Supporting Information

α-Selective Lysine Ligation and Application in Chemical Synthesis of Interferon Gamma

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I. General Information

Materials and Methods

HPLC grade tetrahydrofuran, methylene chloride, diethyl ether, toluene, and DMF were purchased from Fisher, Acros, J&K, and Oceanpak, and were purified and dried by passing through a PURE SOLV® solvent purification system (Innovative Technology, Inc.) when anhydrous solvents were required. Other reagent grade solvents for chromatography were purchased from Beijing Tongguang Fine Chemicals Company. Molecular sieves (4 Å, power) were pre-activated in an oven at 65 ºC overnight and further flame-dried before being used in the reactions. All other reagents and relevant catalysts were purchased from Sigma-Aldrich, NovaBiochem, GL Biochem, Acros, TCI, Adamas, Innochem, J&K, Alfa, and Energy, and were used without further purification. Ultra-pure argon (≥99.999%) was used when inert reaction conditions were required.

Analytical thin layer chromatography was performed using 0.25 mm silica gel 60-F plates (Merck). Flash chromatography was performed using 200-300 mesh silica gel (Qingdao Haiyang Chemical Co., Ltd.). Yields refer to chromatographically and spectroscopically pure materials unless otherwise stated. ^{1}H NMR spectra were recorded at 400 MHz at ambient temperature with $CDCl_3$ (Cambridge Isotope Laboratories, Inc.) as the solvent unless otherwise stated. ^{13}C NMR spectra were recorded at 100.0 MHz at ambient temperature with $CDCl_3$ as the solvent unless otherwise stated. Chemical shifts are reported in parts per million relative to $CDCl_3$ (^{1}H, δ 7.26; ^{13}C, δ 77.0) and acetone-d_6 (^{1}H, δ 2.05; ^{13}C, δ 206.3). Data for ^{1}H NMR are reported as follows: chemical shift, integration, multiplicity (ovrlp = overlapping, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) and coupling constants. All ^{13}C NMR spectra were recorded with complete proton decoupling. Infrared spectra were recorded on a Nicolet Nexus 470 FT-IR spectrophotometer. High-resolution mass spectra were obtained in the Chemical Instrumentation Center, Peking University Health Science Center using a Waters Q-TOF mass spectrometer (Xevo G2 Q-TOF). Optical rotations were recorded on an AUTOPOL

VI digital polarimeter at 589 nm and are recorded as $[\alpha]_D^{25}$ (concentration in grams/100 mL solvent). Melting points were recorded on a Melting Point-M560 (Buchi). Low-resolution mass spectra analyses were performed on a Waters SQD mass spectrometer. Circular dichroism (CD) spectra was performed on a JASCO J-810 Spectrometer (Japan), Ultraviolet-Visible Spectroscopy was performed on a WPA Biowave II Spectrophotometer. The absorbance of cell viability was detected on a Multiskan™ GO (Thermo Scientific™).

HPLC

All HPLC separations involved a mobile phase of 0.05% (*v/v*) TFA in water (solvent A) and 0.04% (*v/v*) TFA in MeCN (solvent B) unless otherwise stated.

Analytical LC-MS chromatographic separations were performed using a Waters Alliance e2695 Separations Module, an SQ Detector, and a Waters 2489 UV/Visible (UV/Vis) Detector equipped with an Agilent C18 column (5.0 μm, 4.6 × 150 mm) at a flow rate of 0.4 mL/min or a Higgins Analytical PROTO-300 C4 (5.0 μm, 2.1 × 150 mm) at a flow rate of 0.2 mL/min. The wavelengths of UV-detector were set to 210 nm and 220 nm.

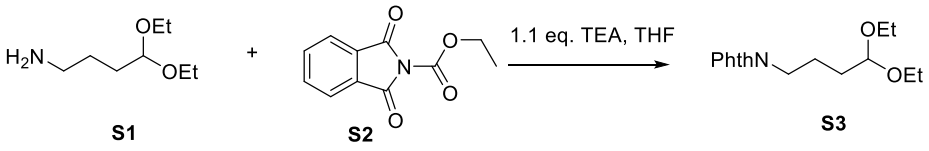
Analytical HPLC chromatographic separations were performed using an Agilent Technologies 1260 Infinity LC system equipped with an Agilent C18 column (5.0 μm, 4.6 × 150 mm) at a flow rate of 0.4 mL/min or a Higgins Analytical PROTO-300 C4 (5.0 μm, 2.1 × 150 mm) at a flow rate of 0.2 mL/min. The wavelengths of UV-detector were set to 210 nm and 220 nm.

Preparative HPLC separations were performed using a Hanbon Sci. & Tech. NP7005C solvent delivery system and a Hanbon Sci. & Tech. NU3010C UV detector equipped with an Agilent Eclipse XDB-C18 column (7.0 μm, 21.2 × 250 mm) or a Proto 300 C4 column (10.0 μm, 20 × 250 mm) at a flow rate of 16 mL/min. The wavelengths of UV-detector were set to 210 nm and 220 nm.

II. Experimental Procedures and Characterization of Unnatural Amino Acids and Derivatives

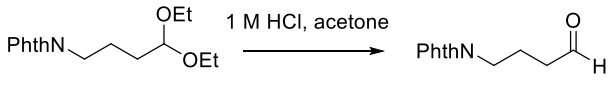
2.1 Procedures for preparing amino acids and derivetives:

Preparation of (2S, 3S)-2,6-di-(tert-butoxycarbonylamino)-4-methyldithio-hexanoic acid (13a)



S1 **S2** **S3**

To a solution of 4,4-diethyloxybutylamine (2.0 mL, 11.3 mmol) and *N*-carbethoxyphthalimide (3.17 g, 14.5 mmol) in THF (23.0 mL) was added triethylamine (1.7 mL, 12.2 mmol) at 0 ˚C dropwise. The reaction was stirred at room temperature for 24 h and then quenched with 1 M aqueous HCl solution at 0 ˚C. The resulting mixture was extracted three times with EtOAc, and the combined extracts were washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude residue was purified using silica gel column chromatography (petroleum ether/EtOAc = 3:1) to afford known compound **S3** as a white solid (3.32 g, 98%).[S1]

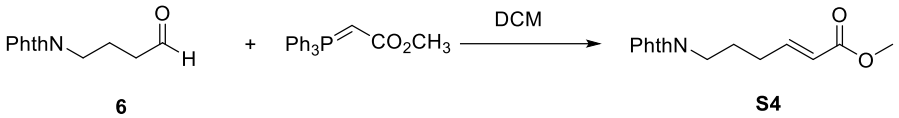


S3 **6**

To a solution of compound **S3** (3.32 g, 11.4 mmol) in acetone (22.0 mL) was added 1 M aqueous HCl solution (20.0 mL) dropwise. The resulting solution was stirred at room temperature for 20 min and then concentrated under reduced pressure to remove most of the acetone. The resulting mixture was extracted three times with EtOAc, and the combined extracts were washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude

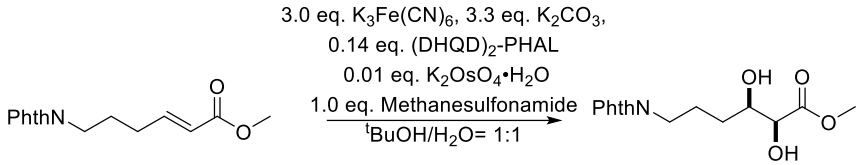
[S1] Storz, M. P.; Maurer, C. K.; Zimmer, C.; Wagner, N.; Brengel, C.; de Jong, J. C.; Lucas, S.; Musken, M.; Haussler, S.; Steinbach, A.; Hartmann, R. W. *J. Am. Soc. Chem.* **2012**, *134*, 16143-16146.

residue was purified using silica gel column chromatography (petroleum ether/EtOAc = 4:1) to afford known compound **6** as a white solid (2.21 g, 90%).[S1]



6 **S4**

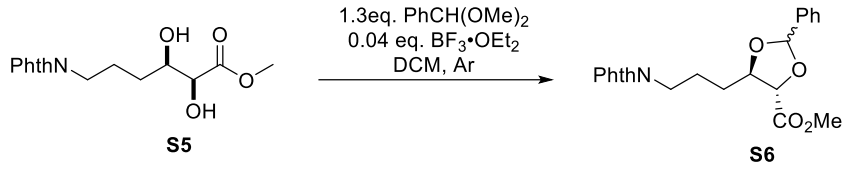
To a solution of compound **6** (1.82 g, 8.4 mmol) in DCM (15.0 mL) was added the solution of methyl(triphenylphosphoranylidene)acetate (2.94 g, 8.8 mmol) in DCM (15.0 mL), and the reaction was stirred at room temperature for 20 h. After concentrated *in vacuo*, the crude residue was purified using silica gel column chromatography (petroleum ether/EtOAc = 4:1) to afford known compound **S4** as a white solid (2.07 g, 90%).[S2]



S4 **S5**

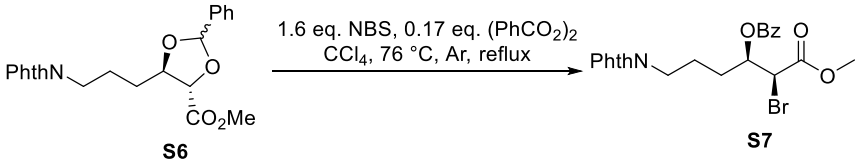
A solution of methanesulfonamide (2.13 g, 22.4 mmol), potassium ferricyanide (22.95 g, 69.7 mmol), $(DHQD)_2$-PHAL (262.8 mg, 3.37 mmol) and potassium osmate(VI) dehydrate (90.1 mg, 0.24 mmol) in $^tBuOH/H_2O$ (80.0 mL/80.0 mL) was stirred at 0 ˚C for 30 min, and a solution of compound **S4** (6.35 g, 23.2 mmol) in DCM (20.0 mL) was added dropwise at 0 ˚C. The resulting solution was stirred at room temperature for 24 h. The reaction was quenched with Na_2SO_3 (30 g, 0.24 mol) and then stirred for 1 h until the color turned to yellow. The organic phase was removed *in vacuo* and the water phase was extracted three times with EtOAc, the combined extracts were washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was dissolved in DCM and recrystallized at -20 ˚C for 12 h to afford known compound **S5** as a white solid (5.71 g, 80%).[S2]

[S2] Hughes, P. F.; Smith, S. H.; Olson, J. T. *J. Org. Chem.* **1994**, *59*, 5799-5802.



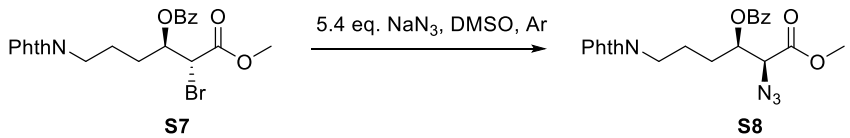
S5 **S6**

To a solution of compound **S5** (1.10 g, 3.6 mmol) and benzaldehyde dimethyl acetal (0.7 mL, 4.7 mmol) in anhydrous DCM (13.0 mL) was added boron trifluoride diethyl etherate (20 µL, 0.16 mmol) at 0 ˚C. The resulting solution was stirred at room temperature under an argon atmosphere for 16 h and then quenched with water. The reaction was diluted with DCM and water and then extracted three times with DCM, and the combined extracts were washed with saturated aqueous $NaHCO_3$ solution, brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (petroleum ether/EtOAc = 2:1) to afford known compound **S6**[S2] as a white solid (1.1 g, 75%) and recovered starting materials **S5** (250 mg, 20%).



S6 **S7**

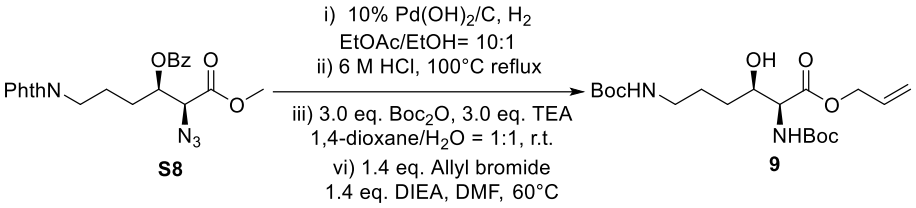
To a solution of compound **S6** (2.65 g, 6.7 mmol) and *N*-bromosuccinimide (1.92 g, 10.9 mmol) in anhydrous CCl_4 (80.0 mL) was added benzoyl peroxide (272 mg, 1.12 mmol). The resulting solution was refluxed under an argon atmosphere for 2 h and then cooled to room temperature. The CCl_4 was removed under a nitrogen atmosphere and the resulting mixture was diluted with DCM and water and extracted three times with DCM, and the combined extracts were washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude residue was purified using silica gel column chromatography (petroleum ether/EtOAc = 5:1) to afford known compound **S7** as a yellow oil (2.34 g, 75%).[S2]



S7 **S8**

A solution of compound S**7** (2.34 g, 5.0 mmol) and sodium azide (1.8 g, 27.5 mmol) in anhydrous DMSO (11.4 mL) was stirred at room temperature under an argon atmosphere

for 4 h and then dilute with EtOAc and water. The resulting mixture was extracted three times with EtOAc, the combined extracts were washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude residue was purified using silica gel column chromatography (petroleum ether/EtOAc = 4:1) to afford known compound **S8** as a yellow oil (1.65 g, 76%).[S2]



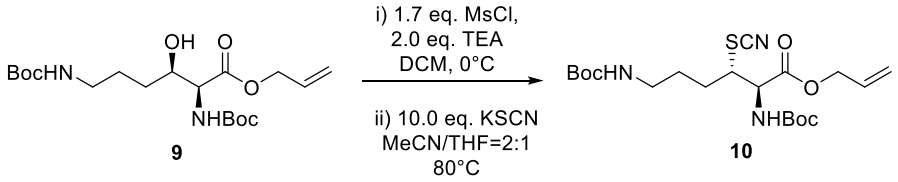
To a solution of compound **S8** (726.7 mg, 1.7 mmol) in EtOAc/EtOH (8.0 mL/0.8 mL) was added powder of palladium hydroxide on carbon (72.7 mg, 10% *w/w*). The reaction was bubbled under a H_2 atmosphere for 30 min. The resulting solution was stirred under a H_2 atmosphere at room temperature for 12 h and filtered through Celite. The filtrate was collected and concentrated *in vacuo* to afford a white solid.

The crude obtained from last step was dissolved in 6 M aqueous HCl (16.0 mL), the resulting mixture was refluxed at 100 ^oC for 24 h and then cooled to room temperature. The mixture was neutralized with saturated aqueous Na_2CO_3 solution at 0 ^oC, and concentrated *in vacuo* to give crude mixture, which was utilized in the next step immediately without further purification.

To a solution of the obtained crude in 1,4-dioxane/H_2O (9.0 mL/9.0 mL) was added di-tert-butyl pyrocarbonate (1.2 mL, 5.2 mmol), then triethylamine (0.65 mL, 4.7 mmol) was added dropwise at 0 ^oC. The reaction was stirred at room temperature for 24 h and then quenched with 1 M aqueous HCl. The mixture was extracted three times with EtOAc, and the combine extracts were washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting mixture was utilized immediately without further purification.

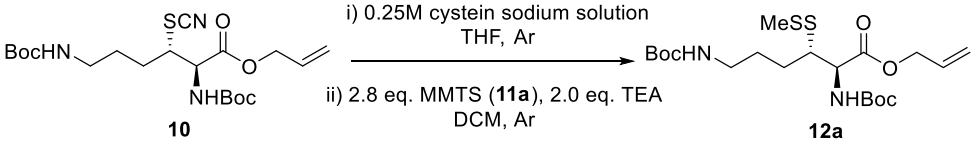
To a solution of the obtained crude and allyl bromide (0.25 mL, 2.0 mmol) in DMF (6.0 mL) was added *N*,*N*-diisopropylethylamine (0.34 mL, 2.0 mmol) at 0 ^oC. The reaction was stirred at 60 ^oC for 24 h and then diluted with EtOAc and water. The resulting mixture was washed three times with EtOAc, and the combined organic phases were washed with water for five times, brine for once, dried over $MgSO_4$, and concentrated *in vacuo*. The

resulting residue was purified using silica gel column chromatography (petroleum ether/ EtOAc = 3:1) to afford compound **9** as a colorless oil (434 mg, 65% over four steps).



A solution of compound **9** (220.0 mg, 0.55 mmol) in DCM (6.0 mL) was cooled to 0 ^{o}C. Triethylamine (152 µL, 1.10 mmol) and methanesulfonyl chloride (72 µL, 0.93 mmol) were added dropwise. The resulting solution was stirred at 0 ^{o}C for 30 min and then quenched with 1 M aqueous HCl at 0 ^{o}C. The resulting mixture was extracted three times with DCM, and the combined extracts were washed with water, brine, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting crude compound was utilized immediately without further purification.

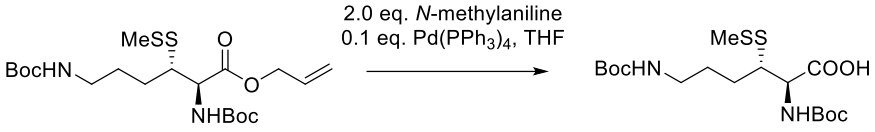
To a solution of the obtained crude in MeCN /THF (4.0 mL/2.0 mL) was added potassium thiocyanate (534.5 mg, 5.5 mmol). The reaction was stirred at 80 ˚C for 24 h and then concentrated *in vacuo*. The resulting residue was diluted with DCM and water, then extracted three times with DCM, and the combined extracts were washed with water, brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (petroleum ether/ EtOAc = 3:1) to afford compound **10** as a yellow oil (134 mg, 55% over two steps).



A solution of cysteine (90.0mg, 0.75 mmol) and sodium hydroxide (30.0 mg, 0.75 mmol) in degassed H_2O (3.0 mL) was added into the solution of compound **10** (50.0 mg, 0.11 mmol) in anhydrous degassed THF (2.0 mL) at 0 ˚C. The reaction was stirred at room temperature for 2 h and then quenched with 1 M aqueous HCl at 0 ˚C. The resulting mixture was extracted three times with EtOAc, the combined extracts were washed with water,

brine, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting crude residue was utilized immediately without further purification.

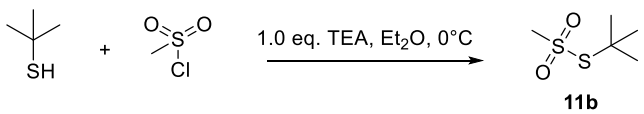
To a solution of the obtained crude in DCM (2.0 mL) was added methanethiosulfonic acid *S*-methyl ester (**11a**) (31 µL, 0.32 mmol) and triethylamine (31 µL, 0.23 mmol) at 0 ˚C. The reaction was stirred at room temperature under an argon atmosphere for 12 h and then quenched with 1 M aqueous HCl at 0 ˚C. The resulting mixture was extracted three times with DCM, and the combined extracts were washed with water, brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (DCM/EtOAc = 20:1) to afford compound **12a** as a colorless oil (33.0 mg, 65% over two steps).



12a **13a**

To a solution of compound **12a** (82.8 mg, 0.18 mmol) and tetrakis (triphenylphosphine) palladium (22.4 mg, 0.018 mmol) in anhydrous THF (2.0 mL) was added *N*-methylaniline (37 µL, 0.36 mmol) at 0 ˚C. The resulting solution was stirred at room temperature under an argon atmosphere for 30 min and then diluted with EtOAc and H_2O. The resulting mixture was extracted three times with EtOAc, and the combined extracts were washed with 1 M aqueous HCl for three times, brine for once, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (DCM/Acetone/AcOH = 90:9:1) to afford compound **13a** as a yellow oil (56.6 mg, 75%).

<u>Preparation of S-tert-Butyl Methanethiosulfonate (11b)</u>

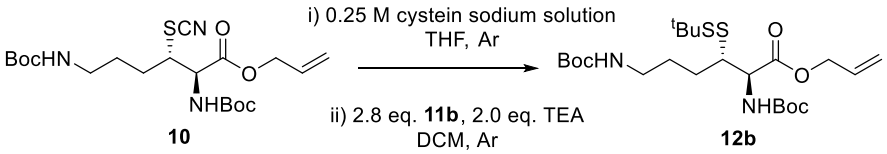


11b

To a solution of tert-butyl thiol (0.11 mL, 1.0 mmol) in EtO_2 (2 mL) was added methanesulfonyl chloride (77 µL, 1.0 mmol) and triethylamine (0.14 mL, 1.2 mmol) at 0

˚C. The reaction was stirred at 0˚C for 30 min and then quenched with 1 M HCl solution at 0˚C. The resulting mixture was extracted three times with EtOAc and the combined extracts were washed with water, brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (petroleum ether/DCM= 5:1) to afford known compound **11b** as a colorless oil (150 mg, 90%).[S3]

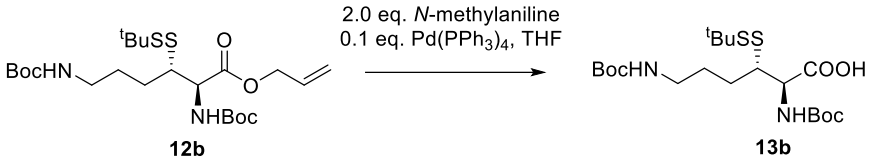
Preparation of (2S, 3S)-2,6-di-(tert-butoxycarbonylamino)-4-tert-butyldithio-hexanoic acid (13b)



A solution of cysteine (90.0mg, 0.75 mmol) and sodium hydroxide (30.0 mg, 0.75 mmol) in degassed H_2O (3.0 mL) was added into the solution of compound **10** (50.0mg, 0.11 mmol) in anhydrous degassed THF (2.0 mL) at 0 ˚C. The reaction was stirred at room temperature for 2 h and then quenched with 1 M aqueous HCl at 0 ˚C. The resulting mixture was extracted three times with EtOAc, and the combined extracts were washed with water, brine, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting crude residue was utilized immediately without further purification.

To a solution of the obtained crude in DCM (2.0 mL) was added reagent **11b** (53.0 mg, 0.32 mmol) and triethylamine (31 μL, 0.23 mmol) at 0 ˚C. The reaction was stirred at room temperature under an argon atmosphere for 12 h and then quenched with 1 M aqueous HCl at 0 ˚C. The resulting mixture was extracted three times with DCM, and the combined extracts were washed with water, brine, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (petroleum ether/EtOAc = 8:1) to afford compound **12b** as a colorless oil (40.0 mg, 72% over two steps).

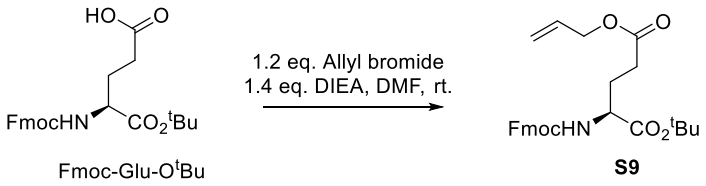
[S3] Pham, H. T.*et al.*, *Phosphorus, Sulfur, and Silicon and the Related Elements* **2015**, *190*, 1934-1941.



12b **13b**

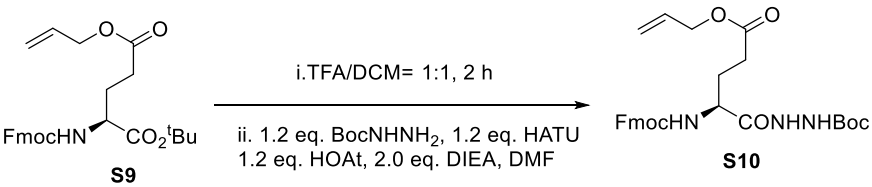
To a solution of compound **12b** (95.0mg, 0.19 mmol) and tetrakis (triphenylphosphine) palladium (22.4 mg, 0.018 mmol) in anhydrous THF (2.0 mL) was added *N*-methylaniline (37 μL, 0.36 mmol) at 0 ˚C. The reaction was stirred at room temperature under an argon atmosphere for 30 min and then dilute with EtOAc and H_2O. The resulting mixture was extracted three times with EtOAc, and the combined extracts were washed with 1 M aqueous HCl for three times, brine for once, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (DCM/Acetone/AcOH = 90:9:1) to afford compound **13b** as a yellow oil (66.0 mg, 75%).

<u>Preparation of 2S-2-((9H-fluoren-9-yl) methoxy carbonyl) amino-1-(tert-butoxycarbo-nyl) hydrazinyl-5-oxopentanoic acid (28)</u>



Fmoc-Glu-O^{t}Bu **S9**

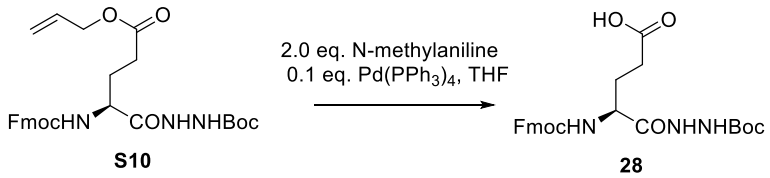
To a solution of Fmoc-Glu-O^{t}Bu (200 mg, 0.47 mmol) and allyl bromide (50 μL, 0.58 mmol) in DMF (2.0 mL) was added *N*,*N*-diisopropylethylamine (0.11 mL, 0.64 mmol) at 0 ^{o}C. The reaction was stirred at room temperature for 24 h and then diluted with EtOAc and water. The resulting mixture was extracted three times with EtOAc, and the combined extracts were washed with water for five times, then brine for once, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (petroleum ether/ EtOAc = 6:1) to afford compound **S9** as a colorless oil (196.7 mg, 90%).



S9 **S10**

Compound **S9** (71.0 mg, 0.15 mmol) was dissolved in a solution of trifluoroacetic acid/DCM (2.0 mL/2.0 mL) and the reaction was stirred at room temperature for 2 h, then the solvent was removed under a nitrogen atmosphere. The resulting residue was co-evaporated with toluene *in vacuo* to get rid of remaining acid and used in the next step without further purification.

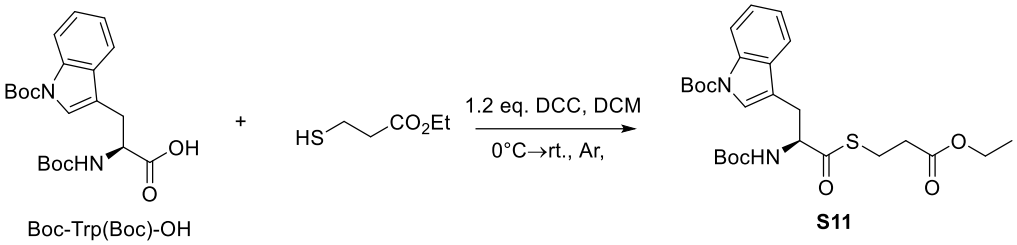
To a solution of crude residue, tert-butyl carbazate (23 mg), HATU (68.0 mg, 0.18 mmol) and HOAt (24 mg, 0.18 mmol) in DMF (2.0 mL) was added *N*,*N*-diisopropylethylamine (52 μL, 0.30 mmol) at 0 ^{o} C. The reaction was stirred at room temperature for 2 h and then diluted with water and EtOAc. The resulting mixture was extracted three times with EtOAc, and combined extracts were washed with water for five times, then brine for once, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (petroleum ether/ EtOAc = 6:1) to afford compound **S10** as a colorless oil (70.0 mg, 89%).



