Synthesis of disulfide surrogate peptides incorporating ethylene glycol bridge

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1.General information

1.1 Materials and Reagents

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), tetrahydrofuran (THF) 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 ℃)

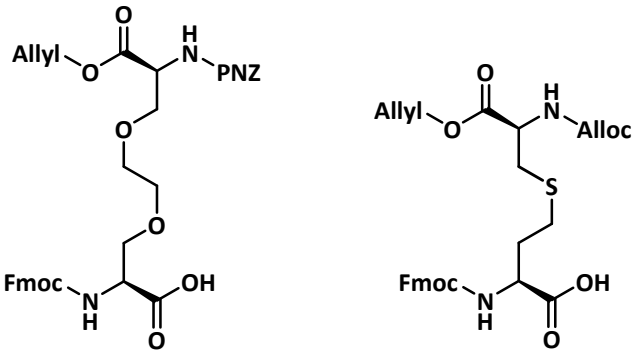
1.2 HPLC

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.). 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). Analytical samples and semi-preparative samples were monitored at 214 nm and 254 nm. Solution A (0.08 % trifluoroacetic acid in acetonitrile) and solution B (0.1 % trifluoroacetic acid in ddH_2O) form the mobile phase.

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 EG-DADA and thioether diaminodiacid



Scheme S1: Structure of EG-DADA and thioether diaminodiacid

2.1 Synthesis of EG-DADA

Synthesis of compound 1-b

Compound **1-a** (2 g, 5.82 mmol) was dissolved in DCM (12 mL), followed by trifluoroacetic acid (12 mL) and triisopropylsilane (1 mL) were added dropwise to the solution. The reaction mixture was stirred at room temperature for 0.5h, monitored by TLC. Trifluoroacetic acid was removed by rotary evaporator. The crude product was used in next step without further purification. After the residue was dissolved in 12 mL acetonitrile, added $(Boc)_2O$ (2.68 mL, 11.64 mmol) and triethylamine (2.42 mL, 17.47 mmol) to the solution. After stirring overnight, the mixture was diluted with water and extracted with EtOAc, the combined organic phase was dried over Na_2SO_4, filtrated and then concentrated under vacuum. The crude product was purified by column chromatography to afford compound **1-b** (1 g, 4.97 mmol, 85%, two steps). R_f = 0.4 (10:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.78 (s, 3H), 3.04 (dd, 1H), 2.53 (dd, 1H), 2.42 (dd, 1H), 1.46 (s, 9H).

Synthesis of compound 2-b

Dissolved the compound **1-b** (0.5 g, 2.48 mmol) and compound **2-a** (488 μL, 3.73 mmol) in 10 mL of DCM at 0℃, and then $BF_3{\cdot}OEt_2$ (31 μL, 0.25 mmol) diluted in 1 mL DCM was added dropwise to the solution. After returning to room temperature and stirring overnight, the mixture was diluted with saturated aqueous sodium bicarbonate and extracted with DCM. The extract organic phase was dried over Na_2SO_4, filtrated and concentrated. The residue was purified by column chromatography to obtain compound **2-b** (0.463 g, 1.45 mmol, 59%). R_f = 0.3 (7:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.66 (d, 1H), 4.40 (m, 1H), 4.02 – 3.96 (m, 1H), 3.75 (s, 3H), 3.70 (dd, 1H), 3.64 – 3.52 (m, 2H), 3.47 (dd, 2H), 1.45 (s, 9H), 1.20 (s, 9H).

Synthesis of compound 2-d

After compound **2-b** (0.4 g, 1.25 mmol) was dissolved in 5 mL THF, 2.5 ml 1 M LiOH was added dropwise to the solution. Stirred for 1 hours, followed by the mixture was acidulated to pH 3 with 1 M HCl. the mixture was extracted with EtOAc and then washed with brine twice. The organic phase was dried over Na_2SO_4, filtrated and evaporated.

The residue was dissolved in 3 mL DCM, and then 3.5 mL trifluoroacetic acid was added to the solution. After the reaction mixture was stirred overnight, the trifluoroacetic acid was removed

using rotary evaporator. The crude compound **2-c** was used in next step without further purification.

At 0°C, the crude compound **2-c**, Fmoc-OSu (0.506 g, 1.5 mmol) and sodium carbonate (0.199 g, 1.875 mmol) were dissolved in 10 mL of 1, 4-dioxane / water (1:1, v:v). After reaction overnight at room temperature, the mixture was diluted with water and extracted with EtOAc. The combined organic phase was dried over Na_2SO_4, filtrated and concentrated to obtain the crude product.

The residue was dissolved in 10 mL DCM / THF (4:1, v:v), and then tert-butyl 2,2,2-trichloroacetimidate (448 μL, 2.5 mmol) was added to the solution. After stirring overnight, the solvent was removed under vacuum. The residue was re-dissolved in DCM and recrystallized, and then the crystals were removed by filtration. The resulting organic phase was dried over Na_2SO_4, filtrated and concentrated. The residue was purified by column chromatography to obtain compound **2-d** (0.227 g, 0.53 mmol, 42%, four steps). Rf = 0.15 (1:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, 2H), 7.62 (d, 2H), 7.40 (t, 2H), 7.31 (t, 2H), 5.87 (d, 1H), 4.51 – 4.32 (m, 3H), 4.23 (t, 1H), 3.88 (dd, 1H), 3.77 – 3.68 (m, 3H), 3.57 (m, 2H), 1.48 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.52, 156.15, 149.01, 143.92, 143.81, 141.30, 127.72, 127.10, 125.17, 119.99, 82.61, 72.77, 71.43, 67.08, 61.62, 54.99, 47.15, 28.02.

Synthesis of compound 3

The compound **2-d** (0.12 g, 0.28 mmol) and compound **1-b** (0.057 g, 0.28 mmol) were dissolved in 1.5 mL DCM, and then $BF_3{\cdot}OEt_2$ (3.5 μL, 0.028 mmol) diluted in 0.5 mL DCM was added dropwise to the solution under ice bath. After stirring overnight at room temperature, the mixture was diluted with saturated aqueous sodium bicarbonate and extracted with DCM. The combined organic phase was washed with brine, dried over Na_2SO_4, filtrated and concentrated. The residue was purified by chromatography to obtain compound **3** (0.074 g, 0.118 mmol, 42%). R_f = 0.1 (4:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, 2H), 7.64 (d, 2H), 7.40 (t, 2H), 7.31 (t, 2H), 5.78 (d, 1H), 5.51 (d, 1H), 4.47 – 4.31 (m, 4H), 4.25 (t, 1H), 3.93 – 3.85 (m, 2H), 3.76 – 3.68 (m, 5H), 3.59 (q, 4H), 1.49 (s, 9H), 1.45 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.16, 169.28, 156.09, 155.56, 143.96, 143.85, 141.29, 127.70, 127.09, 125.24, 119.96, 82.32, 80.05, 71.49, 71.28, 70.82, 70.75, 67.12, 55.02, 54.05, 52.53, 47.14, 29.72, 28.03. HRMS calcd for $C_{33}H_{44}O_{10}N_2$ 628.71900, found $[M+Na]^+$ 651.28955.

Synthesis of compound 4

After compound **3** (0.074 g, 0.118 mmol) was dissolved in isopropyl alcohol/0.8 M aq. $CaCl_2$ (1 mL, 7:3, v/v), an aqueous solution of 1 M NaOH (336 μL) was added. After TLC monitoring showed that the reaction was complete, 1 M HCl was added to acidify the mixture to pH 2. The product was extracted into EtOAc, and then the combined organic phase was washed with brine, dried over Na_2SO_4, filtrated and concentrated. The residues and sodium bicarbonate (15 mg, 0.142 mmol) were dissolved in 2 mL DMF, followed by the dropwise addition of allyl bromide (13 μL, 0.142 mmol). The reaction mixture was stirred at room temperature overnight. After diluting with water, the mixture was extracted with EtOAc and then washed with water to remove the remaining DMF. The organic phase was dried over Na_2SO_4, filtrated and concentrated. The crude product was purified by chromatography to afford compound **4** (0.043 g, 0.066 mmol, 56%, two steps). R_f = 0.18 (4:1, petroleum ether/EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, , 2H), 7.64 (d, 2H), 7.40 (t, 2H), 7.31 (t, 2H), 5.89 (m, 1H), 5.81 (d, 1H), 5.58 – 5.47 (m, 1H), 5.31 (d, 1H), 5.22 (d,

1H), 4.66 (m, 2H), 4.49 – 4.32 (m, 4H), 4.25 (t, 1H), 3.97 – 3.84 (m, 2H), 3.74 – 3.69 (m, 2H), 3.63 – 3.57 (m, 4H), 1.47 (s 9H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.38, 169.29, 156.10, 155.59, 143.96, 141.28, 131.67, 127.70, 127.09, 125.23, 119.96, 118.45, 82.33, 80.05, 72.33, 71.29, 70.79, 67.14, 66.00, 61.77, 55.00, 54.15, 47.14, 28.33, 28.03. HRMS calcd for $C_{35}H_{46}O_{10}N_2$ 654.75700, found $[M+Na]^+$ 677.30585.

