Chemical synthesis of disulfide surrogate peptides by using beta-carbon dimethyl modified diaminodiacids

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1.General information

1.1 Materials and Reagents

2-Chlorotrityl resin, Rink Amide AM resin and Fmoc-amino acids were bought from CS Bio, GL Biochem (Shanghai, China). HCTU, HATU, HOAt, PyAOP, DIEA and 4-Methylmorpholine (NMM) were bought from Adamas (Shanghai, China). Glutathione oxidized (GSSG) and glutathione reduced (GSG) were purchased from Sigma-Aldrich. Dichloromethane (DCM), dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP) and anhydrous diethyl ether were purchased from Sinopharm Chemical Reagent. Trifluoroacetic acid (TFA, HPLC grade) and thioanisole were purchased from J&K Scientific (Beijing, China). Thin-layer chromatography (TLC) was performed on plates pre-coated with silica gel 60 F254 (250 layer thickness). Flash column chromatography was carried out by forced-flow chromatography using Silica Gel (200-300 mesh on small-scale or 300-400 mesh on large-scale). Manual peptide synthesis was performed in a peptide synthesis vessel under a constant temperature shaker (30 ℃)

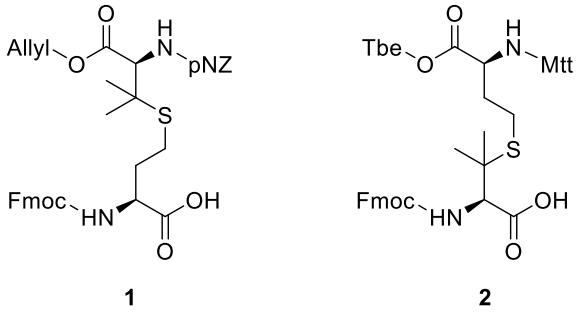
1.2 HPLC

Analytical HPLC was run on a SHIMADZU (Prominence LC-20AT) instrument using a analytical column (Grace Vydac “Protein & Peptide C18”, 250 × 4.6 mm, 5 μm particle size, flow rate 1.0 mL/min, rt.). Analytical samples were monitored at 214 nm and 254 nm. Semi-preparative HPLC was run on a SHIMADZU (Prominence LC-20AT) instrument using a semi preparative column (Grace Vydac “Peptide C18”, 250 × 10 mm, 10 μm particle size, flow rate 4.0 mL/min, rt). The mobile phase consists of solution A (0.08 % trifluoroacetic acid in acetonitrile) and solution B (0.1 % trifluoroacetic acid in ddH2O).

1.3 Mass spectrometry and NMR

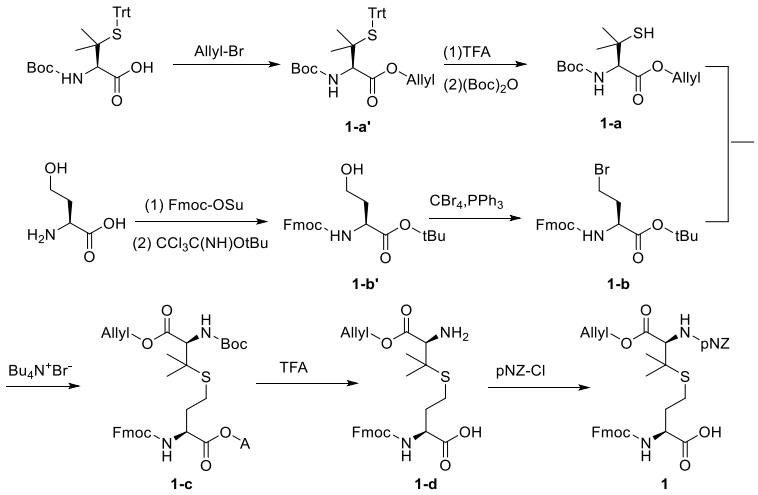
ESI-MS spectra were recorded on a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Fisher Scientific. USA) which was equipped with a standard ESI ion source. Data acquisition and analysis were done with the Xcalibur (version 2.0, Thermo quest Finnigan) software package. ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer using deuteriochloroform ($CDCl_3$) as the solvent ($CDCl_3$: 7.26 ppm, as internal reference) unless otherwise stated. ^{13}C-NMR spectra were recorded with ^{1}H-decoupling on a Brucker 101 MHz spectrometer.

2 Synthesis of Cys Cβ dimethyl modified diaminodiacid 1 and 2



Scheme S1. Structure of Cys Cβ dimethyl modified diaminodiacid 1 and 2

2.1 Synthesis of C-Pen diaminodiacid 1



Scheme S2. Synthesis route of diaminodiacid 1

Synthesis of compound 1-a'

After Boc-Pen(Trt)-OH (2.0 g, 4.07 mmol) and sodium bicarbonate(0.41 g, 4.88 mmol) were dissolved in DMF (30 mL), allyl bromide (0.43 mL, 4.88 mmol) was added. The reaction mixture was stirred overnight followed by diluted with water and extracted with EtOAc. The combined organic phase was washed with water, dried over Na_2SO_4, filtrated and concentrated in vacuo. The crude product was purified by chromatography to obtain compound **1-a'** (1.69 g, 3.18 mmol, 78.2%). R_f= 0.45 (10:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 – 7.58 (m, 5H), 7.32 – 7.23 (m, 7H), 7.19 (t, 3H), 5.88 (m, 1H), 5.40 – 5.28 (m, 2H), 5.21 (m, 1H), 4.60 (m,2H), 3.56 (d, 1 H), 1.45 (s, 9H), 1.04 (d, 6H).

Synthesis of compound 1-a

After compound **1-a'** (1.8 g, 3.39 mmol) was dissolved in DCM (14 mL), trifluoroacetic acid (14 mL) and triisopropylsilane (0.5 mL) were added dropwise to the solution. The reaction mixture was stirred at room temperature for 0.5h. Trifluoroacetic acid was removed by azeotroping with DCM under reduced pressure. Without further purification, the residue and $(Boc)_2O$ (0.86 mL, 3.73 mmol) were dissolved in 40 mL THF / water (1:1, v:v) and alkalified to pH 8 with 1M NaOH. After stirred for 8h, the mixture was concentrated under vacuum to remove THF and then extracted with EtOAc.

The combined organic phase was washed with water thrice. The crude product was purified by chromatography to afford compound **1-a** (0.91 mg, 3.14 mmol, 92.6%). R_f = 0.51(10:1, petroleum ether/EtOAc).

Synthesis of compound 1-b'

At 0°C, L-homoserine (1.0 g, 8.40 mmol), Fmoc-OSu (3.4 g, 10.1 mmol) and sodium carbonate (1.1 g, 10.1 mmol) were dissolved in 48 mL 1, 4-dioxane / water (1:5, v:v). After heated to room temperature, the reaction mixture was stirred overnight. Then, the mixture was diluted with water and washed with petroleum ether twice. After acidulated to pH 2 with 4 M HCl, the aqueous phase was extracted with EtOAc. The combined organic phase was dried over Na_2SO_4, filtrated and evaporated. The residue was then dissolved in DCM/THF (24 mL/6 mL), followed by tert-butyl-2, 2, 2-trichloroacetimidate (3.0 mL, 16.8 mmol) added. After stirred overnight, the reaction was concentrated under vacuum. The residue was dissolved in DCM and recrystallized. The precipitate was removed via filtration and the liquid was concentrated. The crude product was purified by chromatography to afford the expected product **1-b'** (1.67 g, 4.2 mmol, 50%). R_f= 0.35 (1:1, petroleum ether/EtOAc).

Synthesis of compound 1-b

Compound **1-b'** (1.5 g, 3.78 mmol) and carbon tetrabromide (1.5 g, 4.53 mmol) were dissolved in DCM (28 mL) at 0°C, followed by triphenylphosphine (1.2 g, 4.53 mmol) in DCM (2 mL) added dropwise. After heated to room temperature, the reaction mixture was stirred for 3h. The solution was concentrated to ~5 mL by rotary evaporator. Then, phosphine oxide byproducts was precipitated with excess of petroleum ether/EtOAc (1:1, v:v). After filtered and concentrated in vacuo, the crude product was purified by chromatography to obtain compound **1-b** (1.0 g, 2.18 mmol, 57.6%). R_f= 0.5 (5:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, 2H), 7.60 (d, 2H), 7.41 (t, 2H), 7.32 (t, 2H), 5.38 (d, 1H), 4.41 (m, 3H), 4.22 (t, 1H), 3.39 (m, 2H), 2.47 – 2.14 (m, 2H), 1.48 (s, 9H).

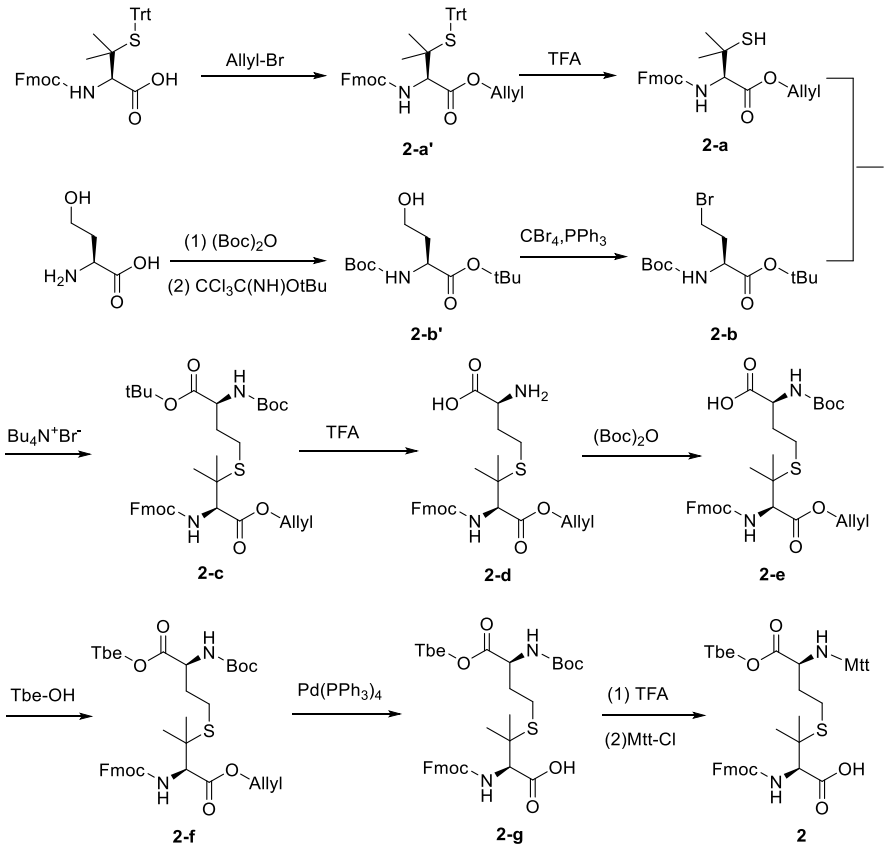
Synthesis of compound 1-c

Compound **1-a** (0.91 g, 3.14 mmol) and compound **1-b** (1.73 g, 3.77 mmol) were dissolved in EtOAc (40 mL), followed by tetrabutylammonium bromide (4.05 g, 12.56 mmol) in saturated $NaHCO_3$ aqueous solution (8 mL) added. After stirred vigorously for 24h at 40°C, the mixture was diluted with water and extracted with EtOAc. The extract organic phase was dried over Na_2SO_4, filtrated and concentrated. The crude product was purified by chromatography to give compound **1-c** (1.35 g, 2.02 mmol, 64.3%). R_f= 0.43(4:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, 2H), 7.62 (d, 2H), 7.40 (t, 2H), 7.31 (t, 2H), 5.91 (m, 1H), 5.62 (d, 1H), 5.44 – 5.32 (m, 2H), 5.25 (d, 1H), 4.70 – 4.55 (m, 2H), 4.38 (d, 2H), 4.31 (dd, 2H), 4.24 (t, 1H), 2.60 (t, 2H), 2.08 – 2.00 (m, 1H), 1.97 – 1.85 (m, 1H), 1.46 (d, 18H), 1.36 (d, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.97, 170.58, 155.93, 155.41, 143.91, 143.86, 141.29, 131.49, 127.71, 127.09, 125.22, 119.98, 119.25, 82.43, 80.14, 67.06, 66.03, 60.42, 53.80, 47.86, 47.16, 29.34, 28.31, 28.02, 25.75. HRMS calcd for $C_{36}H_{49}O_8N_2S$ 668.31314, found $[M+H]^+$ 669.32202.

