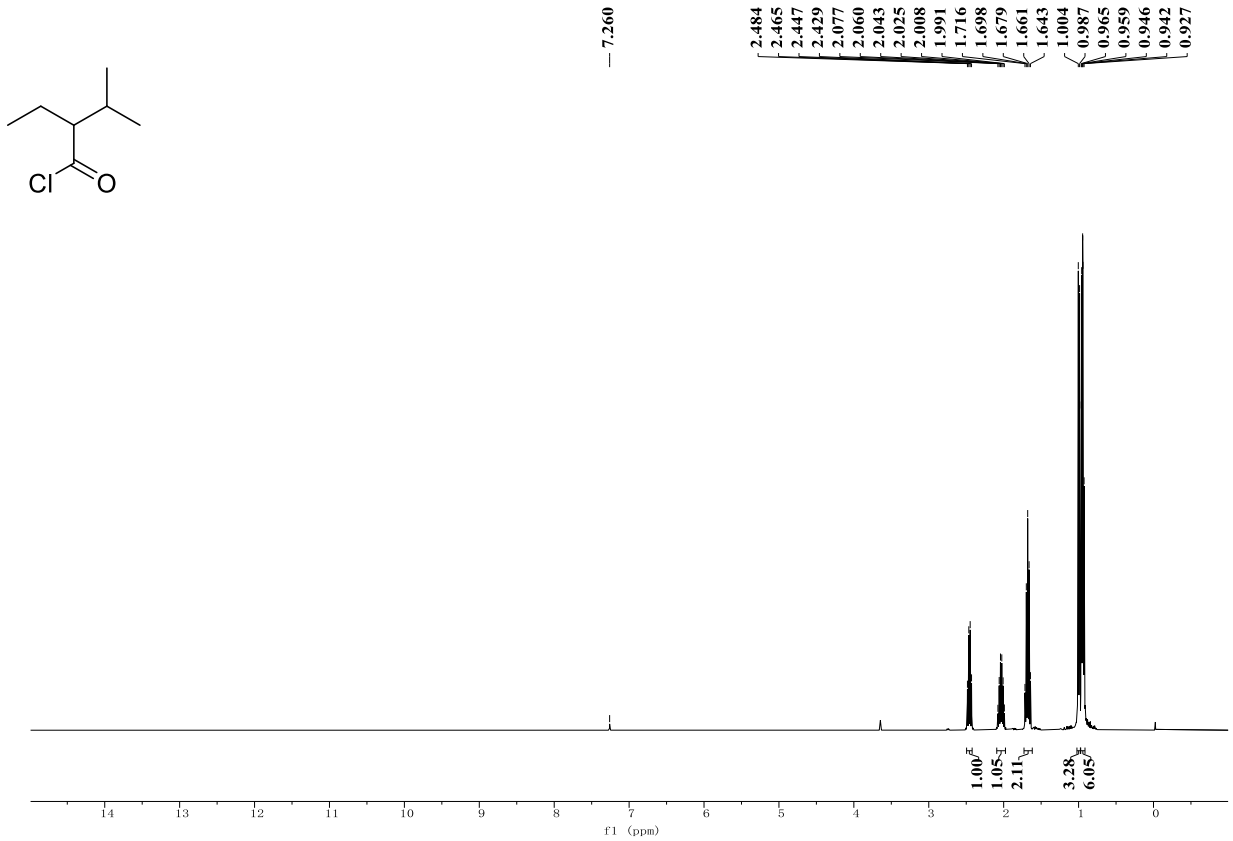
Ac-D-2-Nal-D-Phe(4-Cl)-3-D-Pal-Ser-Aph(Hor)-D-Phe(4-NHCONH$_2$)-Leu-Lys(IPr)-Pro-D-Ala-NH$_2$ (degarelix 14) (^{1}H NMR, 400 MHz, DMSO-d$_6$, 298 K)



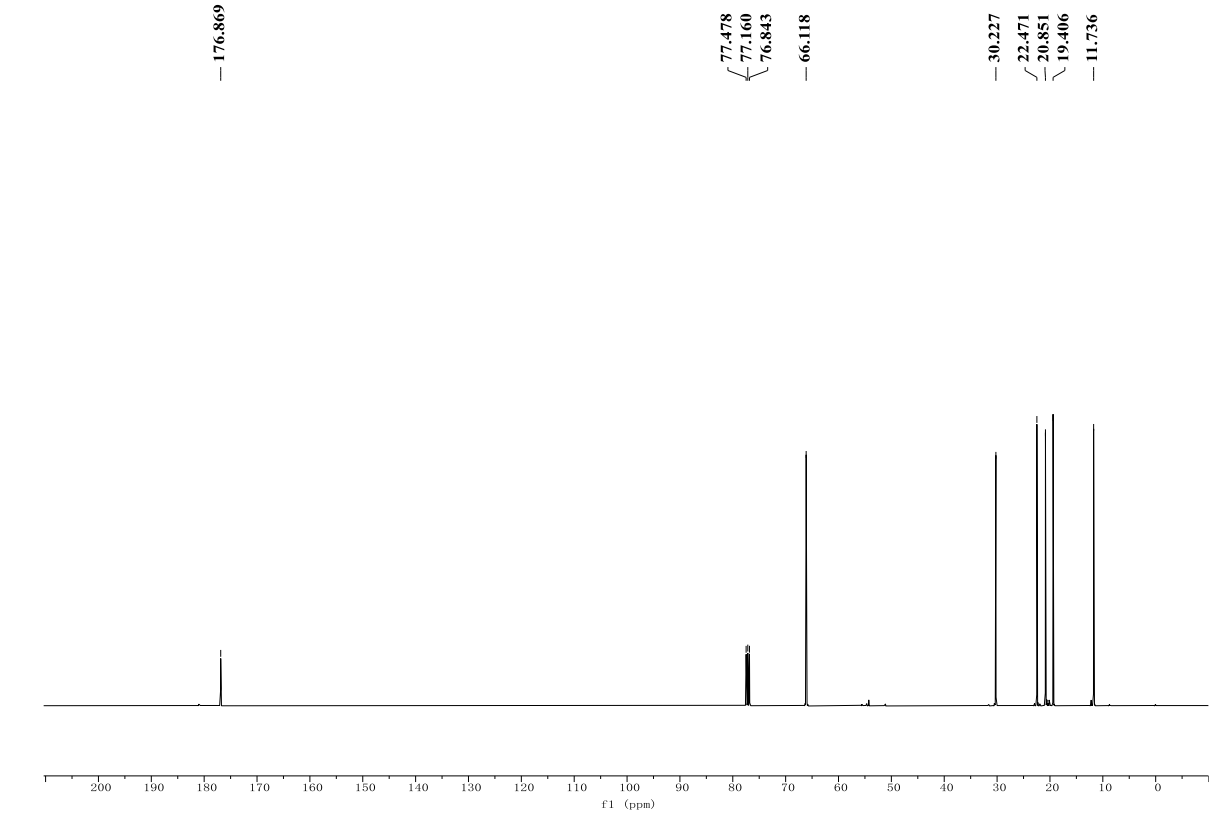
Ac-D-2-Nal-D-Phe(4-Cl)-3-D-Pal-Ser-Aph(Hor)-D-Phe(4-NHCONH$_2$)-Leu-Lys(IPr)-Pro-D-Ala-NH$_2$ (dagarelix 14) (^{13}C NMR, 101 MHz, DMSO-d$_6$, 298 K)



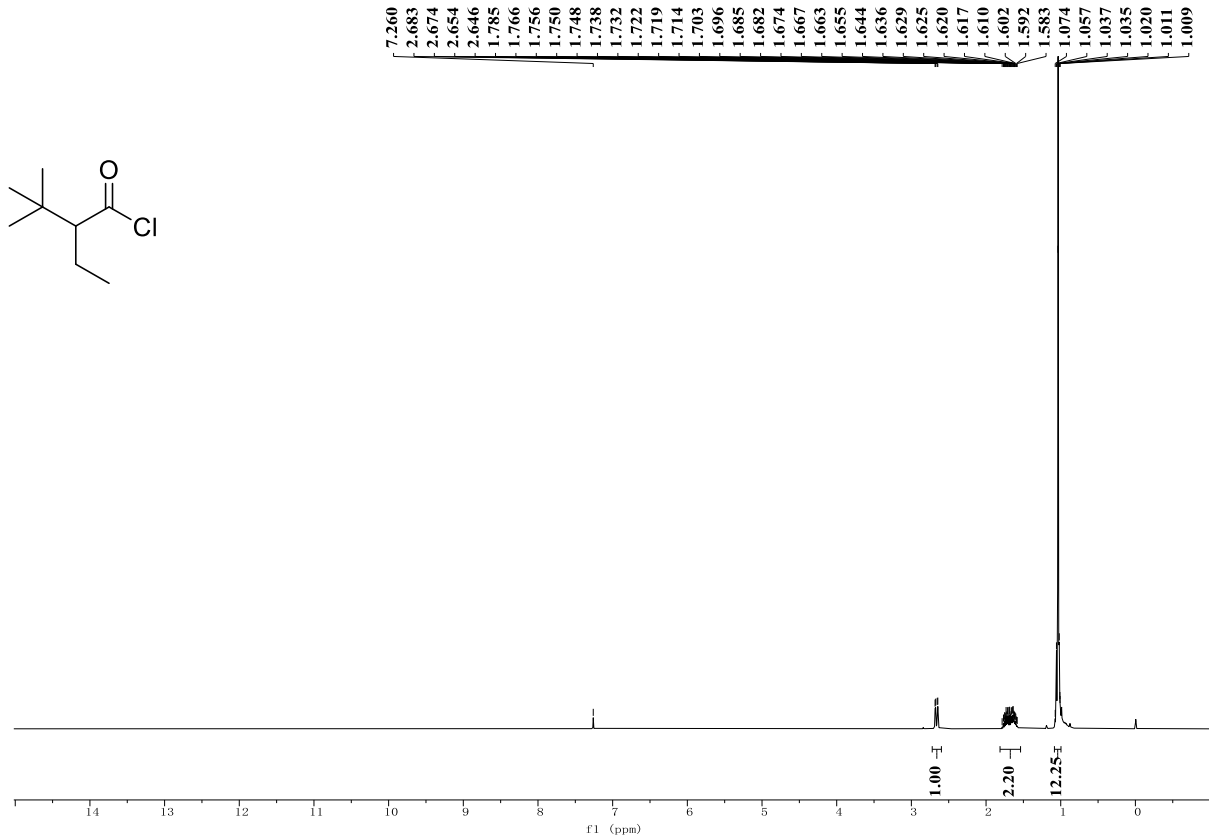
2-ethyl-3-methylbutanoyl chloride (R1) (^{1}H NMR, 400 MHz, $CDCl_3$)



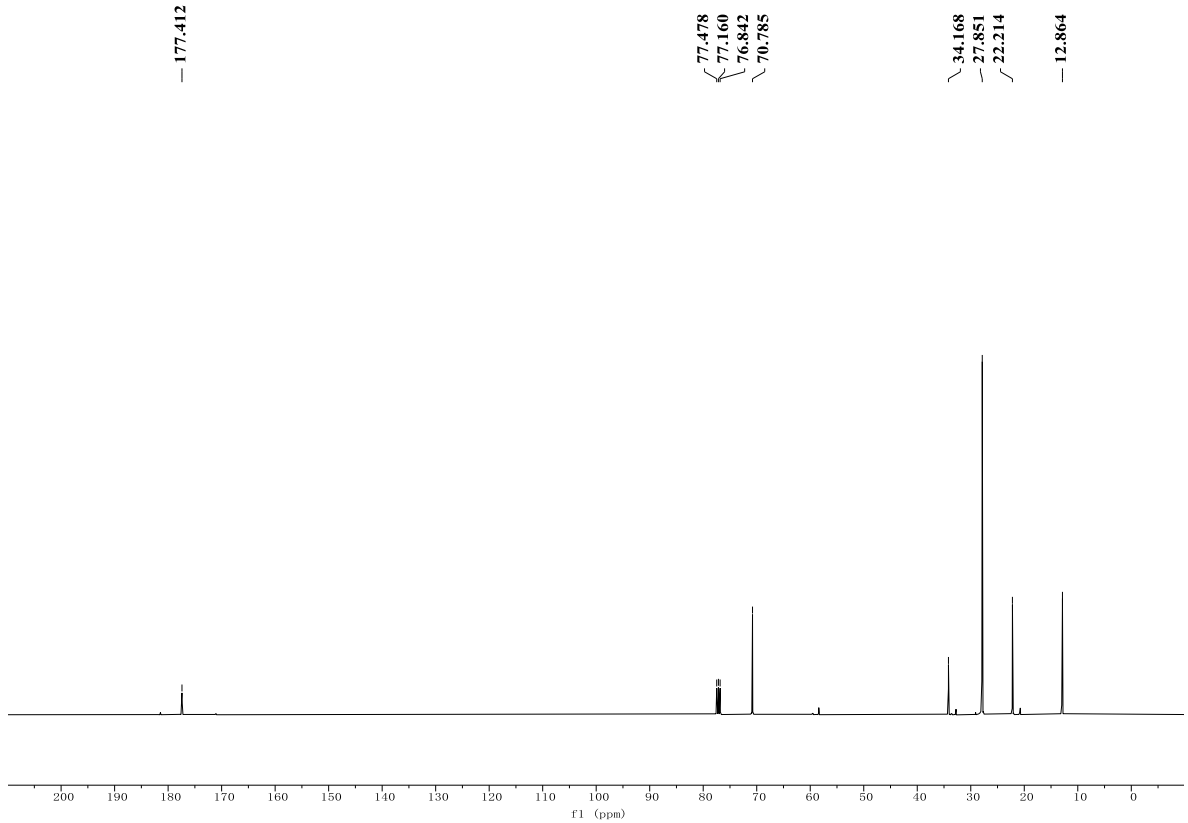
2-ethyl-3-methylbutanoyl chloride (R1) (^{13}C NMR, 101 MHz, $CDCl_3$)