S10 **28**

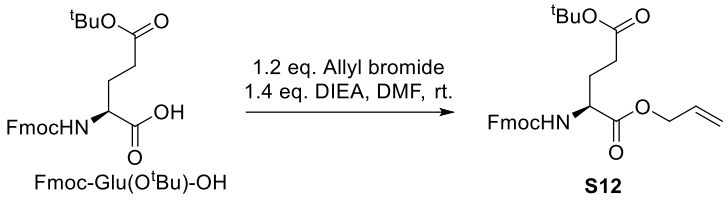
To a solution of compound **S10** (60.0mg, 0.12 mmol) and tetrakis (triphenylphosphine) palladium (15.4 mg, 0.012 mmol) in anhydrous THF (2.0 mL) was added *N*-methylaniline (27 μL, 0.24 mmol) at 0 ˚C. The resultant solution was stirred at room temperature under an argon atmosphere for 30 min and then dilute with EtOAc and H_2O. The resulting mixture was extracted three times with EtOAc, and the combined extracts were washed with 1 M aqueous HCl for three times, brine for once, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (petroleum ether/EtOAc/AcOH = 66:33:1) to afford compound **28** as a yellow oil (43.0 mg, 75%).

Preparation of N,N-di-(tert-butoxycarbonyl)-S-ethoxypropanoate-L-tryptophan thioester (S11)



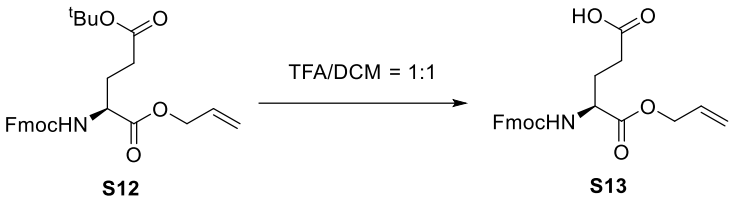
To a solution of Boc-Trp(Boc)-OH (200.0mg, 0.50 mmol) and ethyl 3-mercaptopropionate (74 µL, 0.6 mmol) in anhydrous DCM (2.0 mL) was added dicyclohexylcarbodiimide (124 mg, 0.6 mmol) at 0 ˚C. The reaction was stirred at 0 ˚C under an argon atmosphere for 30 min and then warmed to room temperature and reacted for 1 h. The reaction was quenched with AcOH (0.2 mL) then filtered through Celite and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (petroleum ether/EtOAc = 10:1) to give compound **S11** as a colorless oil (237.5 mg, 95%).

Preparation of 4-(((9H-fluoren-9-yl)methoxy carbonyl)amino)-5-(allyloxy)-5-oxopentanoic acid (S13)



To a solution of Fmoc-Glu(O^{t}Bu)-OH (200 mg, 0.47 mmol) and allyl bromide (49 µL, 0.58 mmol) in DMF (2.0 mL) was added *N*, *N*-diisopropylethylamine (0.11 mL, 0.64 mmol) at 0 ^{o}C. The reaction was stirred at room temperature for 24 h and then diluted with EtOAc and water. The resulting mixture was extracted three times with EtOAc, and the combined extracts were washed with water for five times, brine for once, dried over $MgSO_4$, and concentrated *in vacuo*. The crude was purified using silica gel column chromatography (petroleum ether/ EtOAc = 6:1) to afford the known compound **S12** as a colorless oil (203 mg, 93%).[S4]

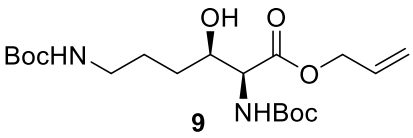
[S4] Fluxa, V. S.; Maillard, N.; Page, M. G. P.; Reymond, J.-L. *Chem. Commun.* **2011**, *47*, 1434–1436.



S12 S13

Compound **S12** (203mg, 0.44 mmol) was dissolved in a solution of trifluoroacetic acid/DCM (2.0 mL/2.0 mL), the solution was stirred at room temperature for 2 h and the solvent was removed under a nitrogen atmosphere. The crude was purified using silica gel column chromatography (petroleum ether/ EtOAc/ AcOH = 66:33:1) to afford the known compound **S13** as a colorless oil (107 mg, 60%).[S4]

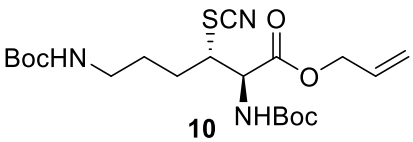
2.2 Characterization of amino acids and derivatives



9

(2*S*, 3*R*)-Allyl-2,6-di-(*tert*-butoxycarbonylamino)-4-hydroxyhexanoate (9)

R_f = 0.2 (petroleum ether/EtOAc=2:1); IR (thin film): ν_{max} 3385, 2978, 2935, 1717, 1525, 1367, 1252, 1167, 558 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.90 (tdd, *J* = 10.4Hz, *J* = 10.8 Hz, *J* = 8.6 Hz, 1H), 5.40 (br, 1H), 5.30 (d, *J* = 40.3 Hz, 1H), 5.26 (d, *J* = 33.4 Hz, 1H), 4.75 (br, 1H), 4.64 (d, *J* = 5.4 Hz 2H), 4.29 (d, *J* = 8.2 Hz, 1H), 4.12 (br, 1H), 3.23-3.04 (m, 3H), 1.68-1.50 (m, 4H), 1.43 (s, ovlp, 9H), 1.42 (s, ovlp, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) : δ 171.207, 156.385, 156.197, 131.595, 118.711, 80.017, 79.320, 71.921, 66.049, 57.898, 40.119, 30.628, 28.412, 28.298, 26.473; HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{19}H_{35}N_2O_7$, 403.2444; found, 403.2435. $[\alpha]^{24.9}_D$ = - 2.40° (*c* = 1.0, MeOH).

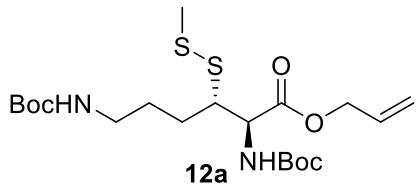


10

(2*S*, 3*S*)-Allyl-2,6-di-(*tert*-butoxycarbonylamino)-4-thiocyanate-hexanoate(10)

R_f = 0.48 (petroleum ether/EtOAc=3:2); IR (thin film): ν_{max} 3364, 2977, 2933, 1712, 1517, 1366, 1164, 1019, 913, 745 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.95 (tdd, *J* = 22.6 Hz, *J* = 11.4 Hz, *J* = 5.6 Hz, 1H), 5.50 (d, *J* = 7.1 Hz, 1H), 5.68 (d, *J* = 29.6 Hz, 1H), 5.34 (d, *J* = 22.8 Hz, 1H), 4.77-4.68 (m, 3H), 4.65 (br, ovlp, 1H), 3.52 (dt, *J* = 9.08 Hz, 4.54 Hz, 1H), 3.18 (m,

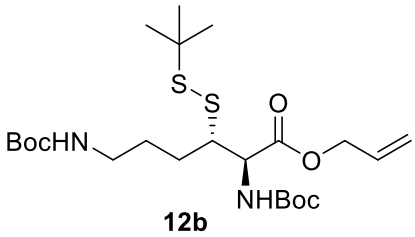
2H), 2.00-1.65 (m, 4H), 1.47 (s, 9H), 1.45 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) : δ 168.395, 156.982, 154.904, 130.897, 120.038, 109.960, 80.967, 79.393, 66.942, 57.238, 52.315, 39.622, 28.908, 28.398, 28.243, 27.768; HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{20}H_{34}N_3O_6S$, 444.2168; found, 444.2166. $[\alpha]^{24.9}_D$ = - 5.20˚ (*c* = 1.0, MeOH).



12a

(2*S*, 3*S*)-Allyl-2,6-di-(*tert*-butoxycarbonylamino)-4-methyldithio-hexanoate (**12a**)

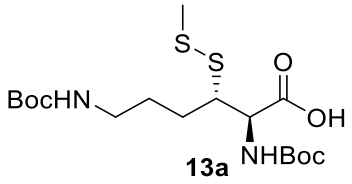
R_f = 0.39 (DCM/EtOAc=5:1); IR (thin film): ν_{max} 2977, 1709, 1165, 913, 742 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.92 (tdd, *J* = 14.0 Hz, *J* = 10.7 Hz, *J* = 8.2 Hz , 1H), 5.34 (d, *J* = 17.2 Hz, 1H), 5.26 (d, *J* = 10.24 Hz, 2H), 4.70-4.58 (m, 3H), 4.57 (br, 1H), 3.03-3.18 (m, 3H), 2.40 (s, 3H), 1.81-1.67 (m, 2H), 1.67-1.55 (m, 2H), 1.43 (s, 18H); ^{13}C NMR ($CDCl_3$, 100 MHz) : δ 170.390, 155.944, 155.225, 131.452, 119.177, 80.248, 79.220, 66.214, 56.721, 53.926, 39.983, 28.422, 28.297, 27.802, 27.482, 23.841; HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{20}H_{37}N_2O_6S_2$, 465.2093; found, 465.2091. $[\alpha]^{25.5}_D$ = - 36.40^o (*c* = 1.0, MeOH).



12b

(2*S*, 3*S*)-Allyl-2,6-di-(*tert*-butoxycarbonylamino)-4-*tert*-butyldithio-hexanoate (**12b**)

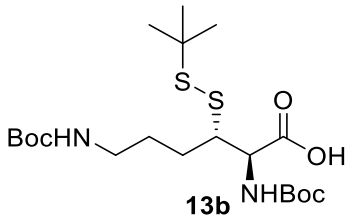
R_f = 0.60 (petroleum ether/EtOAc=2:1); IR (thin film): ν_{max} 2975, 2932, 1716, 1508, 1456, 1392, 1366, 1250, 1168 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.91 (tdd, *J* = 9.52, 11.4, 8.6 Hz , 1H), 5.34 (d, *J* = 17.2, 1.32 Hz, 1H), 5.28 (d, *J* = 28.2 Hz, 2H), 4.76 (d, *J* = 5.6 Hz, 1H), 4.71-4.52 (m, 3H), 3.20-3.03 (m, 3H), 1.84-1.49 (m, 4H), 1.44 (s, 18H), 1.33 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) : δ 170.444, 155.891, 155.209, 131.448, 119.087, 80.077, 79.217, 66.183, 56.378, 54.977, 48.091, 40.164, 29.961, 28.415, 28.304, 27.909, HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{23}H_{43}N_2O_6S_2$, 507.2563; found, 507.2554. $[\alpha]^{24.9}_D$ = - 89.20^o (*c* = 1.0, MeOH).



13a

(2*S*, 3*S*)-2,6-Di-(*tert*-butoxycarbonylamino)-4-methyldithio-hexanoic acid (**13a**)

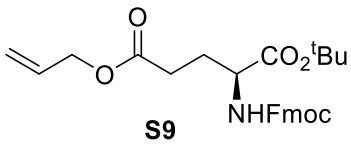
R_f = 0.15 (petroleum ether/EtOAc/AcOH=50:50:1); IR (thin film): ν_{max} 3357, 2978, 2931, 1716, 1509, 1368, 1251, 1167, 1050, 856, 778 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.41 (d, *J* = 25.2, 1 H), 4.72 (d, *J* = 21.2, 2H), 3.29-2.96 (m, 3H), 2.43 (s, 3H), 1.84-1.68 (m, 2H), 1.66-1.52 (m, 2H), 1.45 (s, 18H); ^{13}C NMR ($CDCl_3$, 100 MHz) : δ 176.478, 157.350, 156.319, 79.622, 79.147, 58.641, 53.425, 41.359, 40.355,31.597, 29.708, 28.486; HRMS-ESI (m/z): $[M-H]^+$ calcd for $C_{17}H_{31}N_2O_6S_2$, 423.1624; found, 423.1621. $[\alpha]^{25.0}_D$ = - 35.60° (*c* = 1.0, MeOH).



13b

(2*S*, 3*S*)-2,6-Di-(*tert*-butoxycarbonylamino)-4-*tert*-butyldithio-hexanoic acid (**13b**)

R_f = 0.15 (petroleum ether/EtOAc/AcOH=50:50:1); IR (thin film): ν_{max} 3373, 2973, 2928, 2860, 1709, 1504, 1392, 1366, 1250, 1168, 1051, 1025, 548 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.65 (br, 1H), 5.05 (d, *J* = 9.6, 1H)4.48 (d, *J* = 9.3, 1H), 3.35-2.88 (m, 3H), 2.11-1.54 (m, 4H), 1.43 (s, 18H), 1.31 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) : δ 176.277, 157.403, 156.350, 79.676, 79.182, 57.667, 54.732, 47.566, 40.532, 31.935, 30.019, 29.707, 28.492; HRMS-ESI (m/z): $[M-H]^+$ calcd for $C_{20}H_{37}N_2O_6S_2$, 465.2093; found, 465.2087. $[\alpha]^{25.0}_D$ = - 21.60° (*c* = 1.0, MeOH).

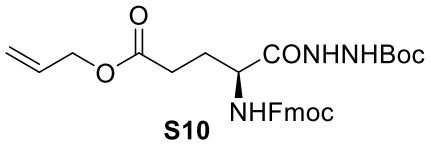


S9

2*S*-2-((9H-fluoren-9-yl) methoxy carbonyl) amino-1-tert-butoxycarbonyl -5-(allyloxy)-5-oxopentanoic acid (S9)

R_f = 0.43 (petroleum ether/EtOAc=3:1); IR (thin film): ν_{max} 3357, 2978, 2931, 1716, 1509, 1368, 1251, 1167, 1050, 856, 778 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.3 Hz, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.32 (t, *J* = 7.4 Hz, 2H), 5.92 (ddt, *J* = 16.2, 10.5, 5.8 Hz, 1H), 5.44 (d, *J* = 7.8 Hz, 1H), 5.32 (dd, *J* = 17.2, 1.4 Hz, 1H), 5.24 (dd, *J* = 10.4, 1.1 Hz, 1H), 4.59 (d, *J* = 5.7 Hz, 2H), 4.40 (d, *J* = 7.0 Hz, 2H), 4.31 (dd, *J* = 12.6, 7.7 Hz, 1H), 4.22 (t, *J* = 7.0 Hz, 1H), 2.55 – 2.33 (m, 2H), 2.29 – 2.17 (m, 1H), 2.06 – 1.92 (m, 1H),

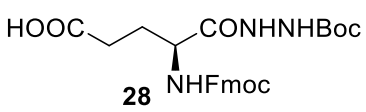
1.48 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz) : δ ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.425, 170.999, 155.946, 143.942, 143.793, 141.324, 132.068, 127.714, 127.079, 125.114, 119.989, 118.374, 82.532, 67.034, 65.345, 53.843, 47.219, 30.212, 28.012; HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{27}H_{32}NO_6$, 466.2230; .found, 466.2218. $[\alpha]^{24.9}_D$ = - 18.00° (*c* = 1.0, MeOH).



S10

2*S*-2-((9H-fluoren-9-yl) methoxy carbonyl) amino-1-(*tert*-butoxycarbonyl) hydrazinyl-5-(allyloxy)-5-oxopentanoic acid (**S10**)

R_f = 0.19 (petroleum ether/EtOAc =2:1); IR (thin film): ν_{max} 3296, 2981, 2935, 1716, 1526, 1479, 1247, 1162, 760, 741cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): δ8.58 (br, 1H), 7.73 (d, *J* = 7.5 Hz, 2H), 7.56 (dd, *J* = 7.3, 3.3 Hz, 2H), 7.37 (t, *J* = 7.5 Hz, 2H), 7.28 (t, *J* = 7.4 Hz, 2H), 6.71 (br, 1H), 5.88 (ddd, *J* = 22.9, 10.9, 5.6 Hz, 2H), 5.29 (dd, *J* = 17.2, 1.3 Hz, 1H), 5.21 (dd, *J* = 10.4, 1.0 Hz, 1H), 4.56 (d, *J* = 5.6 Hz, 2H), 4.35 (t, *J* = 10.5 Hz, 3H), 4.17 (t, *J* = 6.9 Hz, 1H), 2.63 – 2.42 (m, 2H), 2.19 (dd, *J* = 13.3, 6.3 Hz, 1H), 2.00 (dt, *J* = 19.2, 9.5 Hz, 1H), 1.44 (s, 9H), ^{13}C NMR ($CDCl_3$, 100 MHz) : δ 173.030, 171.064, 156.390, 155.204, 143.778, 143.653, 141.284, 131.945, 127.729, 127.101, 125.105, 119.964, 118.482, 81.842, 67.279, 65.495, 52.580, 47.094, 30.216, 28.144, 27.838; HRMS-ESI (m/z): $[M+NH_4]^+$ calcd for $C_{28}H_{37}N_4O_7$, 541.2662; found, 541.2656. $[\alpha]^{24.9}_D$ = - 28.4° (*c* = 1.0, MeOH).

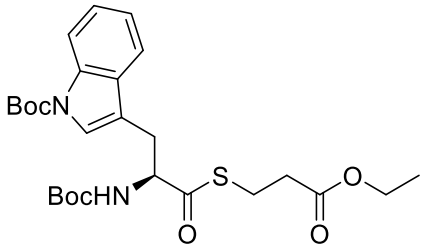


28

2*S*-2-((9H-fluoren-9-yl) methoxy carbonyl) amino-1-(*tert*-butoxycarbonyl) hydrazinyl-5-oxopentanoic acid (28)

R_f = 0.15 (petroleum ether/EtOAc/AcOH=50:50:1); IR (thin film): ν_{max} 3357, 2978, 2931, 1716, 1509, 1368, 1251, 1167, 1050, 856, 778 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 10.38 (br, 1H), 9.26 (br, 1H), 7.69 (d, *J* = 7.2 Hz, 2H), 7.52 (d, *J* = 5.9 Hz, 2H), 7.33 (t, *J* = 7.4 Hz, 2H), 7.24 (d, *J* = 7.4 Hz, 2H), 7.02 (br, 1H), 6.13 (d, *J* = 7.2 Hz, 1H), 4.48 (br, 1H), 4.37-4.23 (m, 2H), 4.11 (t, *J* = 6.6 Hz, 1H), 2.54-2.39 (m, 2H), 2.20-2.05 (m, 1H), 2.02-1.89 (m, 1H), 1.39 (s, 9H), ^{13}C NMR ($CDCl_3$, 100 MHz) : δ 176.602, 171.350, 156.573, 155.602, 143.708, 143.612, 141.222, 132.195, 128.665,

128.543, 127.720, 127.114, 125.164, 119.944, 82.202, 67.384, 52.141, 46.955, 29.654, 28.108; HRMS-ESI (m/z): $[M-H]^+$ calcd for $C_{25}H_{28}N_3O_7$, 482.1927; found, 482.1917. $[\alpha]^{25.1}_D$ = - 35.6º (*c* = 1.0, MeOH).



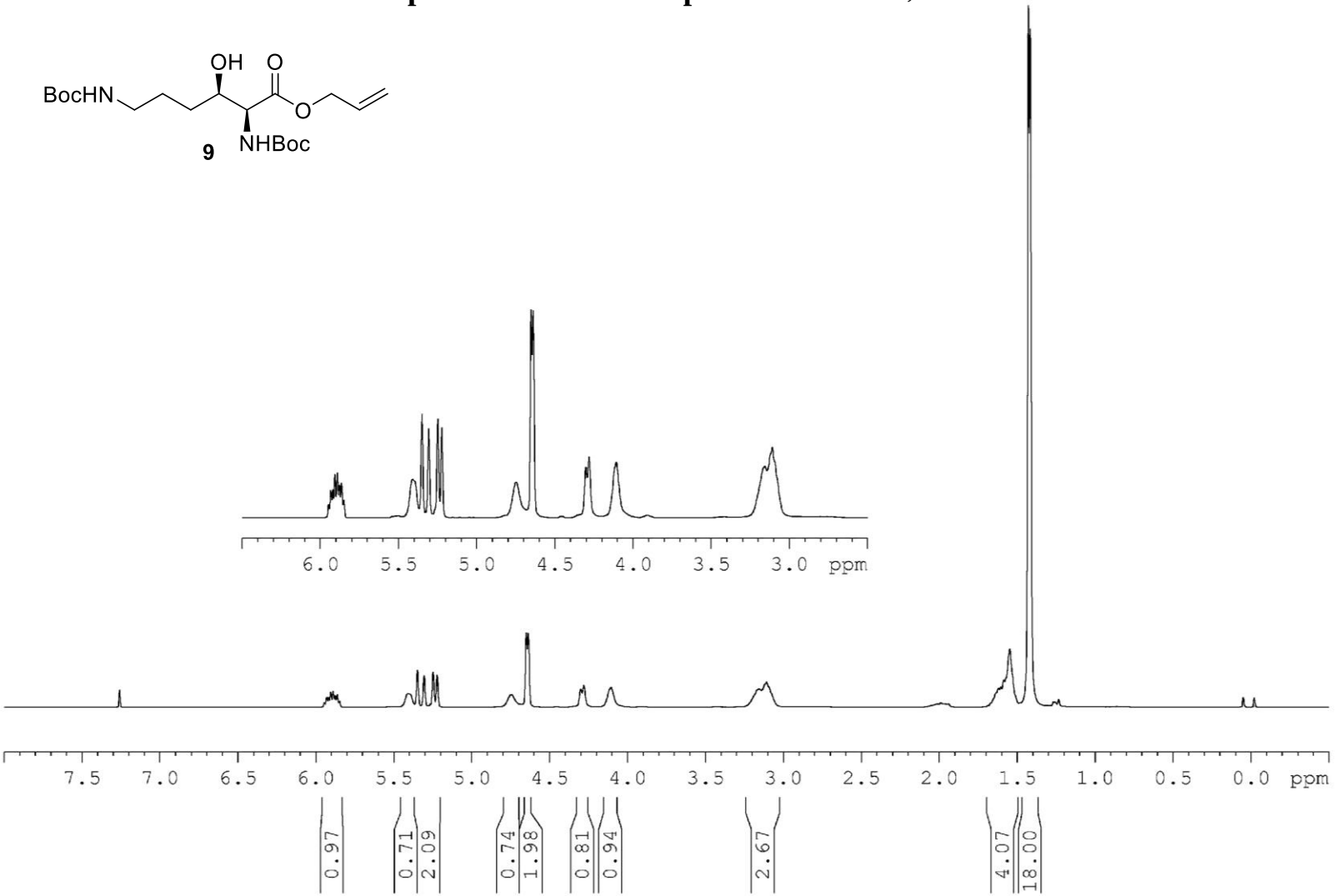
S11

***N,N*-Di-(tert-butoxycarbonyl)-*S*-ethoxypropano-ate-L-tryptophan thioester (S11)**

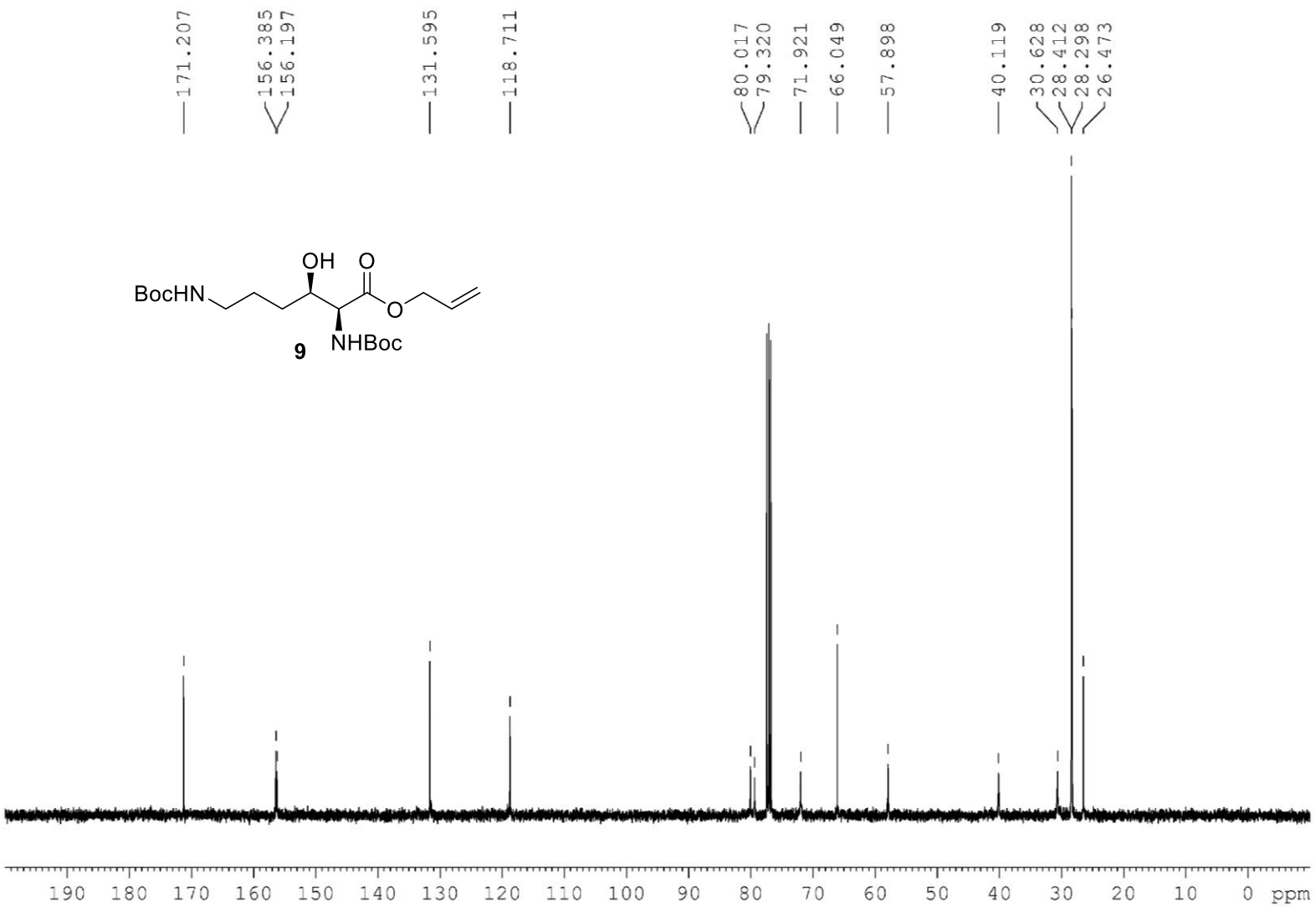
R_f = 0.45 (petroleum ether/EtOAc=3:1); IR (thin film): ν_{max} 3346, 2979, 2933, 2857, 1732, 1454, 1370, 1255, 1160, 1088, 1018, 858, 768, 747, cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): ^1H NMR (400 MHz, $CDCl_3$) δ 8.12 (s, 1H), 7.49 (d, *J* = 7.7 Hz, 1H), 7.43 (d, *J* = 8.8 Hz, 1H), 7.31 (t, *J* = 7.5 Hz, 1H), 7.23 (t, *J* = 7.4 Hz, 1H), 5.02 (d, *J* = 8.7 Hz, 1H), 4.80-4.64 (m, 1H), 4.12 (q, *J* = 7.1 Hz, 2H), 3.28-2.89 (m, 4H), 2.55 (m, 2H), 1.66 (s, 9H), 1.40 (d, *J* = 10.5 Hz, 9H), 1.24 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) : δ ^{13}C NMR (101 MHz, $CDCl_3$) δ 201.056, 171.569, 154.995, 149.535, 135.394, 130.361, 124.641, 124.233, 122,674. 118.971, 115.264, 114.691, 83.737, 80.427, 60. 783, 60. 039, 34.112, 32.543, 28.284, 28.198, 27.923, 14.187; LRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{26}H_{37}N_2O_7S_1$, 521.22; found, 521.26. $[\alpha]^{24.9}_D$ = - 22.40º (*c* = 1.0, MeOH).