Synthesis of DADA1

After compound **1-c** (0.262 g, 0.39 mmol) was dissolved in DCM (2 mL), trifluoroacetic acid (2 mL) was added. The reaction mixture was stirred vigorously for 6h at room temperature. Then, trifluoroacetic acid was removed by azeotroping with DCM under vacuo. The residue was dissolved in 2 mL saturated $NaHCO_3$ aqueous solution and 2.5 mL EtOAc, followed by pNZ-Cl (0.101 g, 0.47 mmol) added. After stirred for 3 hours at room temperature, the mixture was diluted with water and acidulated to pH 2 with 4 M HCl, followed by extracted with EtOAc. The combined organic phase was dried over Na_2SO_4, filtrated and concentrated. The crude product was purified by chromatography to give DADA **1** (0.197 g, 0.28 mmol, 71.8%). R_f= 0.32 (10:1, DCM/CH_3OH). ^1H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, 2H), 7.75 (d, 2H), 7.59 (d, 2H), 7.45 (t, 2H), 7.38 (t, 2H), 7.29 (t, 2H), 5.95 – 5.71 (m, 3H), 5.34 (dd, 1H), 5.25 (d, 1H), 5.17 (d, 2H), 4.69 – 4.57 (m, 2H), 4.52 – 4.32 (m, 4H), 4.21 (dd, 1H), 2.70 – 2.53 (m, 2H), 2.11 – 1.97 (m, 2H), 1.38 (s, 3H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, CDCl3) δ 175.48, 170.17, 156.18, 155.74, 147.59, 143.78, 143.68, 143.49, 141.28, 131.19, 129.94, 129.89, 128.09, 128.01, 127.77, 127.09, 125.12, 124.63, 123.74, 120.02, 119.65, 67.23, 66.35, 65.67, 60.89, 53.07, 52.93, 47.92, 47.10, 29.33, 27.22, 25.45. HRMS calcd for $C_{35}H_{38}O_{10}N_3S$ 691.21997, found $[M+H]^+$ 692.22723.

2.2 Synthesis of diaminodiacid 2



Scheme S3. Synthesis route of diaminodiacid **2**

Synthesis of compound 2-a

Fmoc-Pen(Trt)-OH (2 g, 3.26 mmol) and sodium bicarbonate(328 mg, 3.91 mmol) were dissolved in DMF (150 mL), followed by allyl bromide (338 μL, 3.91 mmol) added dropwise. The reaction mixture was stirred at room temperature overnight. After diluted with water, the mixture was extracted with EtOAc and then washed with water to remove remaining DMF. The organic phase was dried over Na_2SO_4, filtrated and concentrated. The crude product was purified by chromatography to afford compound **2-a'** (1.8 g, 2.75 mmol, 85.7%). R_f = 0.45 (6:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, 2H), 7.61 (d, 7H), 7.39 (t, 2H), 7.27 (m, 9H), 7.19 (t, 3H), 5.88 (m, 1H), 5.62 (d, 1H), 5.32 (d, 1H), 5.21 (d, 1H), 4.61 (d, 2H), 4.41 – 4.29 (m, 2H), 4.24 (t, 1H), 3.63 (d, 1H), 1.13 (s, 3H), 1.05 (s, 3H).

After compound **2-a'** (1.8 g, 2.75 mmol) was dissolved in DCM (12 mL), 12 mL trifluoroacetic acid and 0.5 mL triisopropylsilane were added dropwise. The reaction was monitored by TLC. After 0.5h, trifluoroacetic acid was removed under vacuum to give **2-a** without further purification.

Synthesis of compound 2-b'

L-Homoserine (4 g, 33.6 mmol) and Na_2CO_3 (8.6 g, 80.6 mmol) were dissolved in water/1, 4-dioxane (60 mL/12 mL) at 0°C. Then, $(Boc)_2O$ (9.2 mL, 40.3 mmol) was added to the mixture. After stirred overnight at room temperature, the mixture was washed with petroleum ether twice. The aqueous phase was acidulated to pH 2 with 4 M HCl and extracted with EtOAc. The combined organic phase was dried over Na_2SO_4, filtrated and evaporated. The residue was dissolved in DCM/THF (60 mL/15 mL). Then, tert-butyl 2, 2, 2-trichloroacetimidate (7 mL,67.2 mmol) was added to the solution. The reaction mixture was stirred at room temperature overnight. After concentrated in vacuo, the residue was redissolved in DCM and recrystallized to remove the byproducts (white crystals). The residue was purified to afford the expected product 2-b' (3.86 g, 16.8 mmol,44%) by column chromatography. R_f= 0.38 (3:1, petroleum ether/EtOAc).

Synthesis of compound 2-b

Compound **2-b'** (1.3 g, 4.72 mmol) and carbon tetrabromide (1.88 g, 5.67 mmol) were dissolved in DCM (13 mL) at 0°C, followed by triphenylphosphine (1.59 g, 6.05 mmol) in DCM (2 mL) added dropwise. After heated to room temperature, the reaction mixture was stirred for 3h. Then, the solution was concentrated to ~5 mL by rotary evaporator. Phosphine oxide byproducts was precipitated with excess of petroleum ether/EtOAc (1:1). After filtered and concentrated in vacuo, the crude product was purified by chromatography to obtain compound **2-b** (1.13 g, 2.95 mmol, 62.4%). R_f= 0.5 (6:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.11 (s, 1H), 4.28 (s, 1H), 3.47 – 3.38 (m, 2H), 2.38 (dd, 1H), 2.23 – 2.11 (m, 1H), 1.46 (d, 18H).

Synthesis of compound 2-c

Compound **2-a** (1.8 g, 2.75 mmol) and compound **2-b** (1.02 g, 3.02 mmol) were dissolved in EtOAc (40 mL). Then, tetrabutylammonium bromide (4.01 g, 12.44 mmol) in saturated $NaHCO_3$ solution (8 mL) was added. After stirred vigorously for 24h at 40°C, the mixture was diluted with water and extracted with EtOAc. The organic phase was dried over Na_2SO_4, filtrated and concentrated. The crude product was purified by chromatography to give compound **2-c** (1.2 g, 2.42 mmol, 66.7%). R_f= 0.37 (4:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, 2H), 7.61 (d, 2H), 7.40 (t, 2H), 7.32 (t, 2H), 5.94 (m, 1H), 5.69 (t, 1H), 5.33 (dd, 2H), 5.16 (d, 1H), 4.70

– 4.59 (m, 2H), 4.34 (m, 5H), 2.58 (t, 2H), 1.88 – 1.76 (m, 2H), 1.49 – 1.43 (m, 18H), 1.41 (s, 3H), 1.36 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.27, 170.26, 156.03, 155.40, 143.76, 141.30, 131.41, 127.74, 127.11, 125.19, 120.01, 119.42, 82.19, 79.79, 67.27, 66.17, 61.07, 53.50, 47.77, 47.15, 32.65, 29.72, 28.34, 28.00, 24.20. HRMS calcd for $C_{36}H_{49}O_8N_2S$ 668.31314, found $[M+H]^+$ 669.32208.

Synthesis of compound 2-f

Compound **2-c** (1.2 g, 1.79 mmol) was dissolved in DCM (8 mL) and TFA (8 mL) was added dropwise. After stirred for 6h, the reaction mixture was evaporated under vacuum to remove redundant TFA to give **2-d** without further purification. **2-d** and Na_2CO_3 (0.46 g, 4.31 mmol) were dissolved in water/1, 4-dioxane (20 mL/5 mL) under an ice bath followed by $(Boc)_2O$ (0.50 mL, 2.15 mmol) added to the mixture. After heated to room temperature, the mixture was stirred overnight. The solution was washed with petroleum ether twice. The aqueous phase was acidulated to pH 2 with 4 M HCl and extracted with EtOAc. The combined organic phase was dried over Na_2SO_4, filtrated and evaporated to give **2-e** without further purification. **2-e** and DMAP (0.03 g, 0.3 mmol) was dissolved in DCM (13 mL). Tbe-OH (0.36 g, 2.15 mmol) was dissolved in DCM (2 mL) and added to the mixture under an ice bath, followed by DCC (0.41 g, 2.24 mmol) added. After stirred at room temperature overnight, the reaction mixture was filtered and concentrated under vacuo. The crude product was purified by chromatography to give compound **2-f** (0.96 g, 1.27 mmol, 70.4%, three steps). R_f=0.45 (5:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, 2H), 7.62 (d, 2H), 7.40 (t, 2H), 7.32 (t, 2H), 5.94 (m, 1H), 5.70 (t, 1H), 5.33 (dd, 2H), 5.24 (d, 1H), 4.66 (s, 2H), 4.50 – 4.31 (m, 6H), 4.24 (t, 1H), 2.89 (m, 2H), 2.60 (t, 2H), 2.09 – 2.02 (m, 1H), 1.94 – 1.87 (m, 1H), 1.44 (s, 9H), 1.40 (d, 3H), 1.37 (d, 3H), 1.32 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.03, 170.31, 156.04, 155.37, 143.75, 141.30, 131.38, 127.75, 127.12, 125.19, 120.01, 119.51, 80.04, 67.28, 66.19, 63.63, 60.97, 52.85, 48.05, 47.89, 47.16, 38.12, 32.14, 29.85, 28.33, 24.20. HRMS calcd for $C_{38}H_{53}O_8N_2S_3$ 760.28858, found $[M+H]^+$ 761.29706.

Synthesis of compound 2-g

To a solution of compound **2-f** (0.44 g, 0.58 mmol) in THF (4 mL), Pd $(PPh_3)_4$ (0.13 g, 0.11 mmol) and N-methylaniline (0.12 mL, 1.16 mmol) were added. After stirred vigorously for 1 h, the mixture was directly concentrated in vacuo to remove THF. The residue was diluted with EtOAc and washed with brine, dried over Na_2SO_4, filtrated and concentrated. The crude product was purified by chromatography to give compound **2-g** (0.33 g, 0.46 mmol, 79.6%). R_f = 0.4 (10:1, DCM/CH_3OH).