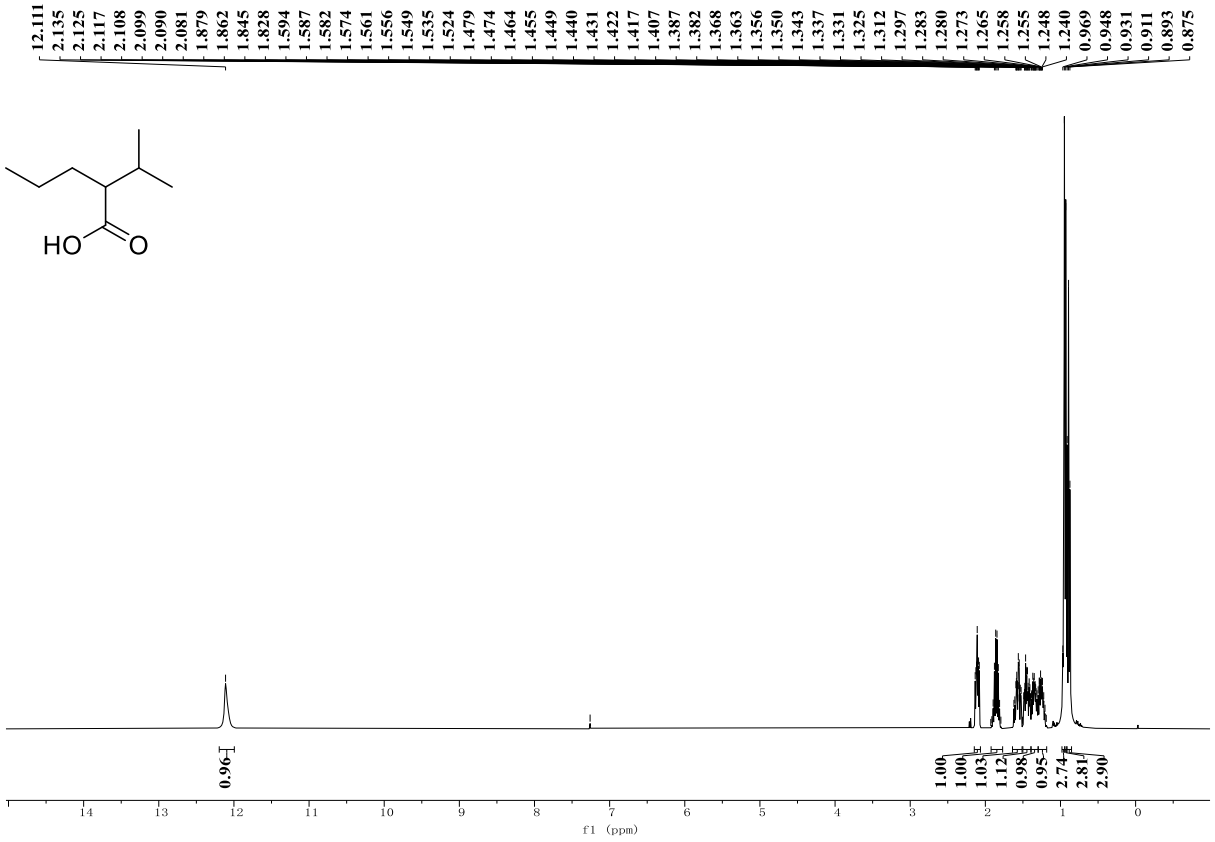
2-ethyl-3,3-dimethylbutanoyl chloride (R2) (^{1}H NMR, 400 MHz, $CDCl_3$)



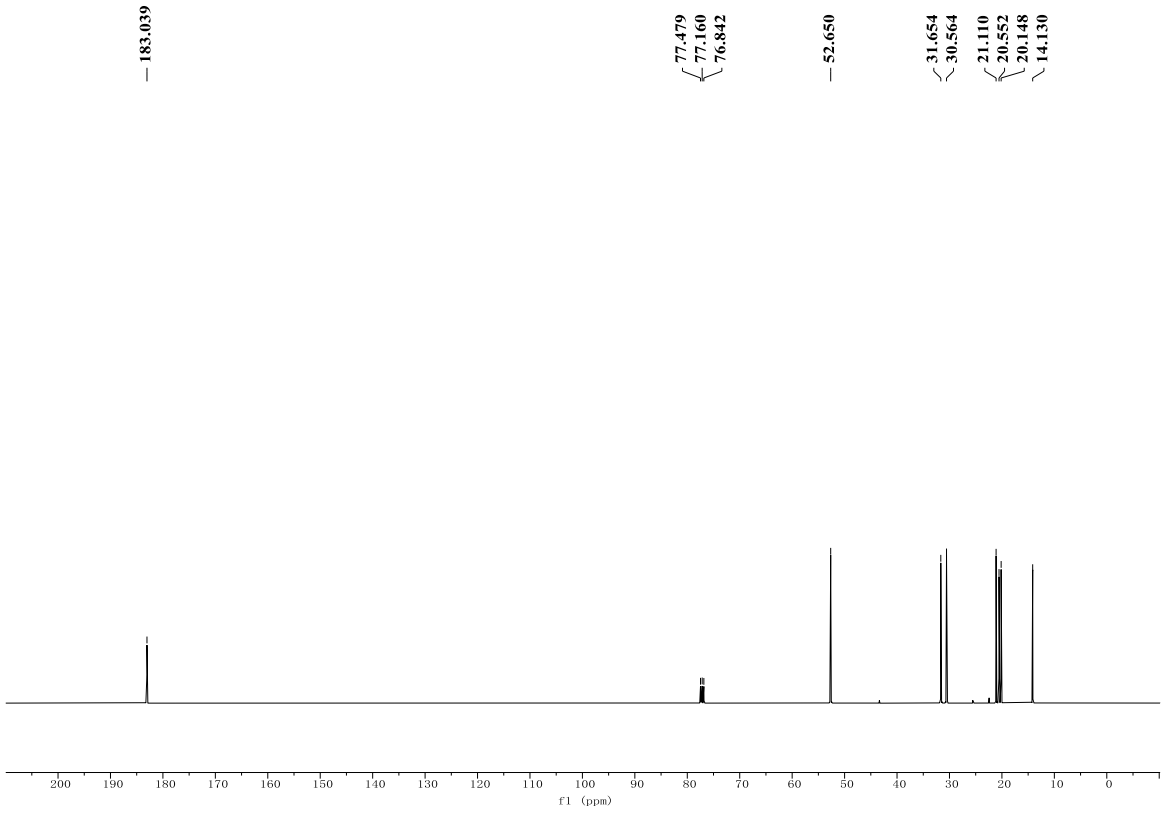
2-ethyl-3,3-dimethylbutanoyl chloride (R2) (^{13}C NMR, 101 MHz, $CDCl_3$)



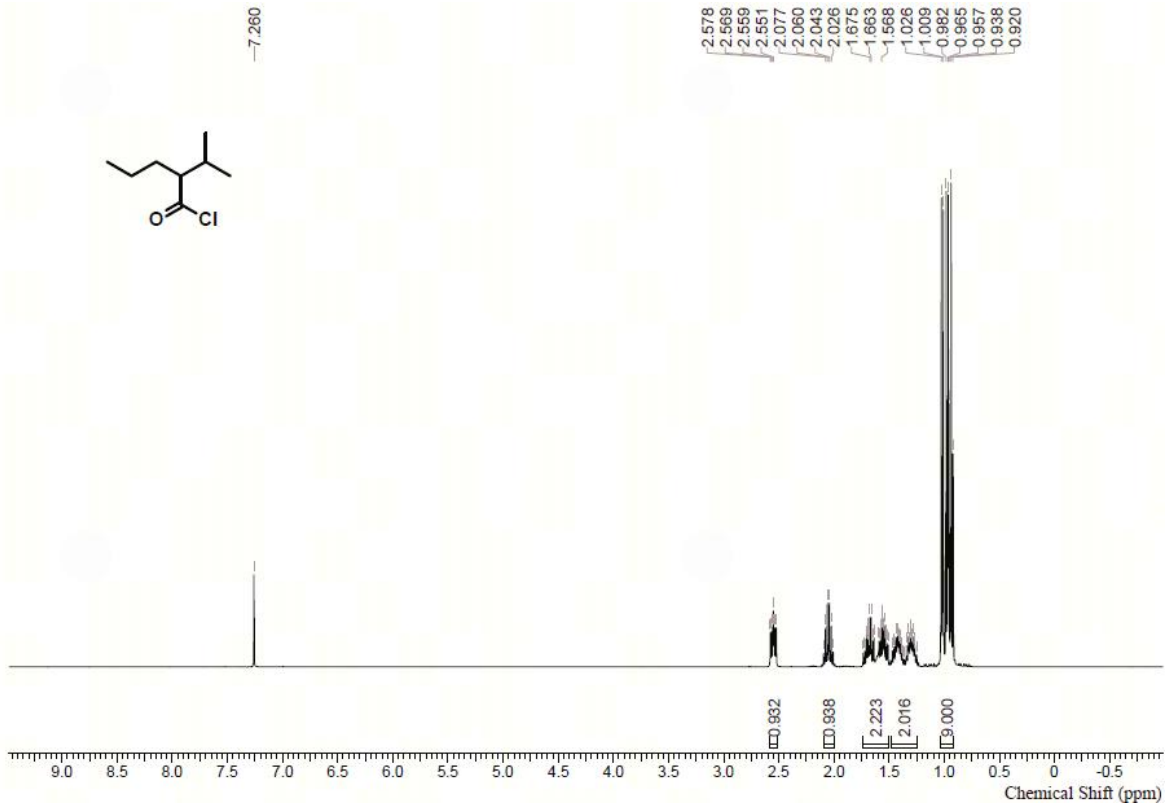
2-isopropylpentanoate (S90) (^{1}H NMR, 400 MHz, $CDCl_3$)