2.3 NMR Spectra. of amino acids and derivatives

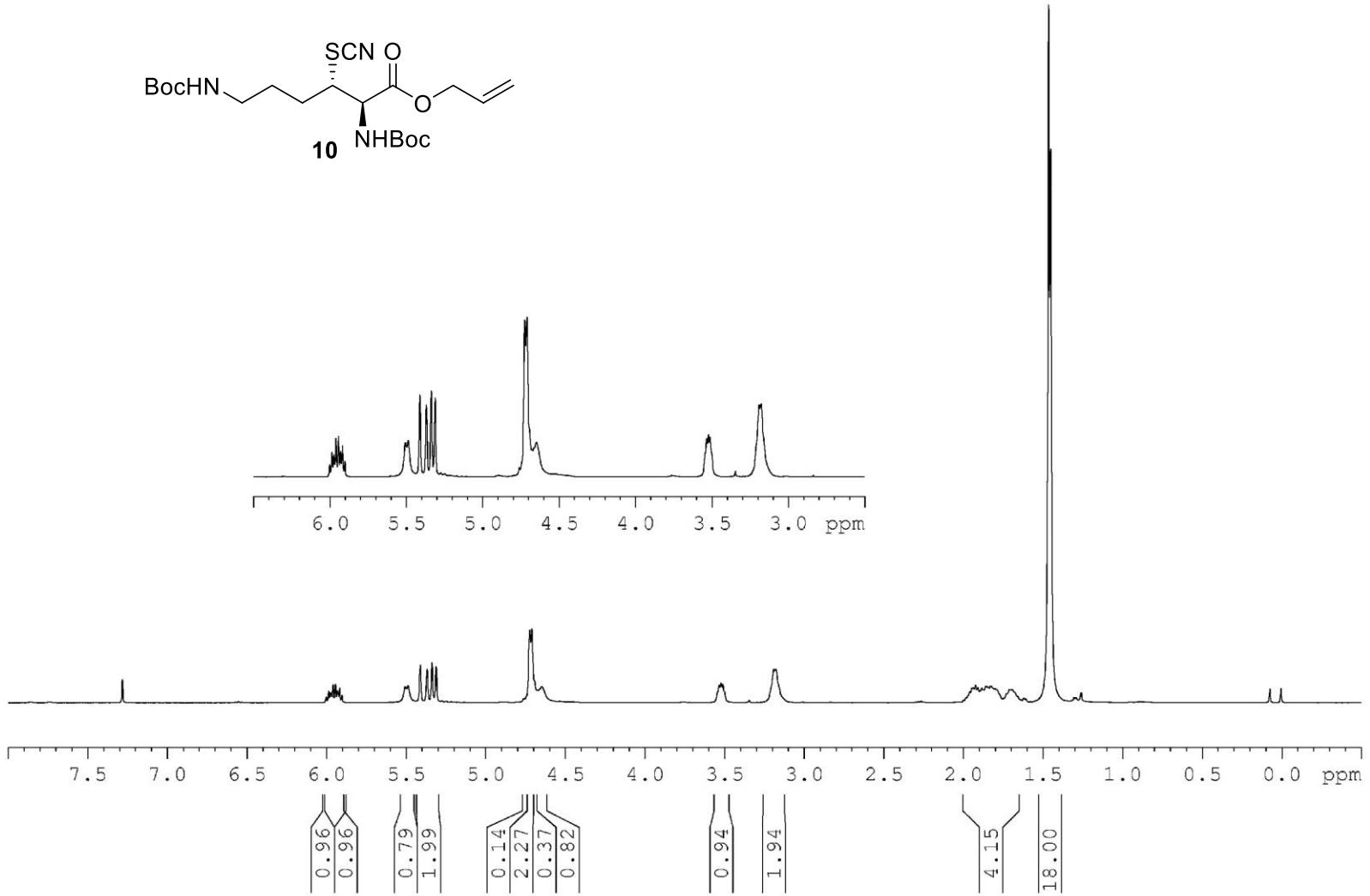
Compound 9 - ^{1}H NMR Spectrum - $CDCl_3$, 400 MHz



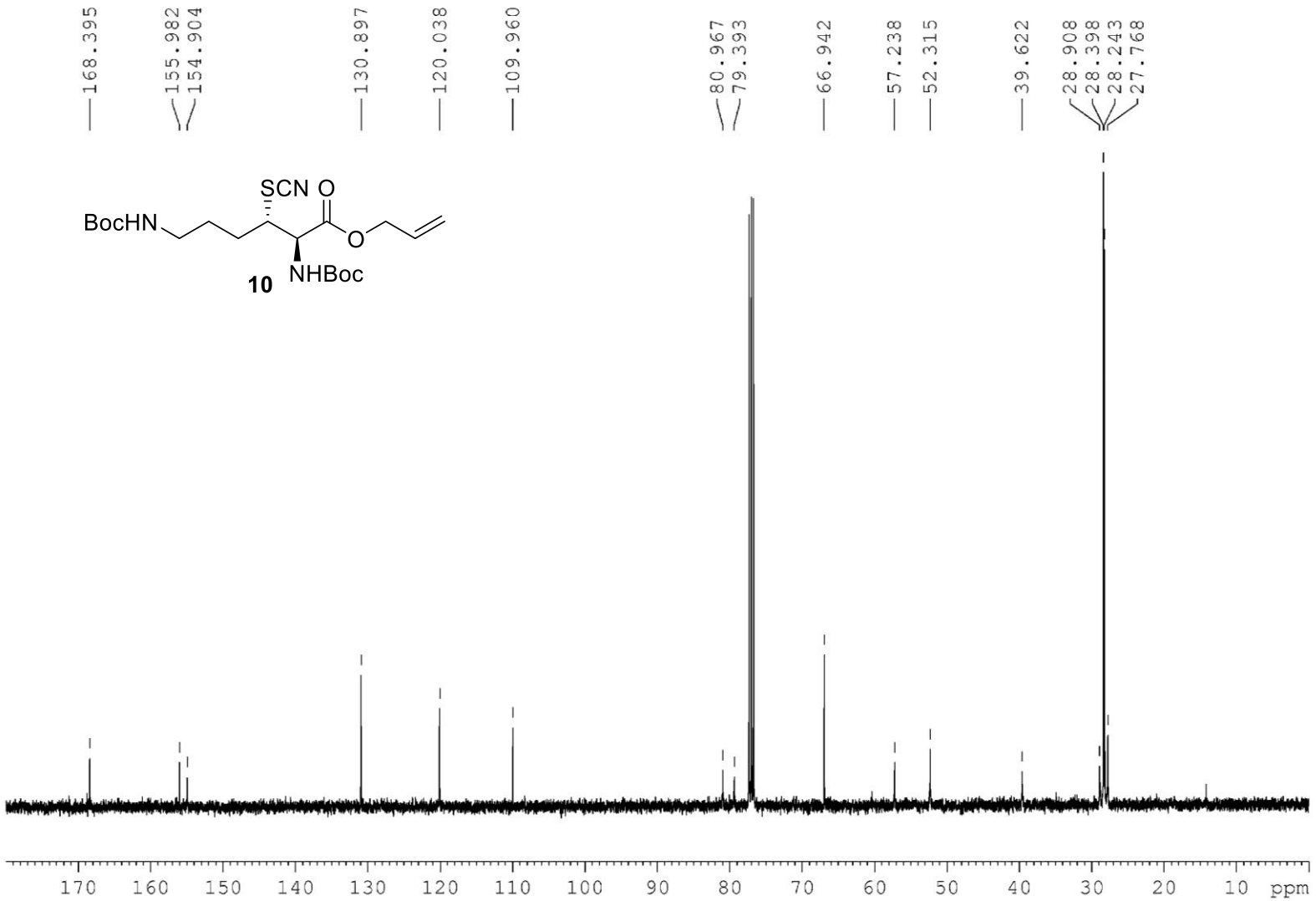
Compound 9 – ^{13}C NMR Spectrum - $CDCl_3$, 100 MHz



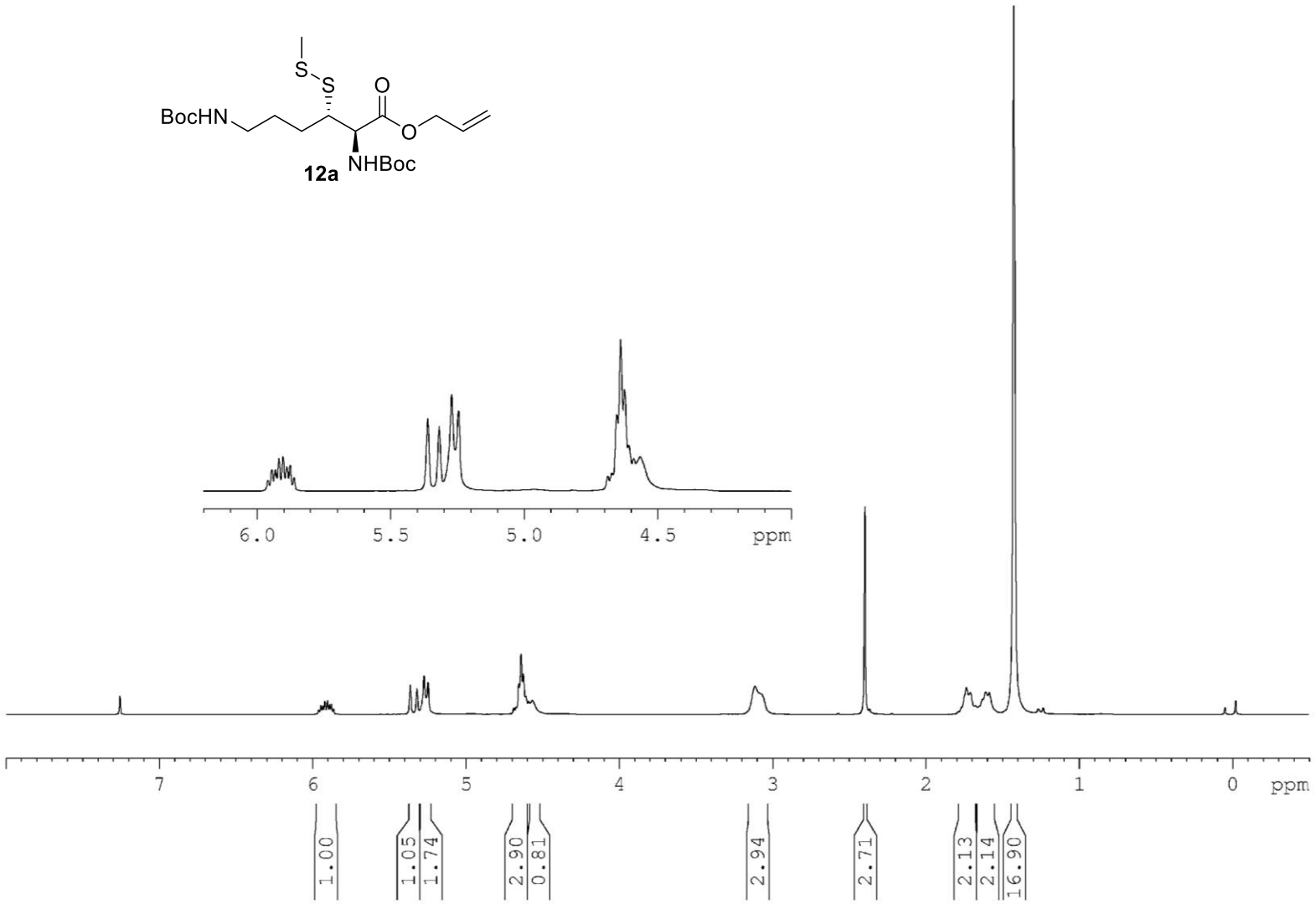
Compound 10 - ^{1}H NMR Spectrum - $CDCl_3$, 400 MHz



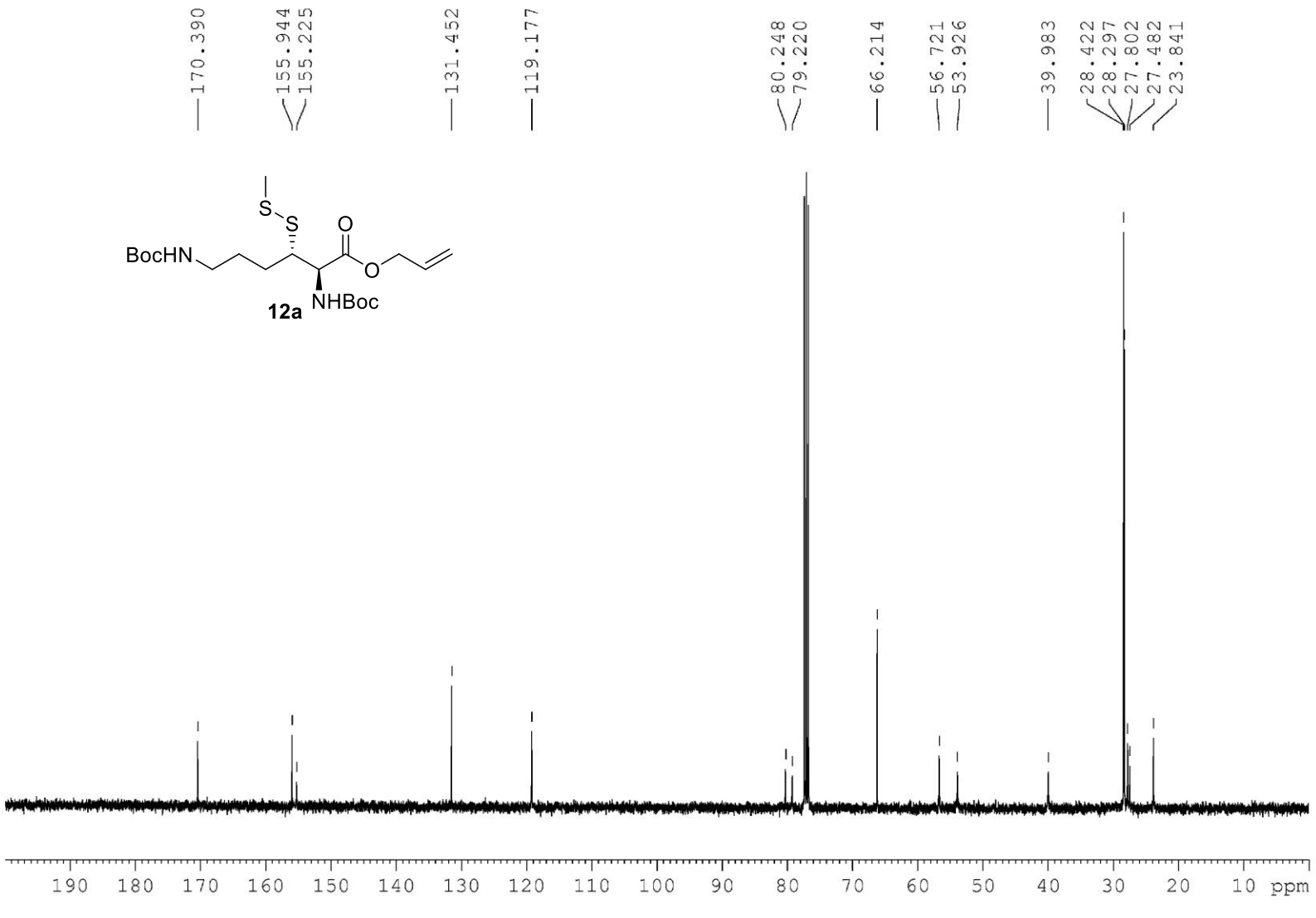
Compound 10 – ^{13}C NMR Spectrum - $CDCl_3$, 100 MHz



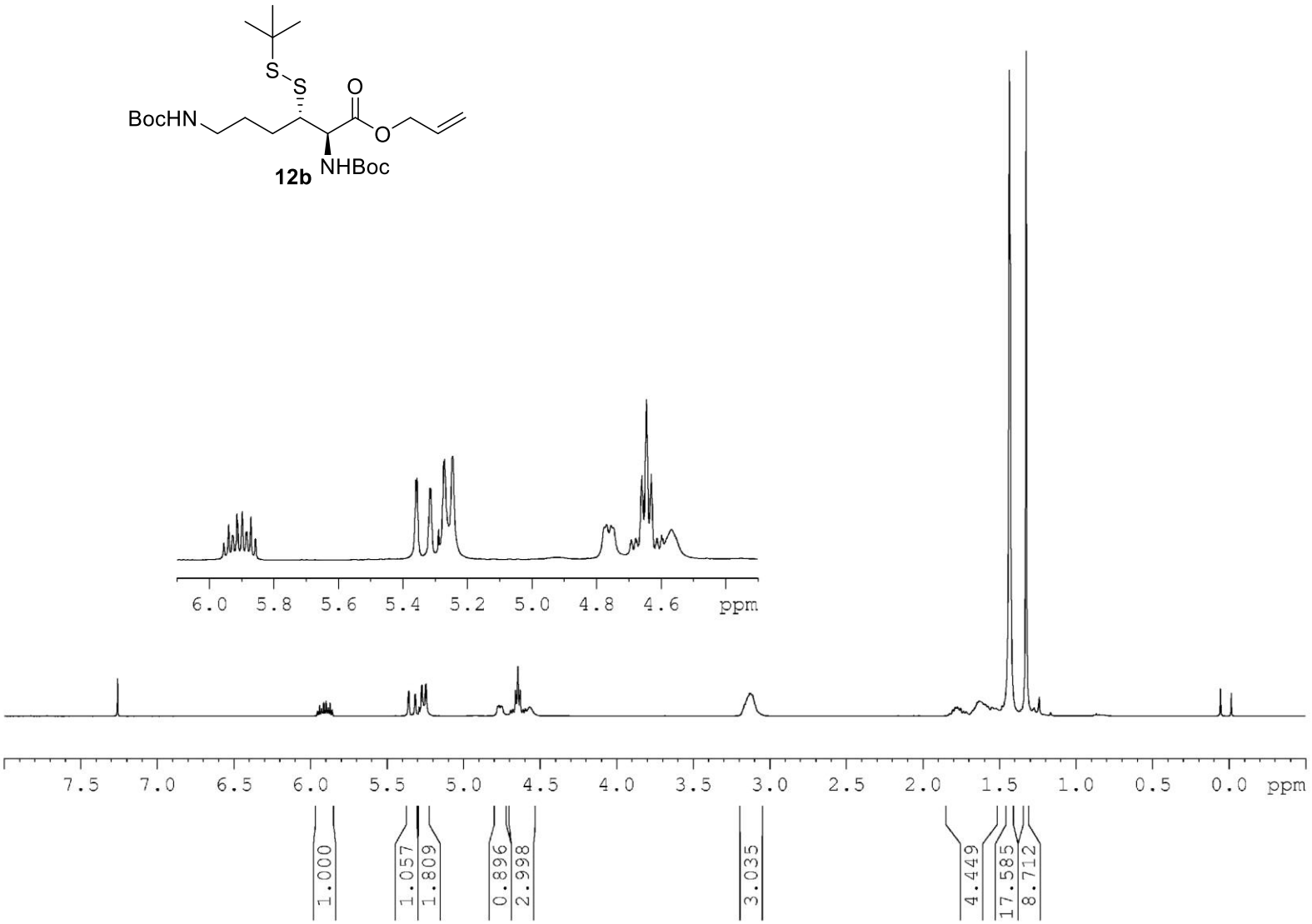
Compound 12a - ^{1}H NMR Spectrum - $CDCl_3$, 400 MHz



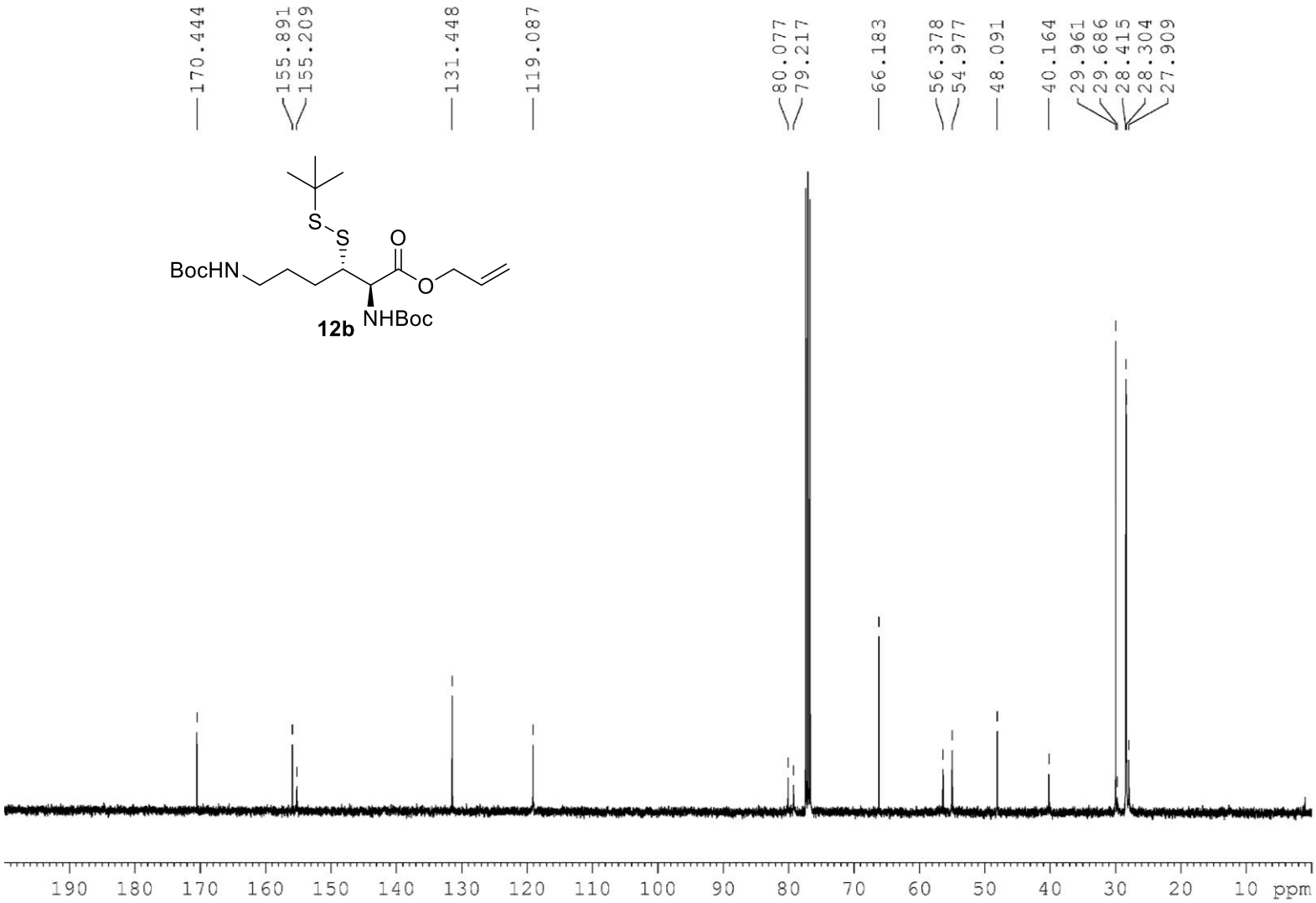
Compound 12a – ^{13}C NMR Spectrum - $CDCl_3$, 100 MHz



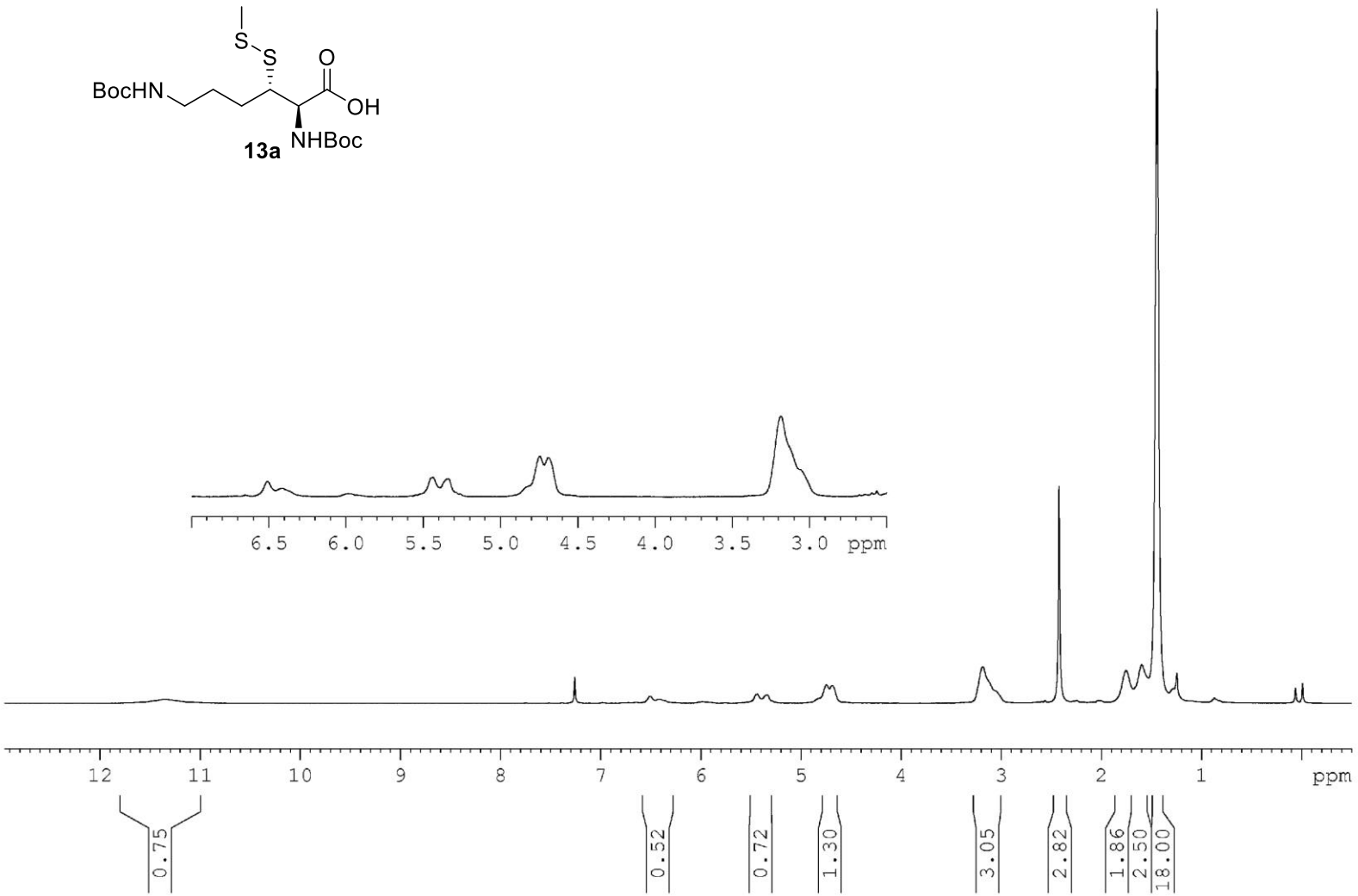
Compound 12b - ^{1}H NMR Spectrum - $CDCl_3$, 400 MHz