Synthesis of DADA 2

After compound **2-g** (0.33 g, 0.46 mmol) was dissolved in DCM (2 mL), TFA (2 mL) was added to the solution. The reaction mixture was stirred vigorously for 1h at room temperature. Then, the residual trifluoroacetic acid was removed by azeotroping with DCM under vacuo. The crude product was dissolved in DCM (4 mL). Under nitrogen atmosphere and room temperature, DIEA (0.24 mL, 1.43 mmol) and trimethylsilyl chloride (0.12 mL, 1.43 mmol) were added. The reaction solution was refluxed at 40 °C for 1h and became homogeneous. Then the reaction system was cooled to 0 °C, DIEA (0.24 mL, 1.43 mmol) and 4-methyltrityl chloride (0.16 g, 0.55 mmol) were added. After stirred overnight at room temperature, methanol (0.5 mL) was added to quench the reaction. After 15 min, the mixture was evaporated under vacuum at 35 °C. The crude product was purified

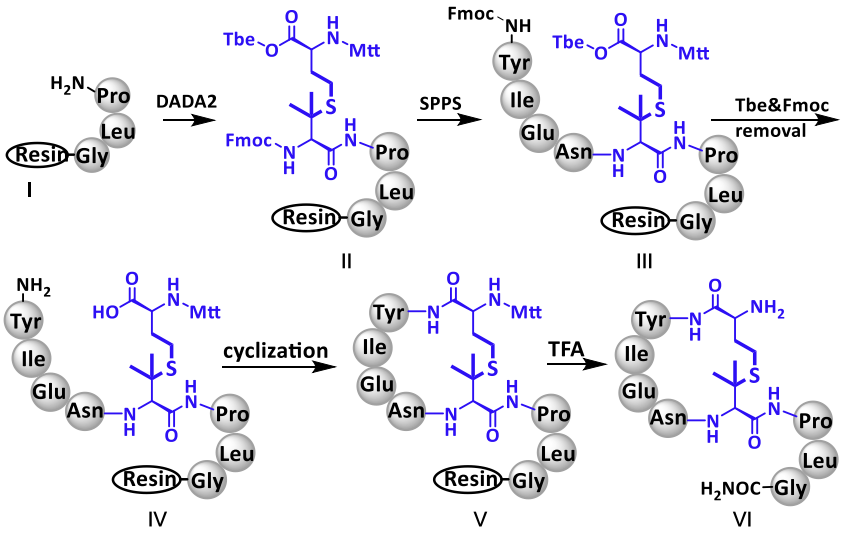
by chromatography to afford DADA**2**(0.21 g, 0.24 mmol, 52%). R_f= 0.42(5:1, petroleum ether/EtOAc with 0.5% AcOH). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, 2H), 7.59 (t, 2H), 7.47 (d, 4H), 7.39 – 7.28 (m, 7H), 7.23 (d, 3H), 7.15 (t, 2H), 7.05 (d, 2H), 5.74 (d, 1H), 5.38 (dd, 1H), 4.49 – 4.17 (m, 6H), 3.83 – 3.64 (m, 2H), 3.47 – 3.39 (m, 1H), 2.64 – 2.58 (m, 2H), 2.28 (s, 3H), 2.02 – 1.96 (m, 2H), 1.28 (s, 9H), 1.25 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.41, 173.38, 156.19, 145.98, 143.81, 142.83, 141.30, 136.03, 128.76, 128.60, 127.88, 127.73, 127.14, 126.45, 125.25, 119.98, 71.00, 67.34, 63.09, 61.06, 55.75, 48.01, 47.16, 45.64, 38.13, 35.46, 29.83, 25.85, 20.96. HRMS calcd for $C_{50}H_{57}O_6N_2S_3$ 876.33005, found $[M+H]^+$ 877.33862.

3. Solid-phase peptide synthesis of disulfide surrogate peptides

3.1 Fmoc-based solid-phase peptide synthesis

Syntheses of peptides began with swelling 2-Chlorotrityl resin or Rink Amide AM resin with DCM/DMF (1/1, v/v) for 30 min. Each coupling cycle included amino acid coupling and Fmoc deprotection. Before each protected amino acid (4.0 equiv. to resin loading) was coupled to the resin, it was pre-activated with 4.0 eq coupling reagent (HCTU or HATU, HATU used for sterically hindered amino acids) and 8.0 eq DIEA in DMF for 0.5-1 min. A 40-min reaction time was costed for each coupling reaction. The Fmoc deprotection was accomplished with 20% piperidine in DMF (5 min+10 min). After each reaction step, the resin was washed with DMF (3 times), DCM (3 times) and DMF (3 times). When the assembly of peptides was finished, the resin was treated with a mixture of TFA/water/phenol/TIPS (88/5/5/2, v/v/v/v) or TFA/ thioanisole/water/1,2-ethanedithiol/ (87/5/5/3, v/v/v/v) for 3h to cleave the completed peptides from the resin. After blowed with N_2, the solution was added an excess of cold ether, followed by centrifugation. The crude peptides were purified by semi-preparative RP-HPLC and analyzed by ESI mass spectra.

3.2 Synthesis and characterization of oxytocin mimic containing DADA2



Scheme S4. Synthesis route of oxytocin mimic.

0.025 mmol Rink amide AM resin (83 mg, 0.3 mmol/g) was used for the synthesis of oxytocin mimic**.** Firstly, Fmoc-Gly-OH, Fmoc-Leu-OH and Fmoc-Pro-OH were assembled to the resin

according to the protocol described in **Fmoc-based solid-phase peptide synthesis** to give **Ⅰ**.Then, PyAOP (2 equiv.), HOAt (2 equiv.) and NMM (4 equiv.) in DMF were used to pre-activate the free -COOH of DADA **2** and the mixture was transferred to the resin. After an overnight reaction, Fmoc groups of **Ⅱ** were removed and Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH and Fmoc-Tyr(tBu)-OH were coupled to the resin in turn to give **Ⅲ**. Next, Tbe and Fmoc groups were removed successively by using 2-mercaptoethanol(1.25M) /DIEA(1.25M) in NMP(1 mL) for 2h x 2 and 20% piperidine in DMF(5min+10min) to give **Ⅳ**. Cyclization was efficiently finished after 12 h to give **Ⅴ** by using a solution of PyAOP (4.0 equiv.), HOAt (4.0 equiv.) and NMM (8.0 equiv.) in DMF. Finally, the resin was treated with a mixture of TFA/water/phenol/TIPS (88/5/5/2, v/v/v/v) for 3h to give **Ⅵ**. After filtration to remove the cleaved resin, the filtrate was concentrated under a stream of N_2, followed by an excess of cold Et_2O added and centrifugation. The crude peptide was then purified by semi-preparative HPLC (a linear gradient from 5% to 90% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, 4 mL/min).

To verify the secondary structure of oxytocin mimic, circular dichroism (CD) spectrum was measured on a Pistar π-180 spectrometer using a quartz cell with a path length of 1 mm at 25°C. The concentration of peptide in H_2O is 0.2 mg/mL. The circular dichroism (CD) spectrum showed a a negative peak at 203nm and a positive band at 219-228nm, which indicates the secondary structure of the product is similar with the structure of the reported native oxytocin.

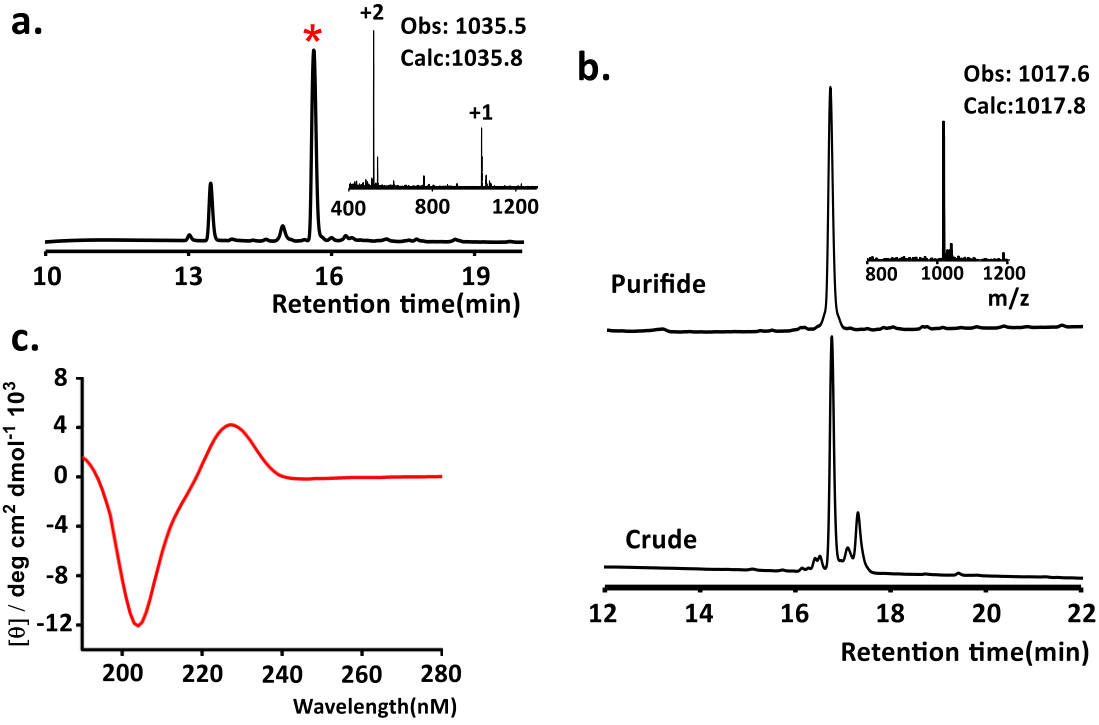


Figure S1. (a) HPLC traces and ESI-MS of **IV**; (b) HPLC traces and ESI-MS of **VI**; (c) CD spectra of oxytocin mimic.

3.3. Synthesis and characterization of μ-conotoxin KIIIA mimic containing DADA1

3.3.1 Synthesis of linear peptide precursor

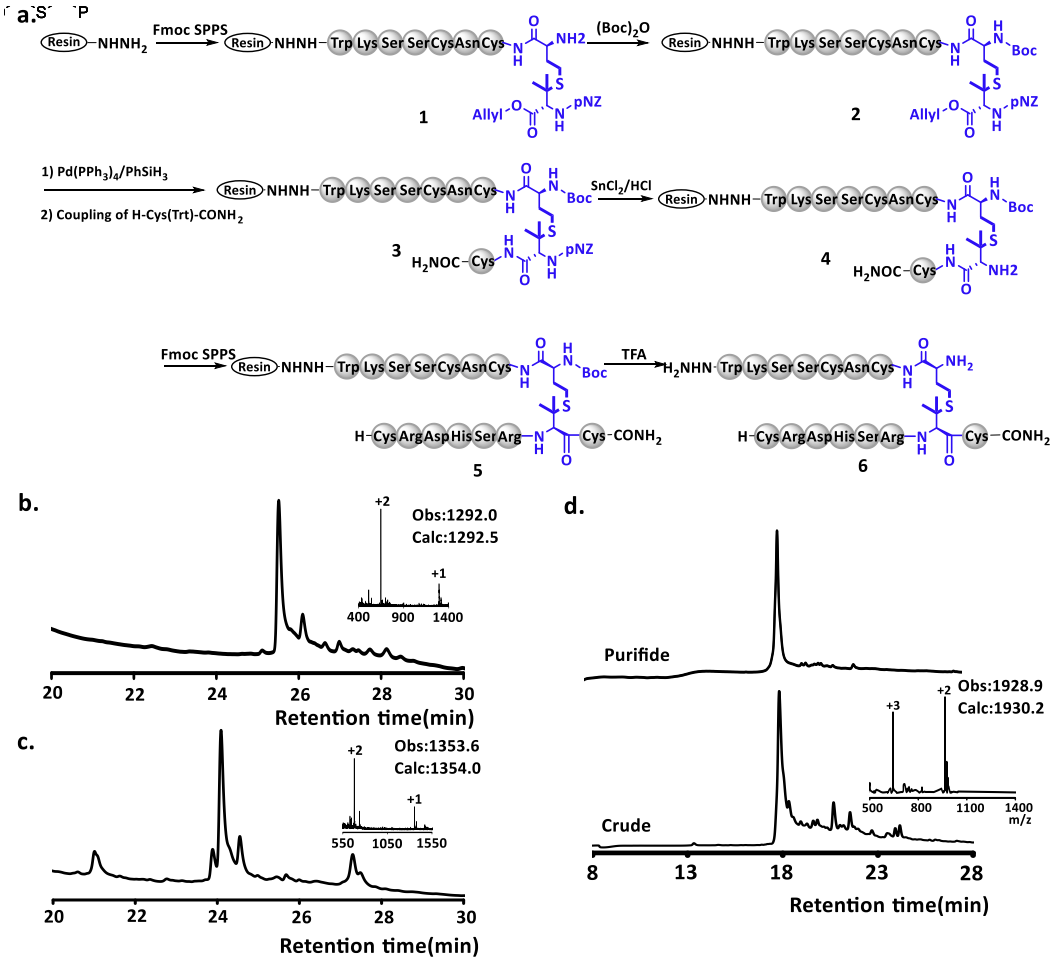


Figure S2. (a) Synthesis route of linear peptide precursor **6**; (b) RP-HPLC and ESI-MS of the crude **2**; (c) RP-HPLC and ESI-MS of the crude **3**;(d) RP-HPLC and ESI-MS of the crude and purified **6**.