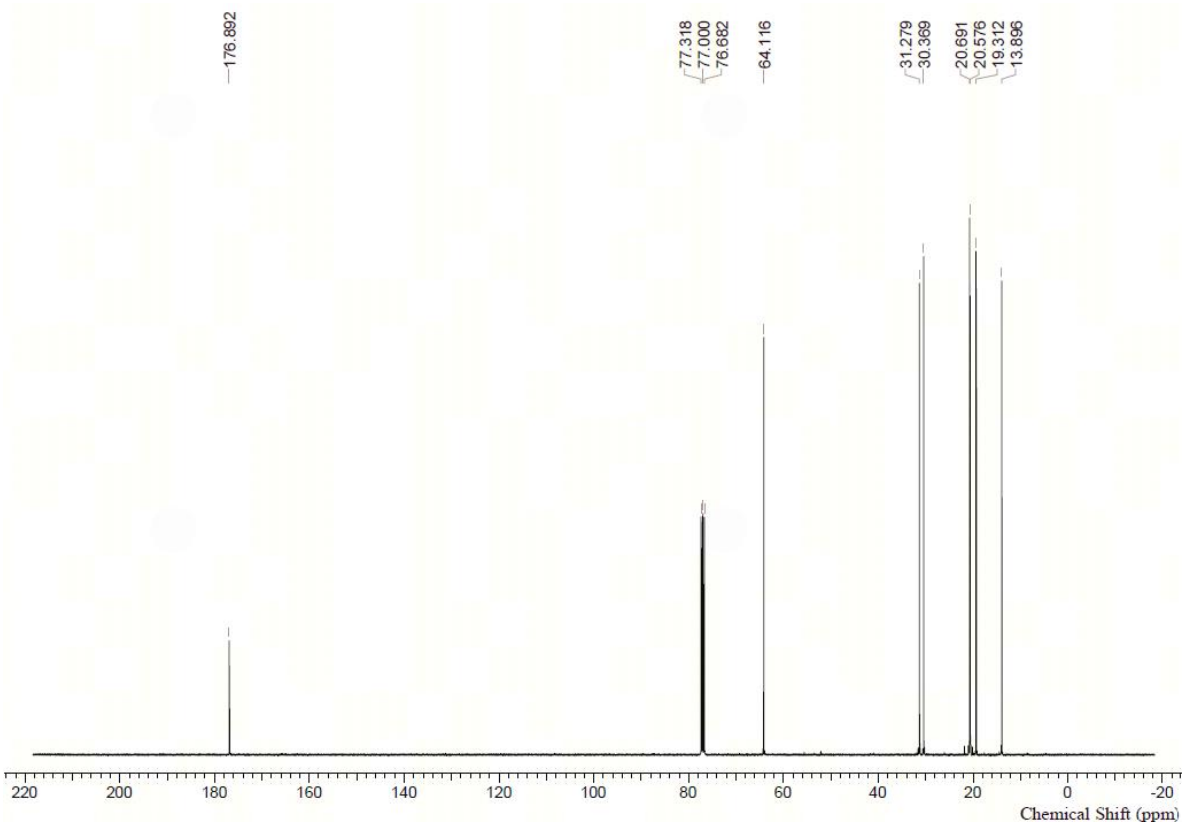
2-isopropylpentanoate (S90) (^{13}C NMR, 101 MHz, $CDCl_3$)



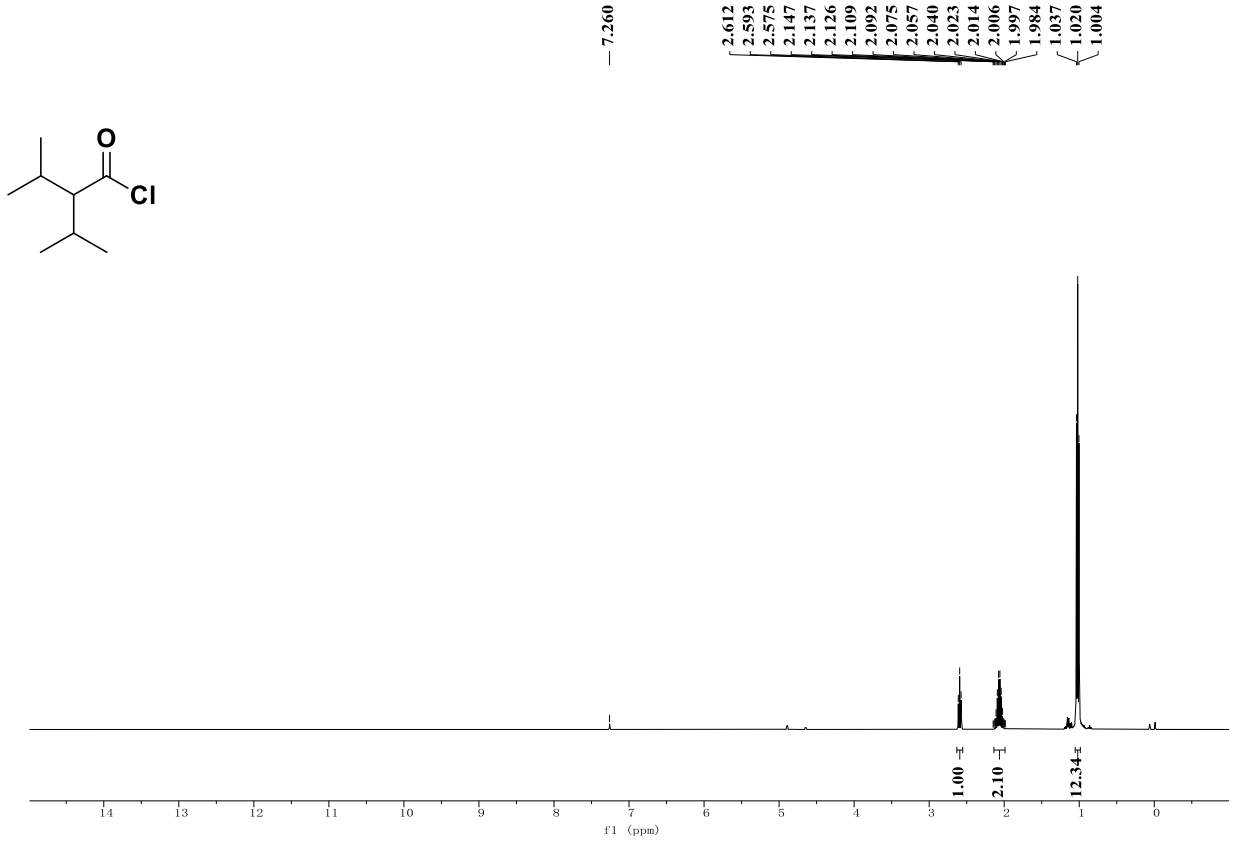
2-isopropylpentanoyl chloride (R3) (^{1}H NMR, 400 MHz, $CDCl_3$, 298 K)



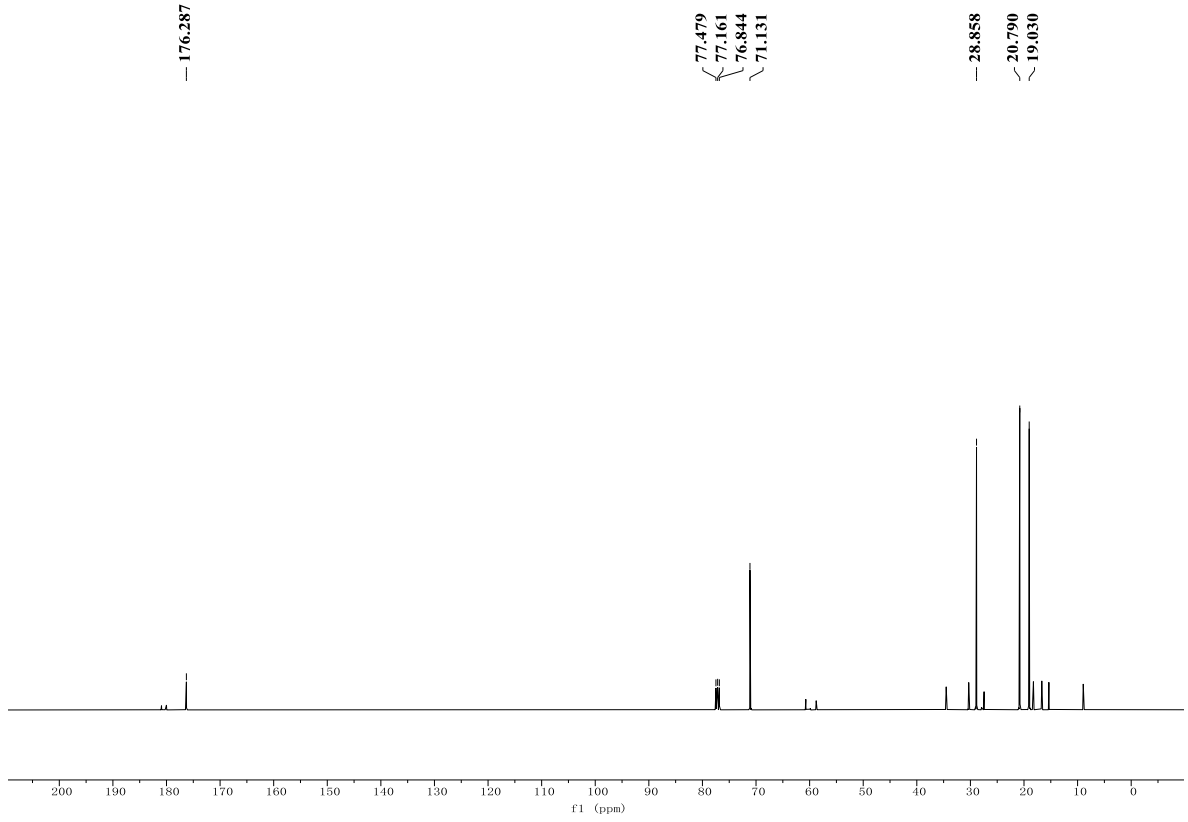
2-isopropylpentanoyl chloride (R3) (^{13}C NMR, 101 MHz, $CDCl_3$, 298 K)



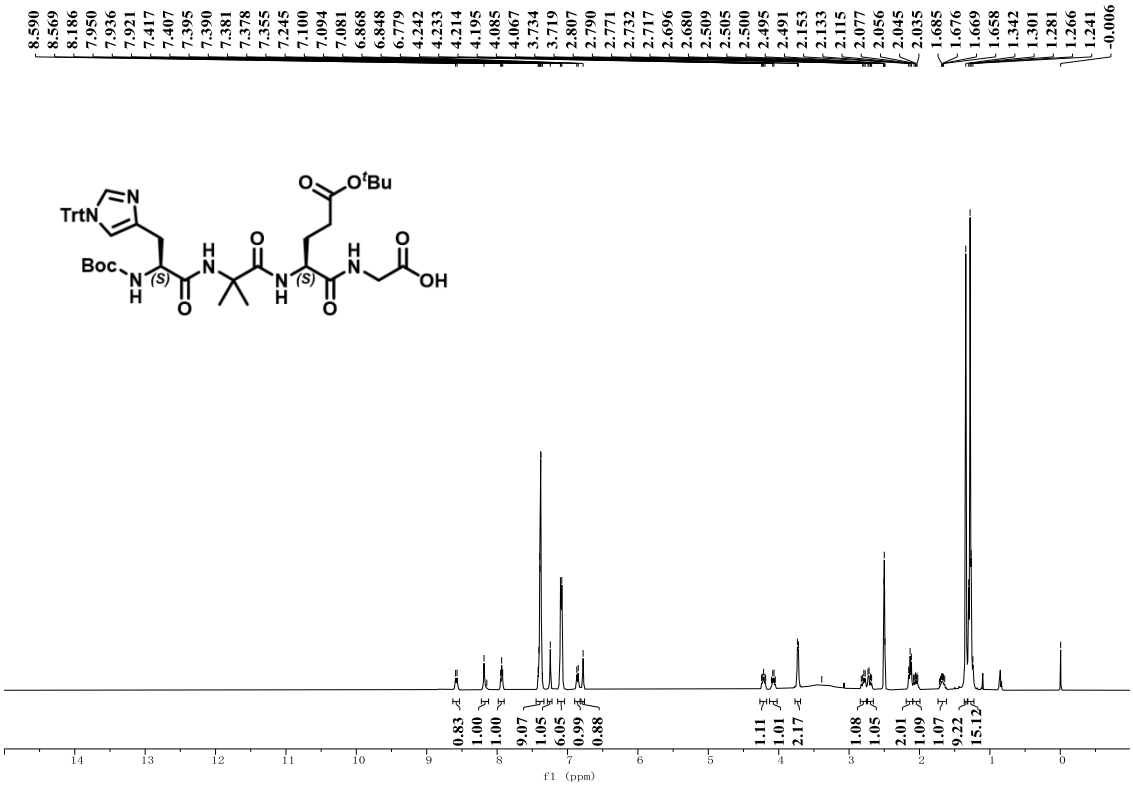
2-isopropyl-3-methylbutanoyl chloride (R4) (^{1}H NMR, 400 MHz, $CDCl_3$)