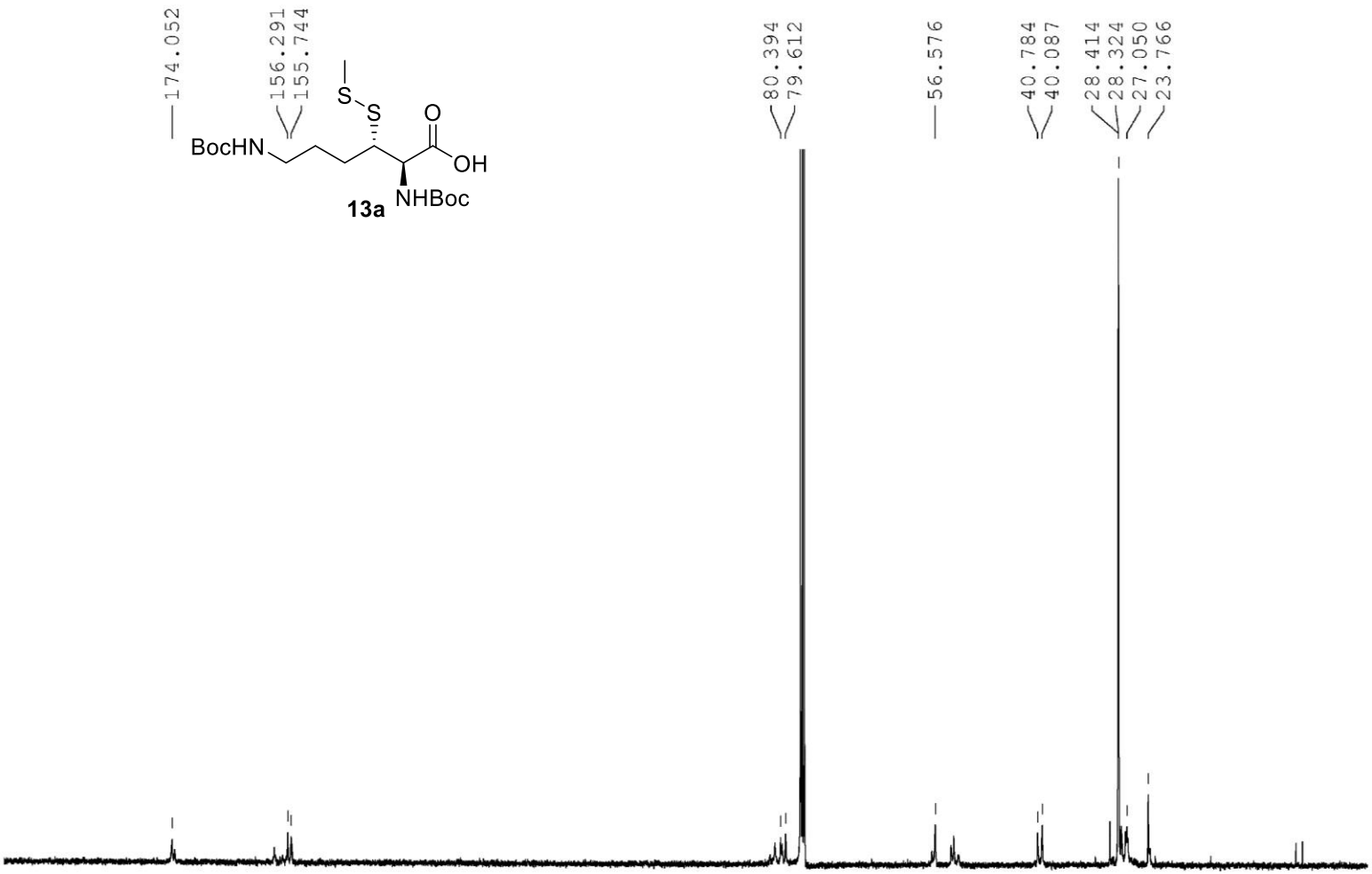
Compound 12b – ^{13}C NMR Spectrum - $CDCl_3$, 100 MHz



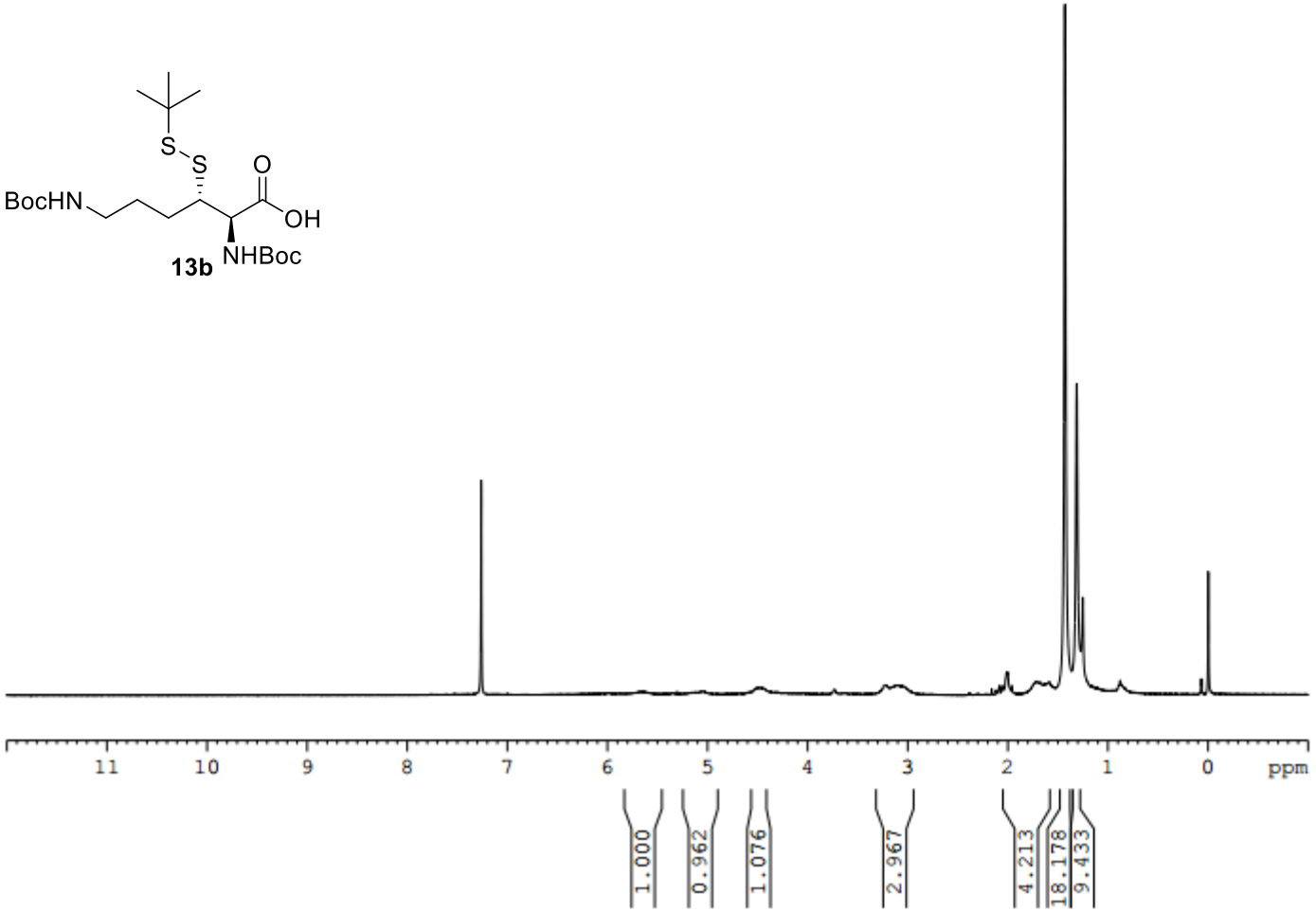
Compound 13a - ^{1}H NMR Spectrum - $CDCl_3$, 400 MHz



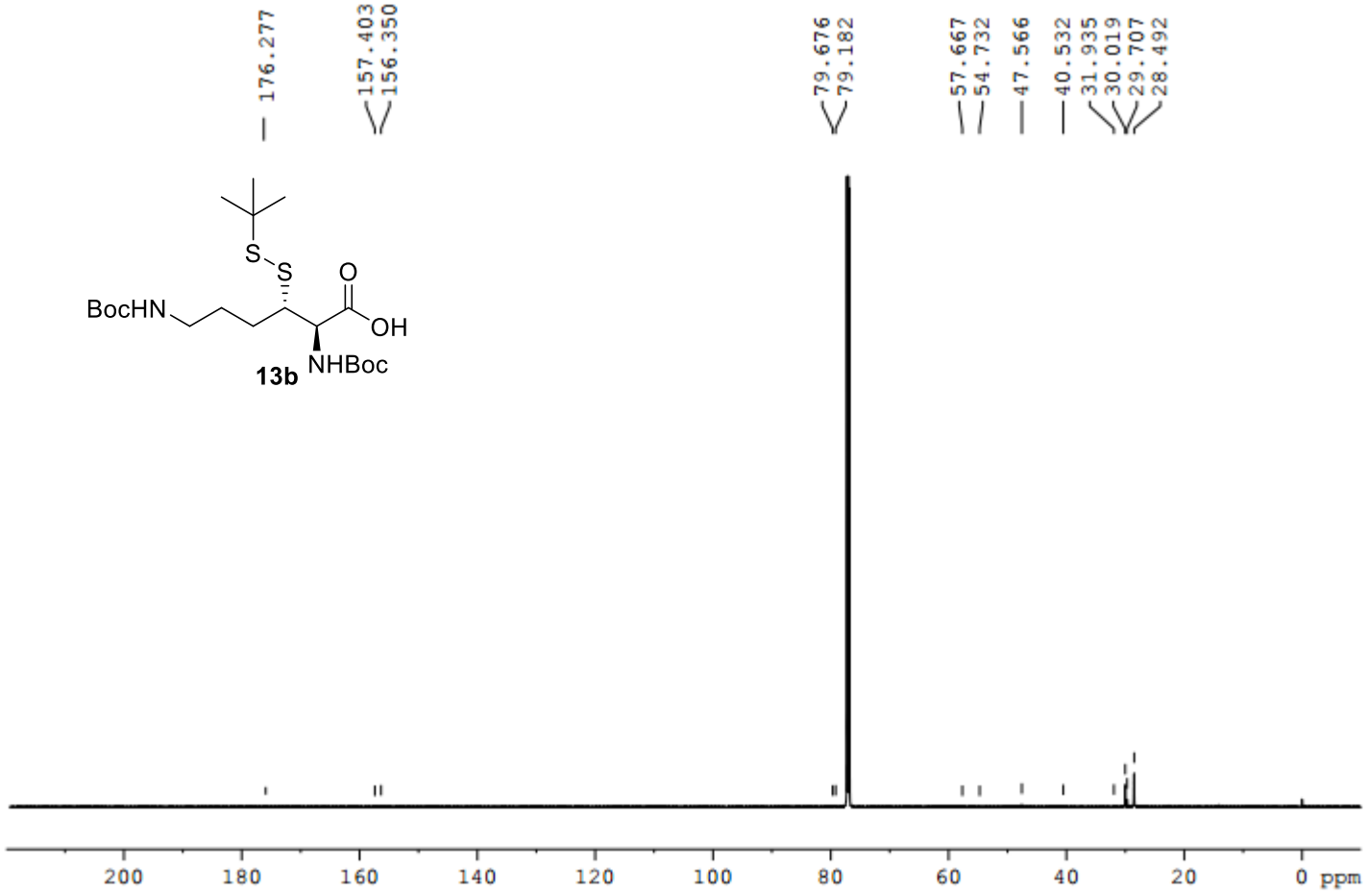
Compound 13a – ^{13}C NMR Spectrum - $CDCl_3$, 100 MHz



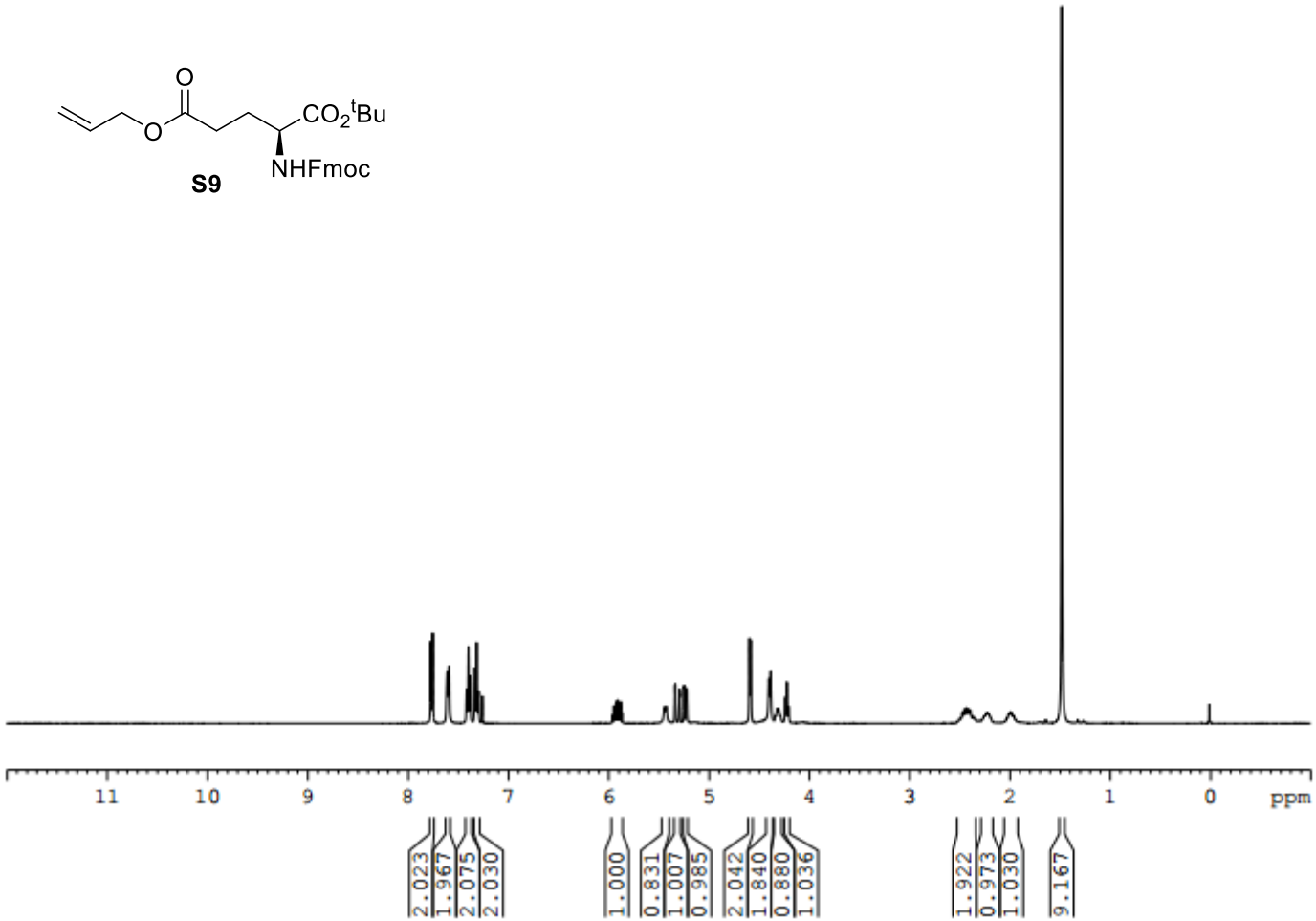
Compound 13b - ^{1}H NMR Spectrum - $CDCl_3$, 400 MHz



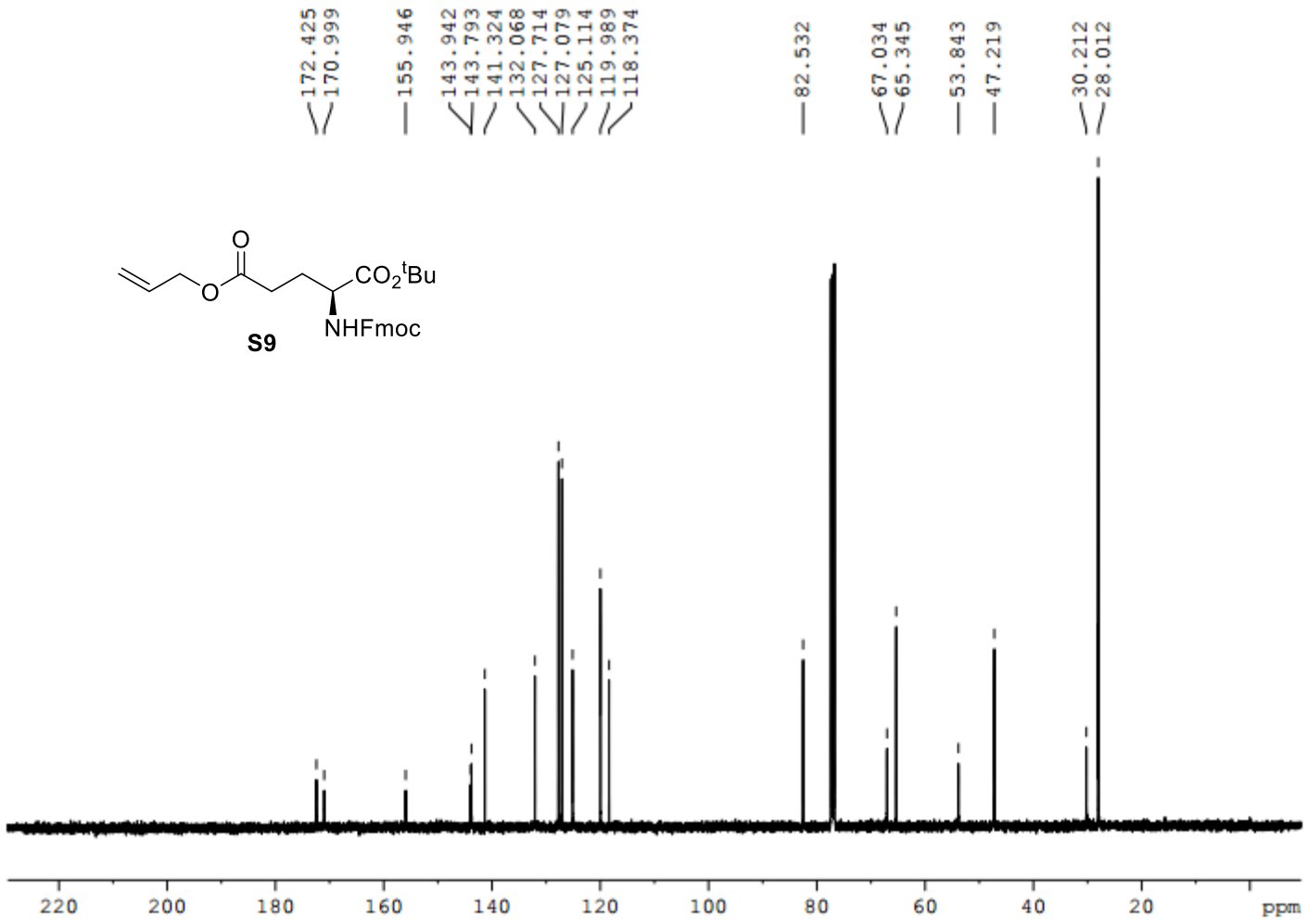
Compound 13b – ^{13}C NMR Spectrum - $CDCl_3$, 100 MHz



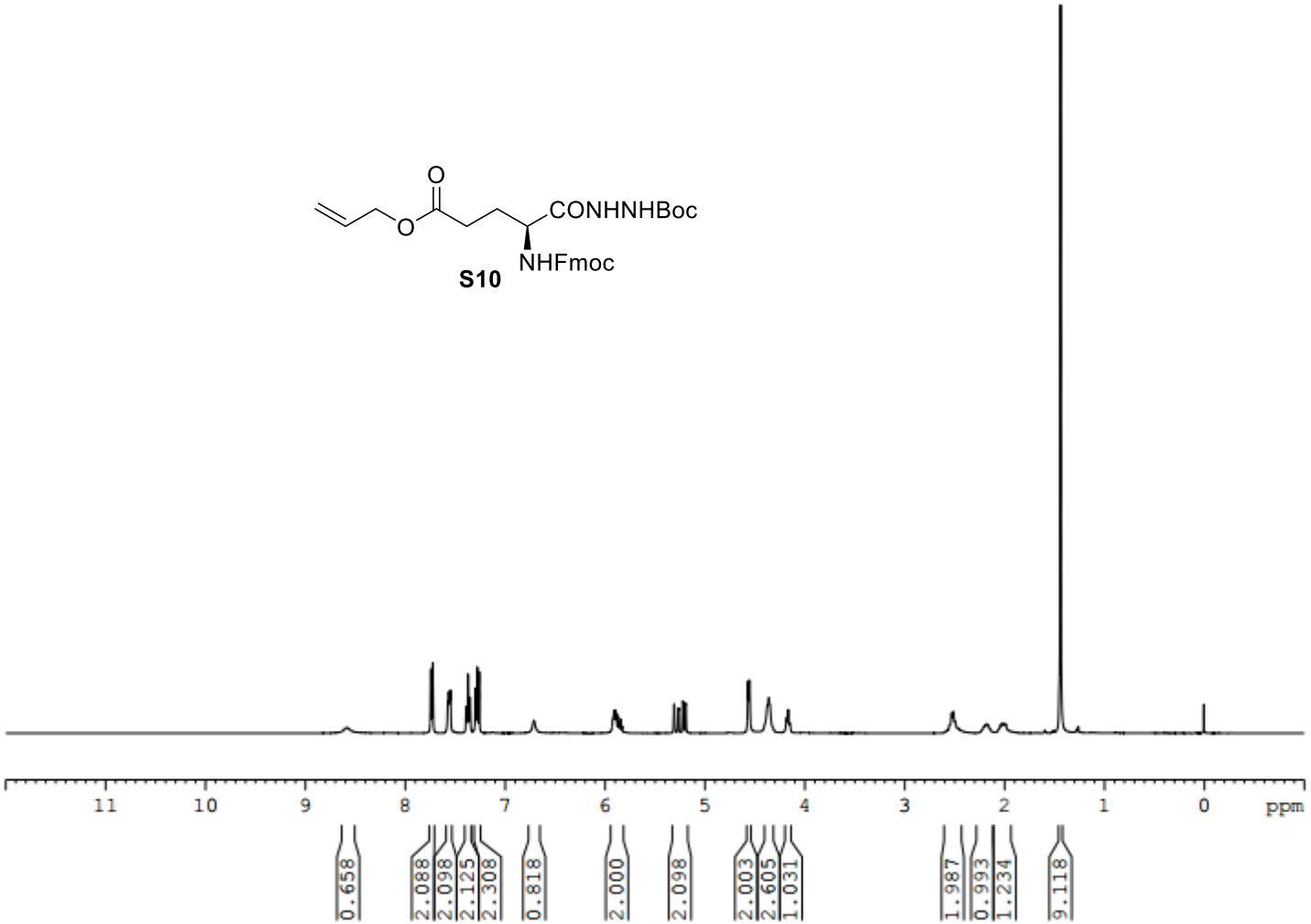
Compound S9 - ^{1}H NMR Spectrum - $CDCl_3$, 400 MHz