0.075 mmol 2-Cl-Trt-$NHNH_2$ resin was prepared by hydrazination of 2-Cl-(Trt)-Cl resin (221 mg, 0.34 mmol/g) according to the reported method, followed by 5% (vol/vol) MeOH/DMF added (10 min ×2) added to cap the unreacted sites. Fmoc-Trp(Boc)-OH was coupled to the resin by using aa/DIC/Oxyma coupling method (resin:aa:DIC:Oxyme=1:4:8:4 (mol/mol/mol/mol), at 37℃ overnight). After Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asn(Trt)-OH and Fmoc-Cys(Trt)-OH coupled to the resin in turn, DADA**1** coupling was carried out by using the same method described in **Synthesis and characterization of oxytocin mimic containing DADA2**. Then the resin was treated with 20% piperidine in DMF to remove the Fmoc groups of DADA to give **1**. The free amine groups were protected again by using $(Boc)_2O$ ($(Boc)_2O$/DIEA/DMF (1:1:8(v/v/v) for 10 min ⋆2 at room temperature) to give **2**. Next, after Allyl groups were removed by using $Pd(PPh_3)_4$ /$PhSiH_3$ (87 mg/91 μL) in DCM for 3 h, H-Cys(Trt)-$CONH_2$ (4 equiv., 109 mg) was coupled to the released acid group of DADA using Pyaop (4 equiv., 156 mg)/HOAT (4 equiv., 41 mg)/NMM(8 equiv., 66 μL) to give intermediate **3**. The pNZ groups were removed by $SnCl_2$ (6 M) and HCl/CH_3OH (5 mM) in DMF (1h ×2) to give **4**. The resin was washed with DMF (3 mL×5 times), H_2O (3 mL×5 times), CH_3OH (3 mL×5 times), DCM (3 mL×5 times) and DMF (3 mL×5 times). After the chain elongation with the remaining amino acids was performed to give **5**, the resin was treated with 5 mL TFA cocktails (thioanisole/water/1,2-ethanedithiol/trifluoroacetic acid = 5/5/3/87, v/v/v/v) for 3h to give linear precursor **6**. The solution

was concentrated under a stream of N_2, followed by excess of cold Et_2O added and centrifugation. Crude peptide was verified by ESI-MS and purified by semi-preparative HPLC (a linear gradient from 5% to 90% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, 4 mL/min) to afford 16mg precursor **6** (11% isolated yield).

3.3.2 Intramolecular NCL for cyclization

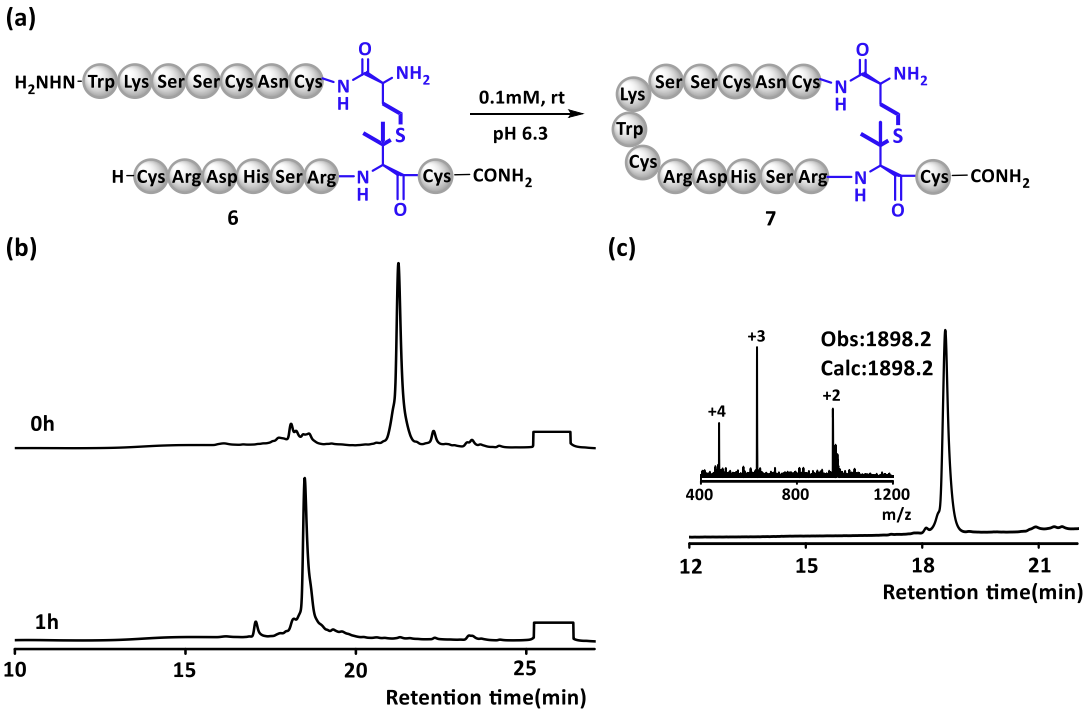


Figure S3. (a) Hydrazide-based NCL for peptide macrocyclization to give **7**; (b) RP-HPLC traces of NCL of **6**; (c) RP-HPLC traces and ESI-MS of purified **7**.

Hydrazide-based NCL approach was carried out to cyclize the precursor **6**. Firstly, 6 mg of **6** was dissolved in 30 mL 0.2 M phosphate buffer (6 M Gn·HCl, pH 3.0). Then, 61 μL of 0.5M $NaNO_2$ aqueous solution was added to oxidize the hydrazide for 20min at -15℃ in the ice/salt bath. Next, MPAA (26 mg, 50 equiv.) was added to the mixture to obtain the corresponding thioesters. The reaction was performed at pH 6.3–6.6 for 1 h. RP-HPLC analysis showed that there was no intermolecular ligation side-product. Peptide **7** (3 mg, 51% isolated yield) was verified by ESI-MS and purified by semi-preparative HPLC (a linear gradient from 10% to 90% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, 4 mL/min).

Folding for 8

Peptide **7** (2 mg) was dissolved in 30 mL water, followed by glutathione oxidized (3 mg, 10equiv.)/glutathione reduced (32 mg, 100 equiv.) added. After adjust the pH to 7.3-7.7, the reaction was performed overnight. The folding mixture was purified by using semi-preparative HPLC (a linear gradient from 10% to 90% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, 4 mL/min) to afford 0.6 mg of the folded product **8** (30% isolated yield).

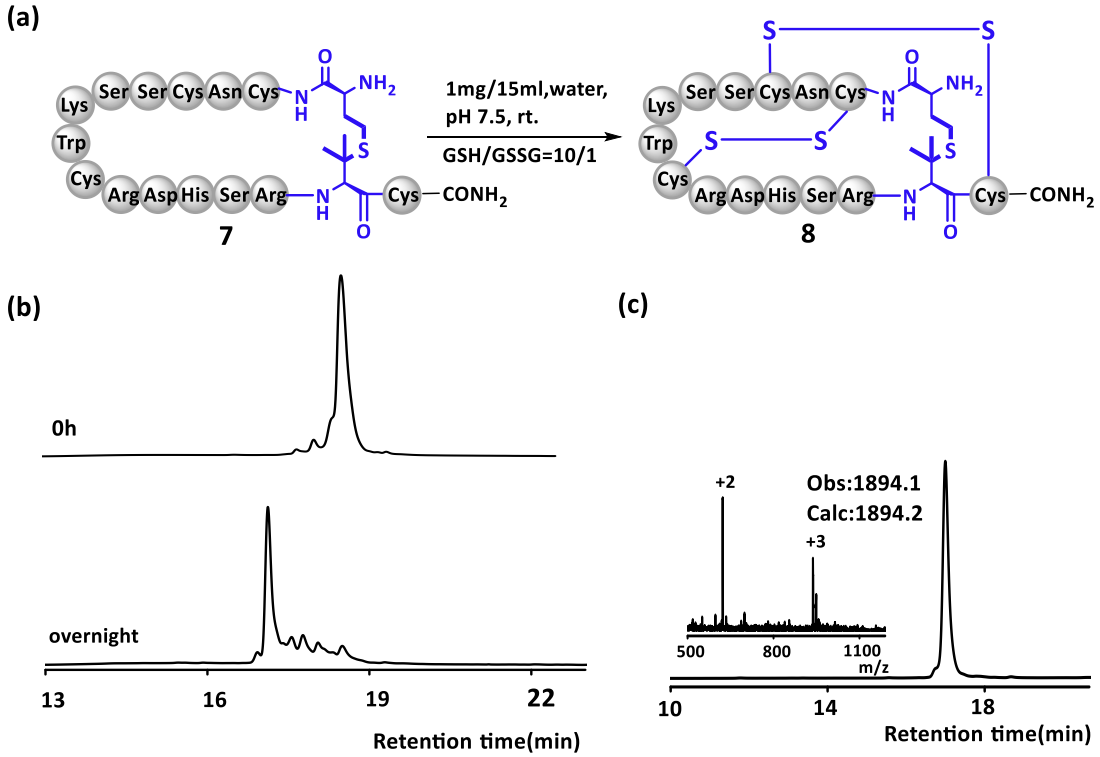
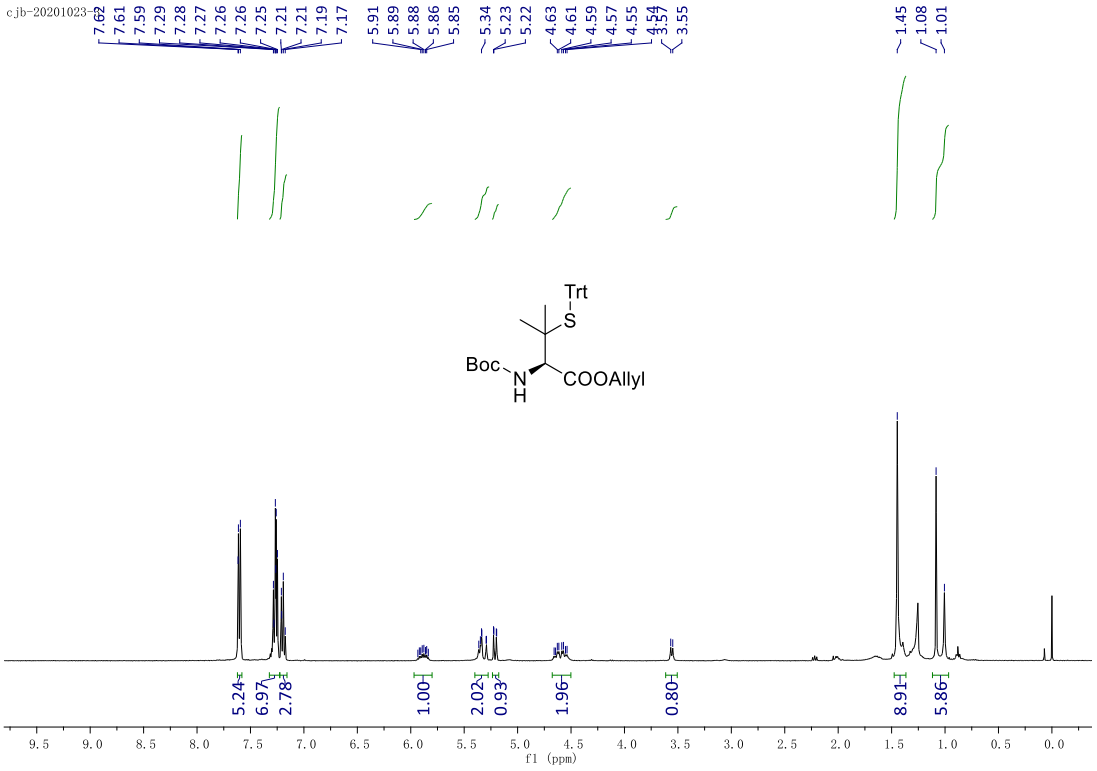
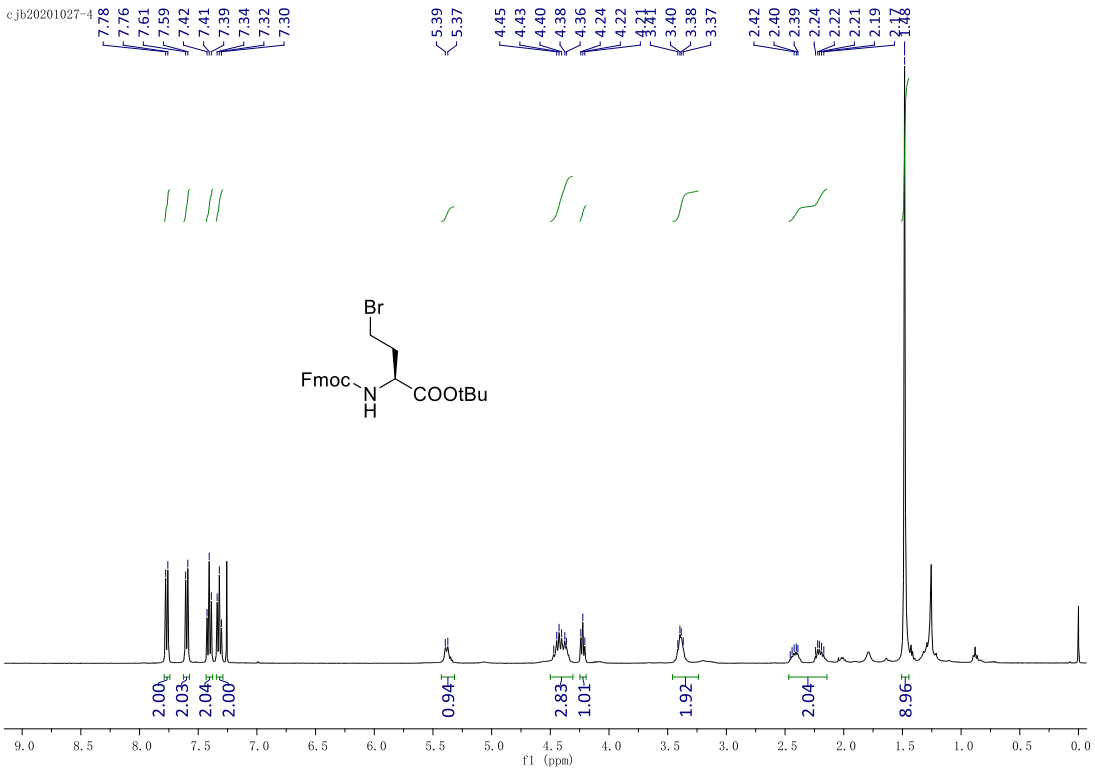