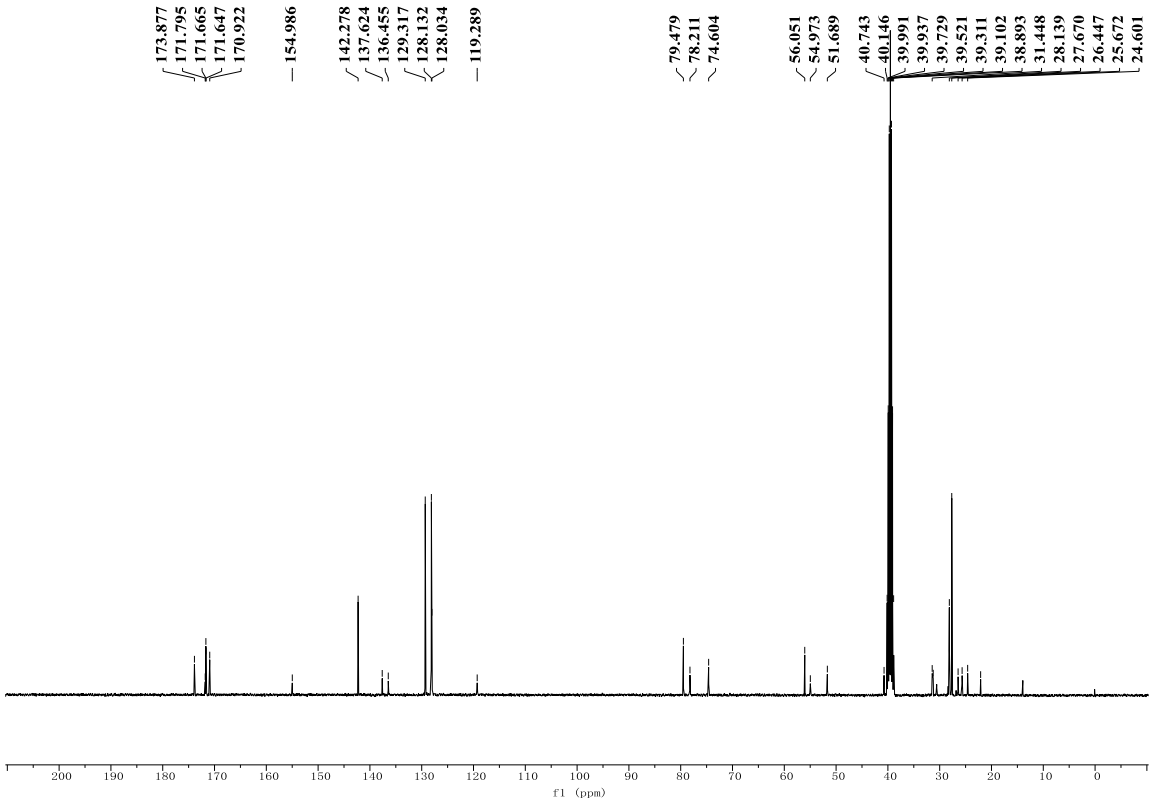
2-isopropyl-3-methylbutanoyl chloride (R4) (^{13}C NMR, 101 MHz, $CDCl_3$)



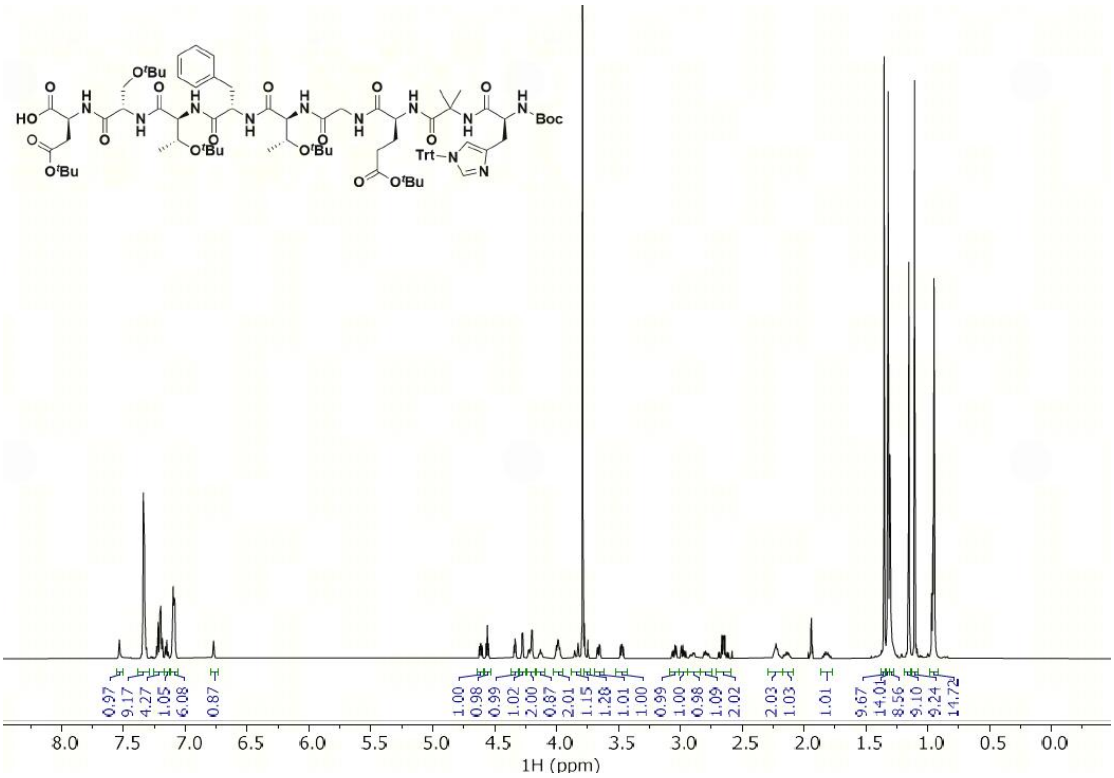
Boc-L-His(Trt)-Aib-L-Glu(5-CO_2tBu)-Gly-OH (S96) (^{1}H NMR, 400 MHz, DMSO-d_6)



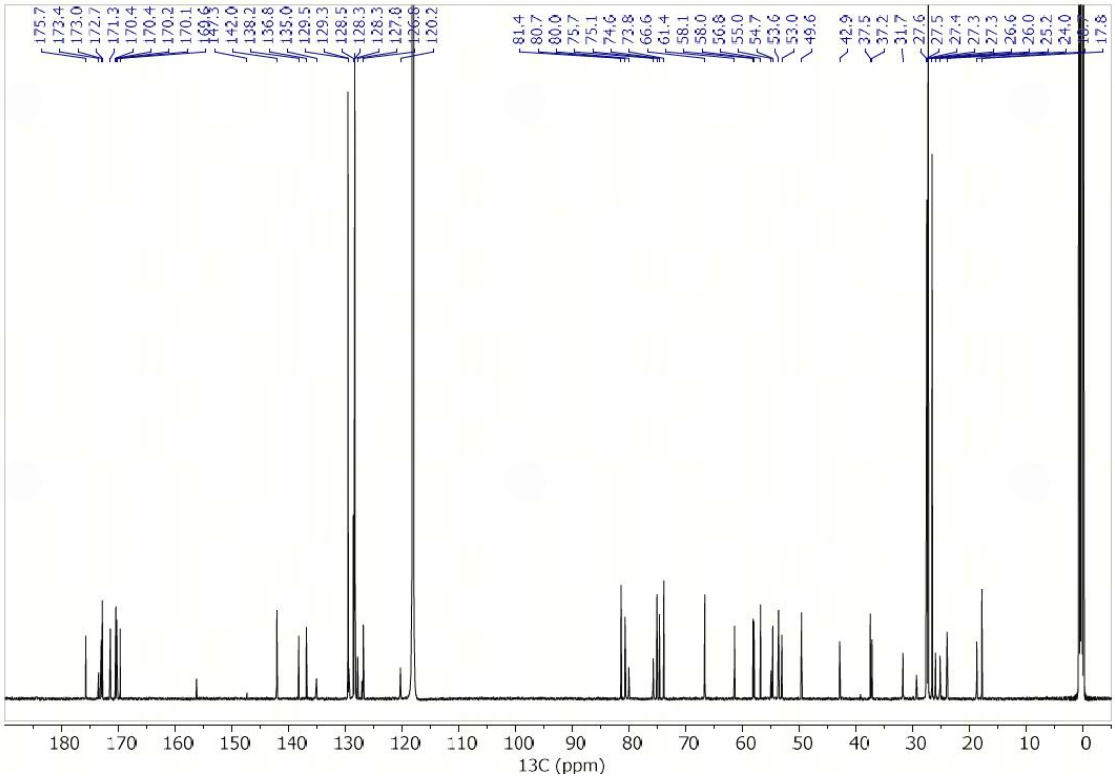
Boc-L-His(Trt)-Aib-L-Glu(5-CO_2tBu)-Gly-OH (S96) (^{13}C NMR, 101 MHz, DMSO-d_6)



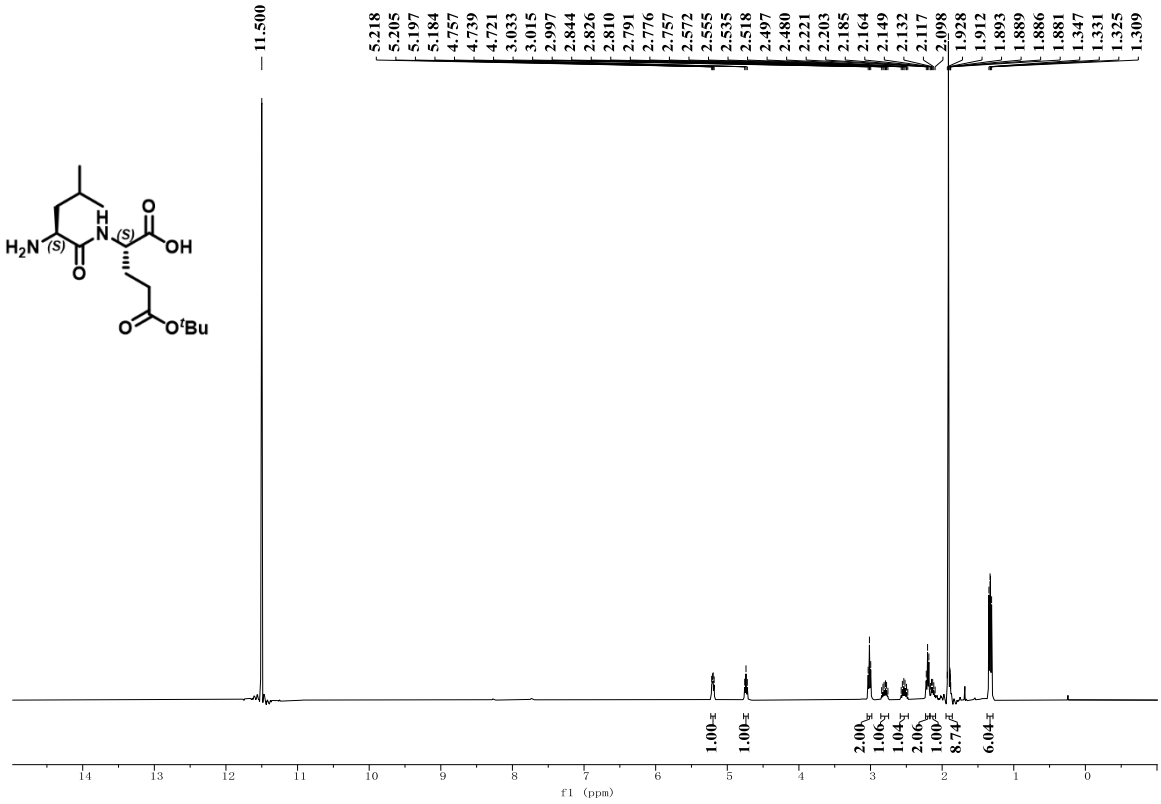
Boc-His(Trt)-Val-Glu(O*t*Bu)-Gly-Thr(*t*Bu)-Phe-Thr(*t*Bu)-Ser(*t*Bu)-Ser(*t*Bu)-OH (15) (^{1}H NMR, 600 MHz, 3:1 CD_3CN/D_2O, 298 K)