Synthesis of EG-DADA

Compound **4** (0.043 g, 0.066 mmol) was dissolved in 0.5 mL DCM, followed by the dropwise addition of 0.5 mL trifluoroacetic acid. The reaction mixture was stirred vigorously overnight at room temperature. Then, trifluoroacetic acid was removed by azeotroping with DCM under reduced pressure. After the residue was dissolved in 0.9 mL of EtOAc / saturated aqueous sodium bicarbonate (1.25:1, v:v), pNZ-Cl (0.017 g, 0.079 mmol) was added to the solution. After stirring for 3 hours at room temperature, the mixture was acidified to pH 2 with 1 M HCl and 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 **EG-DADA** (0.037 g, 0.054 mmol, 82%, two steps). R_f= 0.3 (10:1, DCM/CH_3OH). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (d, 2H), 7.72 (d, 2H), 7.55 (s, 2H), 7.48 – 7.31 (m, 4H), 7.28 (d, 2H), 5.90 – 5.76 (m, 1H), 5.41 – 5.00 (m, 5H), 4.60 (s, 2H), 4.51 (s, 1H), 4.36 (s, 2H), 4.14 (s, 1H), 3.85 (s, 2H), 3.73 (s, 2H), 3.56 (d, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.40, 155.92, 152.27, 147.45, 143.84, 142.45, 141.23, 135.15, 131.49, 129.89, 127.95, 127.72, 127.06, 125.14, 123.97, 123.67, 123.44, 119.97, 118.68, 115.83, 71.50, 70.84, 70.74, 66.93, 66.26, 65.38, 61.13, 55.64, 54.70, 47.11. HRMS calcd for $C_{34}H_{35}O_{12}N_3$ 677.66300, found $[M+Na]^+$ 700.21197.

2.2 Synthesis of thioether diaminodiacid

Thioether diaminodiacid DADA-2 was prepared according to previous work[1]

3. Solid-phase peptide synthesis of disulfide surrogate peptides

3.1 Fmoc-based solid-phase peptide synthesis

Rink Amide AM resin was swelled with DCM/DMF (1/1, v/v) for 30 min. Before the protected amino acid (4.0 equiv to resin loading) was coupled to the resin, it was pre-activated with 3.8 eq coupling reagent (HCTU or HATU/HOAT, HATU/HOAT used for the coupling of sterically hindered amino acids) and 8.0 eq DIEA in DMF for 0.5-1 min. Each coupling reaction needed 40-min reaction time, followed by washing the resin with DMF (3 times), DCM (3 times) and DMF (3 times). Then the Fmoc protecting group was removed with 20% piperidine in DMF (5 min+10 min), followed by washing the resin with DMF (3 times), DCM (3 times) and DMF (3 times). Each coupling cycle was accomplished with the same procedure. After the assembly of peptides was finished, the resin was treated with a mixture of TFA/water/TIPS (92.5/5/2.5, v/v/v/v) or TFA/phenol/thioanisole/water/1,2-ethanedithiol (82.5/5/5/5/2.5, v/v/v/v/v) for 3h. After the TFA-containing solution was blown with N_2, an excess of cold ether was added to the solution, 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 TPI-1

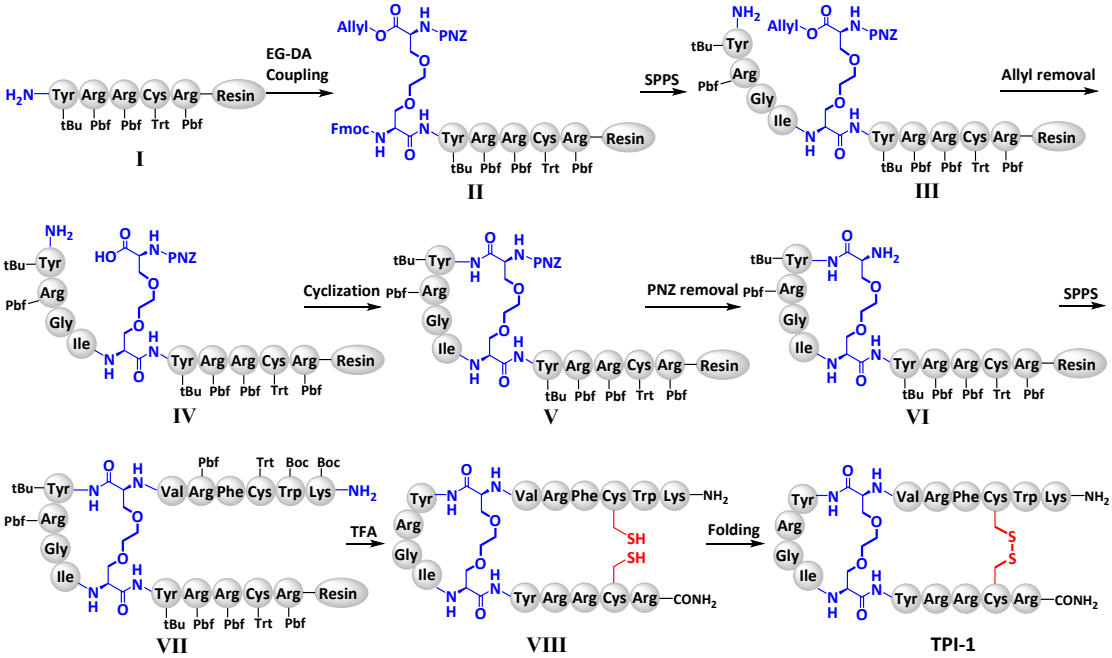


Figure S1. Solid-phase peptide synthesis of **TPI-1**.

0.02 mmol Rink amide AM resin (63 mg, 0.32 mmol/g) was used for the synthesis of **TPI-1.** At first, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Tyr(tBu)-OH were coupling to the resin according to standard **Fmoc-based solid-phase peptide synthesis** to give **I**. Then, pre-activated **EG-DADA** (2 equiv.), prepared using PyAOP (2 equiv.), HOAt (2 equiv.) and NMM (4 equiv.) in 1 mL DMF for 0.5 min, was added the mixture to the resin. After shaking overnight to give **II**, Fmoc-Ile-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Tyr(tBu)-OH were coupled to the resin in sequence to give **III**. After the allyl group was removed using $Pd(PPh_3)_4$(1.0 equiv.) /$PhSiH_3$(10.0 equiv.) in 1 mL DCM for 3 h to give **IV**, under the treatment of a solution of PyAOP (2.0 equiv.), HOAt (2.0 equiv.) and NMM (4.0 equiv.) in 1 mL DMF overnight, cyclization was accomplished efficiently to give **V**. Next, a solution of $SnCl_2$ (4.55 g) and 4 M HCl·1,4-Dioxane (6.5 μL) in 5 mL was used to remove the *p*NZ for 1 h×2 to obtain **VI**, followed by the sequential coupling of Fmoc-Val-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Phe-OH, Fmoc-Cys(Trt)-OH, Fmoc-Trp(Boc)-OH and Fmoc-Lys(Boc)-OH on to **VI** to give **VII**. Then the resin was treated with a mixture of TFA/phenol /water/thioanisole/1,2-ethanedithiol (82.5/5/5/5/2.5, v/v/v/v/v, 4 mL) for 3 h. After filtration, the filtrate was concentrated by nitrogen blowing, followed by an excess of cold Et_2O being added and then centrifugation. The crude peptide was purified by semi-preparative RP-HPLC (a linear gradient from 5% to 90% acetonitrile, 30 min, 4 mL/min) and lyophilized to give 6 mg purified **VIII** (13% isolated yield). Finally, peptide **VIII** (3 mg) was dissolved in 2 mL water and the pH was adjusted to 8, followed by 1,2-di(pyridin-2-yl)disulfane (5 mg) in methanol (80 μL) being added. After letting the solution standing overnight, the folding mixture was purified by using semi-preparative RP-HPLC (a linear gradient from 5% to 90% acetonitrile, 30 min, 4 mL/min) to afford 1.2 mg of the folded product **TPI-1** (40% isolated yield).

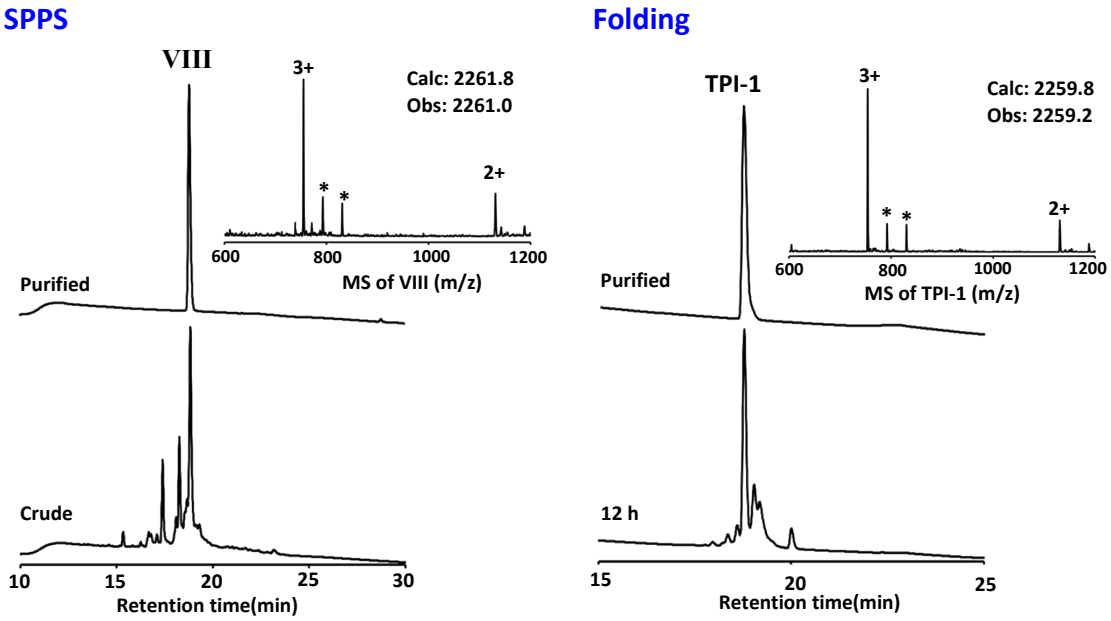


Figure S2. RP-HPLC traces and ESI-MS of **TPI-1** Fmoc-SPPS and folding. "*" corresponded to TFA adduct.