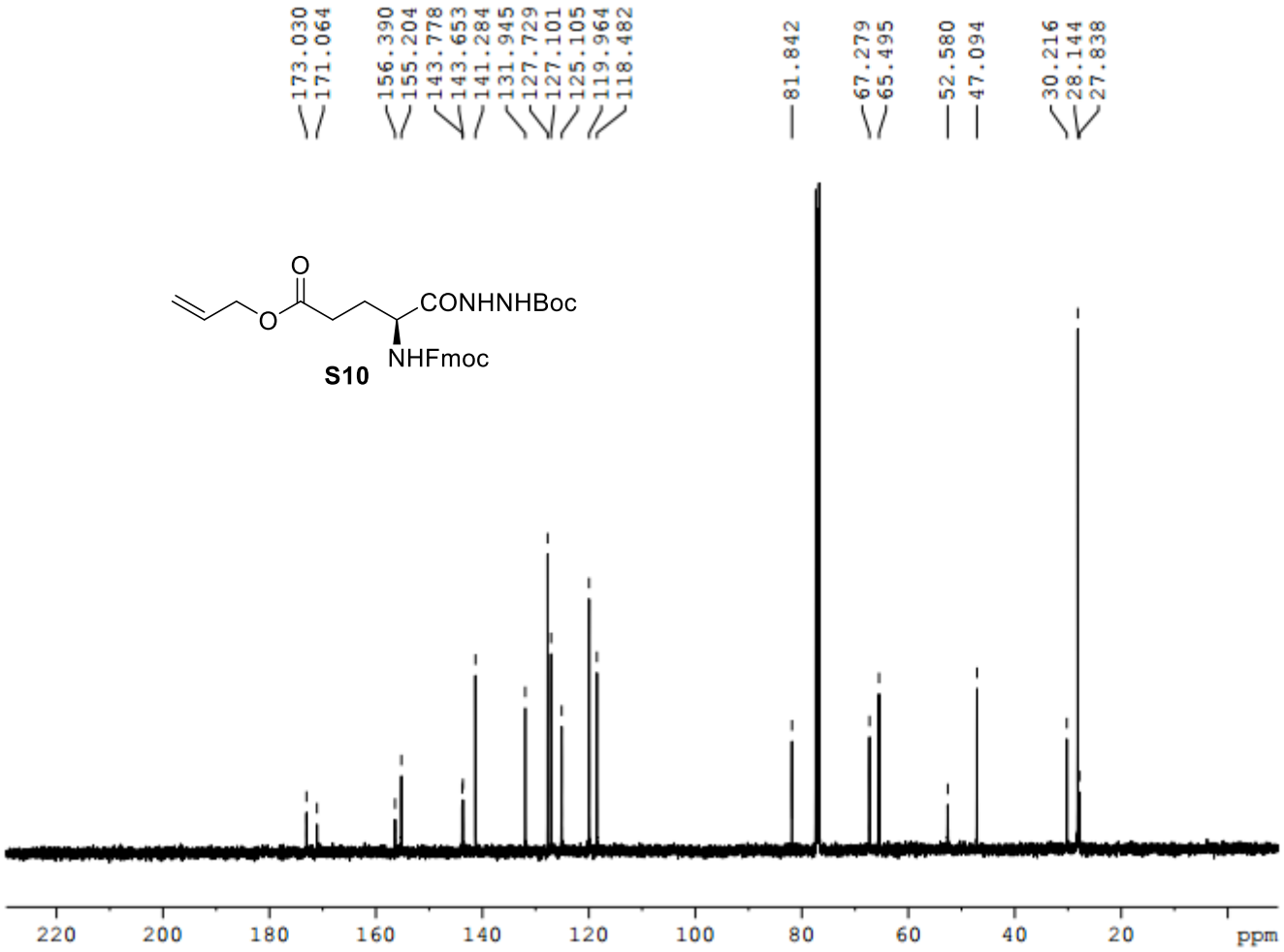
Compound S9 – ^{13}C NMR Spectrum - $CDCl_3$, 100 MHz



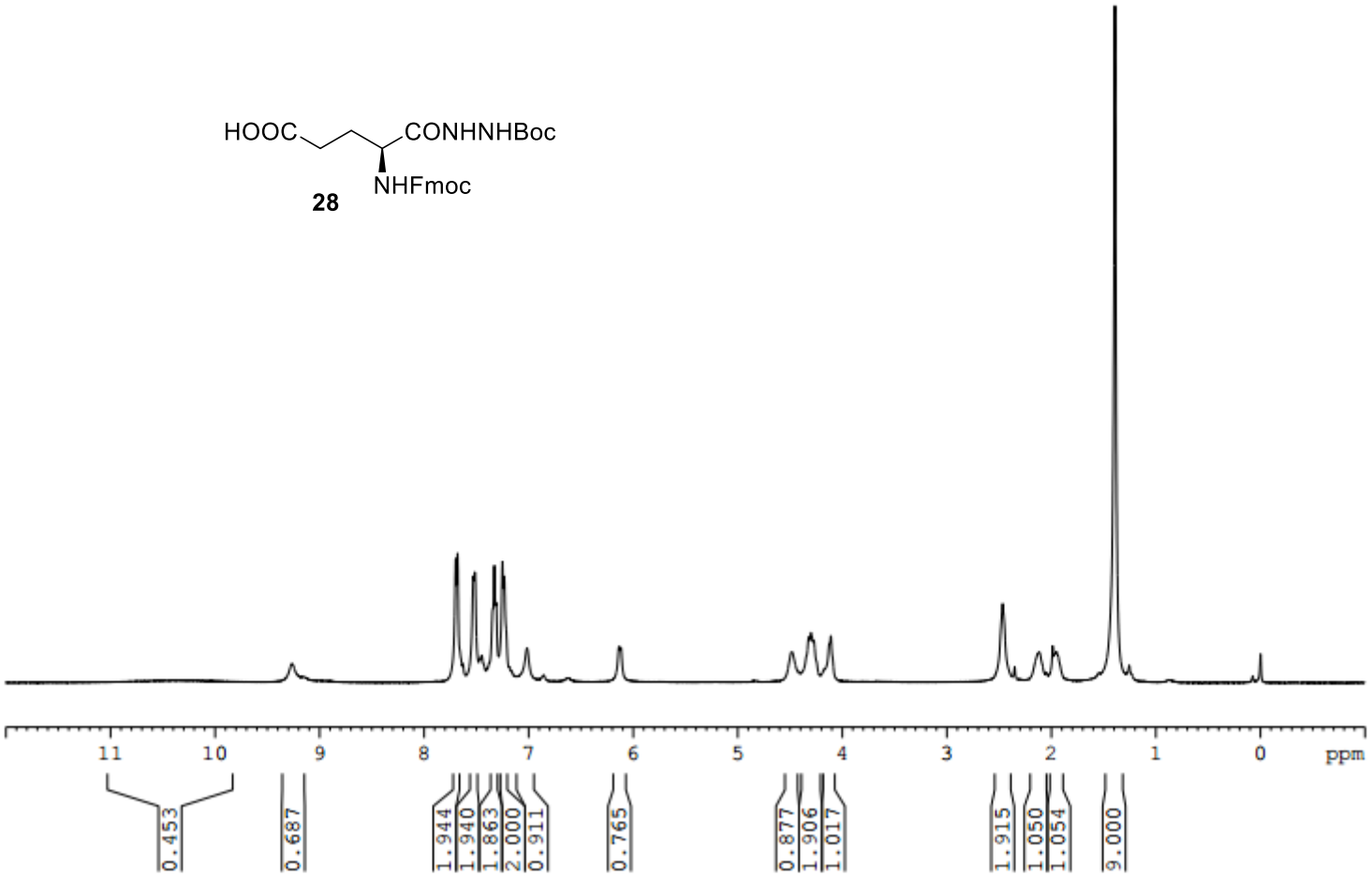
Compound S10 - ^{1}H NMR Spectrum - $CDCl_3$, 400 MHz



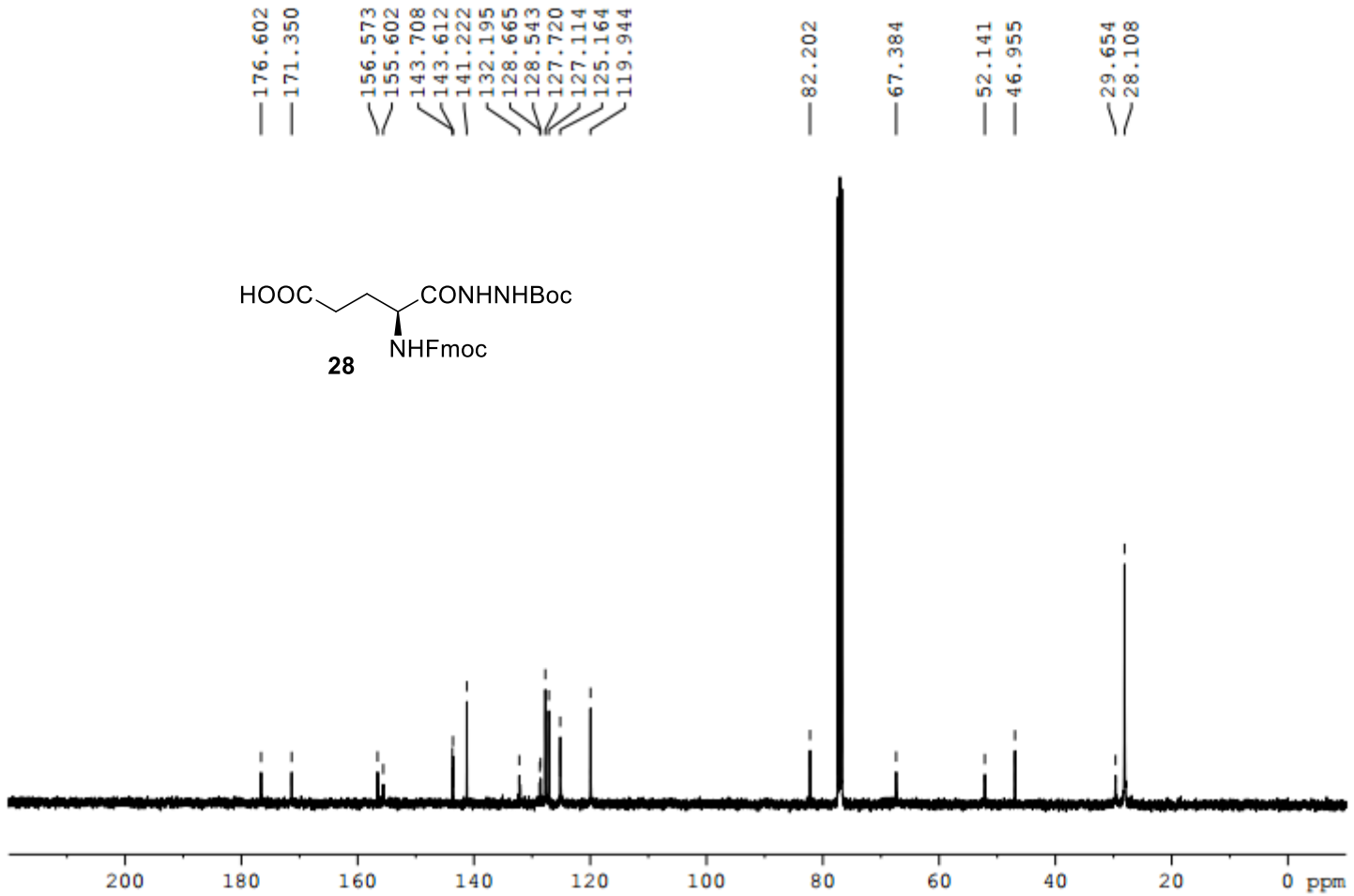
Compound S10 – ^{13}C NMR Spectrum - $CDCl_3$, 100 MHz



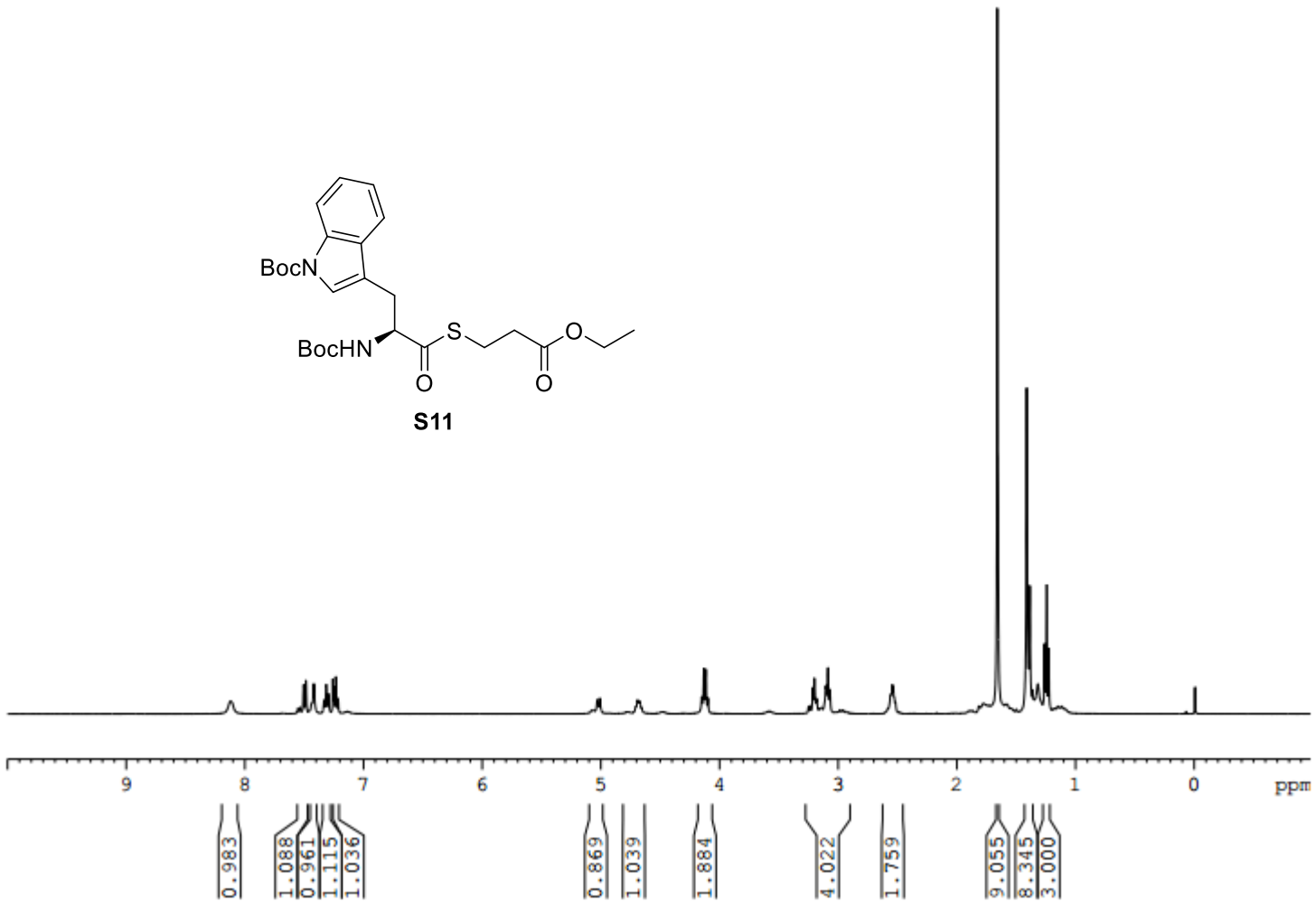
Compound 28 - ^{1}H NMR Spectrum - $CDCl_3$, 400 MHz



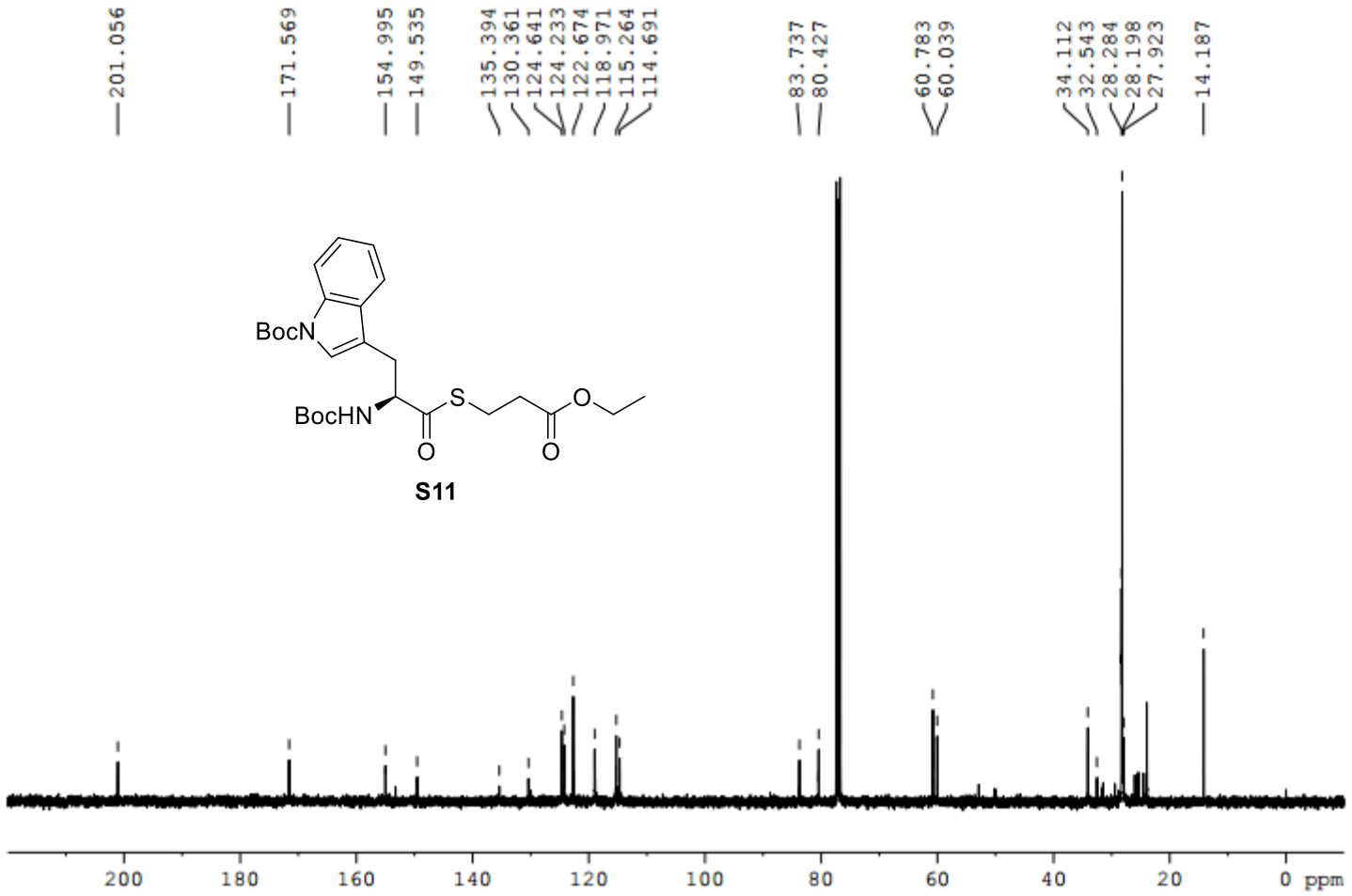
Compound 28 – ^{13}C NMR Spectrum - $CDCl_3$, 100 MHz



Compound S11 - ^{1}H NMR Spectrum - $CDCl_3$, 400 MHz



Compound S11 – ^{13}C NMR Spectrum - $CDCl_3$, 100 MHz



S11

III. General Procedures for Peptide Synthesis

3.1 Preparation of amino acid pre-loaded resin and determination of resin loading[S5]

Pre-load an amino acid to 2-chlorotritylchloride resin

The first Fmoc-amino acid residue was loaded to 2-chlorotritylchloride resin before Fmoc-SPPS following the general procedure below.

To a mixture of Fmoc-amino acid (1.0 equiv) and 2-chlorotritylchloride resin was added dry DCM (approx. 10 mL per gram of resin) and DIEA (4.0 equiv). The reaction was agitated for 2 hours. The resin was collected and washed with 17/2/1 (*v*/*v*/*v*) of DCM/MeOH/DIEA (× 3), DCM (× 3), DMF (× 2), DCM (× 3), and dried *in vacuo* for 12 hours before the loading test.

Determination of resin loading

Dry Fmoc amino-acid resin (approx. 5 μmol with respect to Fmoc) was weighted into a clean test tube, followed by the addition of 2 mL of 2% DBU in DMF. The mixture was agitated gently for 30 min, and then diluted to 10 mL with CH_3CN. 2 mL of the resulting solution was taken out and diluted to 25 mL in a 50 mL centrifuge tube as the test solution. A reference solution was prepared in the same manner without the addition of resin.

The silica UV cell was filled with reference solution to blank the U.V. spectrophotometer. The solution in the silica UV cell was changed to the test solution after washing with the test solution for three times. The optical density at 304 nm was recorded for three times and the average value was calculated as Abs_{sample}. The Fmoc loading of resin could be calculated using the equation below:

$$\text{Fmoc loading: mmol/g} = Abs_{sample} \times 16.4/(\text{mg of resin}).$$

3.2 Solid-Phase Peptide Synthesis

3.2.1 Automated Solid-Phase Peptide Synthesis

Automated peptide synthesis was performed on a Pioneer peptide synthesis system

[S5] Peptide Synthesis, 2010/2011 Catalog, Merck.

(GEN600611) or a CS Bio peptide synthesizer (CX136XT).

Pioneer peptide synthesizer Peptides were synthesized under standard automated Fmoc protocols using DMF as solvent, deblocking for 5 min in piperidine/DBU/DMF (2:2:96, *v/v/v*), coupling for 25 min (standard cycle), or 55 min (extended cycle) for amino acids after steric hindered residues such as prolines, valines, threonines, isoleucines and arginines using HATU as coupling reagent.

CS Bio peptide synthesizer Peptide synthesis was performed following the general protocol using DMF as solvent, deblocking (5 min × 2) in piperidine/DMF (20:80, *v/v*) containing Oxyma (0.1 M), couple for 25 min using HATU/HOBt (1:1) as coupling reagent, for amino acids after steric hindered residues, the coupling cycle was repeated as needed.

3.2.2 Manual Solid-Phase Peptide Synthesis

Manual peptide synthesis was performed when dipeptides (Fmoc-Gly-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Tyr(^{t}Bu)-Ser($\psi^{Me,Me}$Pro)-OH, Fmoc-Asn(Trt)-Ser($\psi^{Me,Me}$Pro)-OH) and synthetic amino acid derivatives (Fmoc-Ser (α-Man-Ac_4)-OH, Fmoc-Asn(Chitobiose-Ac_5)-OH, Boc-Lys$^{\beta\text{-SSMe}}$(Boc)-OH, Boc-Lys$^{\beta\text{-SStBu}}$(Boc)-OH) were involved.

Deprotection: The resin was treated with piperidine/DMF (20:80, *v/v*) containing 0.1 M Oxyma (5 min × 2) and washed with DMF (× 3), DCM (× 3) and DMF (× 3).

Amino acid coupling: Protected amino acid (2 equiv), HATU (2 equiv) and HOAt (2 equiv) was dissolved in 3 mL of DMF, to which DIEA (4 equiv) was added. The amino acid was preactivated for 1 min, then the solution was added to the resin. After agitated for 1 h, the resin was washed with DMF (× 3), DCM (× 3) and DMF (× 3), and same coupling cycle was repeated.

The following $^{\alpha}$*N*-Fmoc or $^{\alpha}$*N*-Boc-protected amino acids and pseudoproline dipeptides from Novabiochem, GL Biochem, CS Bio or Bomaijie were employed in SPPS: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-

Pro-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr-OH, Fmoc-Val-OH, Fmoc-Thr(HPO_3Bzl)-OH, Fmoc-Gly-Thr($\psi^{Me,Me}$Pro)-OH, Fmoc-Tyr(^{t}Bu)-Ser ($\psi^{Me,Me}$Pro)-OH, Fmoc-Asn(Trt)-Ser($\psi^{Me,Me}$Pro)-OH, Boc-Asp(O^{t}Bu)-OH, Boc-Ile-OH, Boc-Leu-OH, Boc-Thr(^{t}Bu)-OH, Boc-Ala-OH, Boc-Trp(Boc)-OH, Boc-Cys(Trt)-OH, *N*-Boc-L-pyroglutamic acid, Fmoc-Thr(HPO_3Bzl)-OH.

The 2-chlorotritylchloride resin (1.147 mmol/g) employed in SPPS was purchased from GL Biochem, and Rink MBHA resin (0.42 mmol/g) was purchased from CS Bio.

3.3 Preparation of Peptidyl Acids and Peptidyl Amides

Upon completion of the automated synthesis on a 0.05 mmol scale, the peptide resin was washed into a peptide synthesis vessel using DCM. Resin cleavage and global deprotection was performed under the treatment of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) solution for 2 hours. The resin was then removed by filtration, and the filtrate was concentrated under a nitrogen atmosphere. The resulting residue was triturated with cold diethyl ether to give a white solid, which was then dissolved in a solution of MeCN and water containing 5% of acetic acid. The resulting solution was ready for HPLC purification after filtration. Utilization of 2-chlorotrityl resin and Rink-MBHA resin afforded the peptidyl acids and peptidyl amides respectively.

3.4 Preparation of Peptidyl Thioesters

3.4.1 Preparation of peptidyl thioester using a side-chain anchoring strategy[S6]

The Rink amide MBHA resin was employed to install the first amino acid derivative, followed by the automated SPPS, where the final amino acid was introduced as Boc-Xaa-OH. After SPPS, the peptide resin was washed into a peptide synthesis vessel using DCM, washed further with DCM, and dried *in vacuo* overnight. Then peptide resin (1.0 equiv based on a scale of 0.05 mmol) was swollen in dry DCM (5 mL), followed by the addition of a solution of $Pd(PPh_3)_4$ (1.0 equiv) and $PhSiH_3$ (40 equiv) in dry DCM (2 mL), agitated for 1 h, and washed with DCM (× 3), DMF (× 3) and DCM (× 3). A solution of ethyl 3-mercaptopropionate (50 equiv), anhydrous HOBt (60 equiv), DIEA (70 equiv) and DIC (60 equiv.) in DCM/DMF (4:1, *v/v*) was added to the vessel, and the resin was agitated for 1 h at room temperature, washed with DCM (× 3), DMF (× 3) and DCM (× 3). Resin cleavage and global deprotection was performed under the

[S6]Ficht, S.; Payne, R. J.; Guy, R. T.; Wong, C.–H. *Chem. Eur. J.* **2008**, *14*, 3620-3629.

treatment of TFA/H_2O/TIPS (95:2.5:2.5, *v/v/v*) solution for 2 hours. The resin was then removed by filtration, and the filtrate was concentrated under a nitrogen atmosphere. The resulting residue was triturated with cold diethyl ether to give a white solid, which was then dissolved in a solution of MeCN and water containing 5% of acetic acid. The resulting solution was ready for HPLC purification after filtration.

3.4.2 Preparation of peptide thioester using a direct thioesterfication strategy[S7]

After SPPS using 2-chlorotritylchloride or Trityl-ChemMatrix resin, where the last residue was introduced as Boc-Xaa-OH, the protected peptidyl acid (1.0 equiv based on a scale of 0.05 mmol) was cleaved from resin using DCM/TFE/AcOH (3:1:1, *v/v/v*) for three times, and the filtrate was concentrated under a nitrogen atmosphere. The resulting residue was dissolved in a solution of 20% MeCN in water, and lyophilized to remove residual acid.

To the obtained dry powder was added PyClock (5 equiv), 4 Å molecular sieves (approx. 40 mg), and the mixture was dissolved in 3 mL of dry DMF. The resulting reaction was cooled to -20 ℃, and ethyl 3-mercaptopropionate (30 equiv) was added, followed by the addition of DIEA (5 equiv). The resulting mixture was stirred at -20 ℃ for 2 h, quenched by adding 0.2 mL of acetic acid, diluted with 20% MeCN in water, and concentrated *via* lyophilization. The resulting residue was treated with 4 mL of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) and stirred at room temperature for 2 h. The mixture was concentrated under a nitrogen atmosphere, and triturated with cold diethyl ether. The resulting residue was dissolved in a solution of MeCN and water containing 5% acetic acid. The resulting solution was ready for HPLC purification after filtration.