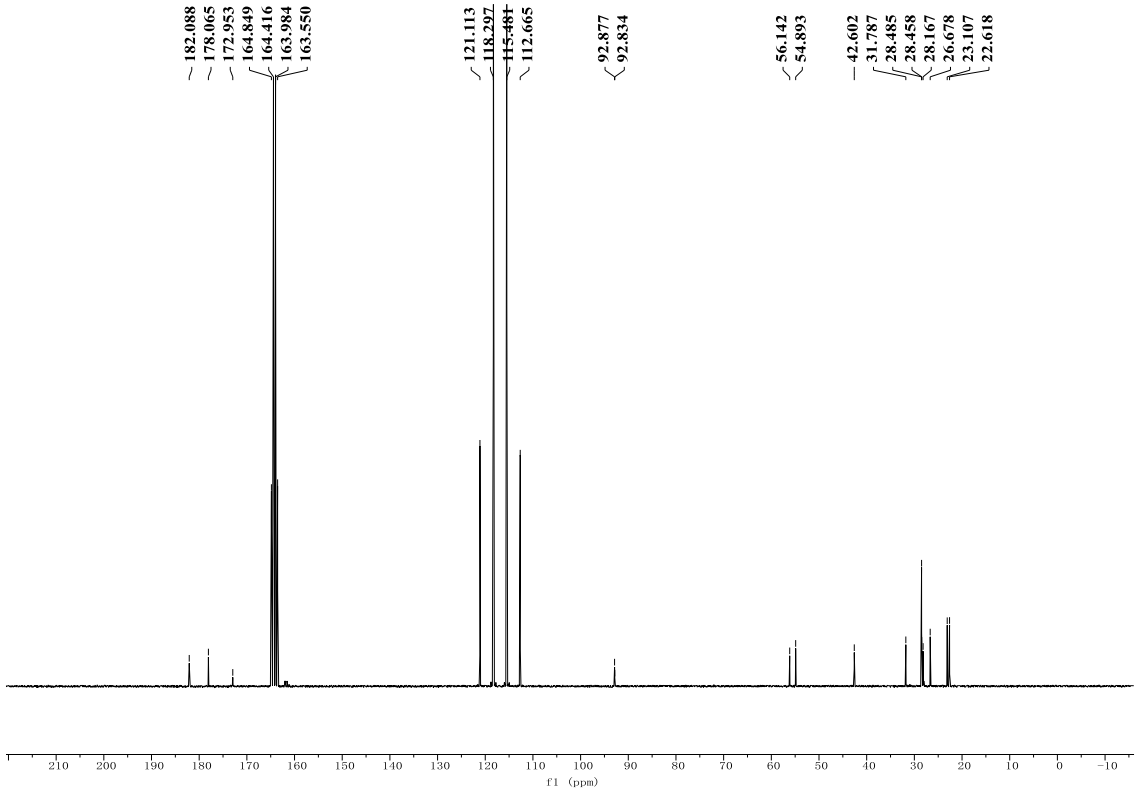
Boc-His(Trt)-Val-Glu(O*t*Bu)-Gly-Thr(*t*Bu)-Phe-Thr(*t*Bu)-Ser(*t*Bu)-Ser(*t*Bu)-OH (15) (^{13}C NMR, 151 MHz, 3:1 CD_3CN/D_2O, 298 K)



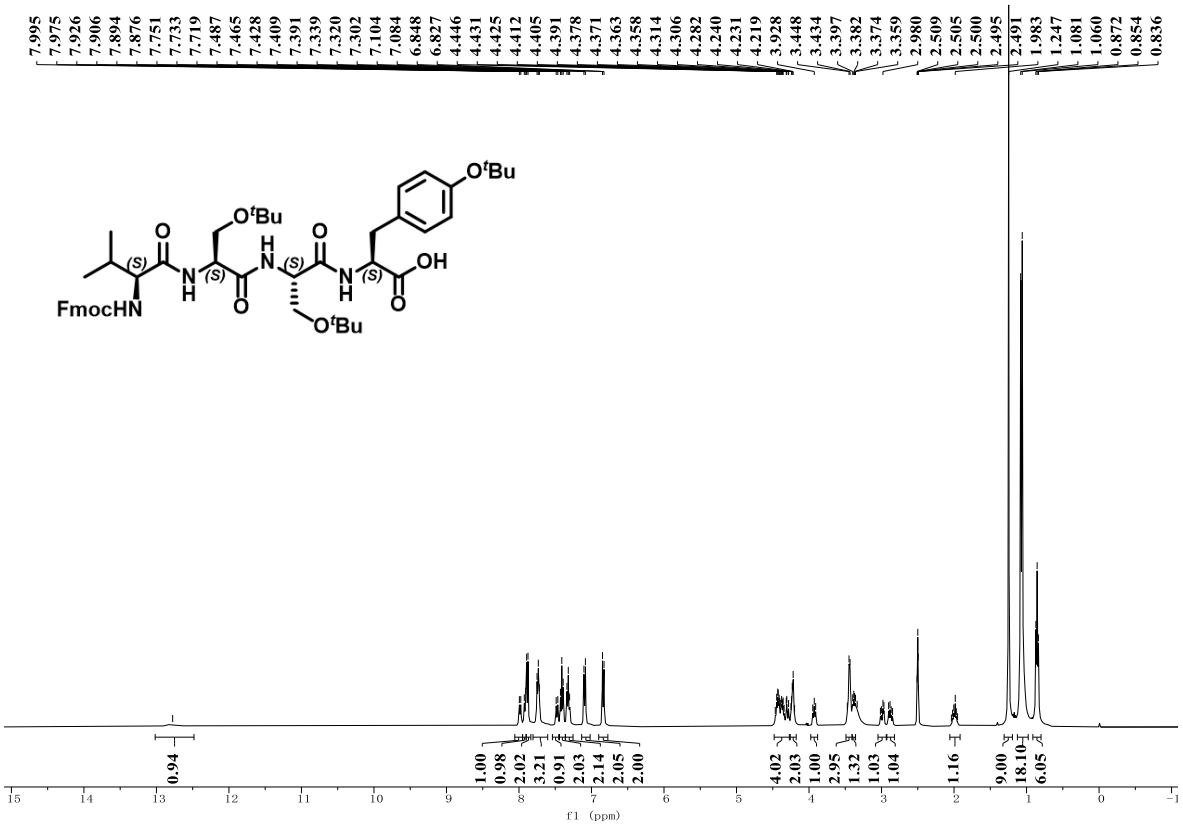
H-L -Leu-L-Glu(CO_2tBu)-OH (S105) (^{1}H NMR, 400 MHz, TFA-*d*)



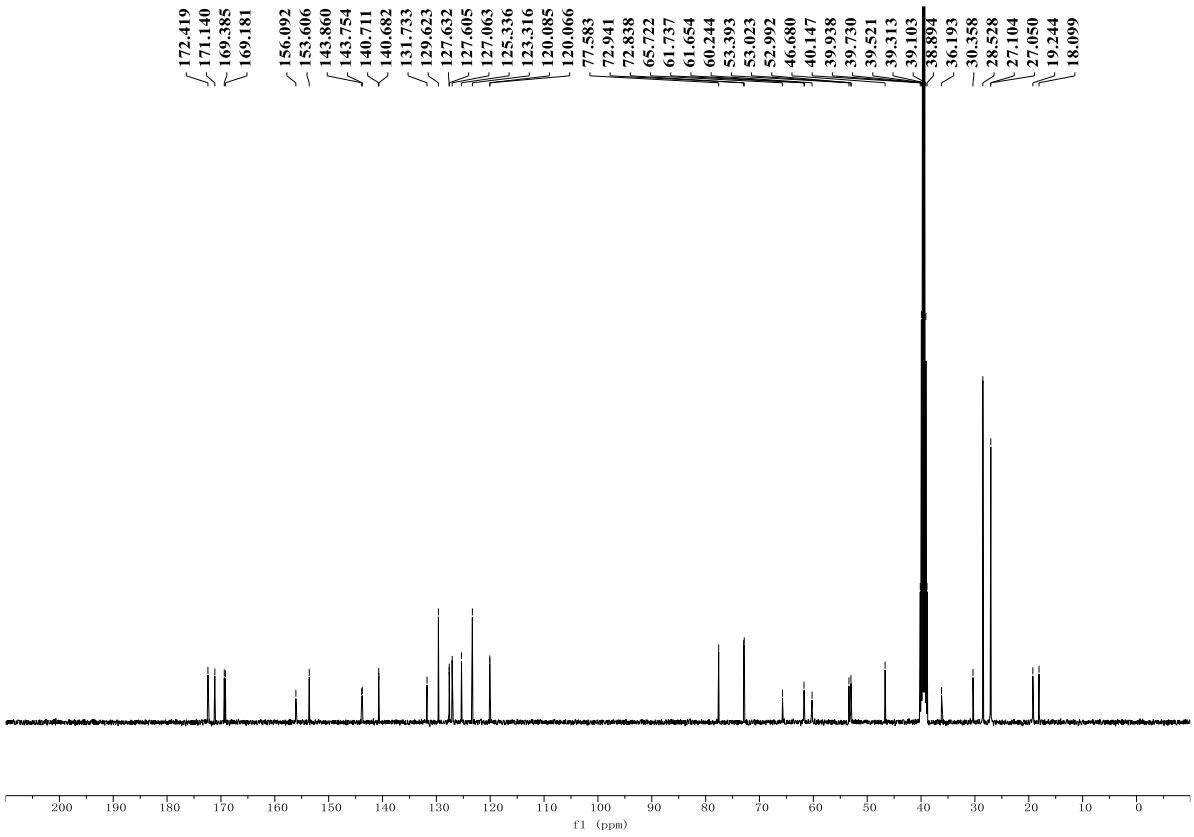
H-L -Leu-L-Glu(CO_2tBu)-OH (S105) (^{13}C NMR, 101 MHz, TFA-*d*)



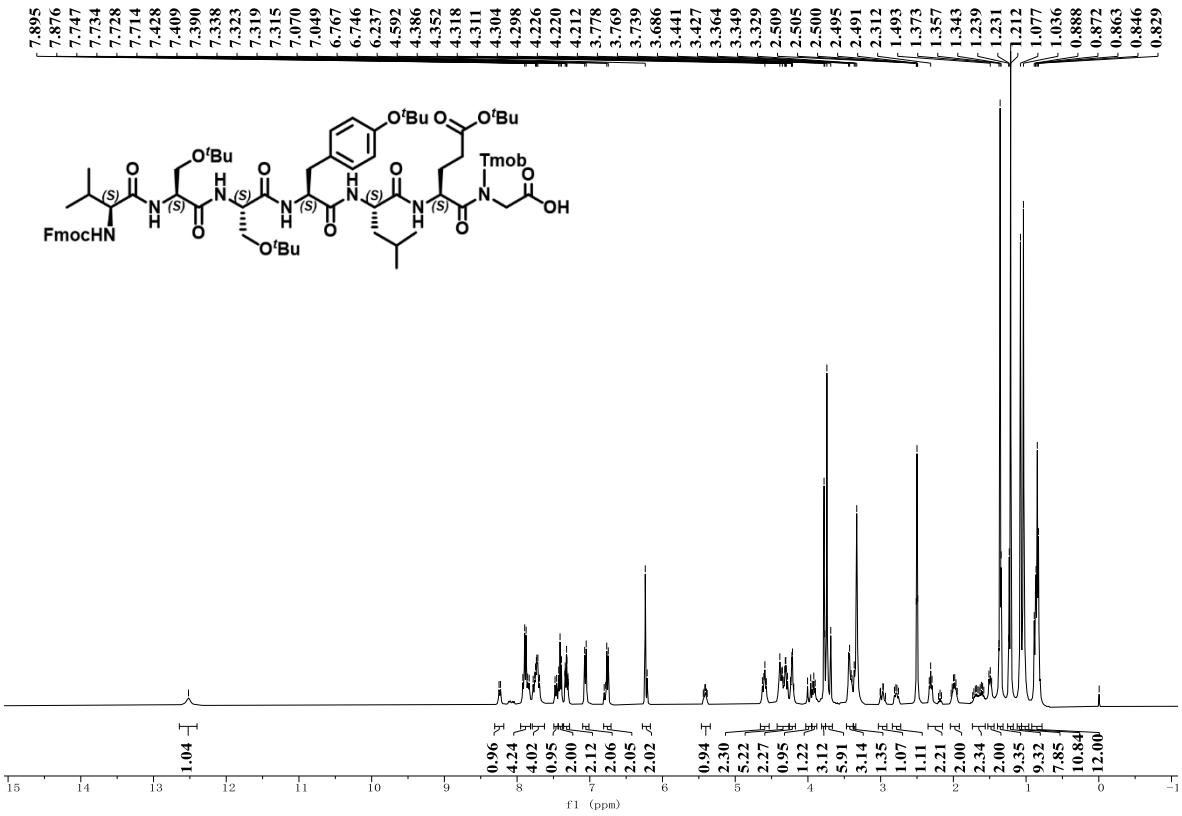
Fmoc-L-Val-L-Ser(O*t*Bu)-L-Ser(O*t*Bu)-L-Tyr(O*t*Bu)-OH (S110) (^{1}H NMR, 400 MHz, DMSO-d_6)