3.3. Synthesis and characterization of α-ImI-1

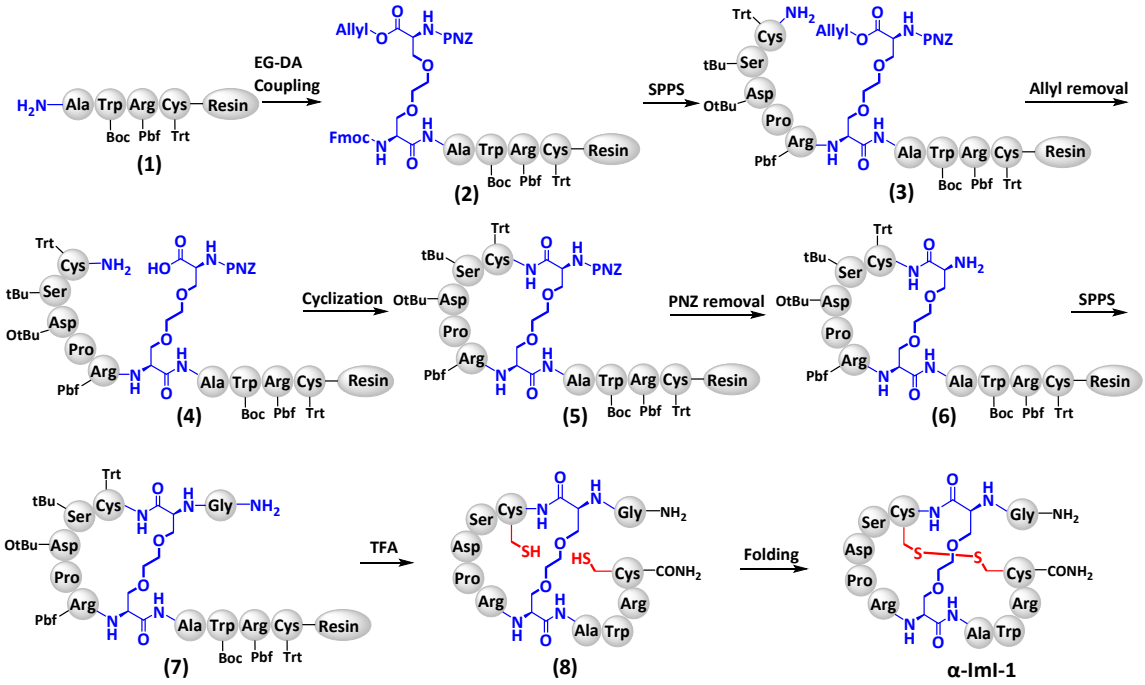


Figure S3. Solid-phase peptide synthesis of **α-ImI-1**.

0.01 mmol Rink amide AM resin (31 mg, 0.32 mmol/g) was swelled for the synthesis of **α-ImI-1.** According to standard **Fmoc-based solid-phase peptide synthesis**, Fmoc-Cys(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Trp(Boc)-OH and Fmoc-Ala-OH were coupling to the resin to give **(1)**. Then, a solution of **EG-DADA** (2 equiv.), PyAOP (2 equiv.), HOAt (2 equiv.) and NMM (4 equiv.) in DMF was added to the resin and shaken overnight to obtain **(2)**. Next, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(tBu)-OH and Fmoc-Cys(Trt)-OH were coupled to **(2)** in turn to give **(3)**. After the allyl protecting group was removed using $Pd(PPh_3)_4$(1.0 equiv.)/$PhSiH_3$(10.0 equiv.) in DCM (1 mL) for 3h to obtain **(4)**, cyclization was accomplished efficiently with PyAOP (2.0 equiv.), HOAt (2.0 equiv.) and NMM (4.0 equiv.) in DMF overnight to give **(5)**. The pNZ group was removed with $SnCl_2$ (4.55 g) and 4 M HCl·1,4-Dioxane (6.25 μL) in 5 mL DMF for 1 h×2 to obtain **(6)**, which was followed by the coupling of Fmoc-Gly-OH to the resin to give **(7)**. Next, the dried resin was treated with a mixture of TFA/phenol/water/thioanisole/1,2-ethanedithiol (82.5/5/5/5/2.5, v/v/v/v/v, 2 mL) for 3 h. The obtained TFA mixture was concentrated by nitrogen blowing, followed by an excess of cold Et_2O being added and centrifugation. Then, the crude peptide was purified by semi-preparative RP-HPLC (a linear gradient from 10% to 70% acetonitrile, 30 min, 4 mL/min) and lyophilized to give 1.44 mg of purified **(8)** (11% isolated yield). Finally, purified **(8)** was dissolved in 25 mL water, then glutathione oxidized (1.55 mg, 10 equiv.)/glutathione reduced (7.75 mg, 100 equiv.) were added. After adjusting the pH to 7.5-8, the mixture was allowed to stand overnight. The folding mixture was purified by using semi-preparative RP-HPLC (a linear gradient from 10% to 70% acetonitrile, 30 min, 4 mL/min) to afford 0.7 mg of the final product **α-ImI-1** (50% isolated yield).

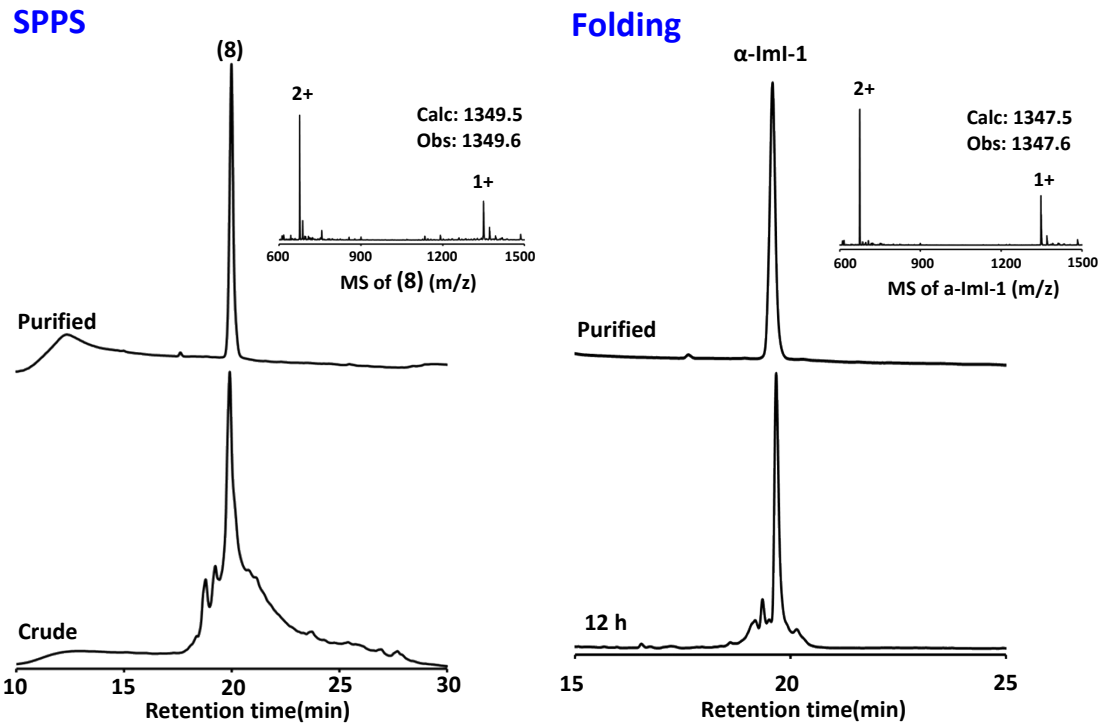


Figure S4. RP-HPLC traces and ESI-MS of **α-ImI-1** Fmoc-SPPS and folding.

3.4. Synthesis and characterization of α-ImI(S-C)

α-ImI(S-C) was synthesized and refolded in a similar manner to **α-ImI-1**. **α-ImI(S-C)** was purified by semi-preparative HPLC (a linear gradient from 10% to 70% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, 4 mL/min) and acquired as white powder.

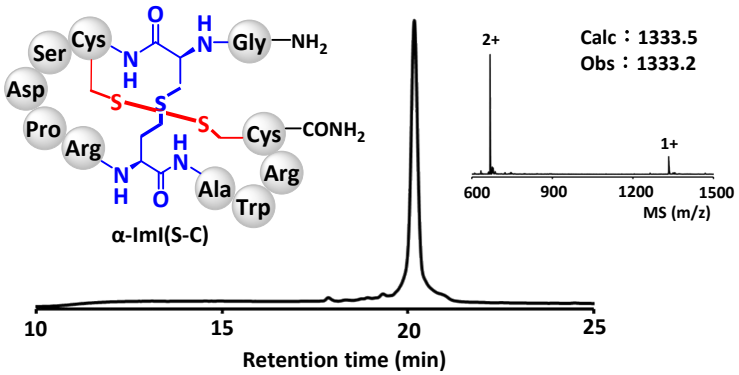


Figure S5. HPLC traces and ESI-MS of purified **α-ImI(S-C)**.

3.5. Synthesis and characterization of native α-ImI

Native α-ImI was synthesized and refolded in a similar manner to **α-ImI-1**. **Native α-ImI** was purified by semi-preparative HPLC (a linear gradient from 10% to 70% acetonitrile in 0.1 % trifluoroacetic acid, 30 min, 4 mL/min) and acquired as white powder.