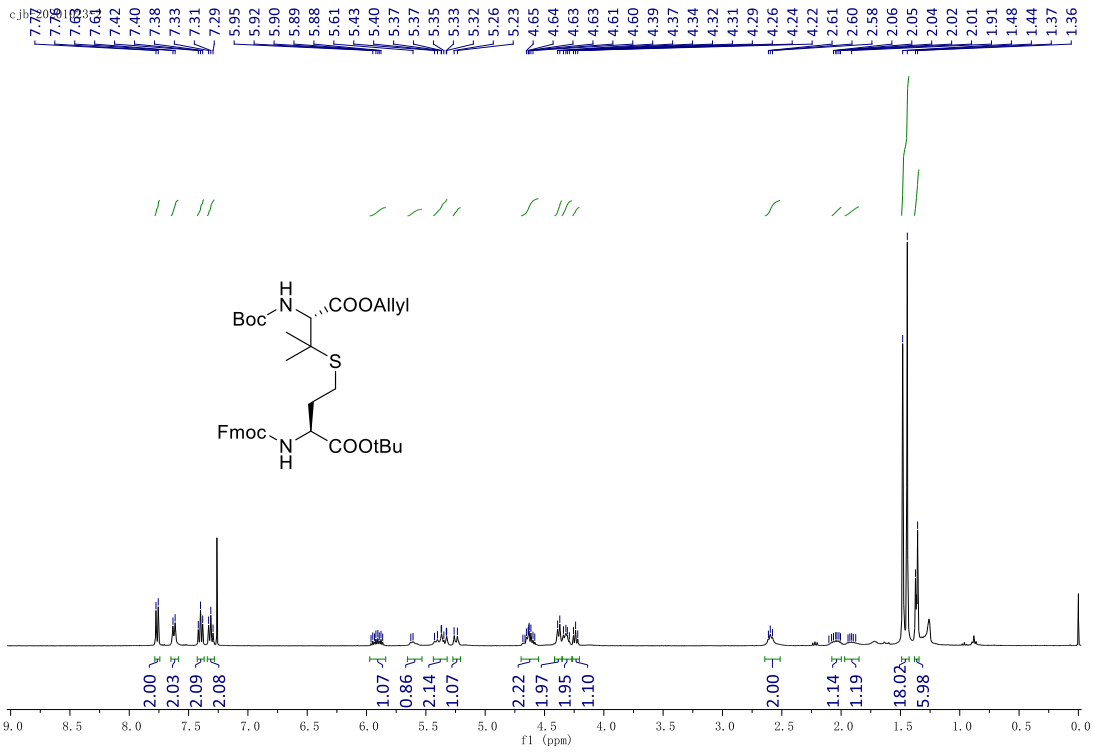
Figure S4. (a) The folding of **7** to give the final product **8**; (b) RP-HPLC traces of the folding of **7**; (c) RP-HPLC traces and ESI-MS of purified **8**.



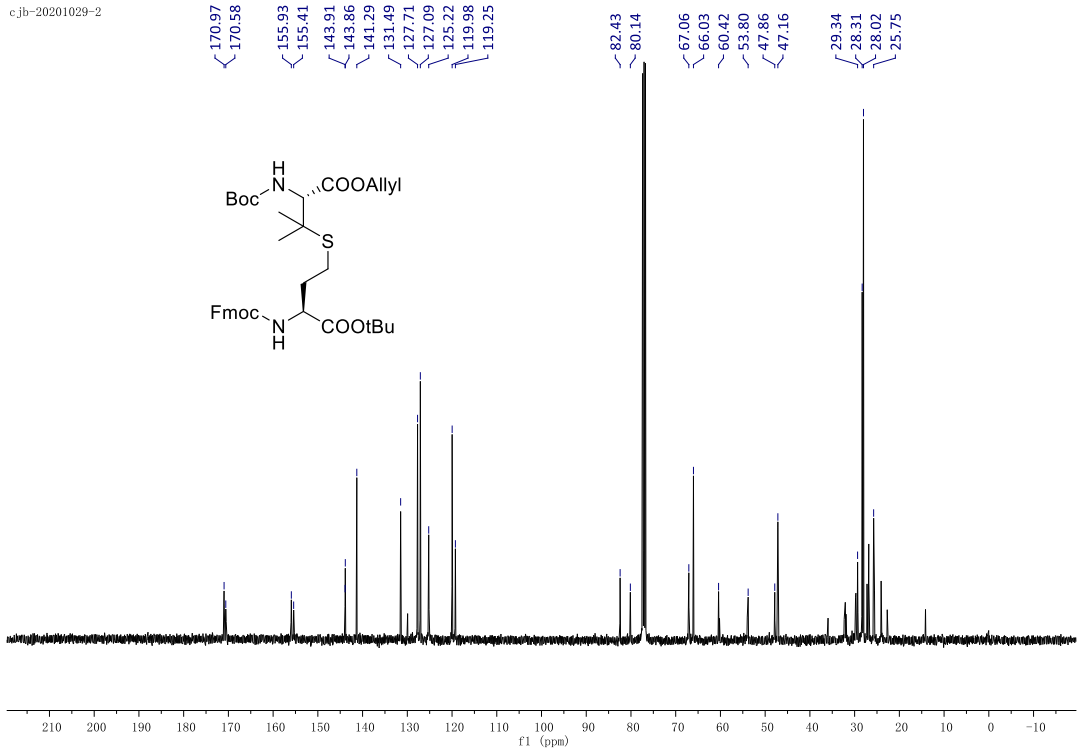
^{1}H NMR spectrum of 1-a'.



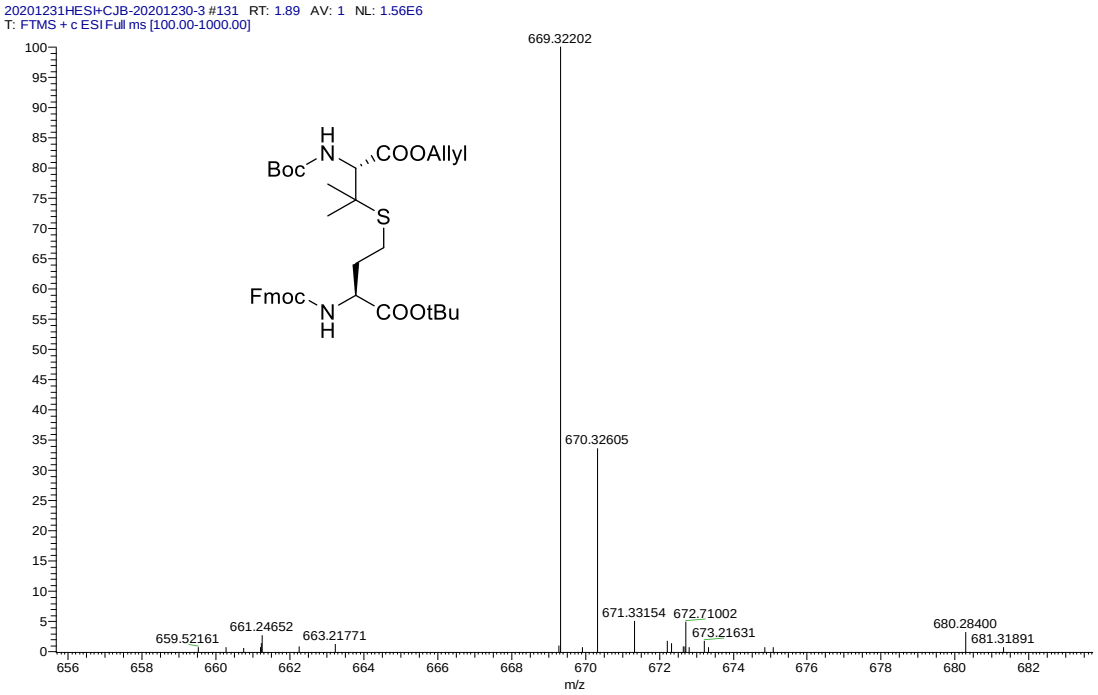
^{1}H NMR spectrum of 1-b.



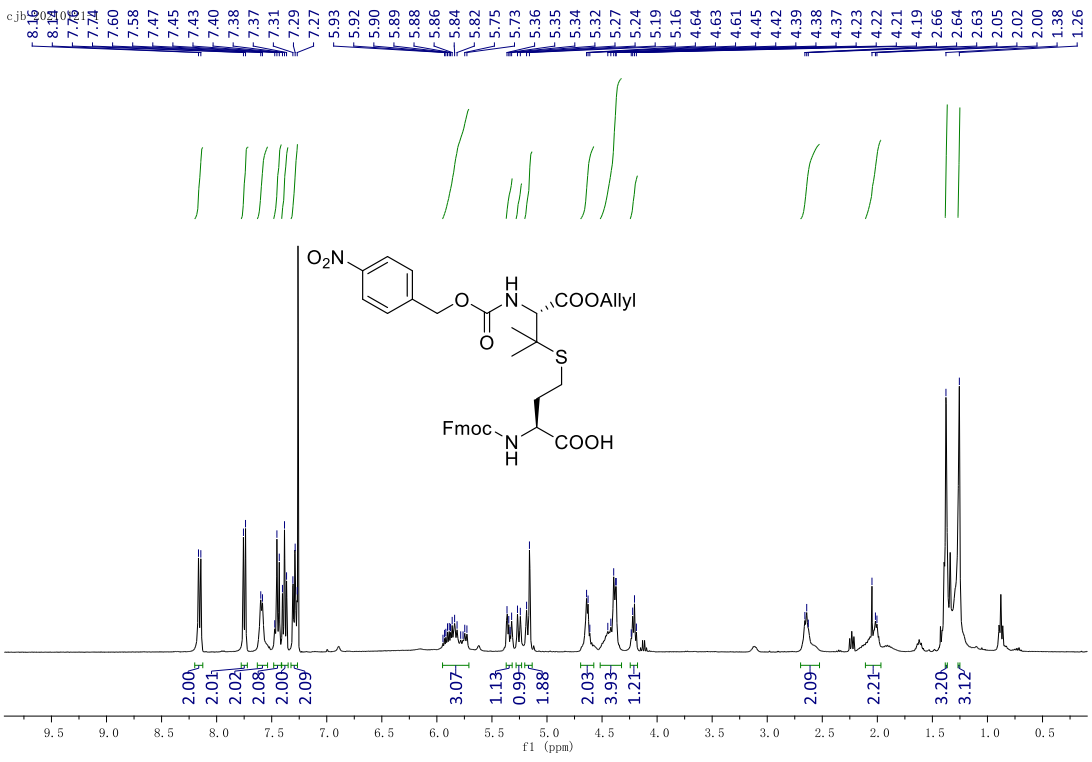
^{1}H NMR spectrum of 1-c.