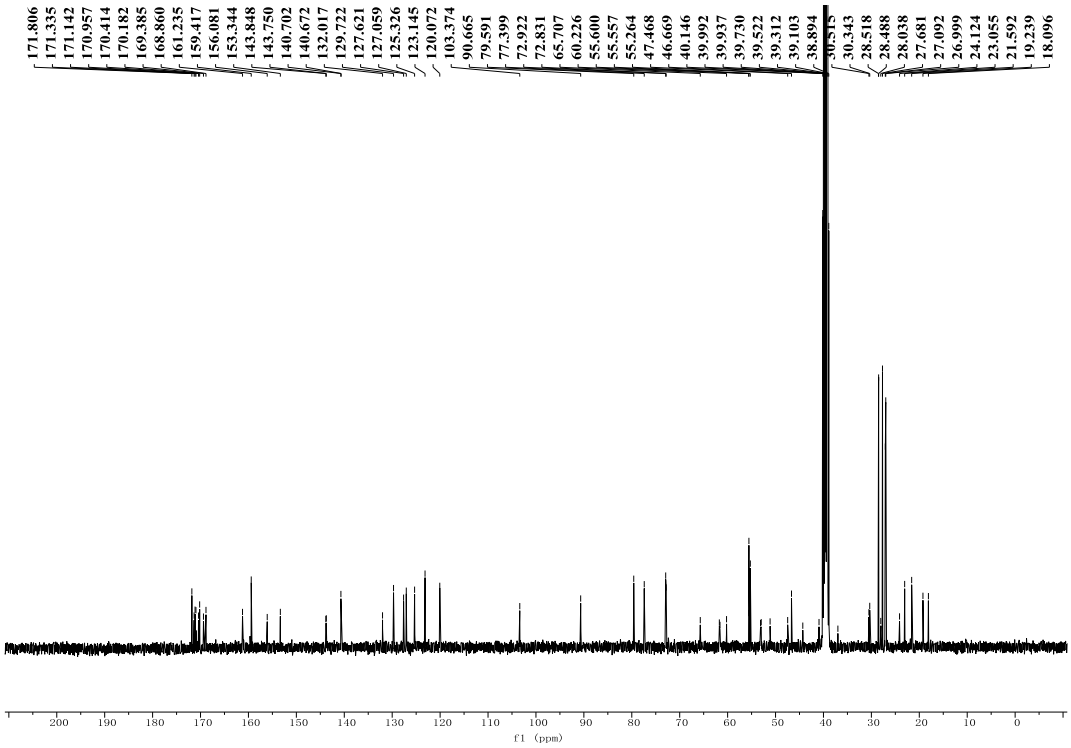
Fmoc-L-Val-L-Ser(O*t*Bu)-L-Ser(O*t*Bu)-L-Tyr(O*t*Bu)-OH (S110) (^{13}C NMR, 101 MHz, DMSO-d_6)



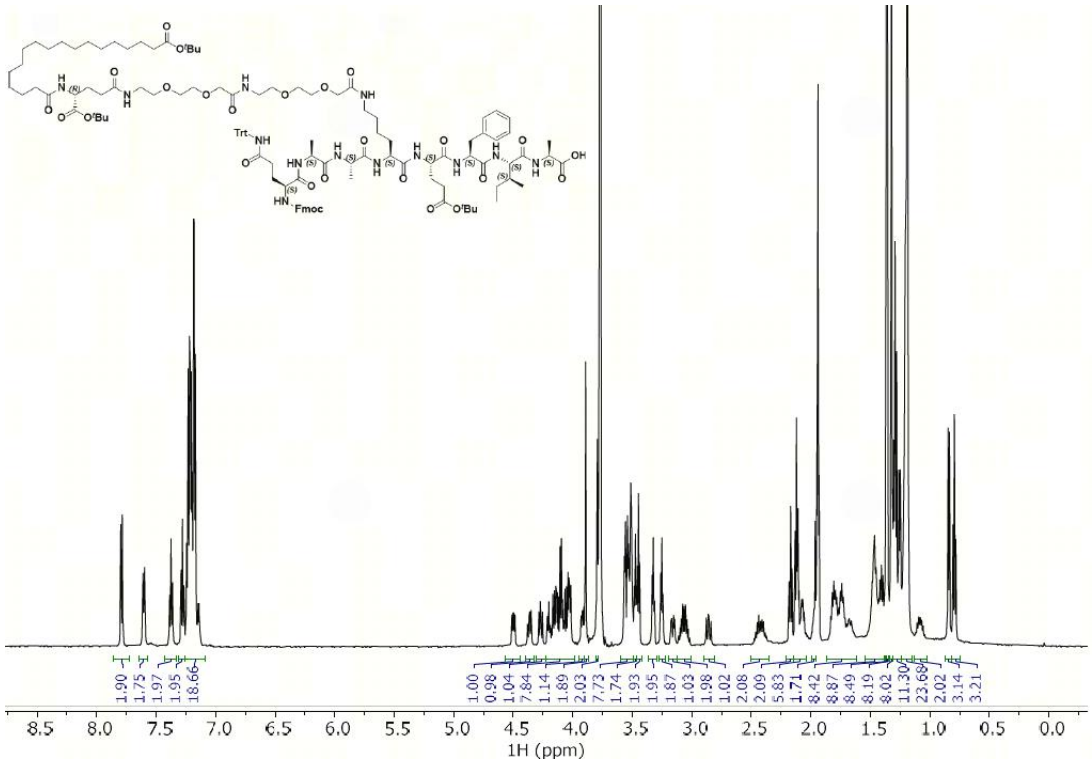
Fmoc-L-Val-L-Ser(O*t*Bu)-L-Ser(O*t*Bu)-L-Tyr(O*t*Bu)-L-Leu-L-Glu(CO_2*t*Bu) -L-Gly(Tmob)-OH (**16**) (^{1}H NMR, 400 MHz, DMSO-d_6)



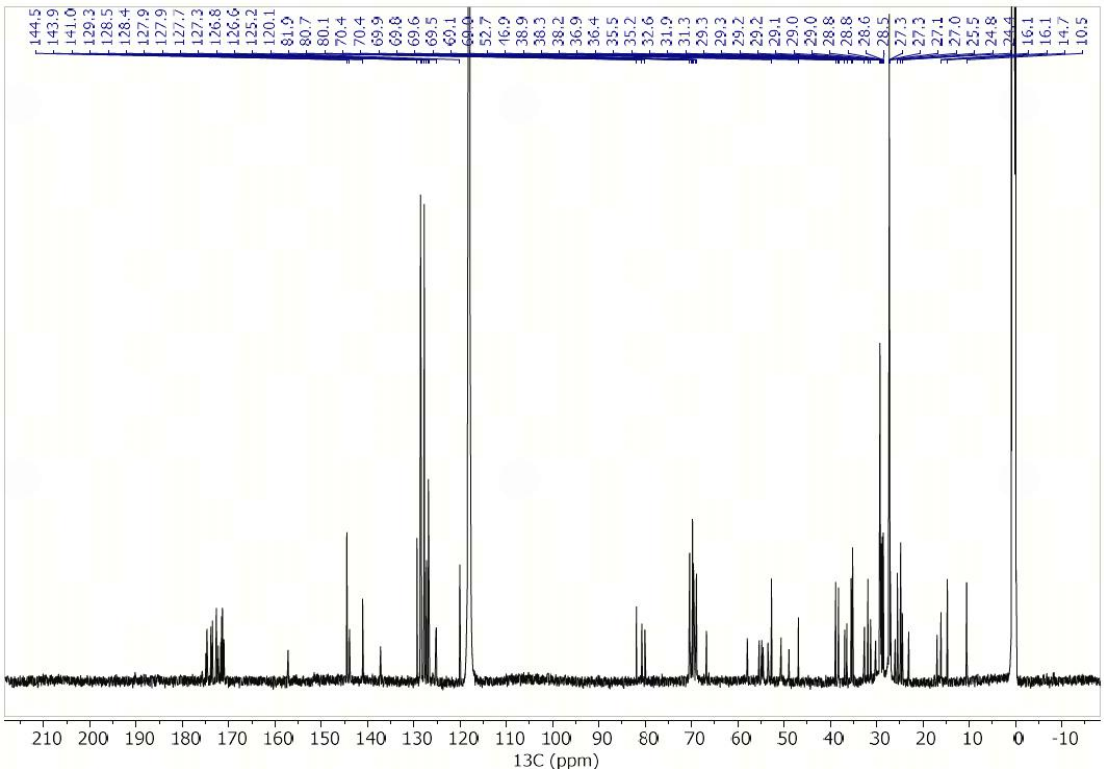
Fmoc-L-Val-L-Ser(O*t*Bu)-L-Ser(O*t*Bu)-L-Tyr(O*t*Bu)-L-Leu-L-Glu(CO_2*t*Bu) -L-Gly(Tmob)-OH (**16**) (^{13}C NMR, 101 MHz, DMSO-d_6)



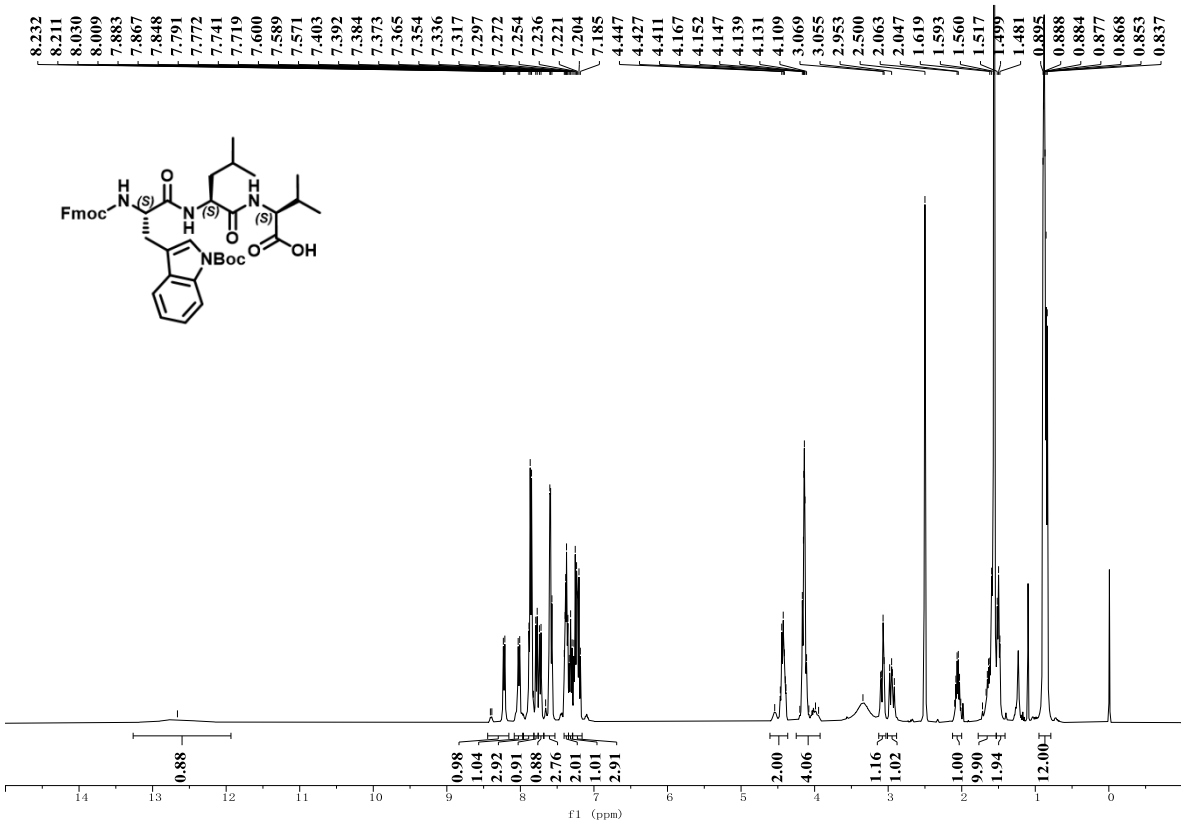
Fmoc-Gln(Trt)-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct(O*t*Bu)]-Glu(O*t*Bu)-Phe-Ile-Ala-OH (17)
(^{1}H NMR, 600 MHz, 3:1 CD_3CN/D_2O, 298 K)