3.5 Native Chemical Ligation

To a mixture of the west-side peptide thioester (1.1 equiv), and east-side peptide containing thio-amino acid at the *N*-terminus (1.0 equiv), were added appropriate volume of ligation buffer (6 M Gn·HCl, 300 mM NaH_2PO_4, 200 mM MPAA, 20 mM TCEP·HCl, pH 7.2) under an argon atmosphere, the concentration of the east-side peptides is approximately 3 mM, and the resulting solution was stirred at room temperature (25 °C) and monitored using LC-MS. The reaction was quenched with H_2O/MeCN/AcOH (90:5:5, *v/v/v*) and purified using preparative HPLC.

[S7] Kajihara, Y.; Yoshihara, A.; Hirano, K.; Yamamoto, N. *Carbohydr. Res.* **2006**, *341*, 1333-1340.

3.6 Metal-free Desulfurization

To a solution of the thiol-containing peptide (3 mM) in appropriate volume of degassed buffer (6 M Gn•HCl, 200 mM Na_2HPO_4, pH 7.2) was added 200 μL of 0.5 M Bond-breaker® TCEP solution (Pierce), 20 μL of 2-methyl-2-propanethiol and 100 μL of radical initiator VA-044 (0.1 M in degassed water). The reaction mixture was stirred at 37 °C and monitored by LC-MS. Upon complete consumption of the thiol-containing peptide, the reaction was quenched with H_2O/MeCN/AcOH (90:5:5, *v/v/v*) and further purified using preparative HPLC.

3.7 One-pot Ligation-Desulfurization

To a mixture of the west-side peptide thioester (1.1 equiv), and east-side peptide containing thio-amino acid at the *N*-terminus (1.0 equiv), was added appropriate volume of ligation buffer (6 M Gn·HCl, 200 mM Na_2HPO_4, 20 mM TCEP·HCl, pH 7.0) under an argon atmosphere, the concentration of the west-side peptides is approximately 3 mM, and the resulting reaction was stirred at room temperature (25 °C) and monitored by LC-MS.

Upon completed consumption of the east-side peptide, 200 μL of 0.5 M Bond-breaker® TCEP solution (Pierce), 20 μL of 2-methyl-2-propanethiol and 100 μL of radical initiator VA-044 (0.1 M in degassed water) were added sequentially to the above reaction mixture carefully under an argon atmosphere. The reaction was stirred at 37 °C and monitored by LC-MS. Upon completion, the reaction was quenched with H_2O/MeCN/ AcOH (90:5:5, *v/v/v*) and further purified using preparative HPLC.

3.8 Hydrazide-based Peptide Ligation[S8]

To a mixture of the west-side peptidyl hydrazide (1.1 equiv), and east-side peptide containing thio-amino acid at the *N*-terminus (1.0 equiv), was added appropriate volume of buffer A (6 M Gn·HCl, 200 mM NaH_2PO_4, pH 3-4), the concentration of the west-side peptides is approximately 4 mM. The reaction mixture was transferred to –a 15 °C bath, and a freshly-prepared solution of 200 mM $NaNO_2$ (7.0 equiv) in water was added dropwise. After oxidized for 20 min at -15 °C, buffer B (6 M Gn·HCl, 200 mM

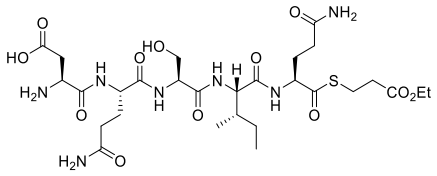
[S8] (a) Fan, G.-M.; Li, Y.-M.; Shen, F.; Huang, Y.-C.; Li, J.-B.; Lin, Y.; Cui, H.-K.; Liu, L. *Angew. Chem., Int. Ed.* **2011**, *50*, 7645 -7649; (b) Zheng, J.-S.; Tang, S.; Qi, Y.-K.; Wang, Z.-P.; Liu, L. *Nat. Protoc*. **2013,** *8*, 2483-2495.

Na_2HPO_4, 200 mM MPAA, pH 7.0-7.2) was added, the solution was adjusted to pH 7.0 by slow addition of 2.0 M NaOH solution, and the final concentration of the west-side peptides is approximately 2 mM.

The resulting reaction was stirred at room temperature (25 °C) and monitored by LC-MS. Upon completed consumption of west-side peptide, 200 mL of buffer C (6 M Gn·HCl, 200 mM Na_2HPO_4, 50 mM TCEP·HCl, pH 7.0-7.2) was added, and reacted for 20 min, then diluted with H_2O/MeCN/AcOH (90:5:5, *v/v/v*) and further purified using preparative HPLC.

IV. Preparation and Characterization of Peptide Segments

Peptidyl thioester 16a



Chemical Formula: $C_{28}H_{47}N_7O_{12}S$
Exact Mass: 705.30
Molecular Weight: 705.78

H - **DQSIQ** - $S(CH_2)_2CO_2Et$ (**16a**)

Peptidyl thioester **16a** was prepared according to General Procedure **2.4.1** using Pioneer peptide synthesizer on a 0.05 mmol scale, and Fmoc-Glu-OAll (**S13**) was applied as the first amino acid residue, the γ-carboxylate was linked to the Rink amide resin. Purification of the crude peptide using preparative HPLC (10 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **16a** as a white solid after lyophilization (16.4 mg, 45%).

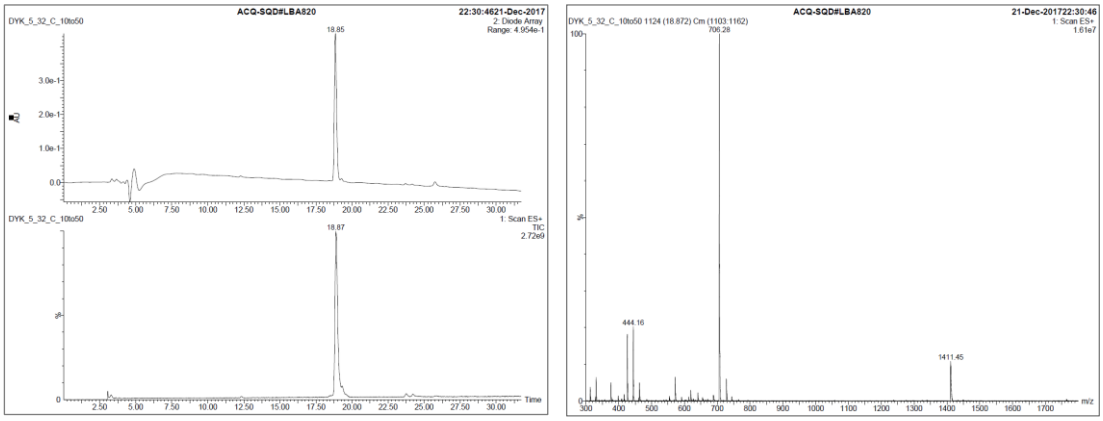
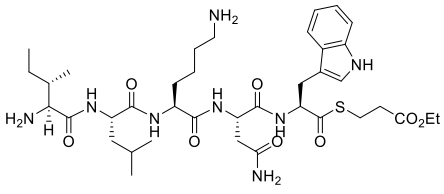


Figure S1. Left: UV and MS traces of the purified peptidyl thioester **16a**. Linear gradient: 10 to 50% solvent B over 30 min, Agilent C18 column, t_R = 18.9 min; Right: ESI-MS data of the purified peptidyl thioester **16a**. Calcd for $C_{28}H_{47}N_7O_{12}S$: 705.78 Da (average isotopes), (*m/z*) $[M+H]^+$: 706.31; found $[M+H]^+$: 706.28.

Peptidyl thioester 16b



Chemical Formula: $C_{38}H_{60}N_8O_8S$
Exact Mass: 788.43
Molecular Weight: 789.01

H - **ILKNW** - $S(CH_2)_2CO_2Et$ (**16b**)

Peptidyl thioester **16b** was prepared according to General Procedure **2.4.2** using

Pioneer peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (20 to 70% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **16b** as a white solid after lyophilization (16.0 mg, 39%).

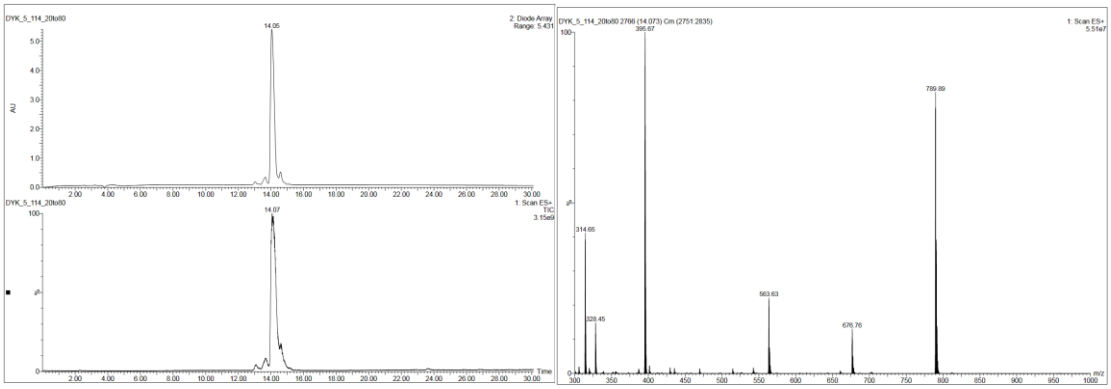
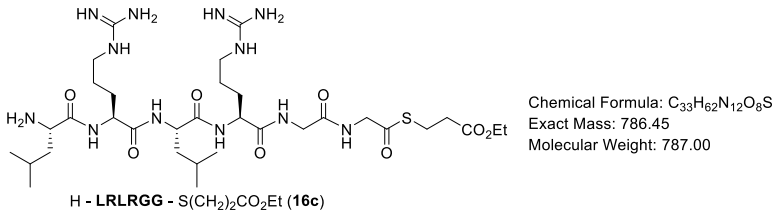


Figure S2. Left: UV and MS traces of the purified peptidyl thioester **16b**. Linear gradient: 20 to 80% solvent B over 30 min, Agilent C18 column, t_R = 14.1 min; Right: ESI-MS data of the purified peptidyl thioester **16b**. Calcd for $C_{38}H_{60}N_8O_8S$: 789.01 Da (average isotopes), (*m/z*) $[M+H]^+$: 789.43, $[M+2H]^{2+}$:395.22; found $[M+H]^+$: 789.89, $[M+2H]^{2+}$: 395.67.

Peptidyl thioester 16c



Peptidyl thioester **16c** was prepared according to General Procedure **2.4.2** using Pioneer peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (55 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **16c** as a white solid after lyophilization (13.9 mg, 35%).

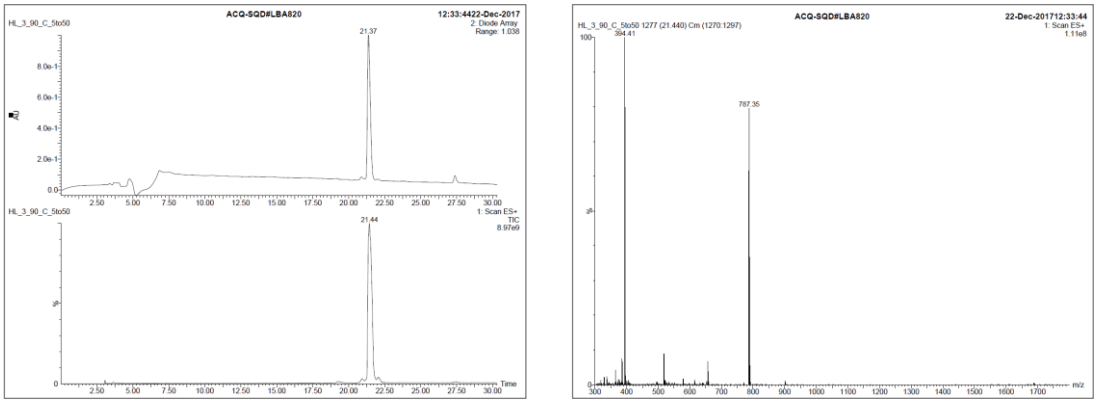
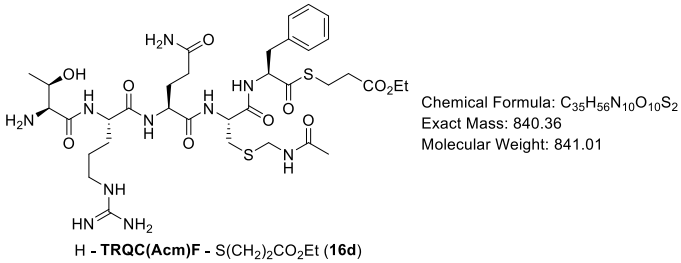


Figure S3. Left: UV and MS traces of the purified peptidyl thioester **16c**. Linear gradient: 5 to 50%

solvent B over 30 min, Agilent C18 column, t_R = 21.4 min; Right: ESI-MS data of the purified peptidyl thioester **16c**. Calcd for $C_{33}H_{62}N_{12}O_8S$: 787.00 Da (average isotopes), (*m/z*) $[M+H]^+$: 787.46, $[M+2H]^{2+}$:394.23; found $[M+H]^+$: 787.35, $[M+2H]^{2+}$: 394.41.

Peptidyl thioester 16d



Peptidyl thioester **16d** was prepared according to General Procedure **2.4.2** using Pioneer peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (20 to 35% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **16d** as a white solid after lyophilization (11.1 mg, 26%).

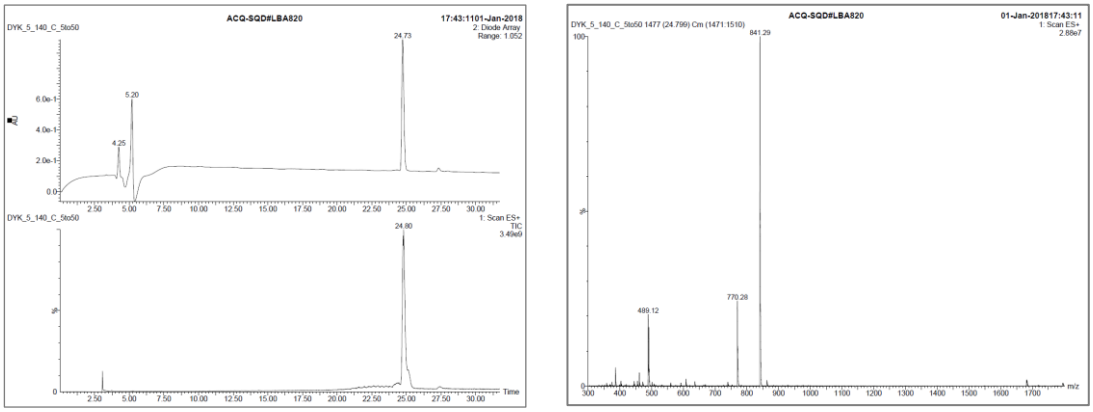
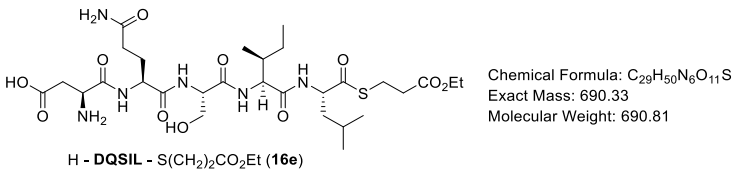


Figure S5. Left: UV trace f of peptidyl thioester **16d**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 24.7 min; Right: ESI-MS data of the purified peptidyl thioester **16d**. Calcd for $C_{35}H_{56}N_{10}O_{10}S_2$: 841.01 Da (average isotopes), (*m/z*) $[M+H]^+$: 841.37; found $[M+H]^+$: 841.29.

Peptidyl thioester 16e



Peptidyl thioester **16e** was prepared according to General Procedure **2.4.2** using Pioneer

peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (20 to 65% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **16e** as a white solid after lyophilization (8.4 mg, 24%).

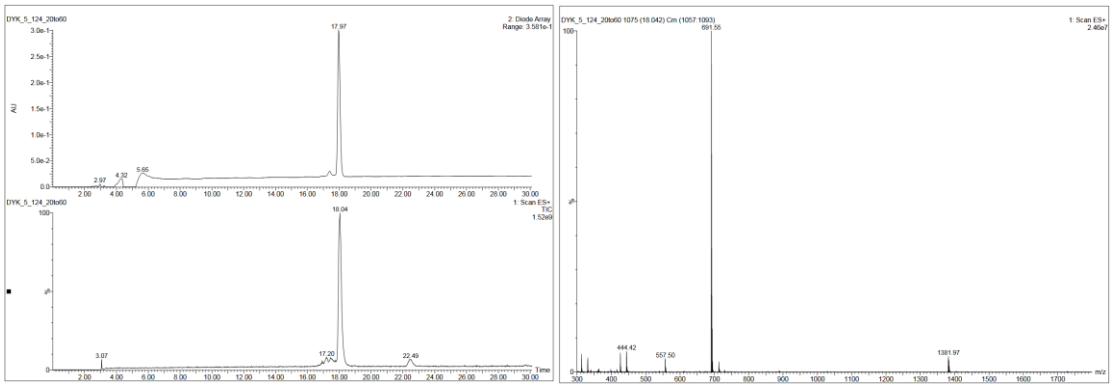
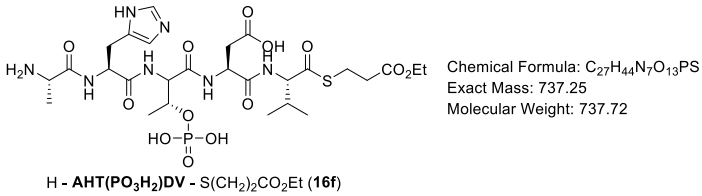


Figure S4. Left: UV and MS traces of the purified peptidyl thioester **16e**. Linear gradient: 20 to 60% solvent B over 30 min, Agilent C18 column, t_R = 18.0 min; Right: ESI-MS data of the purified peptidyl thioester **16e**. Calcd for $C_{29}H_{50}N_6O_{11}S$: 690.81 Da (average isotopes), (*m/z*) $[M+H]^+$: 691.33; found $[M+H]^+$: 691.55.

Peptidyl thioester 16f



Peptidyl thioester **16f** was prepared according to General Procedure **2.4.2** using Pioneer peptide synthesizer on a 0.05 mmol scale, where commercially available Fmoc-Thr(HPO$_3$Bzl)-OH was coupled manually. Purification of the crude peptide using preparative HPLC (15 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **16f** as a white solid after lyophilization (12.0 mg, 33%).

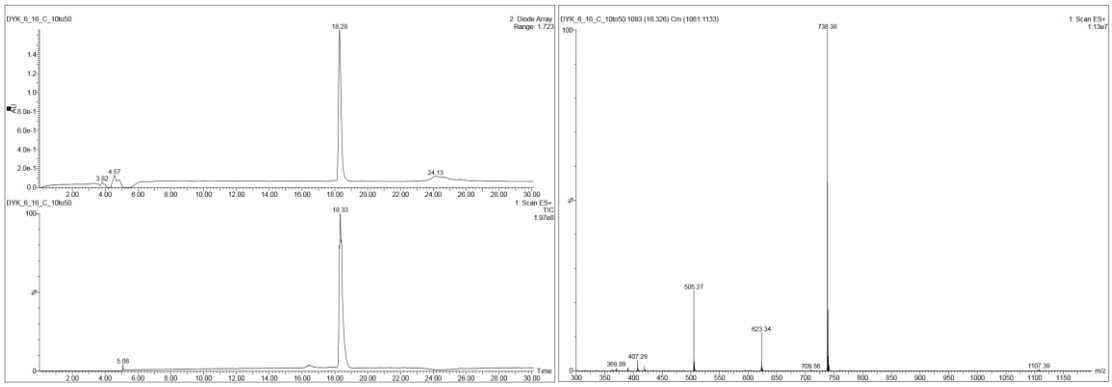
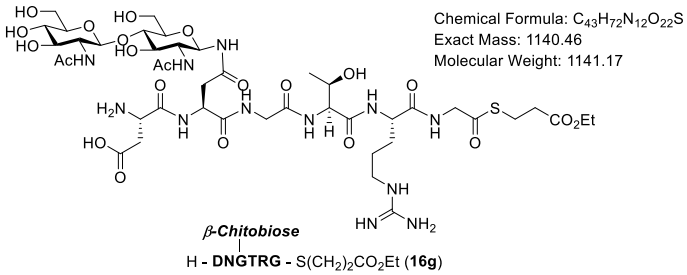


Figure S6. Left: UV and MS traces of the purified peptidyl thioester **16f**. Linear gradient: 10 to 50%

solvent B over 30 min, Agilent C18 column, t_R = 18.3 min; Right: ESI-MS data of the purified peptidyl thioester **16f**. Calcd for $C_{27}H_{44}N_7O_{13}PS$: 737.72 Da (average isotopes), (*m/z*) $[M+H]^+$: 738.25; found $[M+H]^+$: 738.38.

Peptidyl thioester 16g



Peptidyl thioester **16g** was prepared according to General Procedure **2.4.2** using Pioneer peptide synthesizer on a 0.03 mmol scale, where Fmoc-Asn(chitobiose-Ac_5)-OH[S9] (1.5 equiv × 2) and Boc-Asp(O^tBu)-OH (4 equiv × 1) were coupled manually. After the installation of the last amino acid, the acetyl groups on chitobiose were removed by treating the resin with 4 mL of 5% N_2H_4•H_2O in DMF overnight. Then the peptide was cleaved from resin following the general procedure. Purification of the crude peptide using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **16g** as a white solid after lyophilization (2.7 mg, 14%).

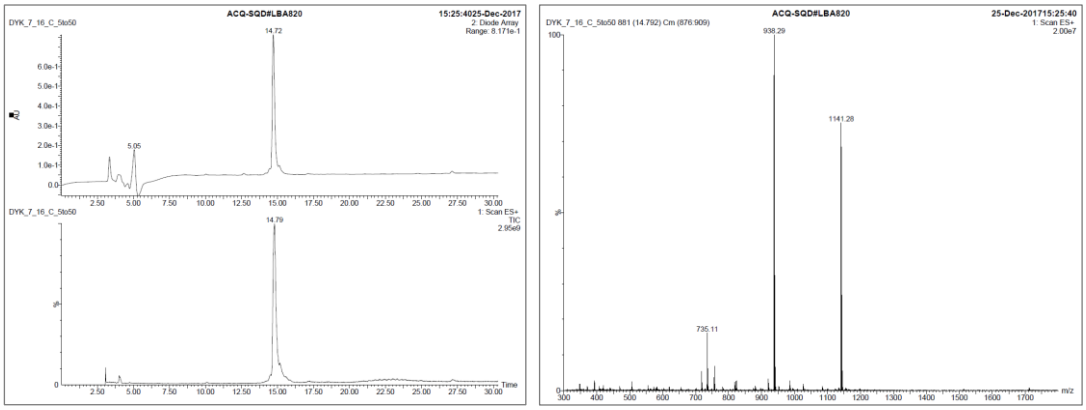
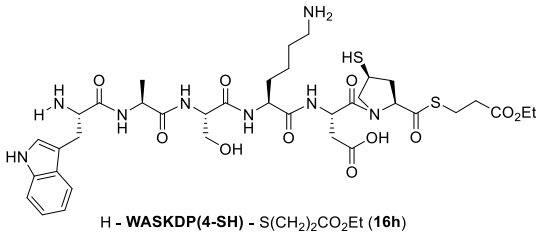


Figure S7. Left: UV and MS traces of the purified peptidyl thioester **16g**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 14.7 min; Right: ESI-MS data of the purified peptidyl thioester **16g**. Calcd for $C_{43}H_{72}N_{12}O_{22}S$: 1141.17 Da (average isotopes), (*m/z*) $[M+H]^+$: 1141.47; found $[M+H]^+$: 1141.28.

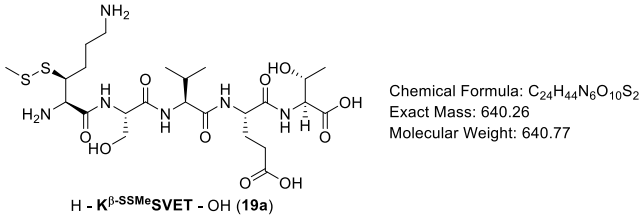
[S9] Wagner, M.; Dziadek, S.; Kunz, H. *Chem. Eur. J.* **2003**, *9*, 6018-6030.

Peptidyl thioester 16h

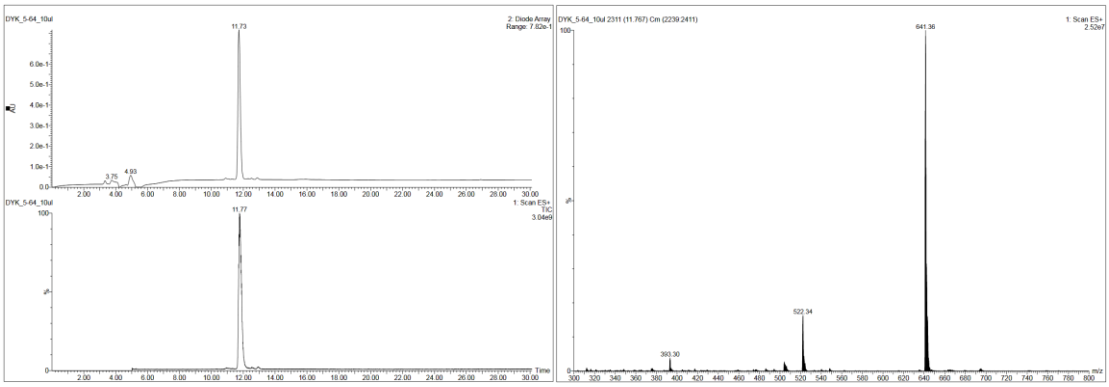


Peptidyl thioester **16h**[S10] was prepared according to General Procedure **2.4.2** using Pioneer peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (18 to 40% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **16h** as a white solid after lyophilization (11.1 mg, 26%). HPLC-MS: t_R = 22.7 min (5 to 50% solvent B over 30 min, Agilent C18 column); ESI-MS: calcd for $C_{37}H_{54}N_8O_{11}S_2$: 851.00 Da (average isotopes), $[M+H]^+$ *m/z* = 852.00, $[M+2H]^{2+}$ m/z = 426.50; observed $[M+H]^+$ *m/z* = 851.90, $[M+2H]^{2+}$ *m/z* = 426.61.

Peptide 19a



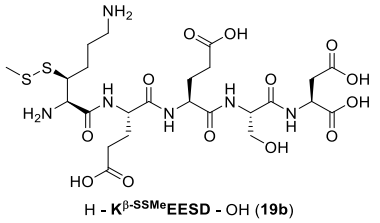
Peptide **19a** was prepared according to General Procedure **2.3** using Pioneer peptide synthesizer on a 0.05 mmol scale, where Boc-Lys$^{\beta\text{-SSMe}}$(Boc)-OH (**13a**) (1.5 equiv) was coupled manually. Purification of the crude peptide using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **19a** as a white solid after lyophilization (17.6 mg, 55%).