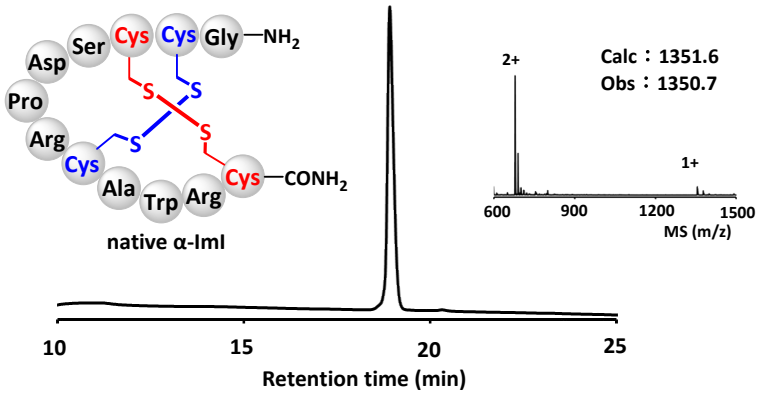


Figure S6. HPLC traces and ESI-MS of purified **native α-ImI**.

4. The CD spectra

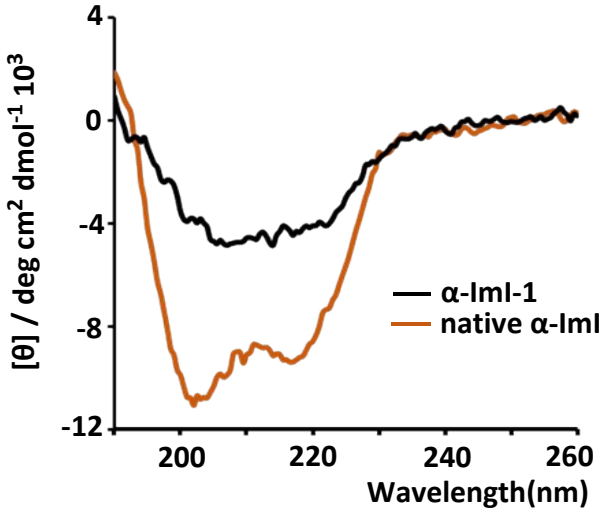


Figure S7. The CD spectra of α-ImI-1 and native α-ImI.

4. The oxidation stability of α-ImI analogs

α-ImI analogs aqueous solution (1 mM) and 0.2% H_2O_2, 0.2% TFA aqueous were mixed in equal volume, followed by the mixture being stirred at room temperature and analyzed after 4 h, 8h and 12 h. After co-incubated for 12 hours, most of **α-ImI(S-C)** is oxidized. Meanwhile, no oxidation by-products of **α-ImI-1**were observed after 12 hours under the same oxidation conditions.

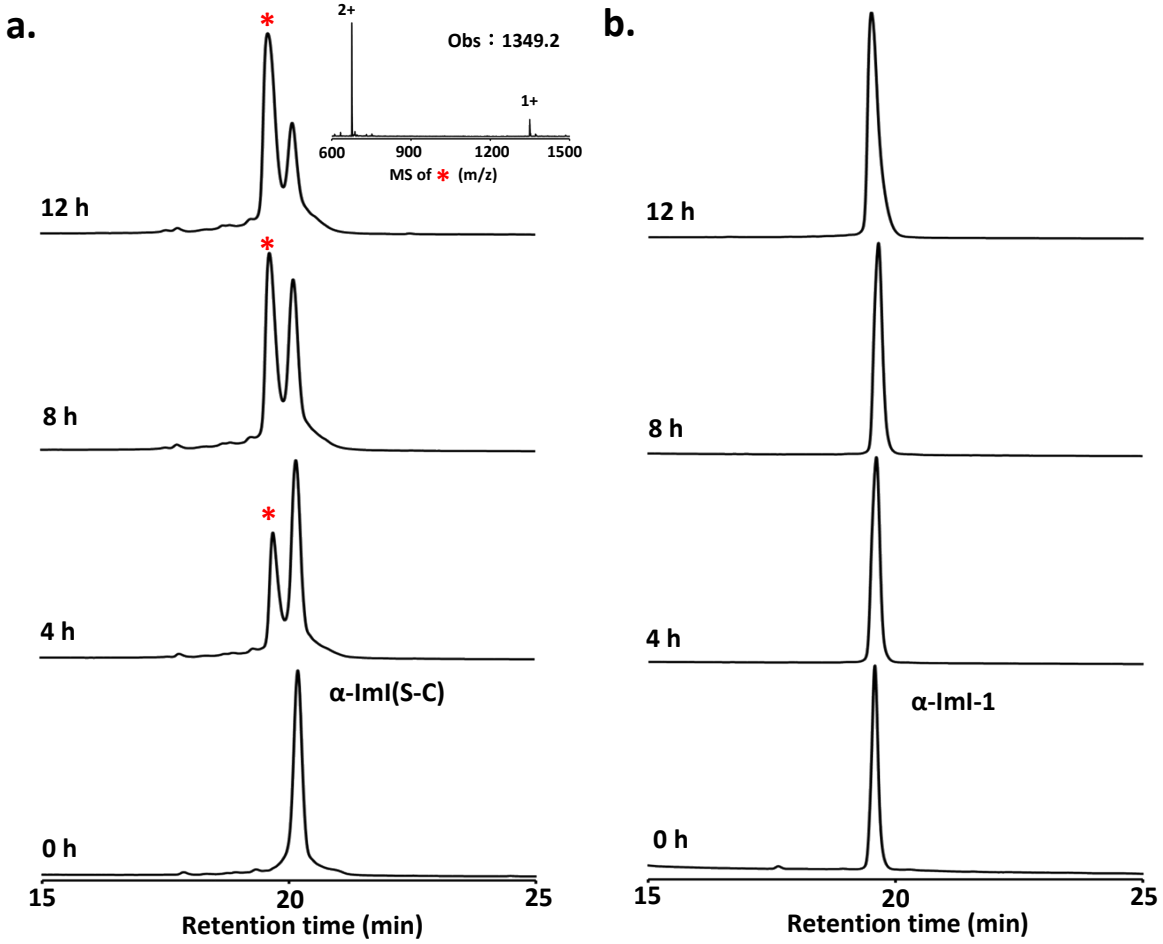


Figure S8. (a) HPLC traces and ESI-MS analysis of oxidative stability studies of **α-ImI(S-C)** (b) HPLC traces and ESI-MS analysis of oxidative stability studies of **α-ImI-1**. "*" refers to **α-ImI(S-C)** in which thioether is oxidized to sulfoxide.

5. The reduction stability of α-ImI-1 and native α-ImI

The 50 μL peptide solution (**α-ImI-1 or native α-ImI**, 1 mg/mL) and 50 μL aqueous dithiothreitol (DTT, 2 M) solution was add to 100 μL of phosphate buffer (1 mM, pH 7.2). Every 20 minutes, 40 μL reaction solution was add to 60 μL buffer (1 mM NaCl, 1% TFA, water: acetonitrile=1:1) and monitored by HPLC.

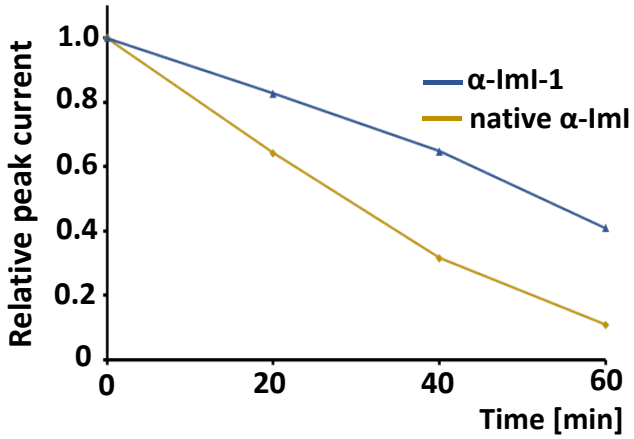


Figure S9. Reductive stability studies on **α-ImI-1** and **native α-ImI** under 500 mM dithiothreitol.