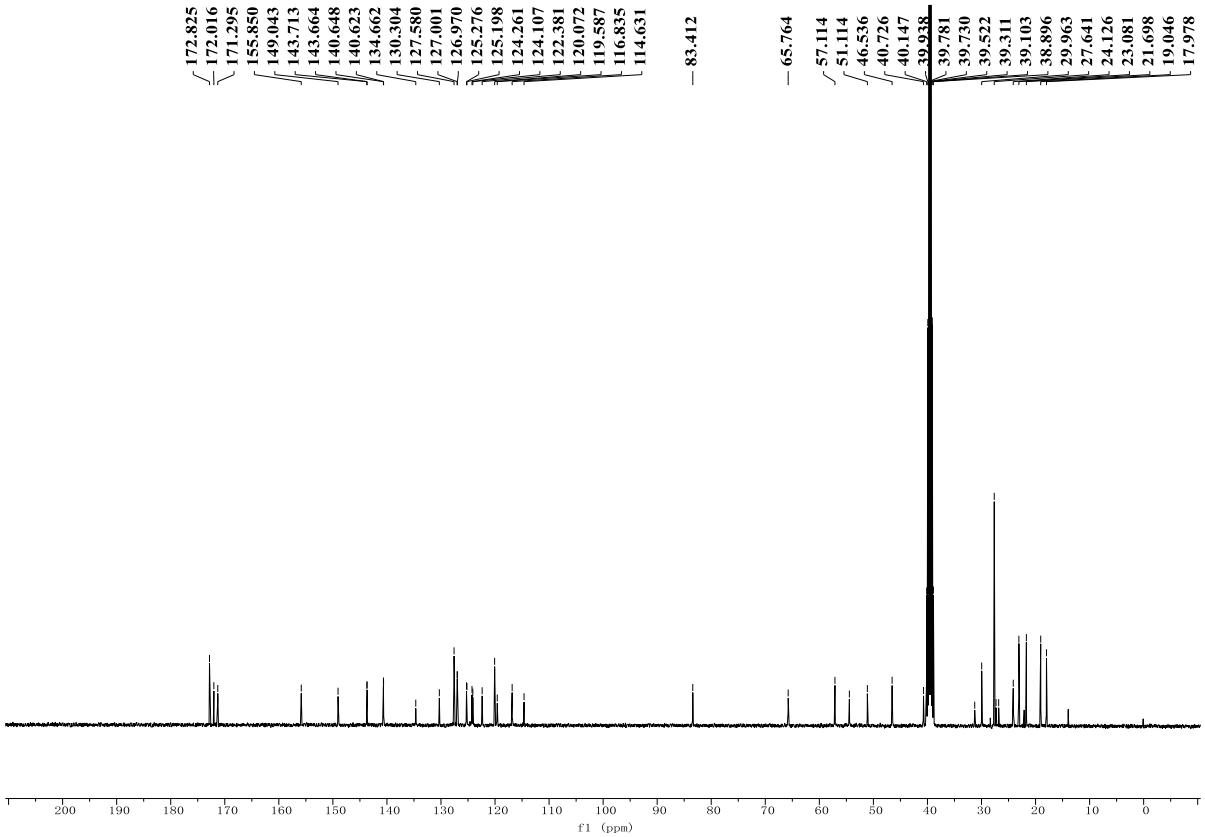
Fmoc-Gln(Trt)-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct(O*t*Bu)]-Glu(O*t*Bu)-Phe-Ile-Ala-OH (17)
(^{13}C NMR, 151 MHz, 3:1 CD_3CN/D_2O, 298 K)



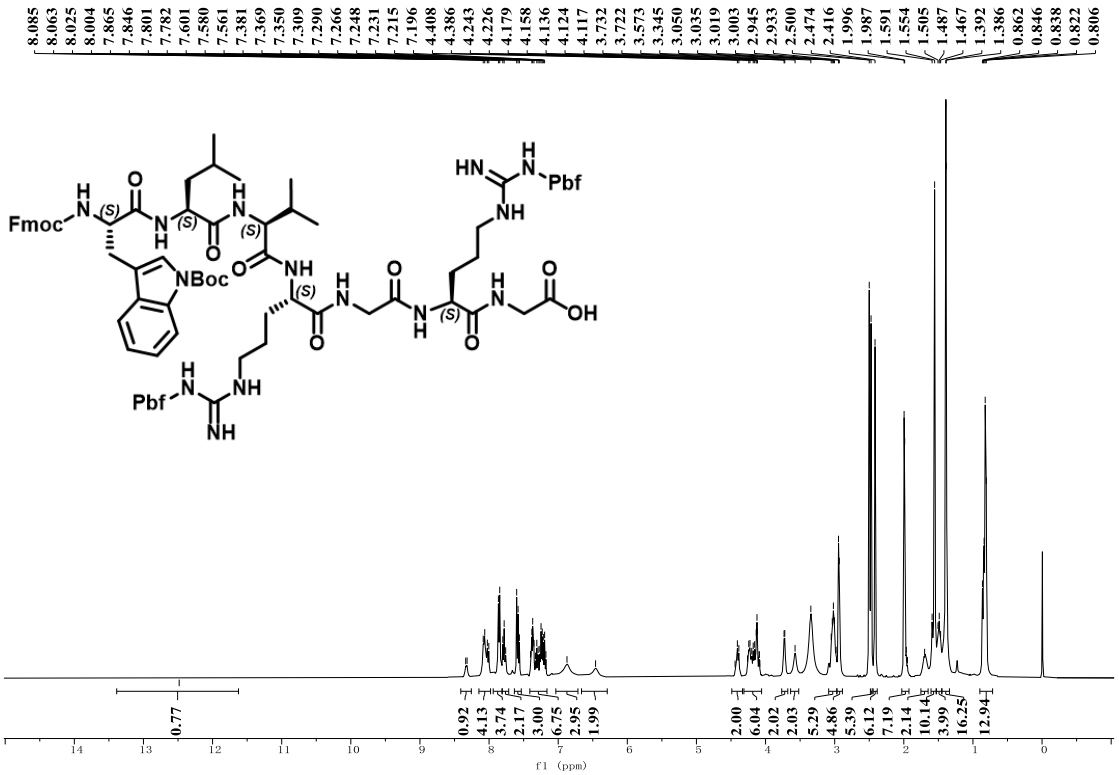
Fmoc-L-Trp(Boc)-L-Leu-L-Val-OH (S125) (^{1}H NMR, 400 MHz, DMSO-d_6)



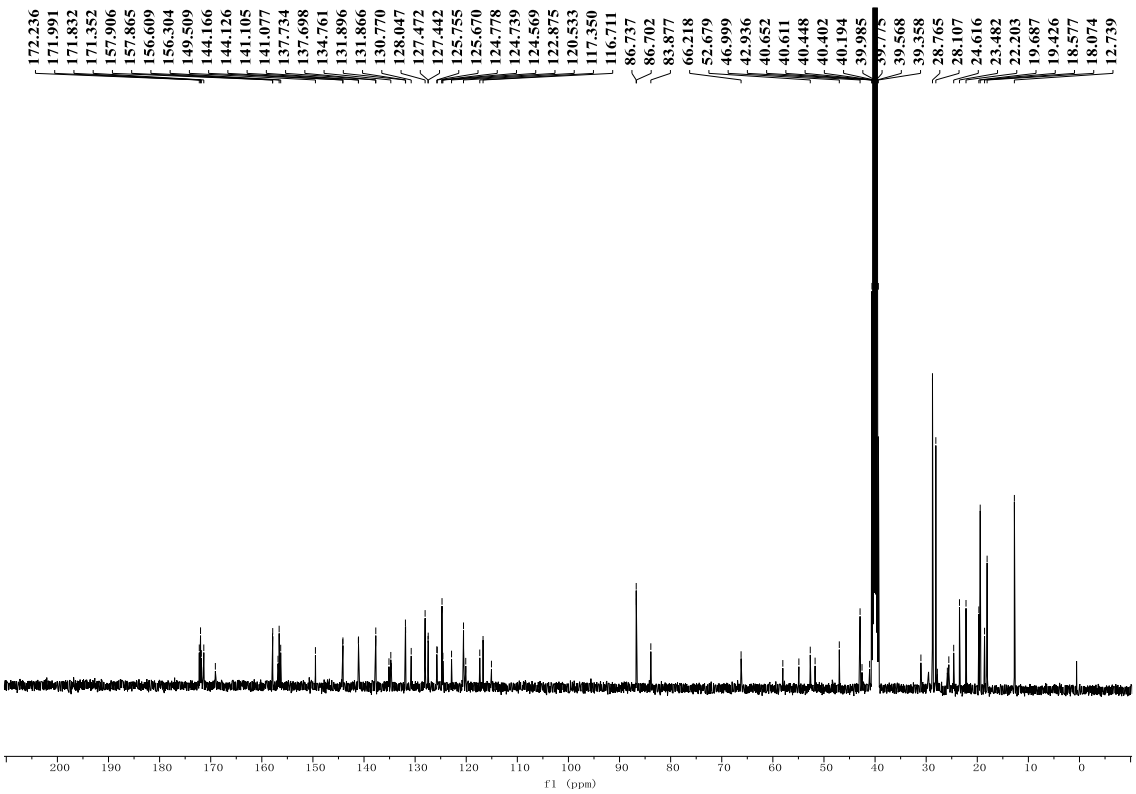
Fmoc-L-Trp(Boc)-L-Leu-L-Val-OH (S125) (^{13}C NMR, 101 MHz, DMSO-d_6)



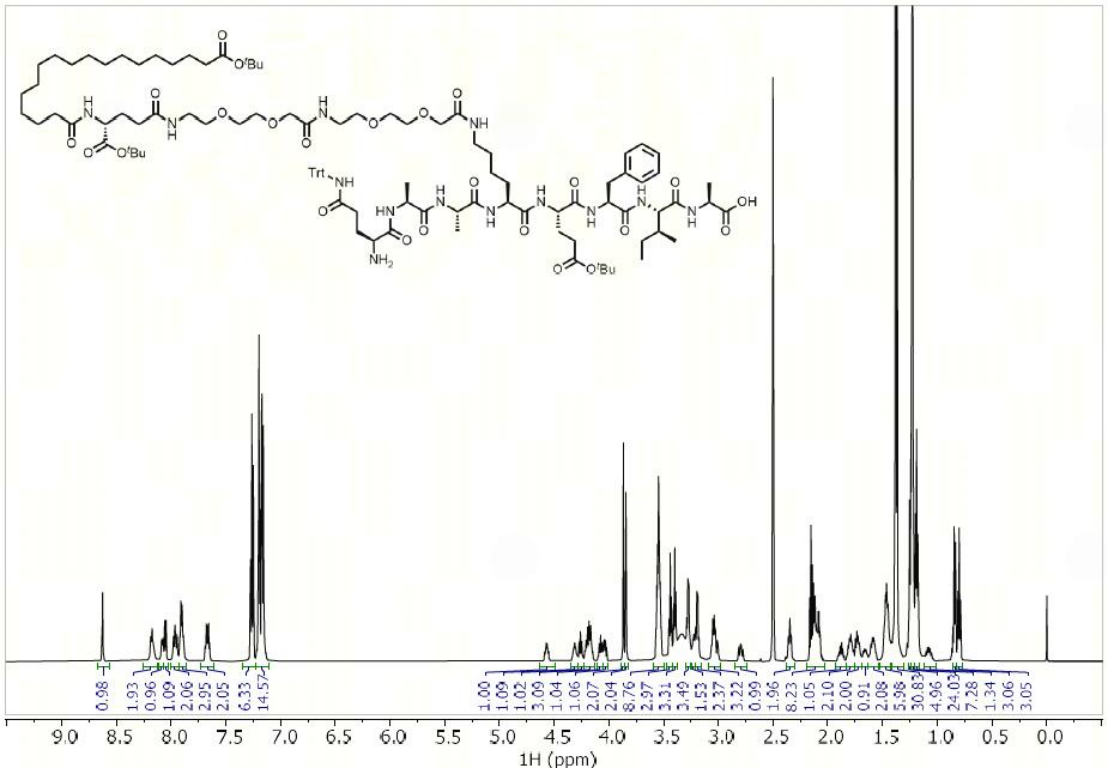
Fmoc-L-Trp(Boc)-L-Leu-L-Val-L-Arg(Pbf)-Gly-L-Arg(Pbf)-Gly-OH (18) (^{1}H NMR, 400 MHz, DMSO-d_6)