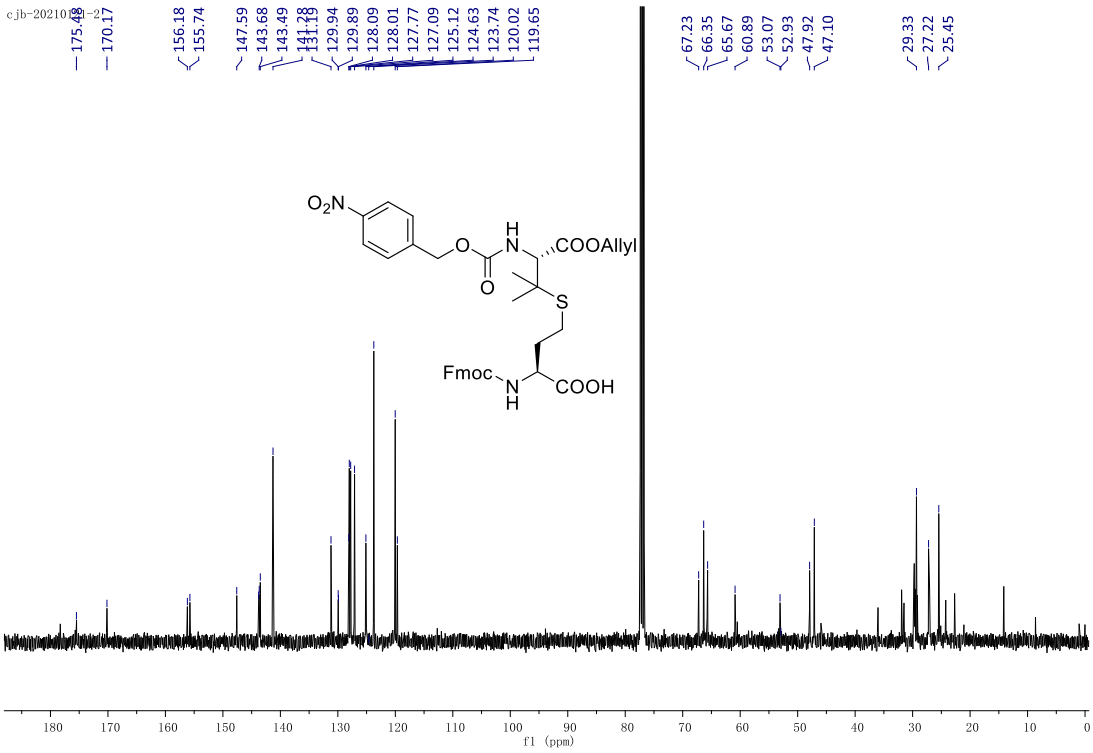
^{13}C NMR spectrum of 1-c.



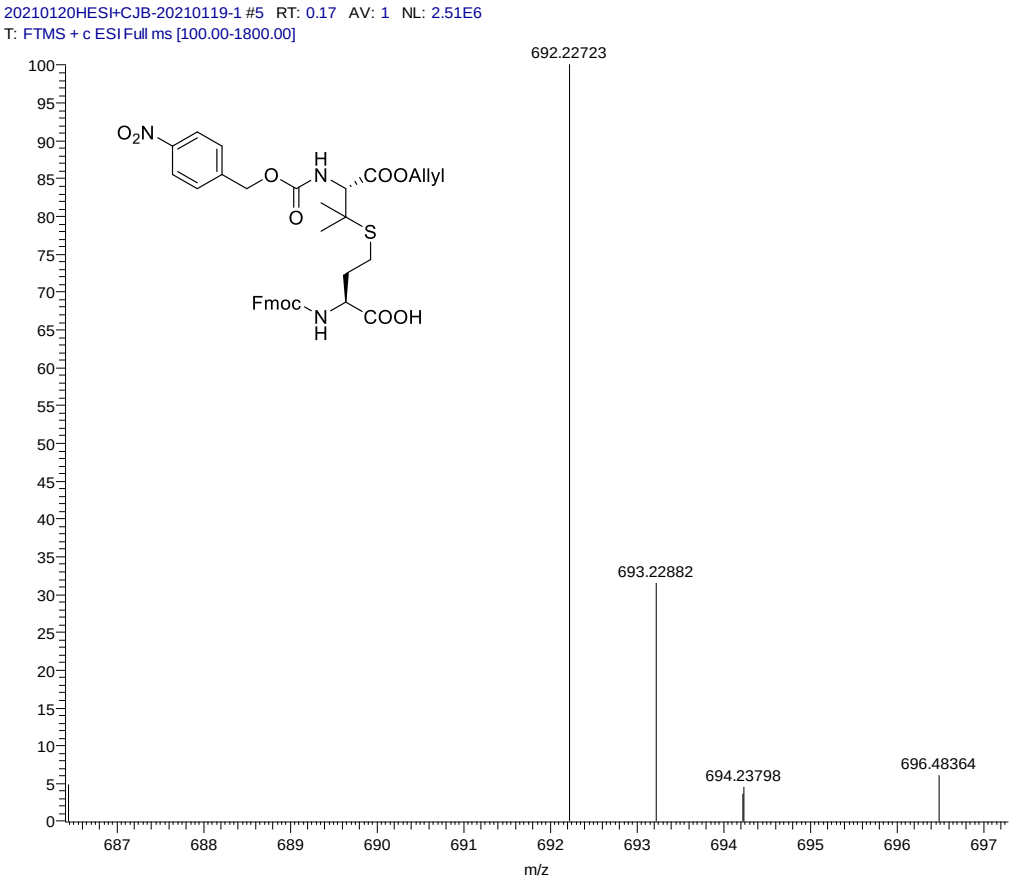
HRMS of compound1-c.



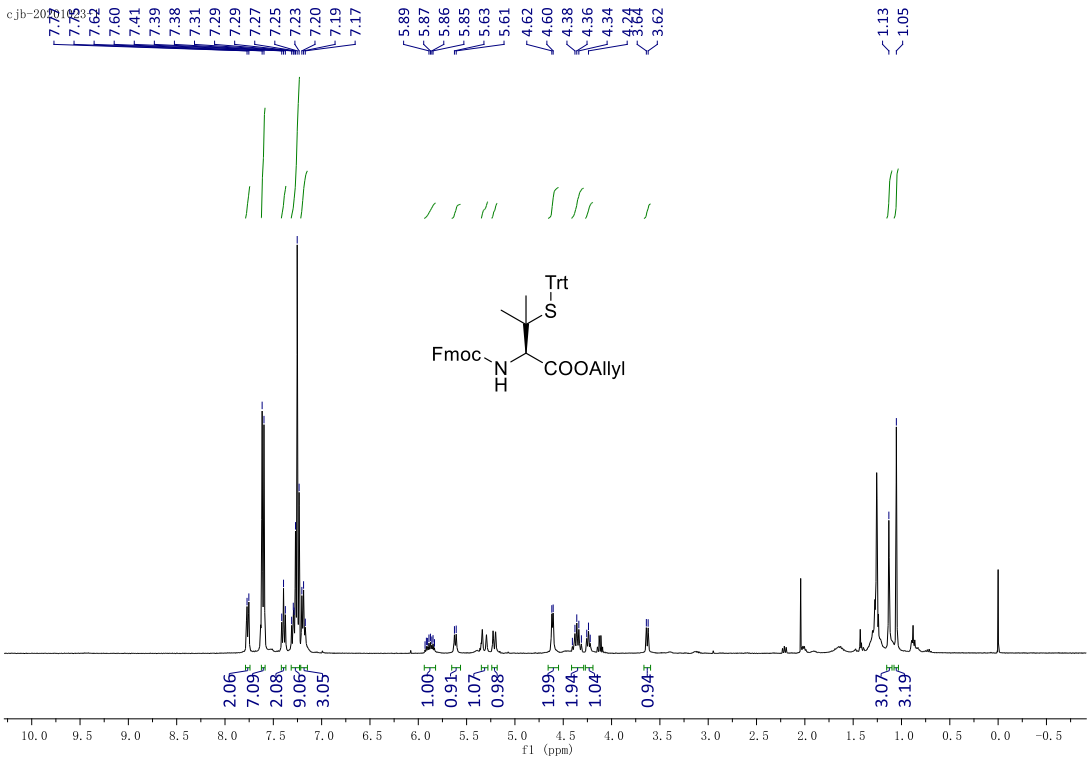
^{1}H NMR spectrum of DADA1.



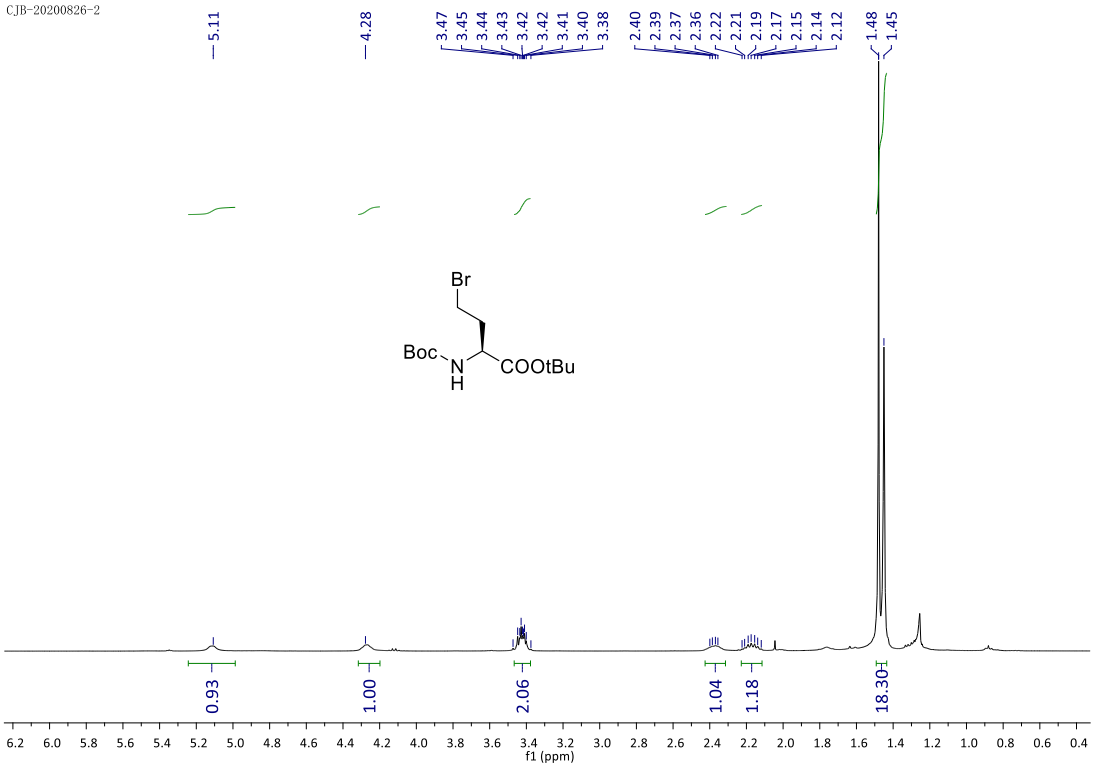
^{13}C NMR spectrum of DADA1.