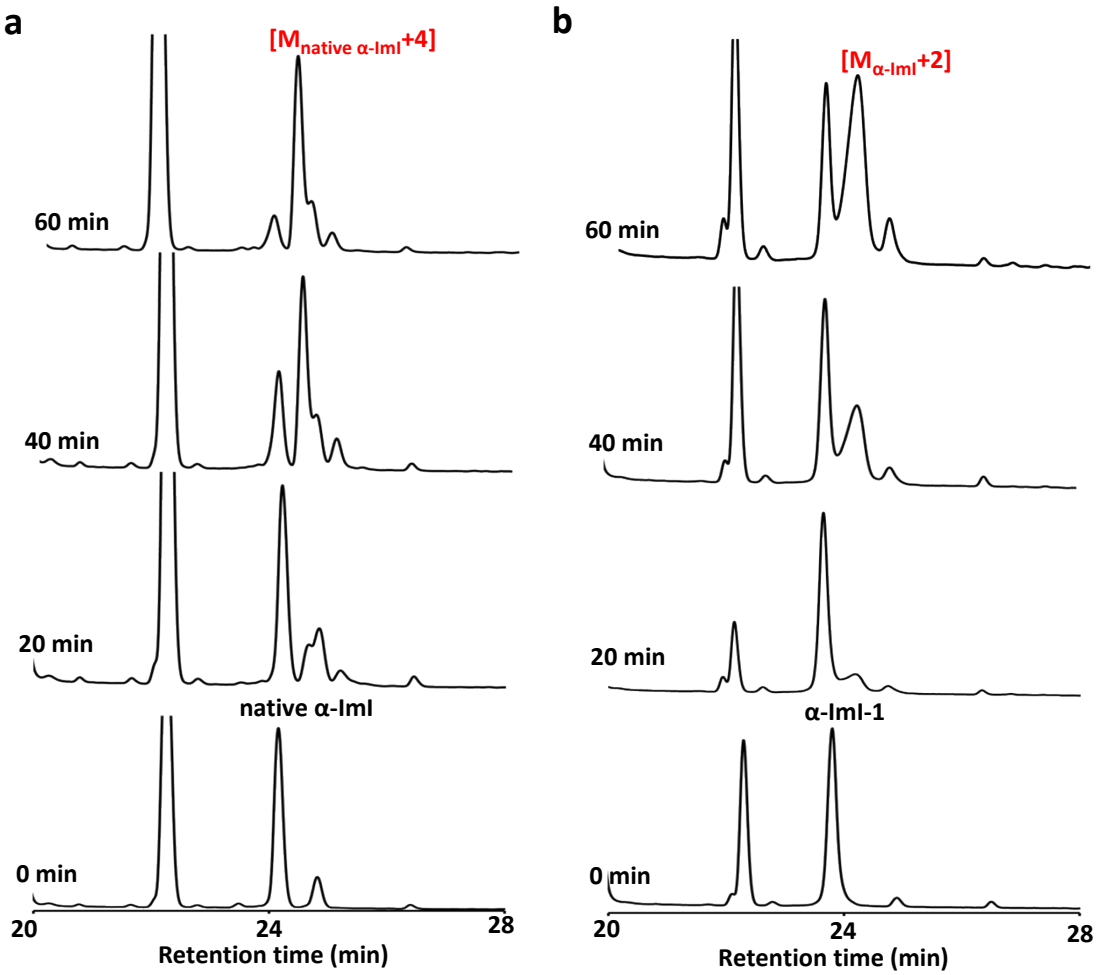
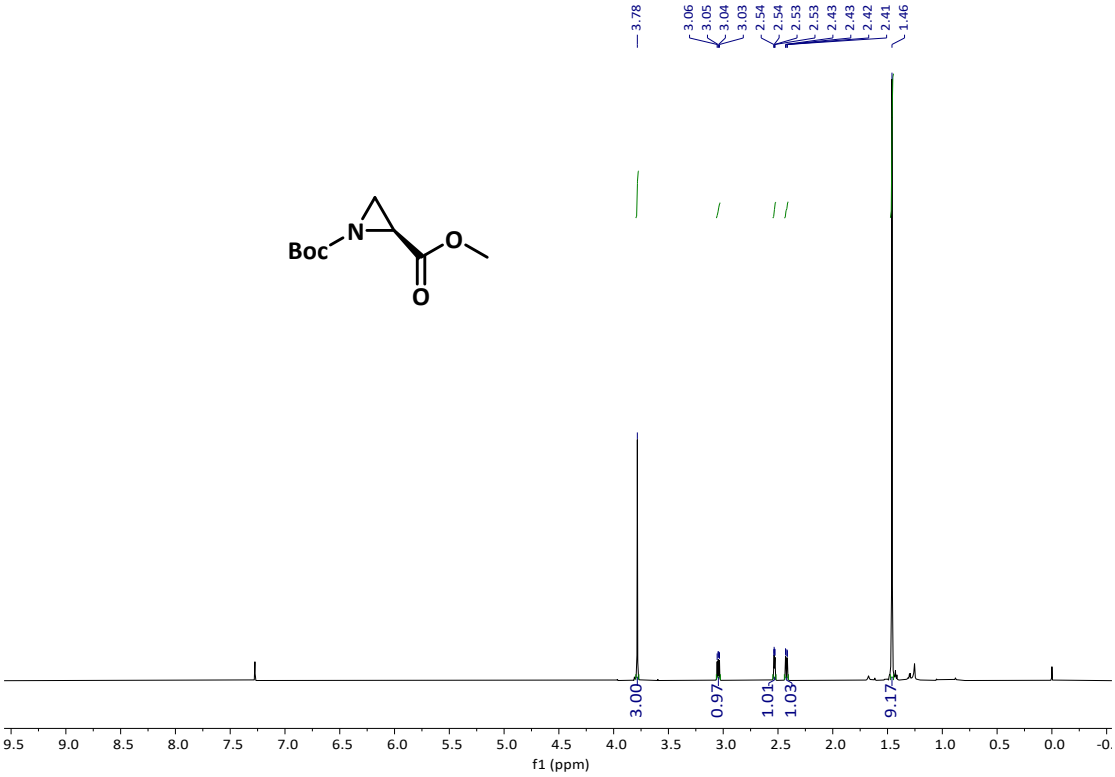


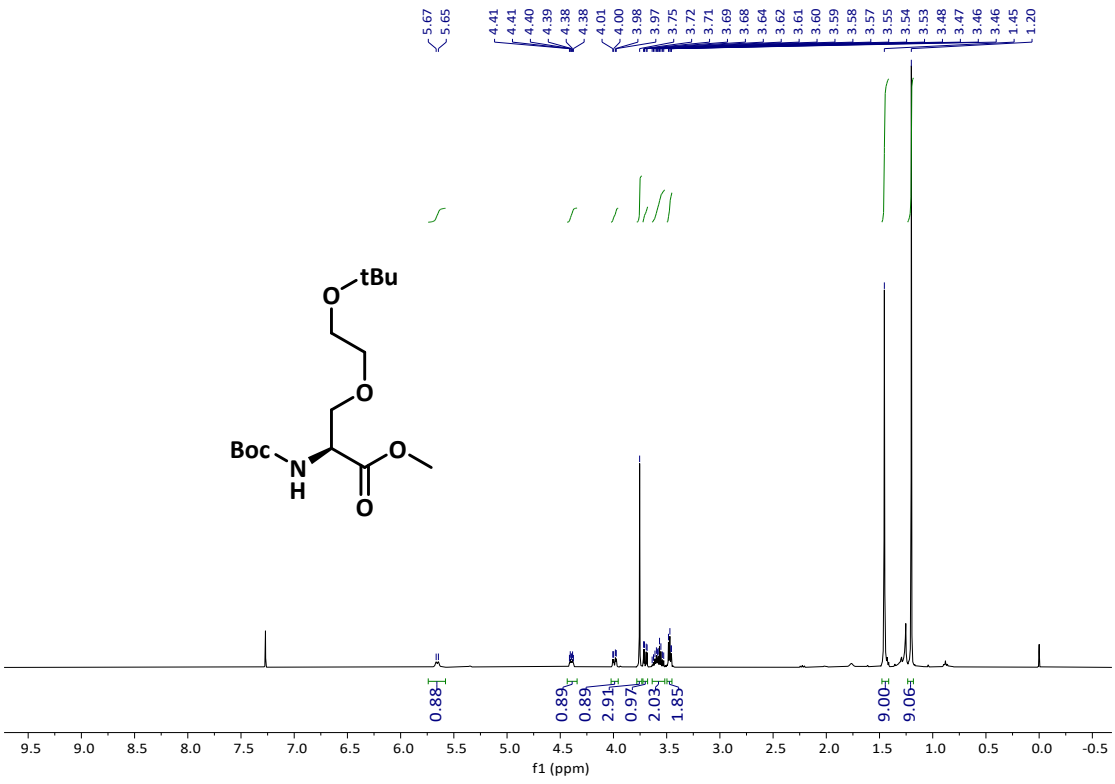
Figure S10. (a) HPLC traces and ESI-MS analysis of reductive stability studies of **native α-ImI** (b) HPLC traces and ESI-MS analysis of reductive stability studies of **α-ImI-1**.

6. NMR and MASS Data for EG-DADA

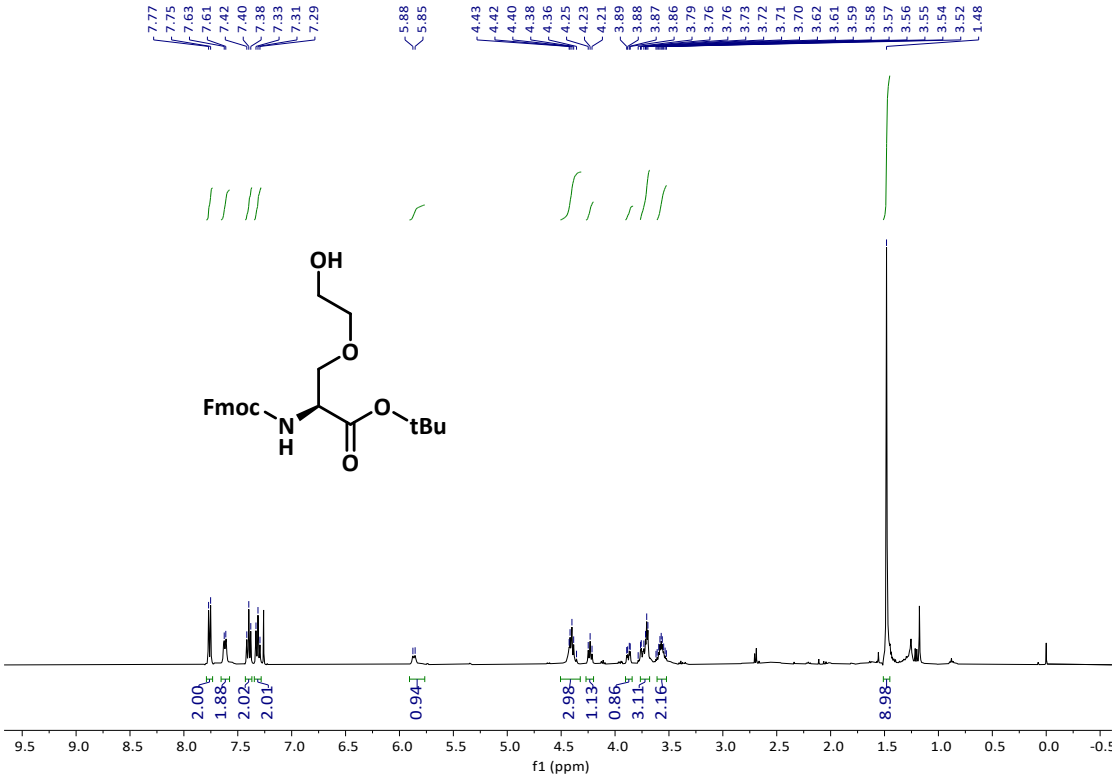
^{1}H NMR spectrum of 1-b.