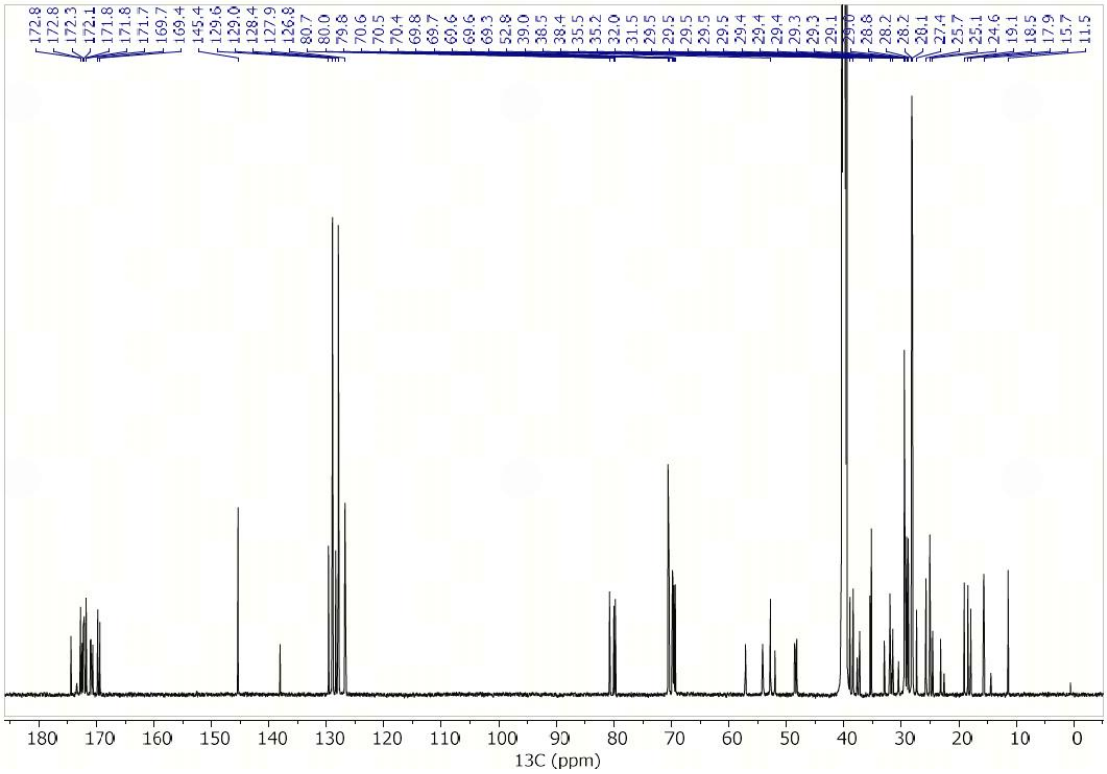
Fmoc-L-Trp(Boc)-L-Leu-L-Val-L-Arg(Pbf)-Gly-L-Arg(Pbf)-Gly-OH (18) (^{13}C NMR, 101 MHz, DMSO-d_6)



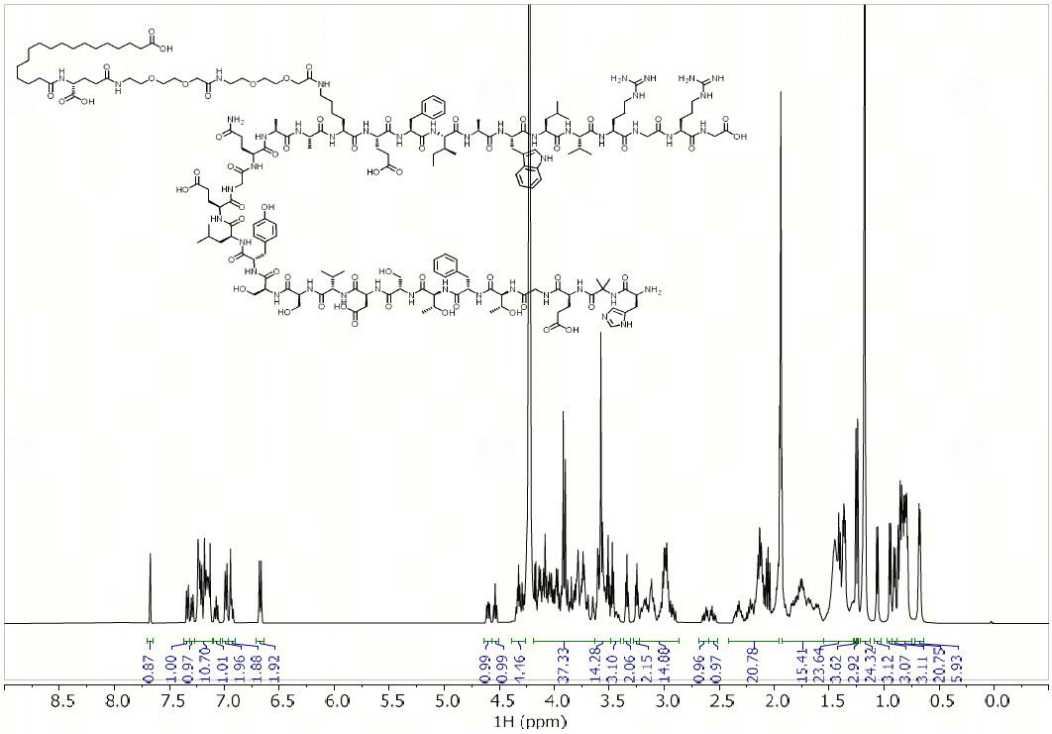
H-Gln(Trt)-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct(O*t*Bu)]-Glu(O*t*Bu)-Phe-Ile-Ala-OH (deFmoc-17, S129) (^{1}H NMR, 600 MHz, DMSO-d6, 298 K)



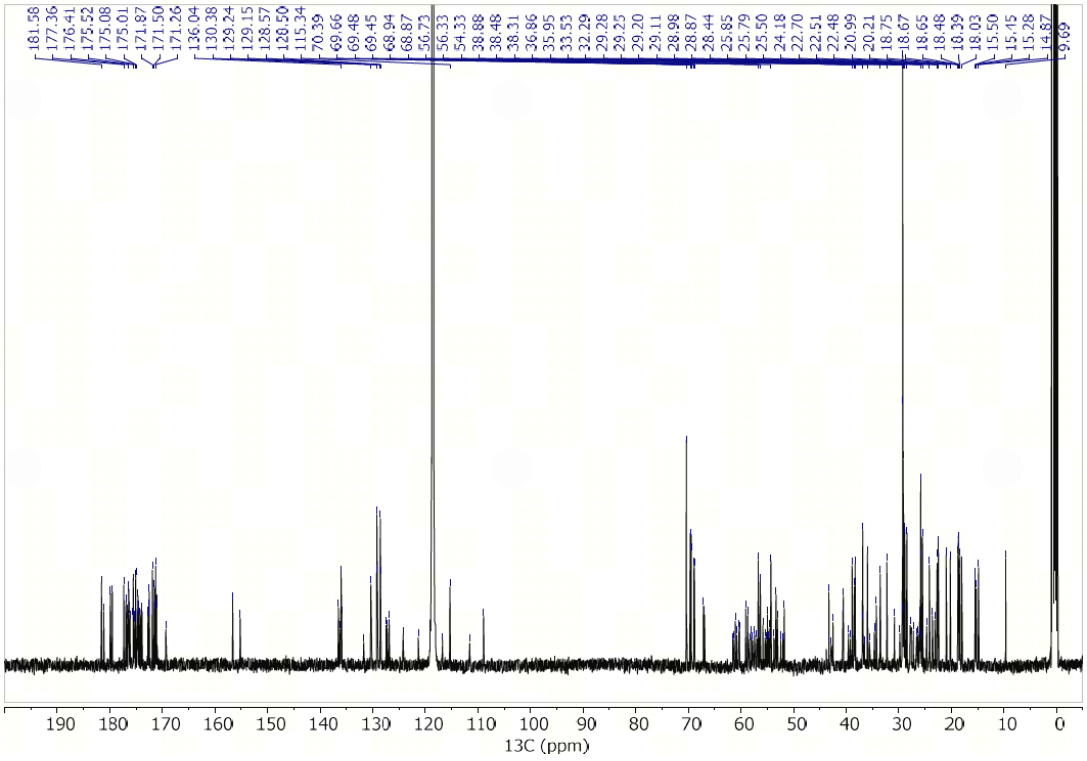
H-Gln(Trt)-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct(O*t*Bu)]-Glu(O*t*Bu)-Phe-Ile-Ala-OH (deFmoc-17, S129) (^{13}C NMR, 151 MHz, DMSO-d6, 298 K)



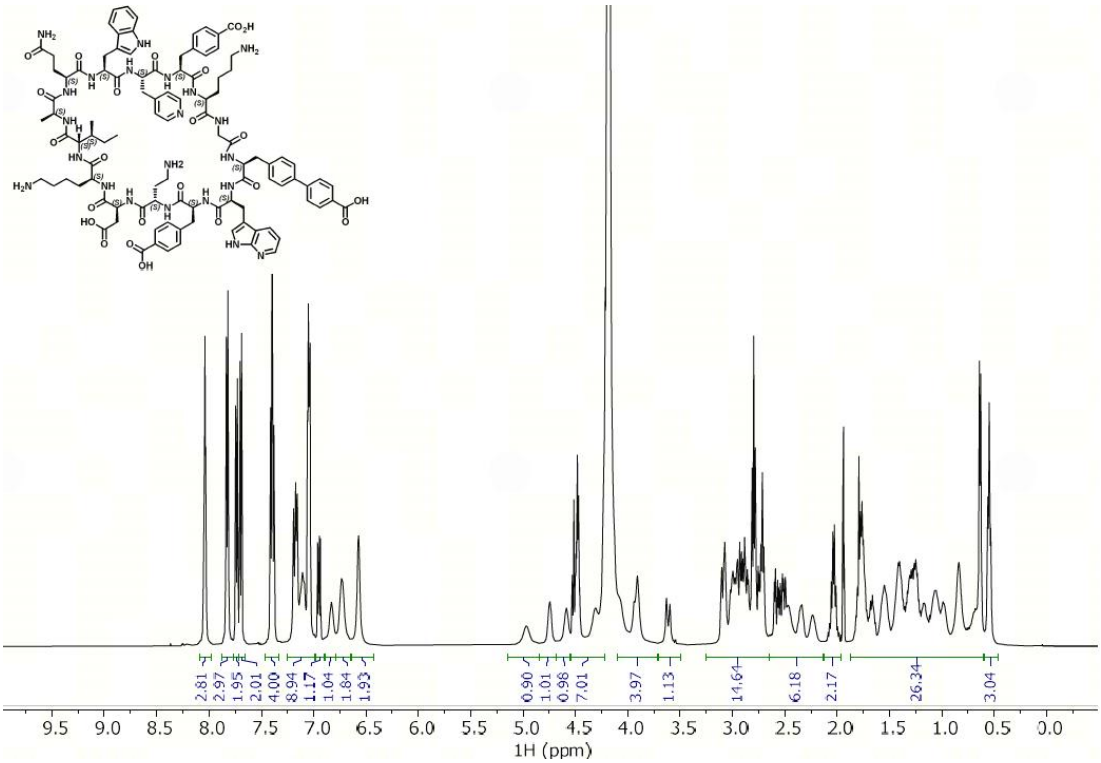
H-His-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Ser-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH (Semaglutide, 20) (^{1}H NMR, 500 MHz, 1:1 CD_3CN/D_2O/NaOH, 298 K)



H-His-Aib-Glu-Gly-Thr-Phe-Thr-Ser-Ser-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys[AEEA2-D-Glu(α-O*t*Bu)-Oct-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH (Semaglutide, 20) (^{13}C NMR, 126 MHz, 1:1 CD_3CN/D_2O/NaOH, 298 K)



H-Bip(4'-Boc)-Pyr(3)-Phe(4-Boc)-Dab(Boc)-Asp(O*t*Bu)-Lys(Boc)-Ile-Ala-Gln(Trt)-Trp(Boc)-Pal(4)-Phe(4-Boc)-Lys(Boc)-Gly-OH (29) (^{1}H NMR, 500 MHz, 1:1 CD_3CN/D_2O/NaOH, 323 K)



H-Bip(4'-Boc)-Pyr(3)-Phe(4-Boc)-Dab(Boc)-Asp(O*t*Bu)-Lys(Boc)-Ile-Ala-Gln(Trt)-Trp(Boc)-Pal(4)-Phe(4-Boc)-Lys(Boc)-Gly-OH (29) (^{13}C NMR, 126 MHz, 1:1 CD_3CN/D_2O/NaOH,323 K)