[S10] Gui, Y.; Qiu, L.; Li, Y.; Li, H.; Dong, S. *J. Am. Chem. Soc*. **2016**, *138*, 4890−4899.

Figure S8. Left: UV and MS traces of the purified peptide **19a**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 11.7 min; Right: ESI-MS data of the purified peptide **19a**. Calcd for $C_{24}H_{44}N_6O_{10}S_2$: 640.77 Da (average isotopes), (*m/z*) $[M+H]^+$: 641.26; found $[M+H]^+$: 641.36.

Peptide 19b



Chemical Formula: $C_{24}H_{40}N_6O_{13}S_2$
Exact Mass: 684.21
Molecular Weight: 684.73

Peptide **19b** was prepared according to General Procedure **2.3** using Pioneer peptide synthesizer on a 0.05 mmol scale, where Boc-Lys$^{\beta\text{-SSMe}}$(Boc)-OH (**13a**) (1.3 equiv) was coupled manually . Purification of the crude peptide using preparative HPLC (2 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **19b** as a white solid after lyophilization (10.5 mg, 29%).

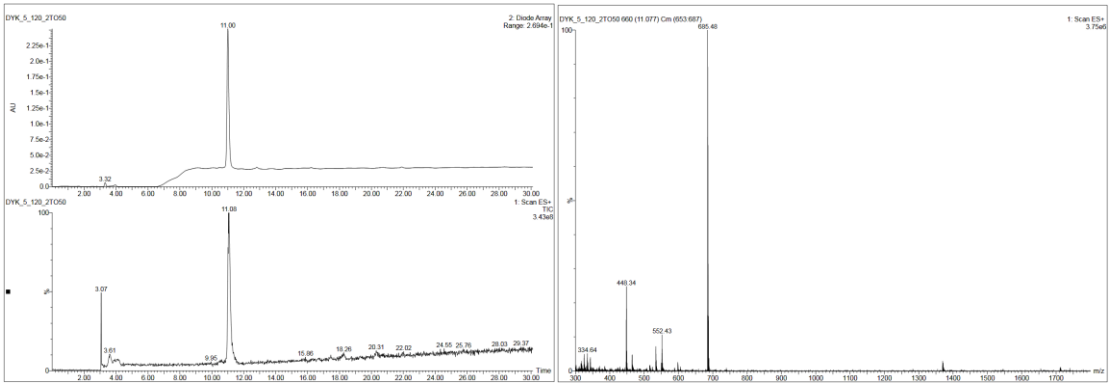
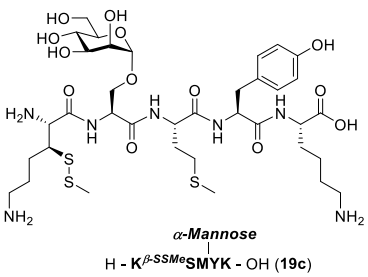


Figure S9. Left: UV and MS traces of the purified peptide **19b**. Linear gradient: 2 to 50% solvent B over 30 min, Agilent C18 column, t_R = 11.0 min; Right: ESI-MS data of the purified peptide **19b**. Calcd for $C_{24}H_{40}N_6O_{13}S_2$: 684.73 Da (average isotopes), (*m/z*) $[M+H]^+$: 685.22; found $[M+H]^+$: 685.48.

Peptide 19c



Chemical Formula: $C_{36}H_{61}N_7O_{13}S_3$
Exact Mass: 895.35
Molecular Weight: 896.10

Peptide **19c** was prepared according to General Procedure **2.3** using Pioneer peptide synthesizer on a 0.05 mmol scale, where Fmoc-Ser(Man-Ac_4)-OH[S11] (1.5 equiv × 2), Boc-Lys$^{\beta\text{-SSMe}}$(Boc)-OH (**13a**) (0.6 equiv × 2) were coupled manually. Upon completion of manual synthesis, the resin was subjected to a cleavage cocktail TFA/TIS/H_2O (95:2.5:2.5, *v/v/v*) for 2 h. The resin was removed *via* filtration, and the solvent was blown off under a nitrogen atmosphere.

The resulting residue was triturated with cold diethyl ether to give a white solid, which was then dissolved in a solution of 20% CH_3CN in H_2O. After lyophilization, the resulting powder was treated with 10 ml of hydrazine solution (hydrazine hydrate/MeOH, 95:5, *v/v*) at rt under an argon atmosphere overnight to remove the acetyl groups on mannose. The reaction was quenched with 10 ml of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*). Purification of the crude peptide using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **19c** as a white solid after lyophilization (10.5 mg, 25%)..

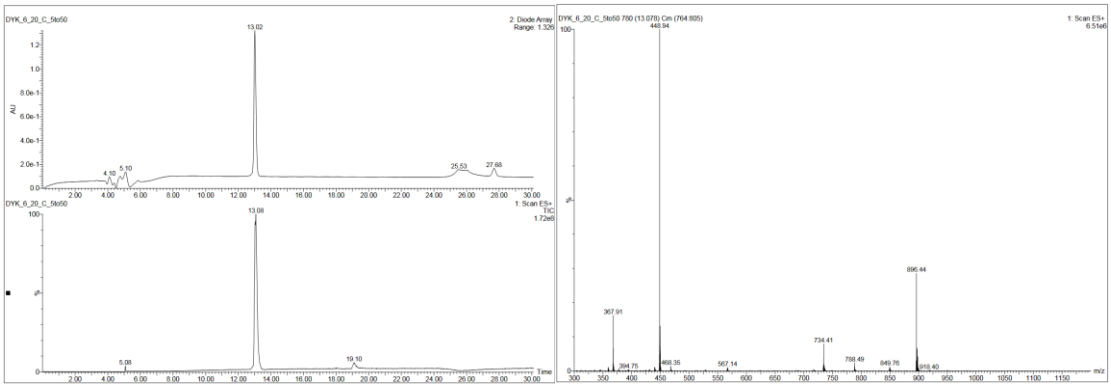
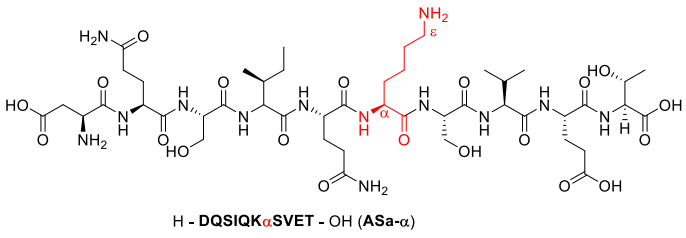


Figure S10. Left: UV and MS traces of the purified peptide **19c**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 13.0 min; Right: ESI-MS data of the purified peptide **19c**. Calcd for $C_{36}H_{61}N_7O_{13}S_3$: 896.10 Da (average isotopes), (*m/z*) $[M+H]^+$: 896.36, $[M+2H]^{2+}$: 448.68; found $[M+H]^+$: 896.41, $[M+2H]^{2+}$: 448.94.

Peptide ASa-α



Chemical Formula: $C_{46}H_{79}N_{13}O_{20}$
Exact Mass: 1133.56
Molecular Weight: 1134.21

[S11] Chen, L.; Tan, Z. *Tetrahedron Lett.* **2013**, *54*, 2190–2193.

Peptide **ASa-α** was prepared according to General Procedure **2.3** using Pioneer peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (10 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **ASa-α** as a white solid after lyophilization (16.9 mg, 30%).

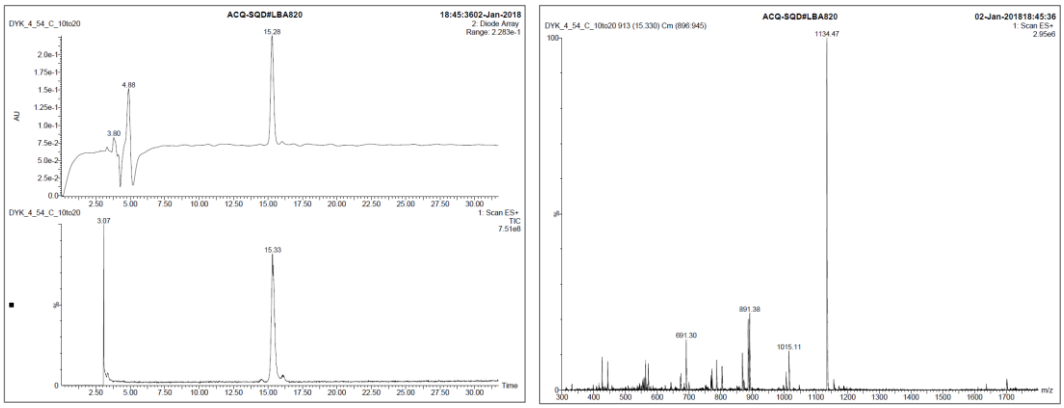
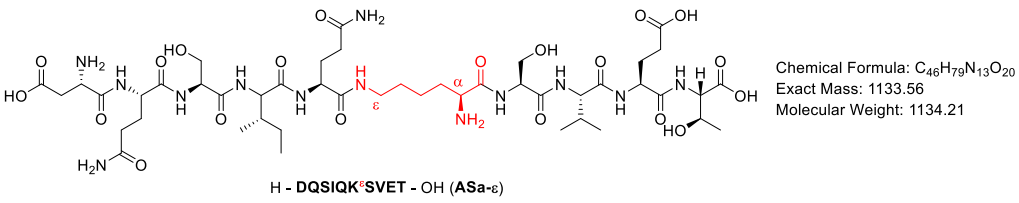


Figure S11. Left: UV and MS traces of the purified peptide **ASa-α**. Linear gradient: 10 to 20% solvent B over 30 min, Agilent C18 column, t_R = 15.3 min; Right: ESI-MS data of the purified peptide **ASa-α**. Calcd for $C_{46}H_{79}N_{13}O_{20}$: 1134.21 Da (average isotopes), (*m/z*) $[M+H]^+$: 1134.56; found $[M+H]^+$: 1134.47.

Peptide ASa-ε



Peptide **ASa-ε** was prepared according to General Procedure **2.3** using Pioneer peptide synthesizer on a 0.05 mmol scale, where Boc-Lys(Fmoc)-OH was used besides other standard Fmoc-amino acids. Purification of the crude peptide using preparative HPLC (10 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **ASa-ε** as a white solid after lyophilization (18.4 mg, 32%).

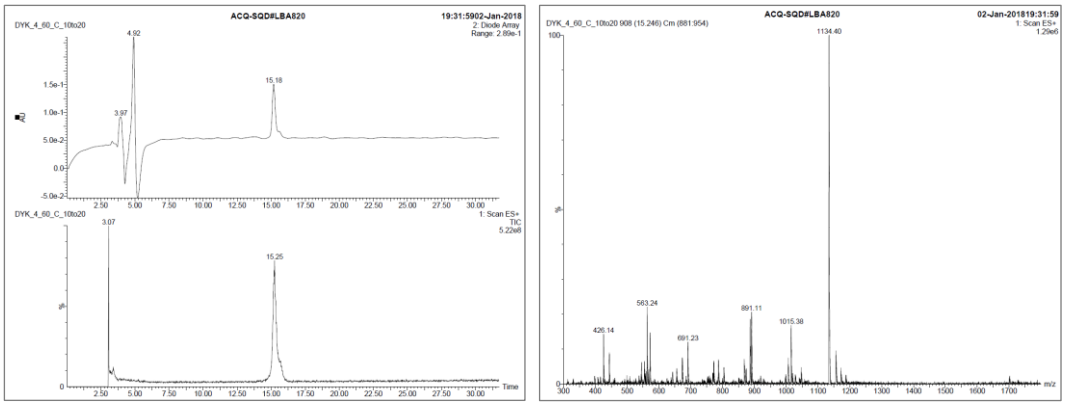
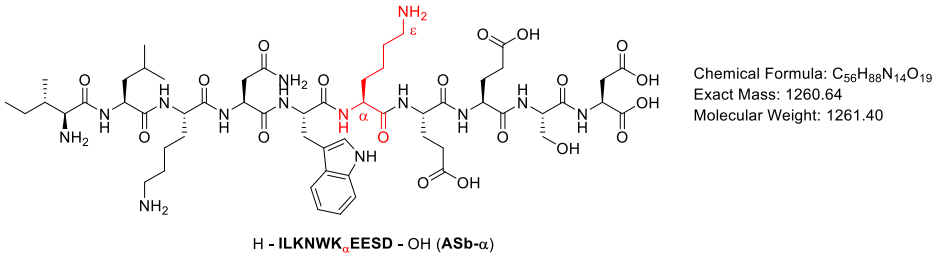


Figure S12. Left: UV and MS traces of the purified peptide **ASa-ε**. Linear gradient: 10 to 20% solvent B over 30 min, Agilent C18 column, t_R = 15.2 min; Right: ESI-MS data of the purified peptide **ASa-ε.** Calcd for $C_{46}H_{79}N_{13}O_{20}$: 1134.21 Da (average isotopes), (*m/z*) $[M+H]^+$: 1134.56; found $[M+H]^+$: 1134.40.

Peptide ASb-α



Peptide **ASb-α** was prepared according to General Procedure **2.3** using Pioneer peptide synthesizer on a 0.05 mmol scale. Purification of the crude peptide using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **ASb-α** as a white solid after lyophilization (27.2 mg, 43%).

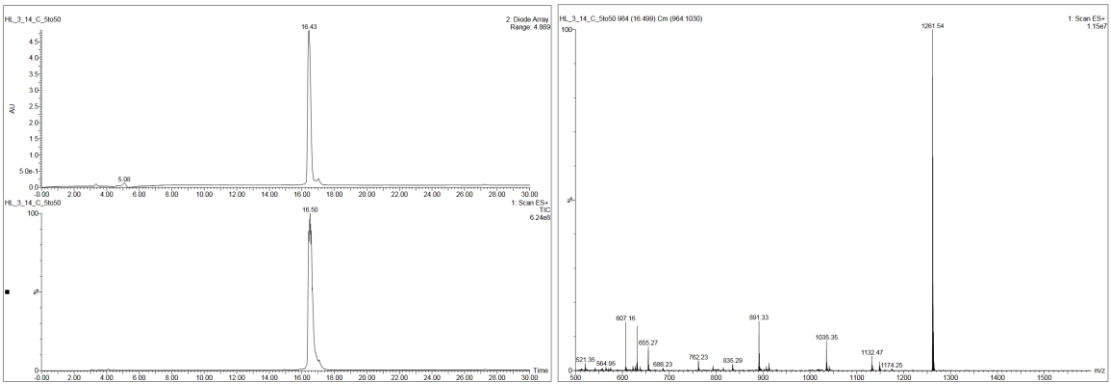
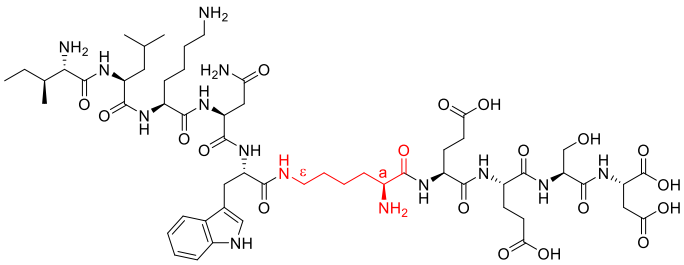


Figure S13. Left: UV and MS traces of the purified peptide **ASb-α**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 16.4 min; Right: ESI-MS data of the purified peptide **ASb-α**. Calcd for $C_{56}H_{88}N_{14}O_{19}$: 1261.40 Da (average isotopes), (*m/z*) $[M+H]^+$: 1261.64; found $[M+H]^+$: 1261.54.

Peptide ASb-ε



Chemical Formula: $C_{56}H_{88}N_{14}O_{19}$
Exact Mass: 1260.64
Molecular Weight: 1261.40

H - **ILKNWK$^{\varepsilon}$EESD** - OH (**ASb-ε**)

Peptide **ASb-ε** was prepared according to General Procedure **2.3** using Pioneer peptide synthesizer on a 0.05 mmol scale, where Boc-Lys(Fmoc)-OH was used besides other standard Fmoc-amino acids. Purification of the crude peptide using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column) afforded peptide **ASb-ε** as a white solid after lyophilization (21.6 mg, 20%).

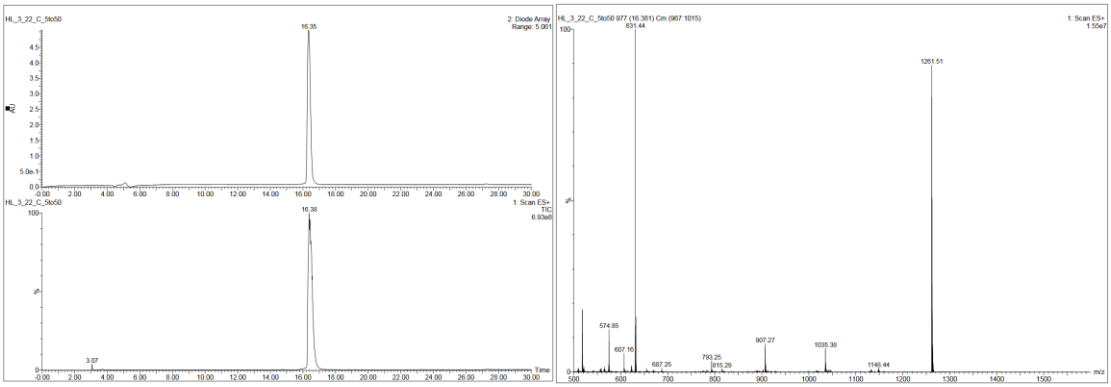
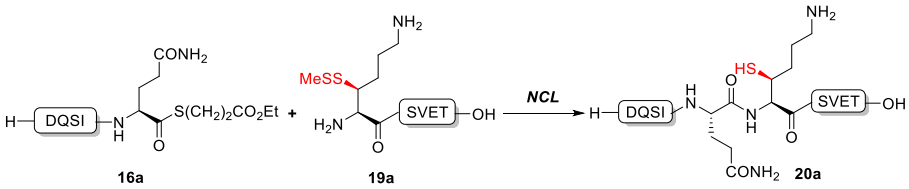


Figure S14. Left: UV and MS traces of the purified peptide **ASb-ε**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 16.4 min; Right: ESI-MS data of the purified peptide **ASb-ε**. Calcd for $C_{56}H_{88}N_{14}O_{19}$: 1261.40 Da (average isotopes), (*m/z*) $[M+H]^+$: 1261.64, $[M+2H]^{2+}$: 631.32; found $[M+H]^+$: 1261.51 $[M+2H]^{2+}$: 631.44.

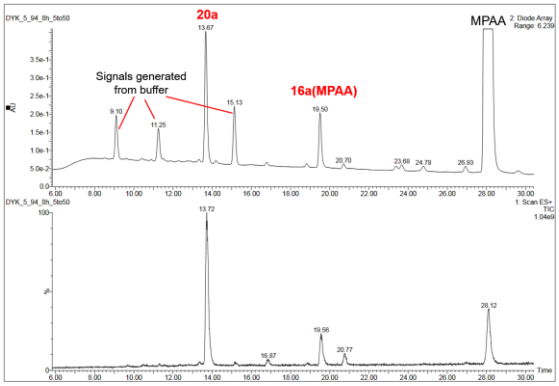
V. Investigation of the Selectivity in β-Thiolysine-mediated Peptide Ligation

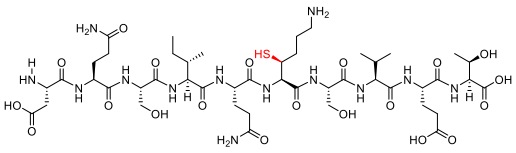
5.1 Investigation of α-ligation at lysine site

<u>Ligation reaction between 16a and 19a</u>



1.12 mg of peptidyl thioester **16a** (1.1 equiv) and 0.92 mg of thiolysine peptide **19a** (1.0 equiv)were subjected to the ligation conditions following General Procedure **3.5** as described previously. The reaction was stirred at room temperature (25 °C) under an argon atmosphere and monitored by LC-MS. Upon the completed consumption of **19a** after 8 h, the reaction was quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **20a** (1.34 mg, 80%) as a white solid.





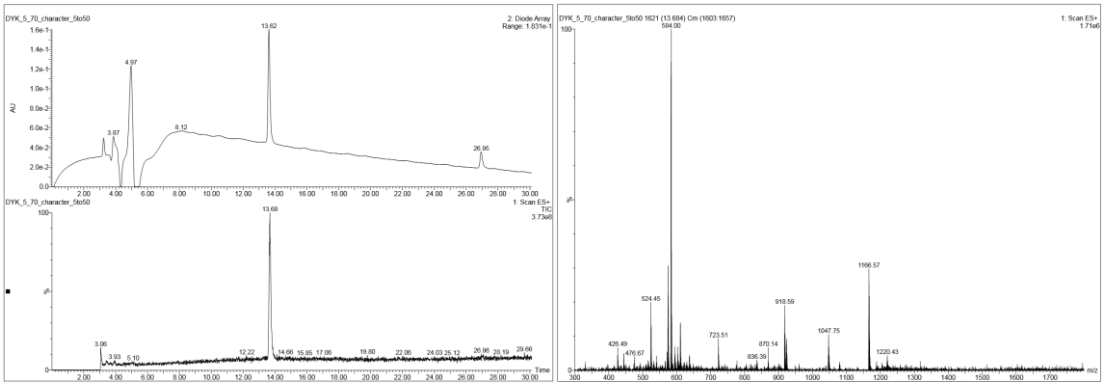
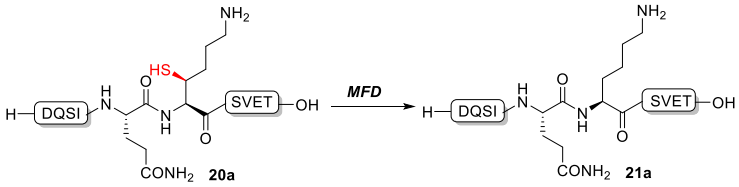
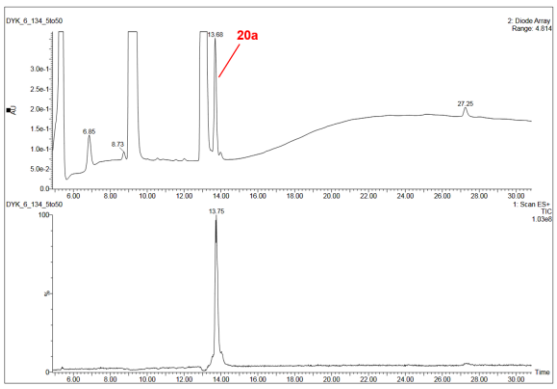


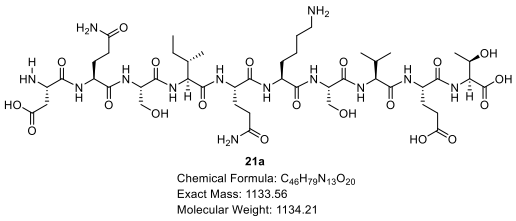
Figure S15. Top left: UV and MS traces of the ligation reaction between peptides **16a** and **19a** after 8 h; Top right: Structure and Structure and calculated data of **20a**; Bottom left: UV and MS traces of the purified product **20a**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 13.6 min; Bottom right: ESI-MS data of the purified product **20a**. Calcd for $C_{46}H_{79}N_{13}O_{20}S$: 1166.27 Da (average isotopes), (*m/z*) $[M+H]^+$: 1166.54, $[M+2H]^{2+}$: 583.77; found: $[M+H]^+$: 1166.57, $[M+2H]^{2+}$: 584.00.

Desulfurization reaction of 20a



1.11 mg of peptide **20a** obtained from the ligation reaction was subjected to the desulfurization conditions following General Procedure **3.6** as described previously. The reaction was stirred at 37 °C under an argon atmosphere and monitored by LC-MS, upon the completed consumption of **20a** after 1 h, the reaction was quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was purified using preparative HPLC (7 to 30% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **21a** (0.76 mg, 70%) as a white solid.





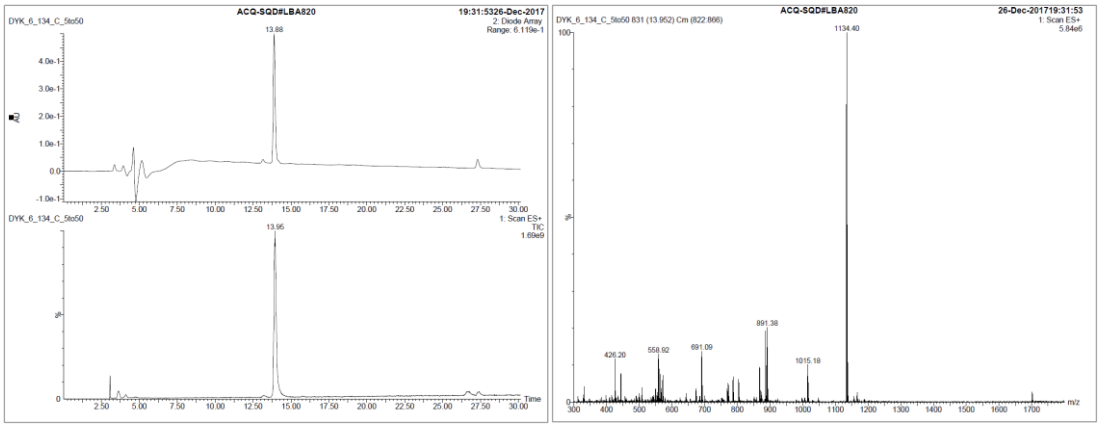


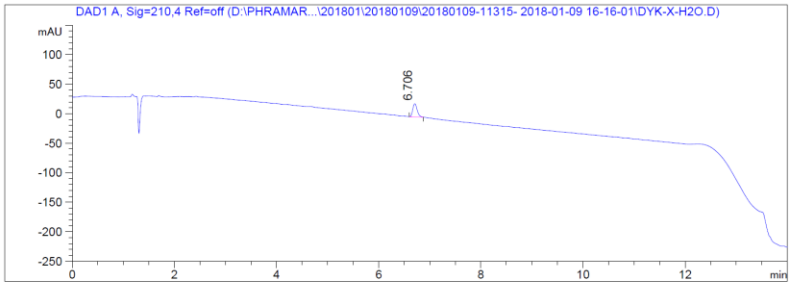
Figure S16. Top left: UV and MS traces of the desulfurization reaction of **20a**; Top right: Structure and calculated data of **21a**; Bottom left: UV trace of the purified product **21a**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 13.9 min; Bottom right: ESI-MS data of the purified product **21a**. Calcd for $C_{46}H_{79}N_{13}O_{20}$: 1134.21 Da (average isotopes), (*m/z*) $[M+H]^+$: 1134.56; found: $[M+H]^+$: 1134.40.