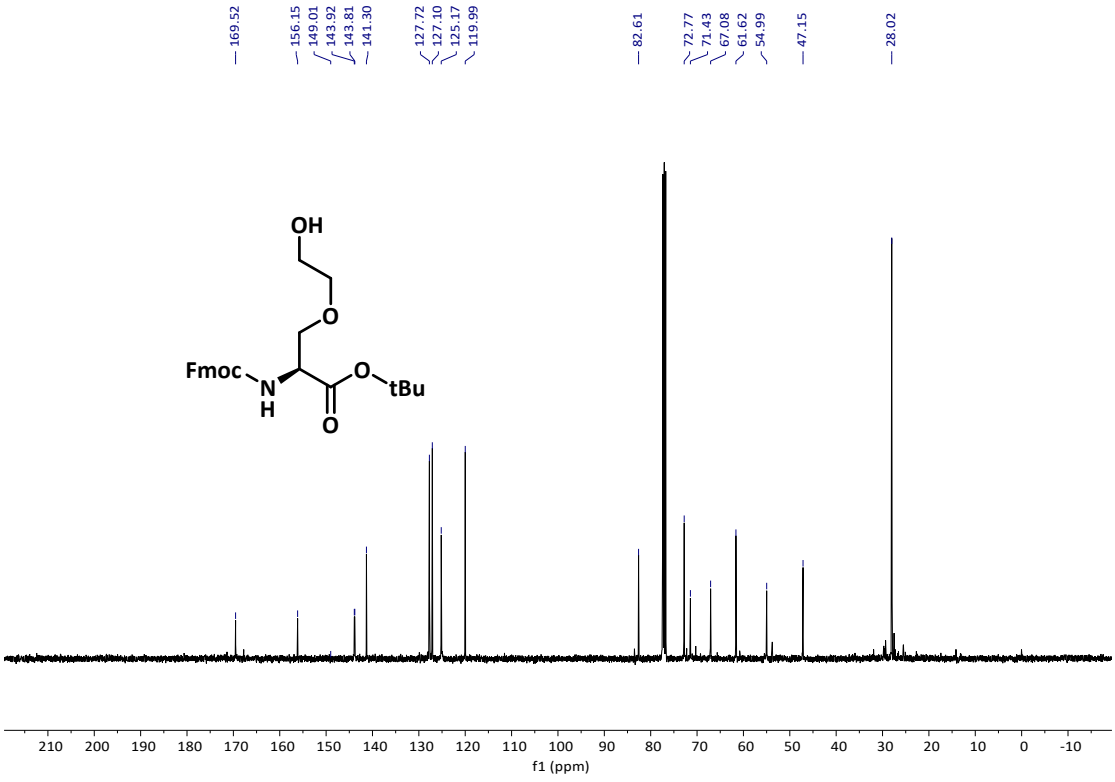
^{1}H NMR spectrum of 2-b.



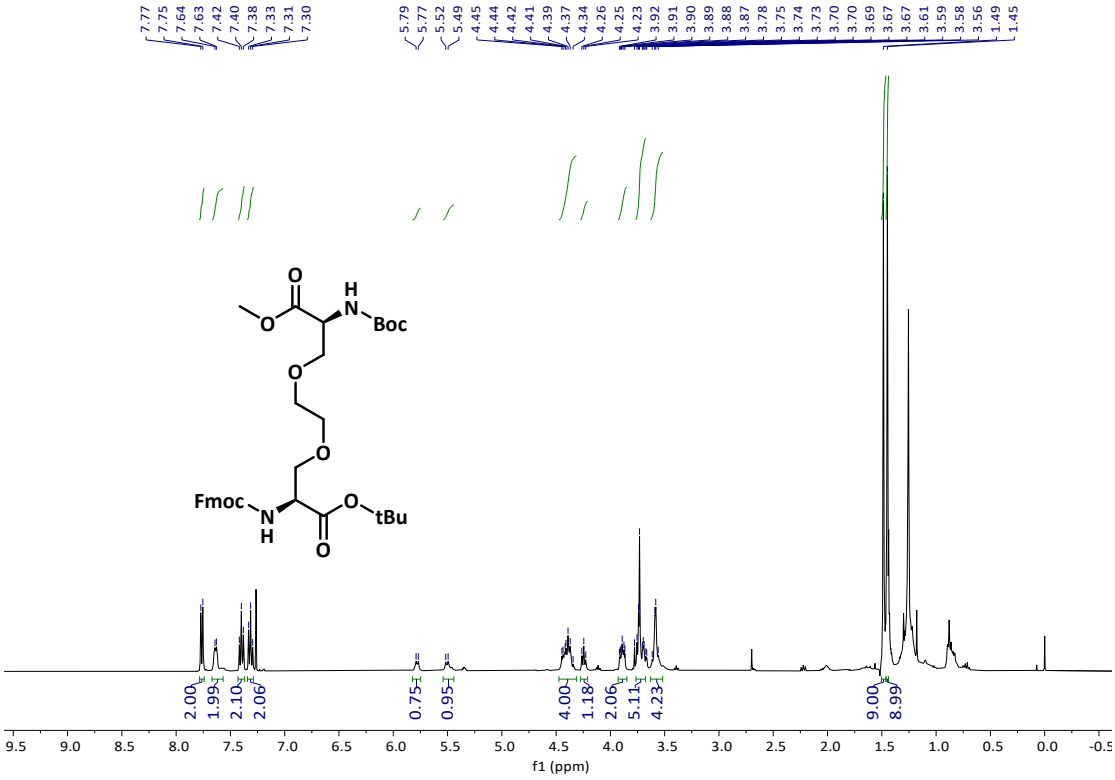
^{1}H NMR spectrum of 2-d.



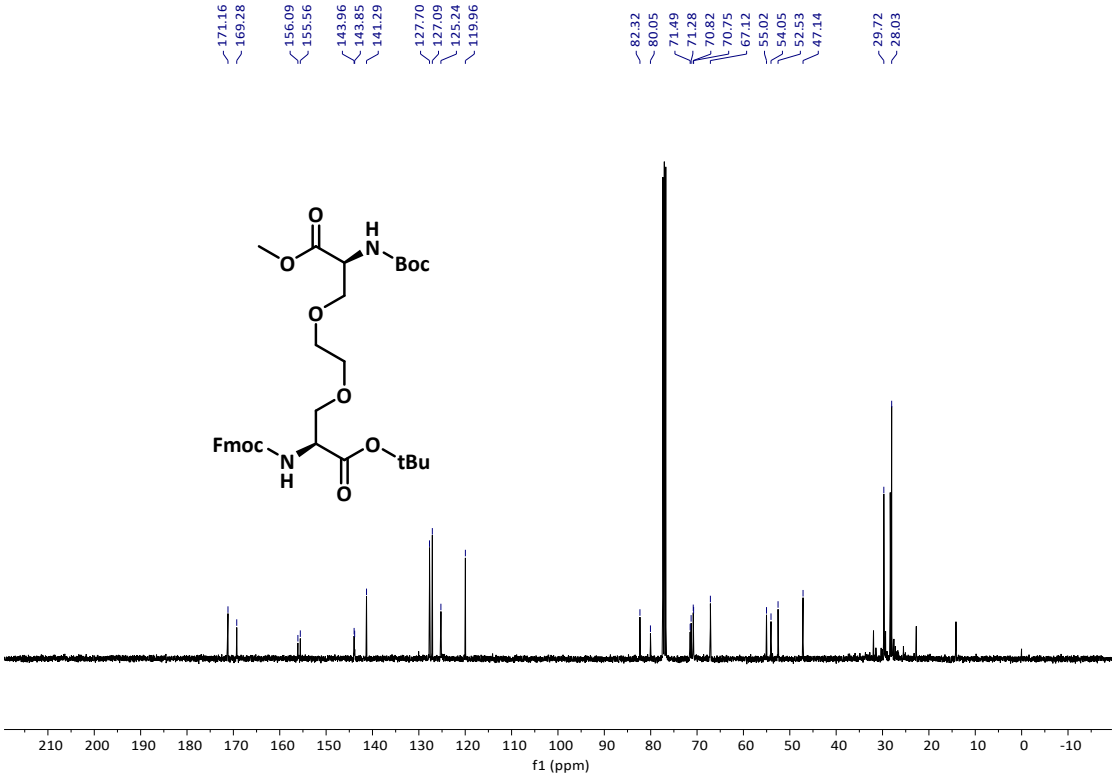
^{13}C NMR spectrum of 2-d.