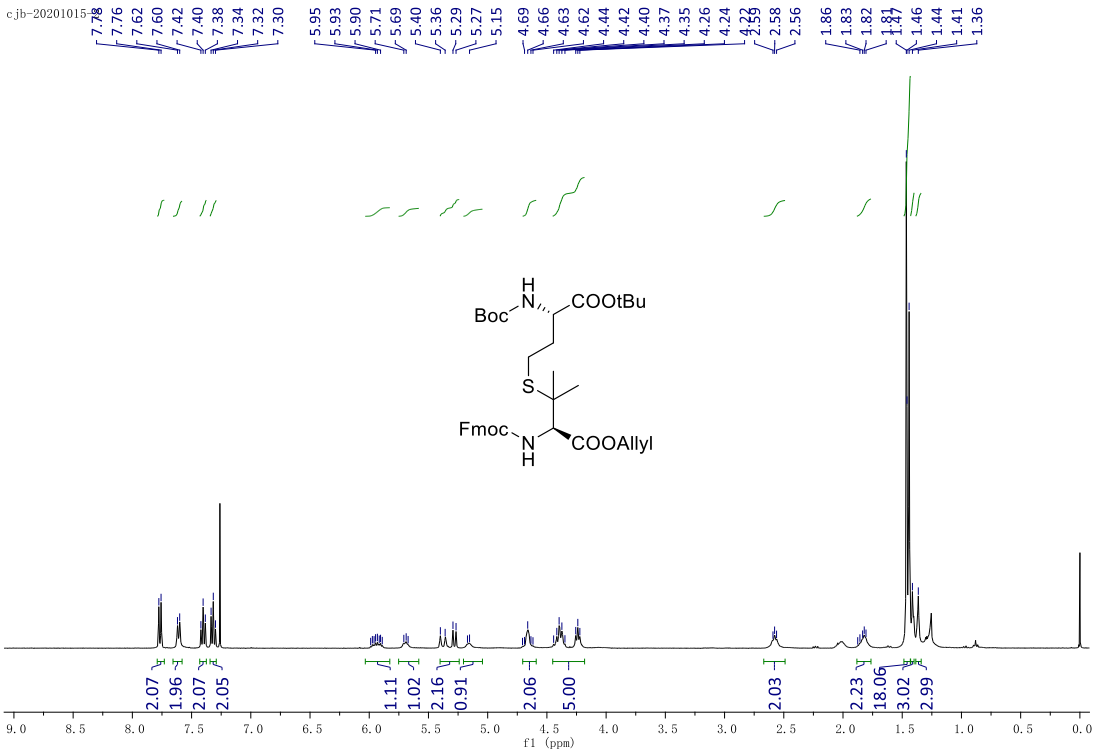
HRMS of DADA1.



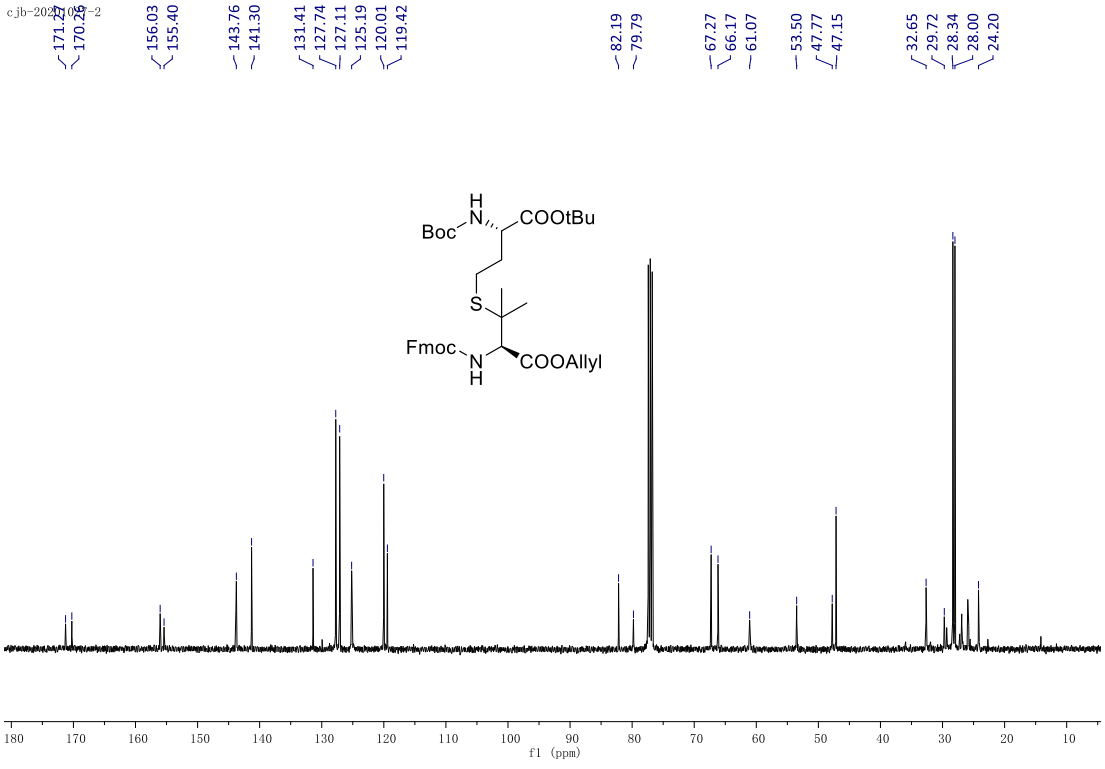
^{1}H NMR spectrum of 2-a'.



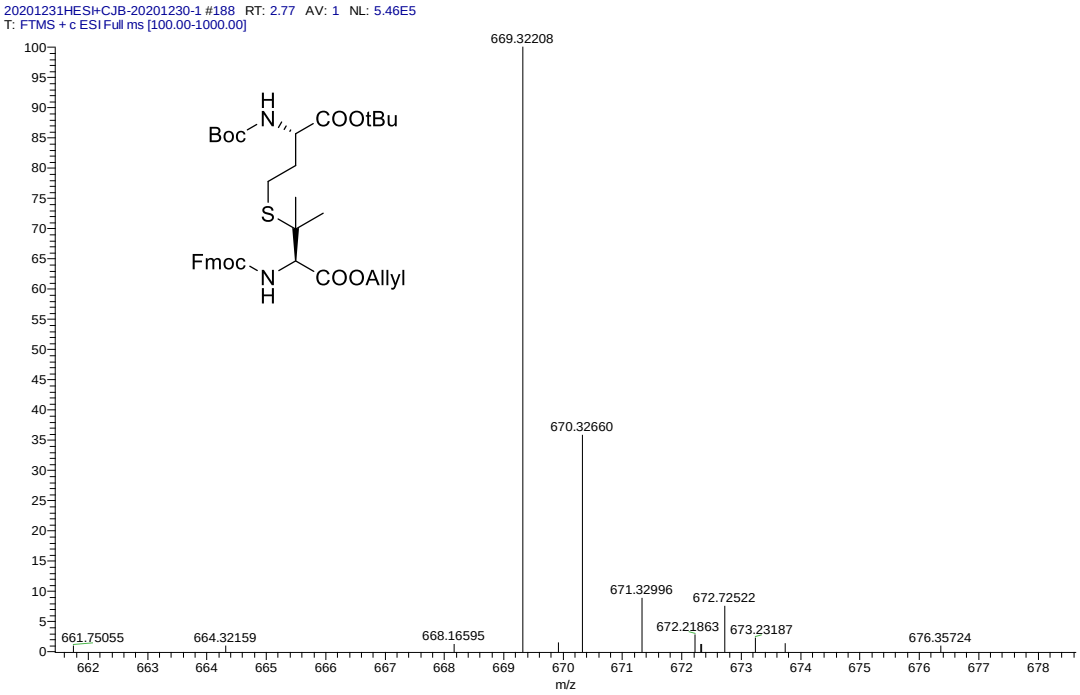
^{1}H NMR spectrum of 2-b.



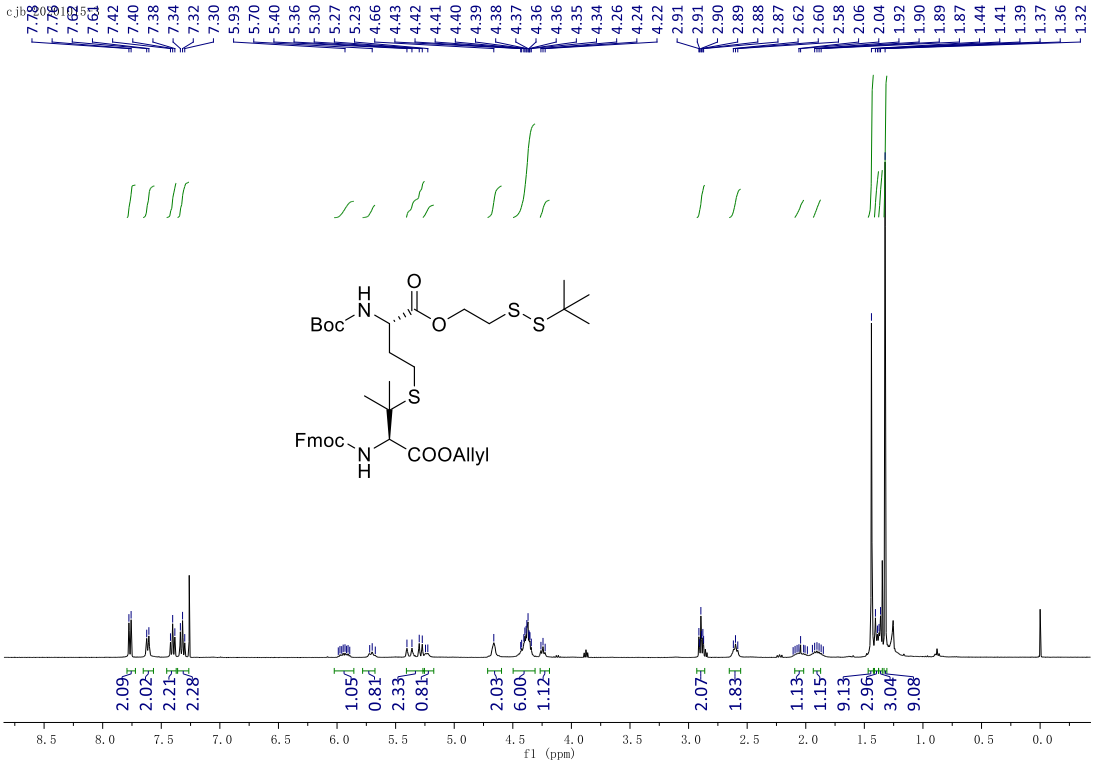
^{1}H NMR spectrum of 2-c.