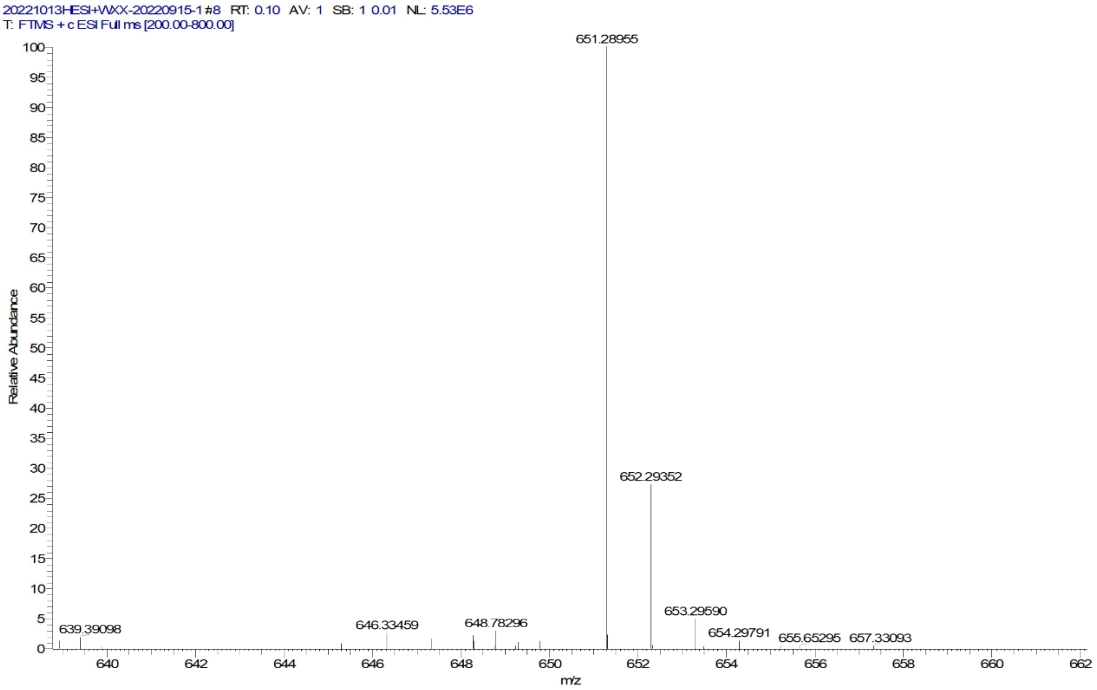
^{1}H NMR spectrum of 3.



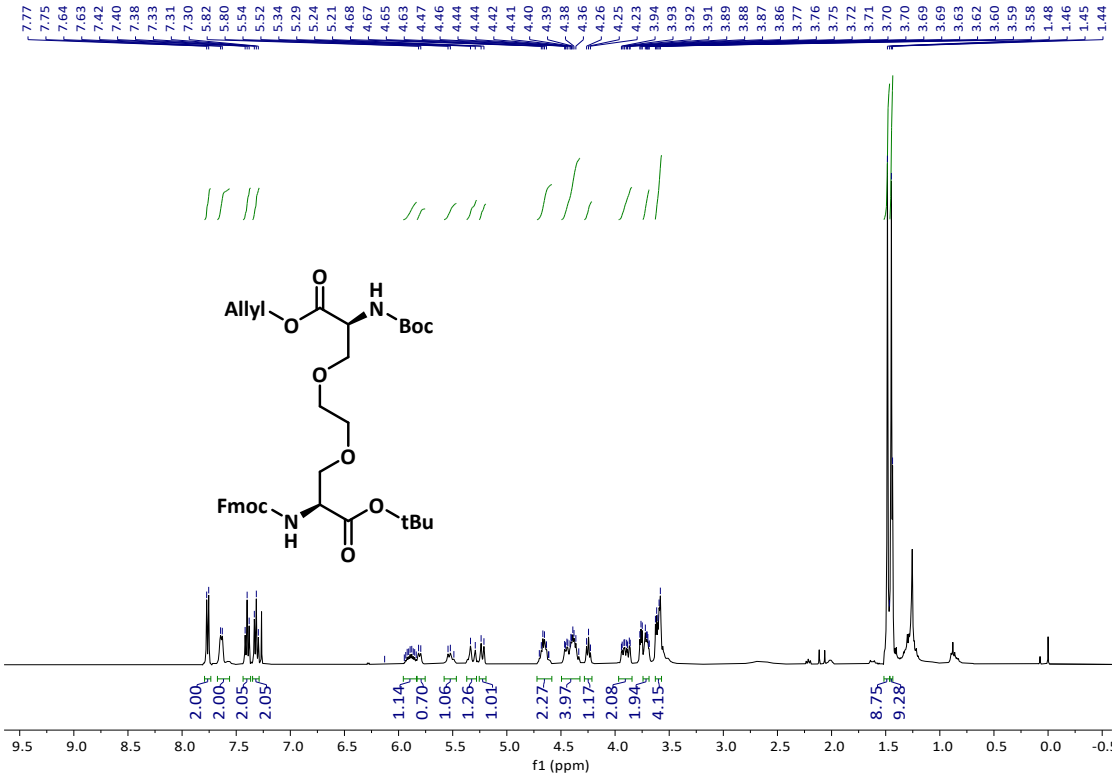
^{13}C NMR spectrum of 3.



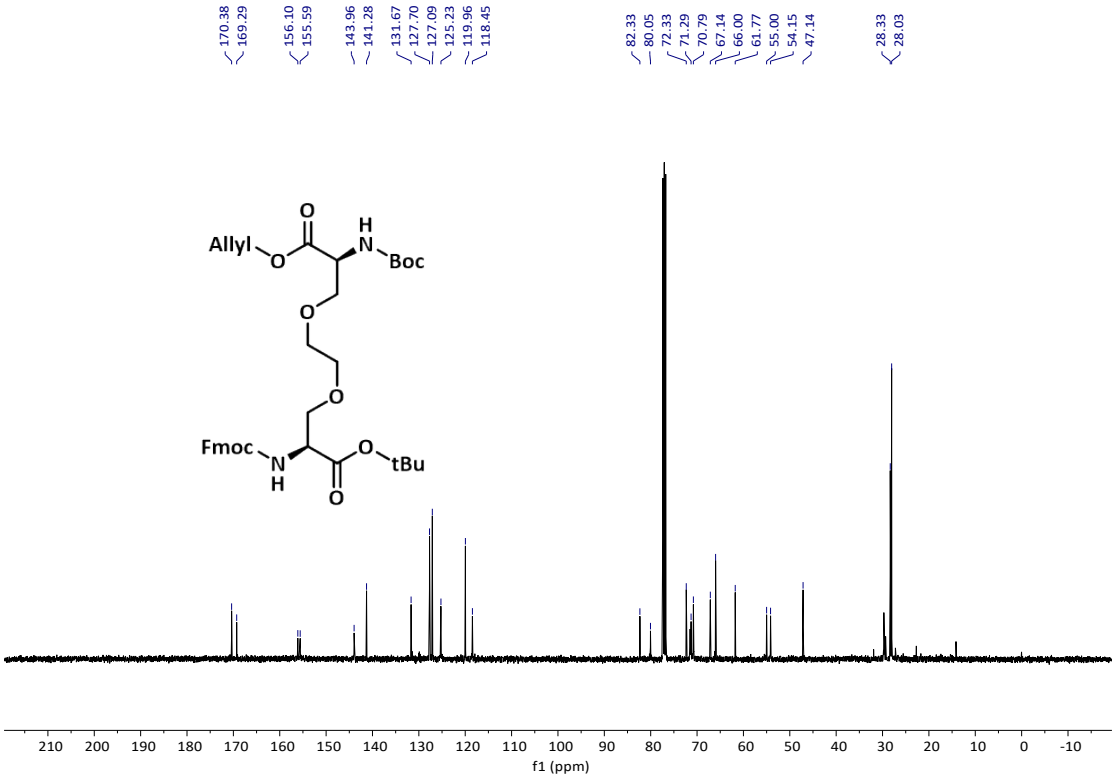
HRMS of compound 3.



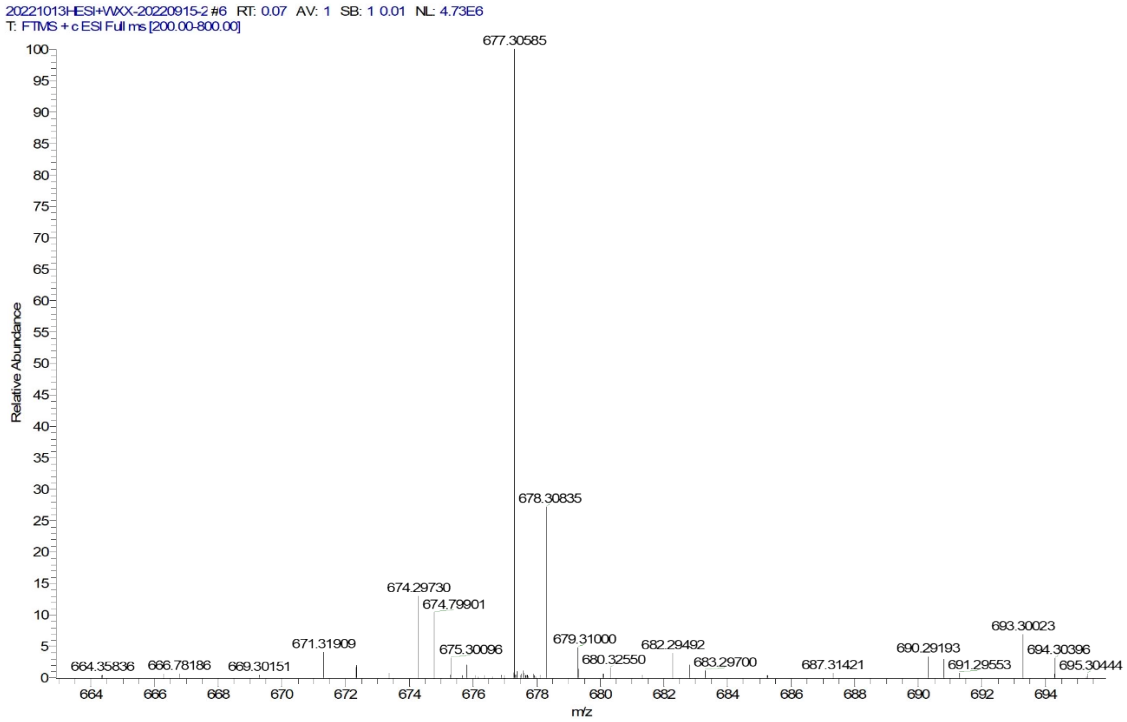
^{1}H NMR spectrum of 4.



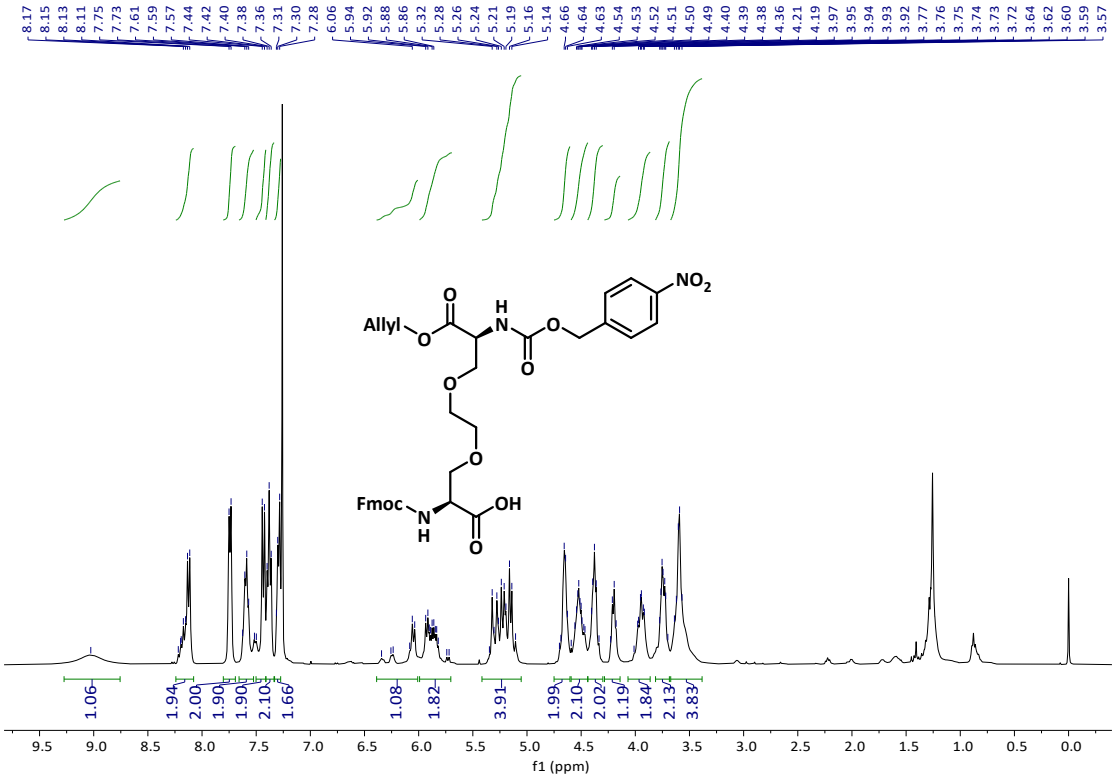
^{13}C NMR spectrum of 4.



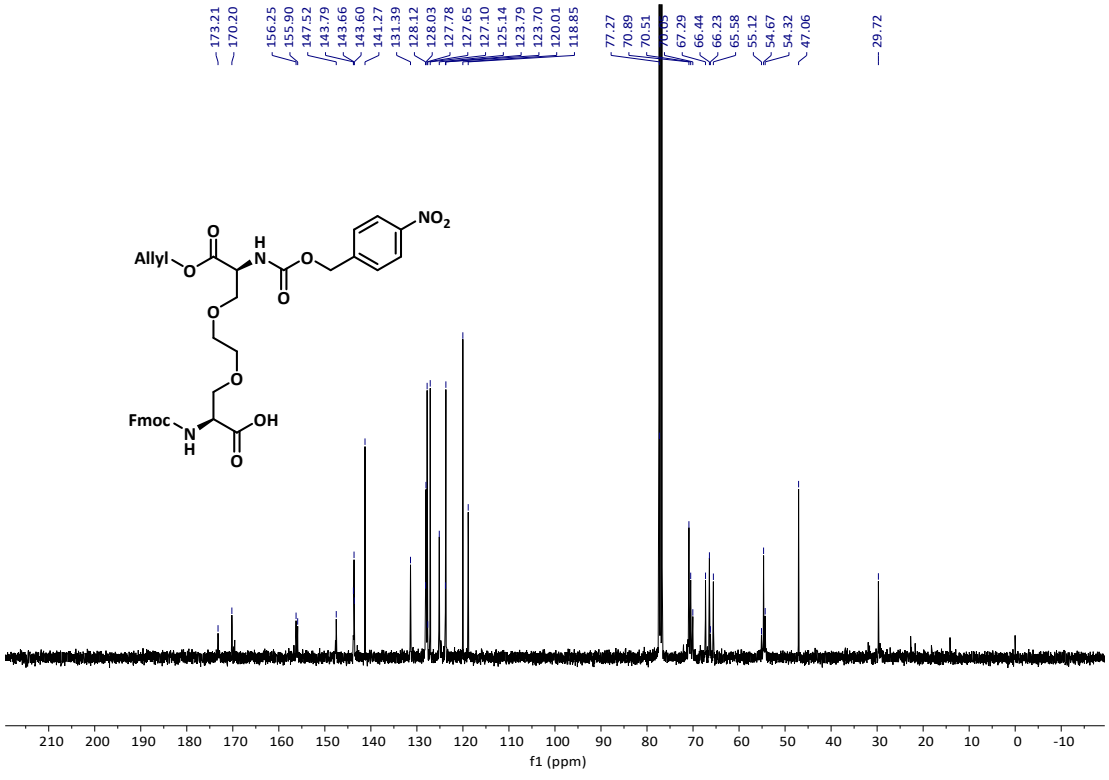
HRMS of compound 4.



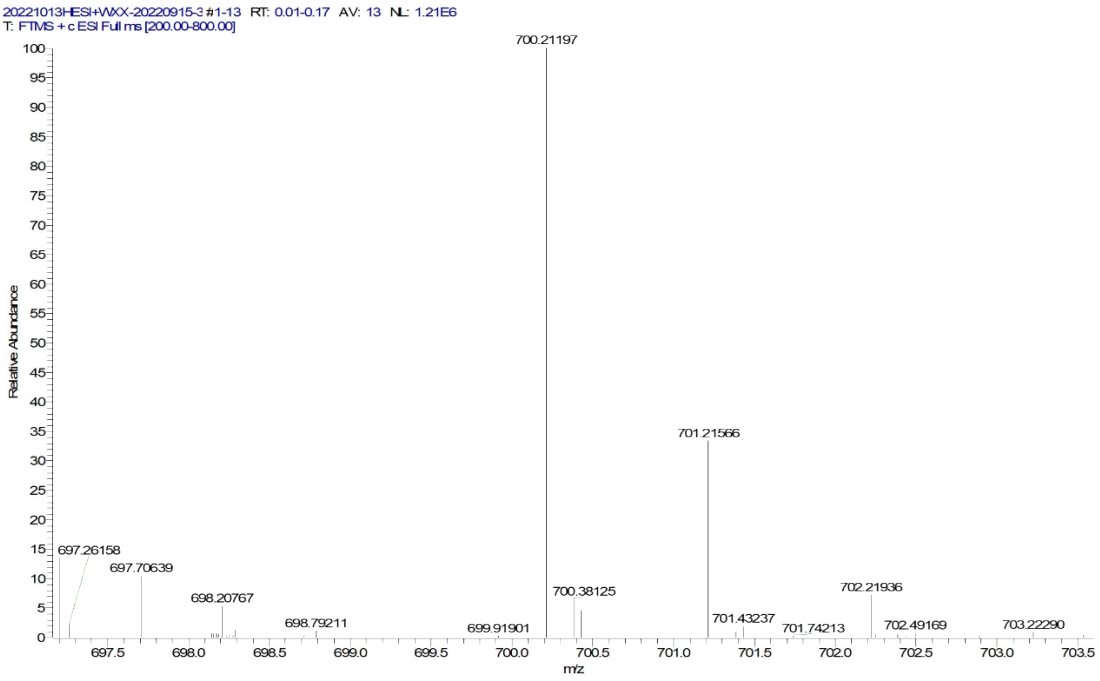
^{1}H NMR spectrum of EG-DADA.



^{13}C NMR spectrum of EG-DADA.



HRMS of EG-DADA.



References

1. H. K. Cui, Y. Guo, F. L. Feng, H. N. Chang, Y. J. Wang, F. M. Wu, C. L. Tian, L. Liu, *Angew. Chem. Int. Ed.* 2013, **52**, 9558-9562.