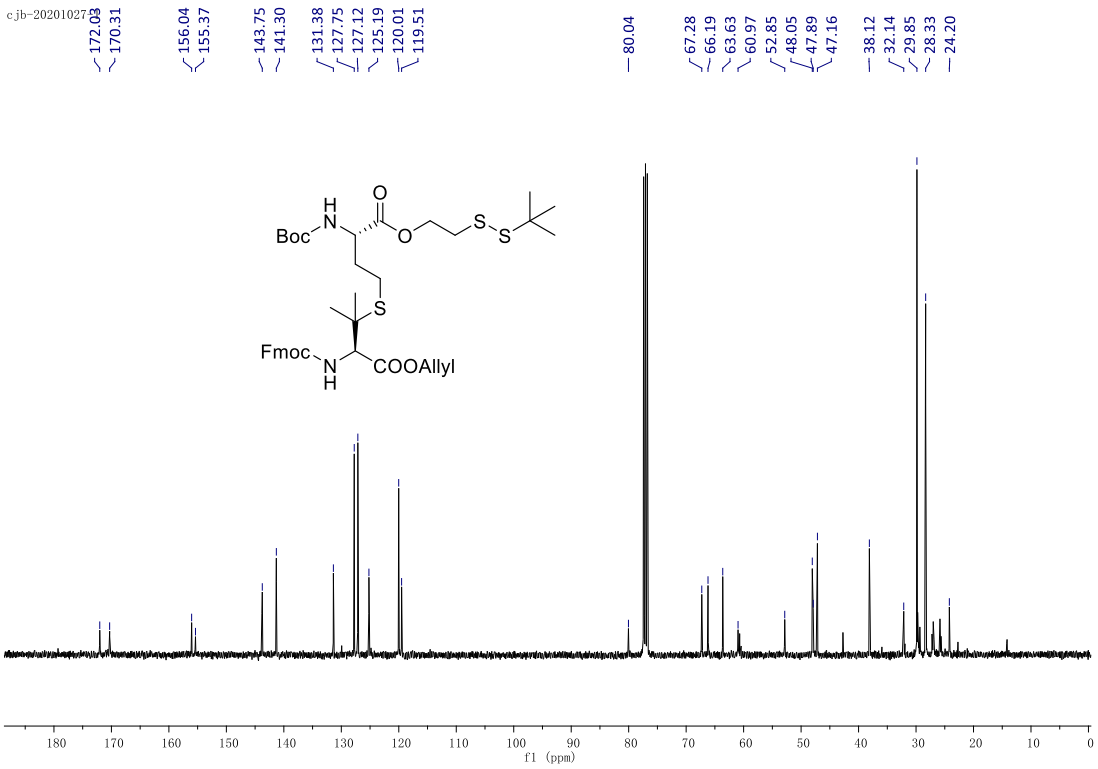
^{13}C NMR spectrum of 2-c.



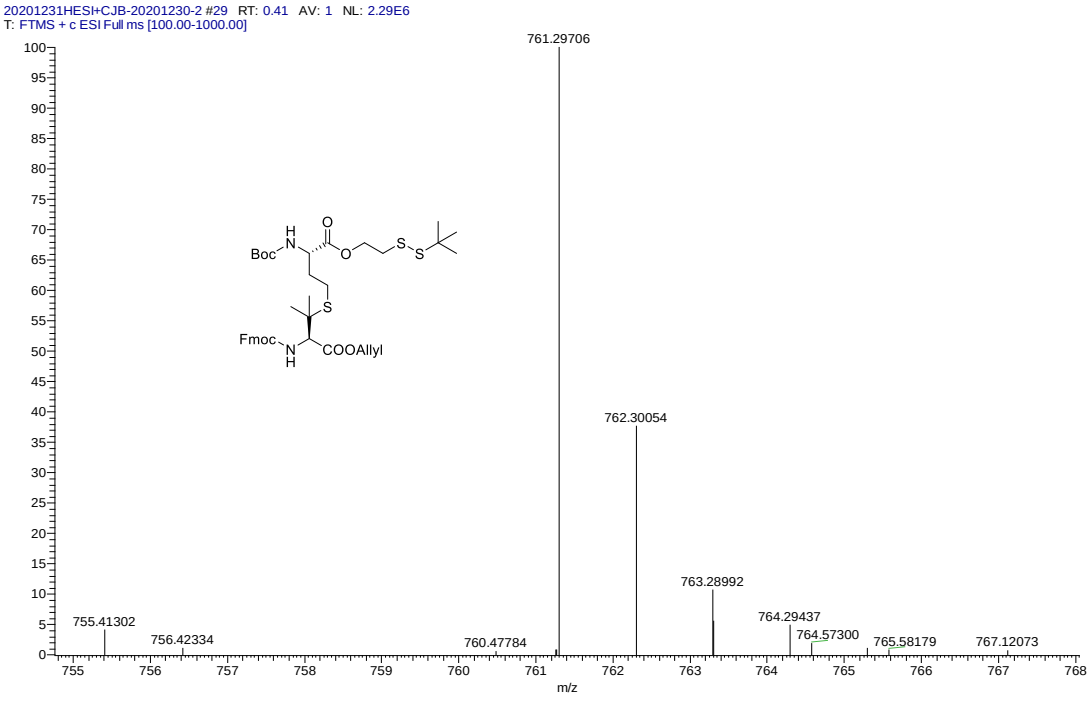
HRMS of 2-c.



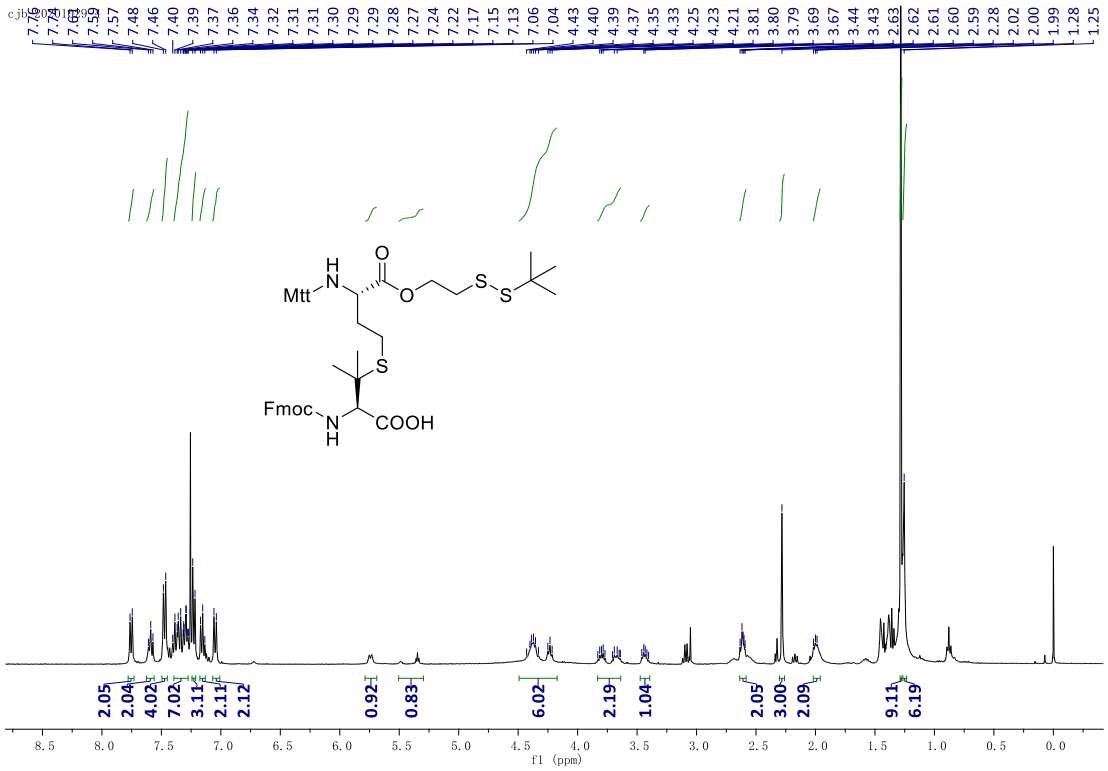
^{1}H NMR spectrum of 2-f



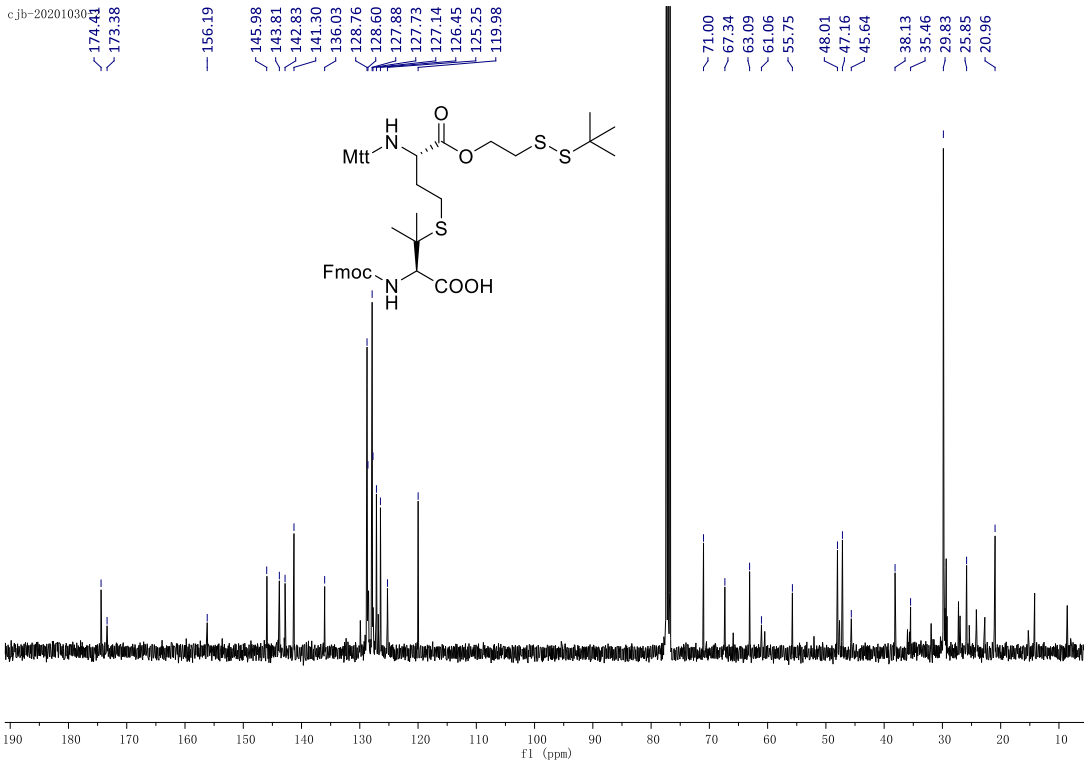
^{13}C NMR spectrum of 2-f.



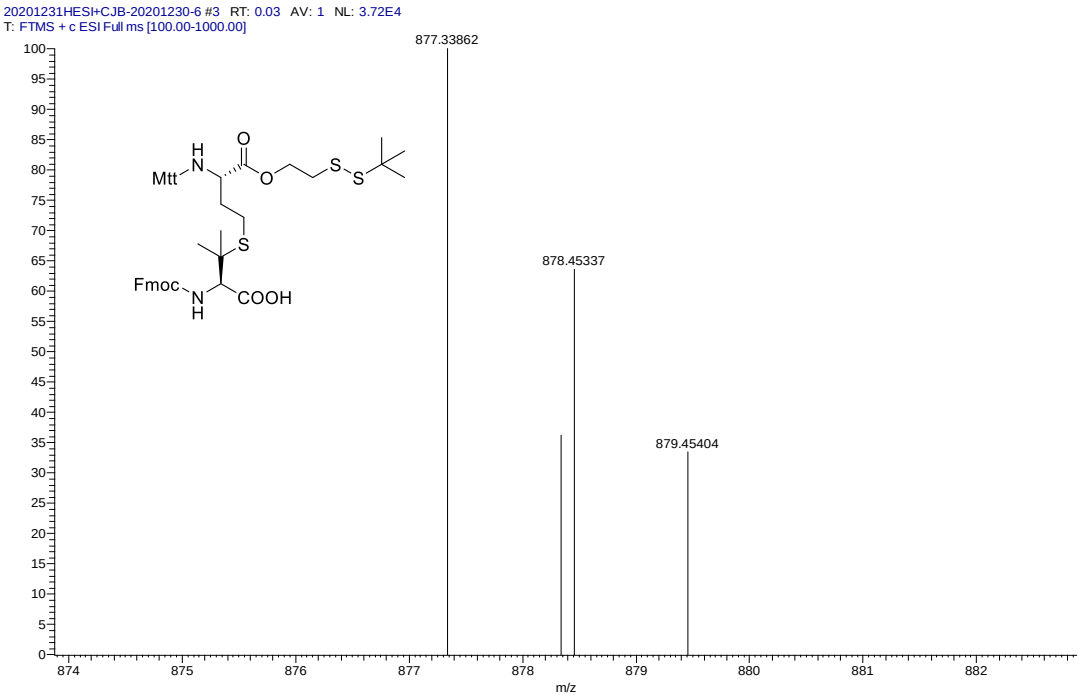
HRMS of 2-f



^{1}H NMR spectrum of DADA2.



^{13}C NMR spectrum of DADA2.



HRMS of DADA2