Structure elucidation of peptide 21a

The analytical samples were prepared by dissolving the desulfurized peptide **21a (DYK-X)**, synthetic authentic peptides **ASa-α (DYK-A)**, **ASa-ε (DYK-B)** and a mixture of **ASa-α** and **ASa-ε (DYK-MIX)** in CH_3CN/H_2O (1:4, *v/v*), respectively. Four samples were injected to analytic UPLC and analyzed according to the conditions described in Table S1. The UV traces indicated that the retention time of **DYK-X** (t_R= 6.71 min) aligned well with that of **DYK-A** (t_R= 6.72 min), which was of significantly difference with the retention time of **DYK-B** (t_R= 7.30 min). These results suggest that the desulfurized peptide **21a** (**DYK-X**) is an α-peptide.

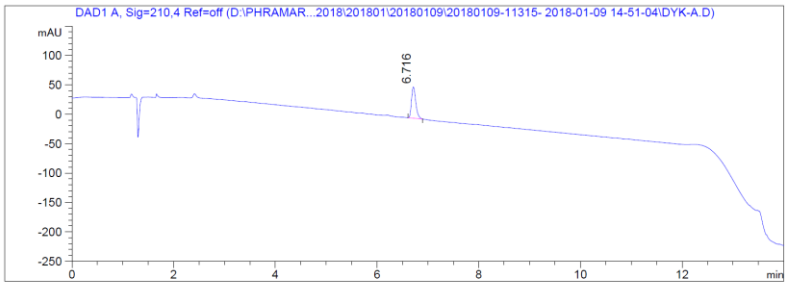
Table S1. UPLC analysis conditions of **21a**, **ASa-α** and **ASa-ε**.

Column	Agilent Zorbax SB-Phenyl column, 100 × 4.6 mm 1.8 μm		
Mobile phase:	Phase A (Water), Phase B (MeOH+0.05% TFA)		
Flow Mode	Time (min)	Phase A	Phase B
	0	90%	10%
	10	70%	30%
	12	5%	95%
	14	5%	95%
Flow	1.0 mL/min		
UV wavelength	210 nm		
Column Temp.	40 °C		



#	Meas. Ret. Time	Height	Height %	Area	Area %
1	6.71	22.24	100.00	132.5011	100.0000

Figure S17. UPLC analysis of desulfurized peptide **21a** (**DYK-X**).



#	Meas. Ret. Time	Height	Height %	Area	Area %
1	6.72	53.07	100.00	310.8501	100.0000

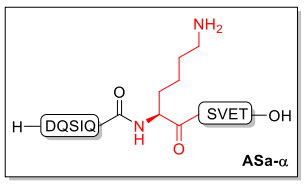
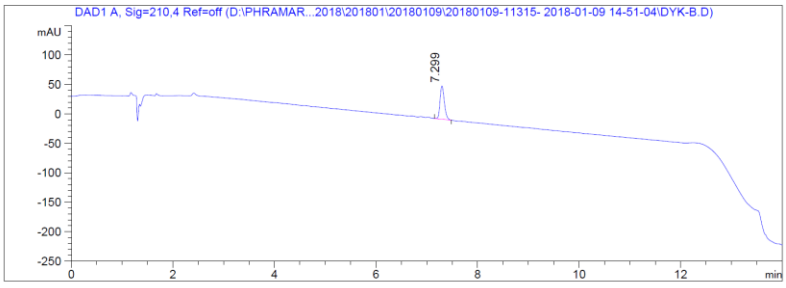


Figure S18. UPLC analysis of synthetic peptide **ASa-α** (**DYK-A**).



#	Meas. Ret. Time	Height	Height %	Area	Area %
1	7.30	56.49	100.00	333.4326	100.0000

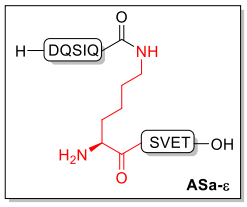
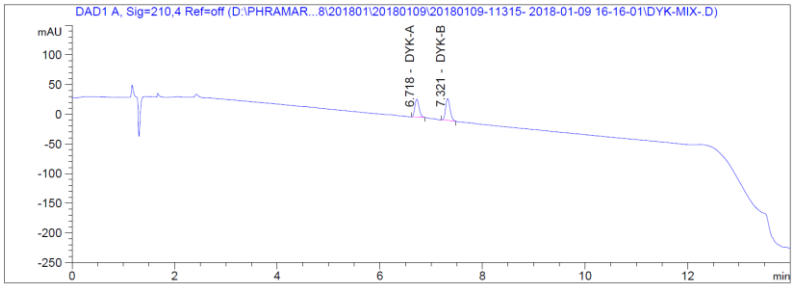


Figure S19. UPLC analysis of synthetic peptide **ASa-ε** (**DYK-B**).



#	Meas. Ret. Time	Height	Height %	Area	Area %
1	6.72	30.25	45.02	175.1598	44.3508
2	7.32	36.94	54.98	219.7816	55.6492

Figure S20. UPLC analysis of mixed peptide **ASa-α** and **ASa-ε** (**DYK-MIX**).

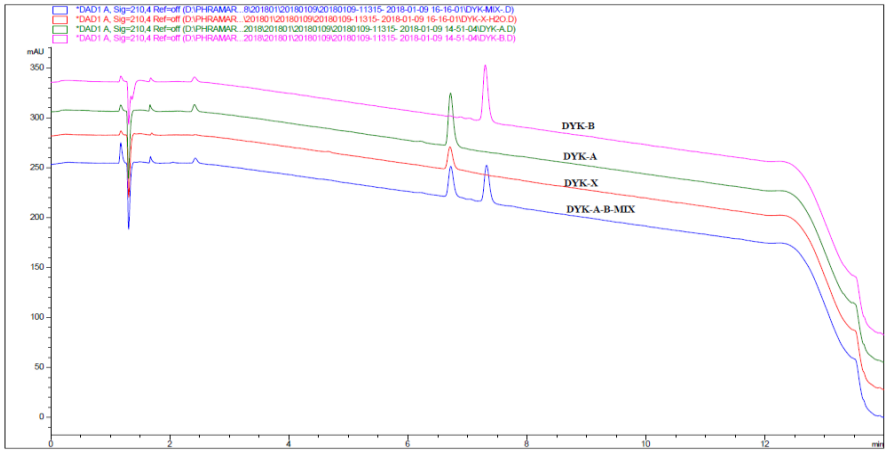
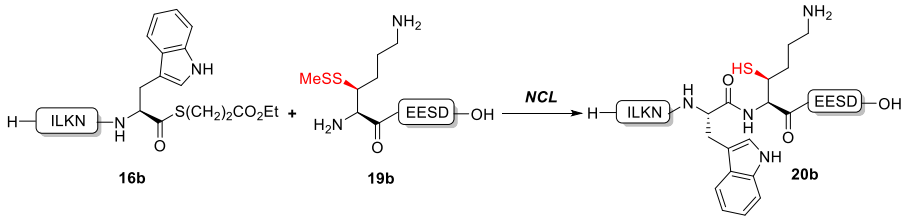


Figure S21. Overlay of the UPLC traces.

Ligation reaction between 16b and 19b



1.28 mg of peptidyl thioester **16b** (1.1 equiv) and 1.0 mg of thiolysine peptide **19b** (1.0 equiv) were subjected to the ligation conditions following General Procedure **3.5** as described previously. The reaction was stirred at room temperature (25 °C) under an argon atmosphere and monitored by LC-MS. Upon the completed consumption of **19b** after 8 h, the reaction was quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **20b** (1.18 mg, 77%) as a white solid.

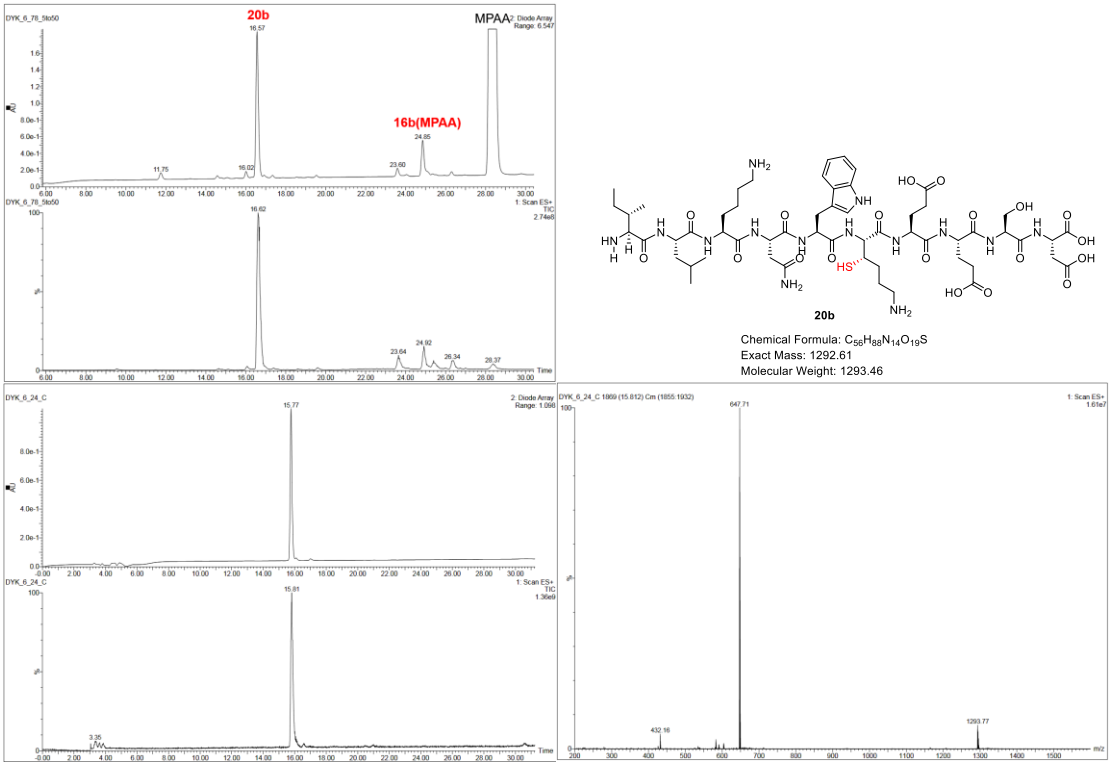
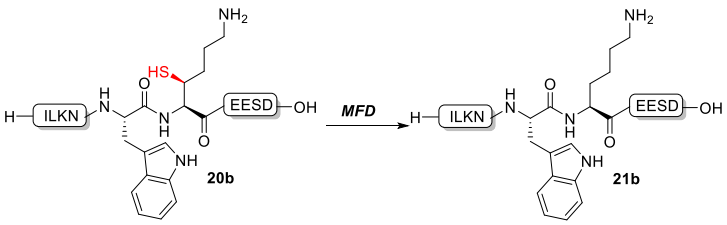


Figure S22. Top left: UV and MS traces of the ligation reaction between peptides **16b** and **19b** after 8 h; Top right: Structure and calculated data of **20b**; Bottom left: UV and MS traces of the purified product **20b**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 15.8 min; Bottom right: ESI-MS data of the purified product **20b**. Calcd for $C_{56}H_{88}N_{14}O_{19}S$: 1293.46 Da (average isotopes), (*m/z*) $[M+H]^+$: 1293.61, $[M+2H]^{2+}$: 647.31, $[M+3H]^{3+}$: 431.88; found: $[M+H]^+$: 1293.70, $[M+2H]^{2+}$: 647.71, $[M+3H]^{3+}$: 432.16.

Desulfurization reaction of 20b



0.70 mg of peptide **20b** obtained from the ligation reaction was subjected to the desulfurization conditions following General Procedure **3.6** as described previously. The reaction was stirred at 37 °C under an argon atmosphere and monitored by LC-MS upon the completed consumption of **20b** after 1 h, the reaction was quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **21b** (0.51 mg, 70%) as a white solid.

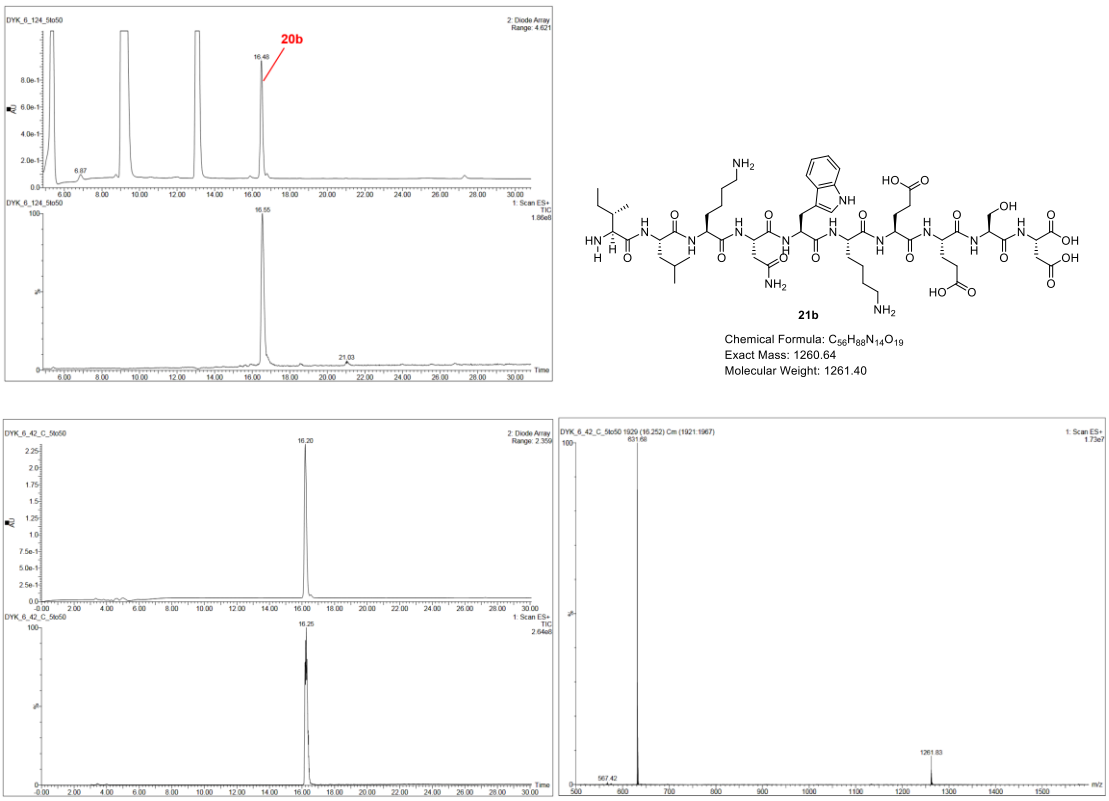


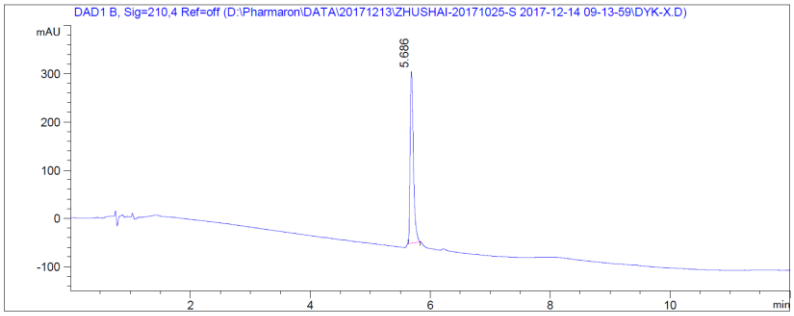
Figure S23. Top left: UV and MS traces of the desulfurization reaction of **20b**; Top right: Structure and calculated data of **21b**; Bottom left: UV trace of the purified product **21b**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 16.2 min; Bottom right: ESI-MS data of the purified product **21b**. Calcd for $C_{56}H_{88}N_{14}O_{19}$: 1261.40 Da (average isotopes), (*m/z*) $[M+H]^+$: 1261.64, $[M+2H]^{2+}$: 631.32; found: $[M+H]^+$: 1261.83, $[M+2H]^{2+}$: 631.68.

Structure elucidation of peptide 21b

The analytical samples were prepared by dissolving the desulfurized peptide **21b (DYK-X)**, synthetic authentic peptides **ASb-α (DYK-A)**, **ASb-ε (DYK-B)** and a mixture of **ASb-α** and **ASb-ε (DYK-MIX)** in CH_3CN/H_2O (1:4, *v/v*), respectively. Four samples were injected to analytic UPLC and analyzed according to the conditions described in Table S2. The UV traces indicated that the retention time of **DYK-X** (t_R= 5.69 min) aligned well with that of **DYK-A** (t_R= 5.68 min), which was of significantly difference with the retention time of **DYK-B** (t_R= 5.98 min). These results suggest that the desulfurized peptide **21b** (**DYK-X**) is an α-peptide.

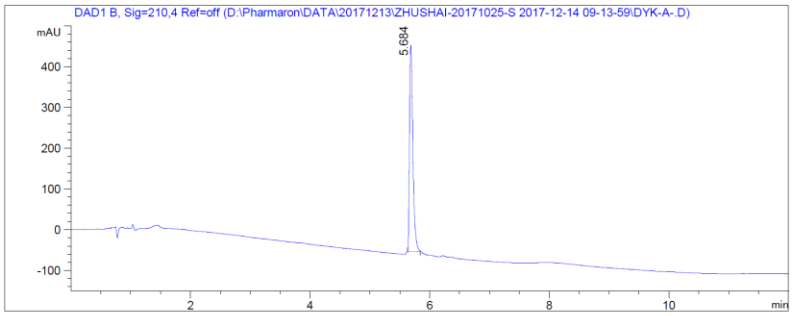
Table S2. UPLC analysis conditions of **ASb-α**, **ASb-ε** and **21b/21b'**.

Column	Waters BEH C18 100 × 2.1 mm 1.7 μm		
Mobile phase:	Phase A (Water+5 mM NH_4Ac), Phase B (MeOH)		
Flow Mode	Time (min)	Phase A	Phase B
	0	95%	5%
	10	15%	85%
	12	15%	85%
Flow	0.3 mL/min		
UV wavelength	210 nm		
Column Temp.	40 °C		



#	Meas. Ret. Time	Height	Height %	Area	Area %
1	5.69	356.30	100.00	1345.0336	100.0000

Figure S24. UPLC analysis of desulfurized peptide **21b** (**DYK-X**).



#	Meas. Ret. Time	Height	Height %	Area	Area %
1	5.68	507.36	100.00	1989.2429	100.0000

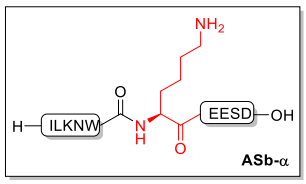
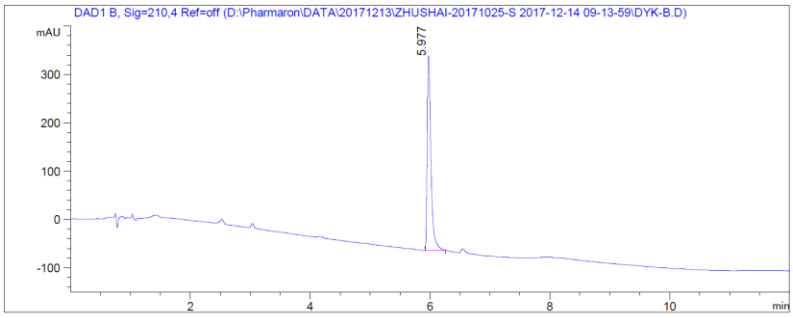


Figure S25. UPLC analysis of synthetic peptide **ASb-α** (**DYK-A**).



#	Meas. Ret. Time	Height	Height %	Area	Area %
1	5.98	402.18	100.00	1751.3956	100.0000

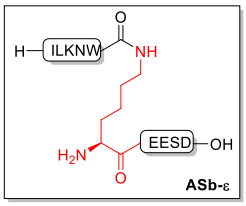
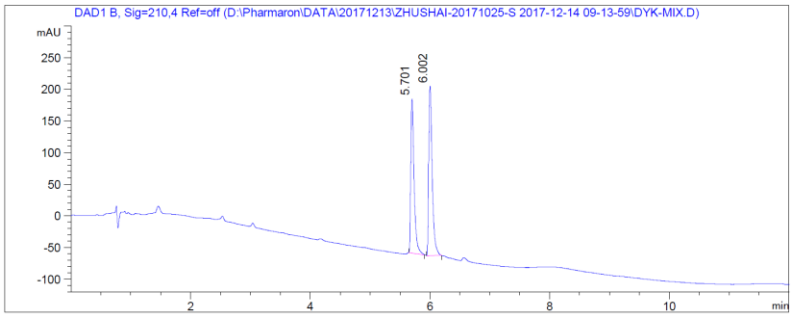


Figure S26. UPLC analysis of synthetic peptide **ASb-ε** (**DYK-B**).



#	Meas. Ret. Time	Height	Height %	Area	Area %
1	5.70	244.31	47.72	939.3489	46.6024
2	6.00	267.66	52.28	1076.3191	53.3976

Figure S27. UPLC analysis of mixed peptide**ASb-α** and **ASb-ε** (**DYK-MIX**).

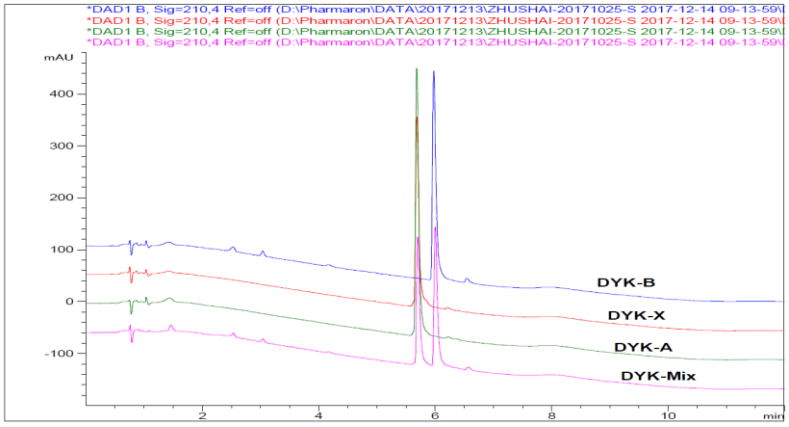
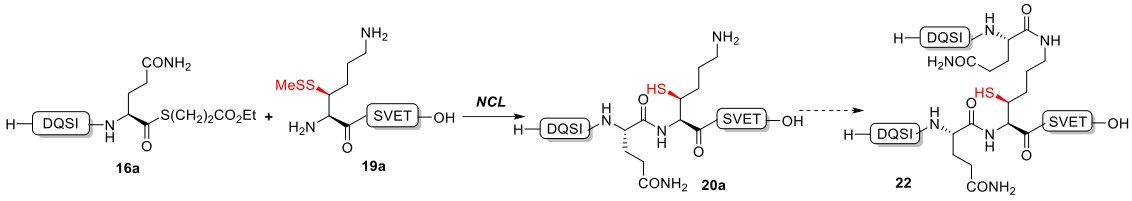


Figure S28. Overlay of the HPLC traces.

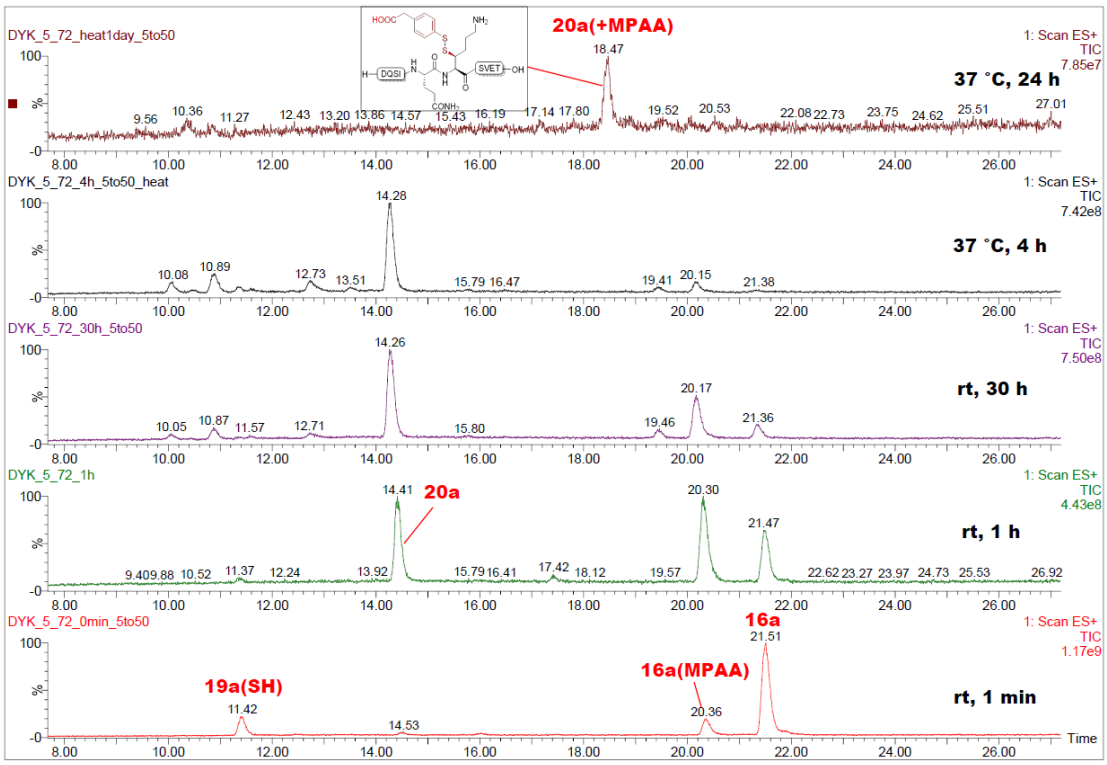
5.2 Attempted ε-ligation at Lysine sites

Ligation reaction between excess 16a and 19a



1.90 mg of peptidyl thioester **16a** (2.2 equiv) and 0.79 mg of thiolysine peptide **19a** (1.0 equiv) were subjected to the ligation conditions following General Procedure **3.5** as described previously. The reaction was stirred at room temperature (25 °C) under an argon atmosphere and monitored by LC-MS (5 to 50% solvent B over 30 min, Agilent C18 column).

As shown in Figure S29, 1 min after dissolved in reaction buffer, peptide **19a** was rapidly reduced to give **19a(SH)**. After 1 h, **19a** was fully converted to ligated product **20a**, but there was no di-ligated peptide **22** detected even after reacting for 30 h. At higher temperature (37 °C), the major product from the reaction did not change after 4 h. Extended reaction time (24 h) led to decomposition, along with MPAA-modified peptide **20a(+MPAA)** as the only identified product.



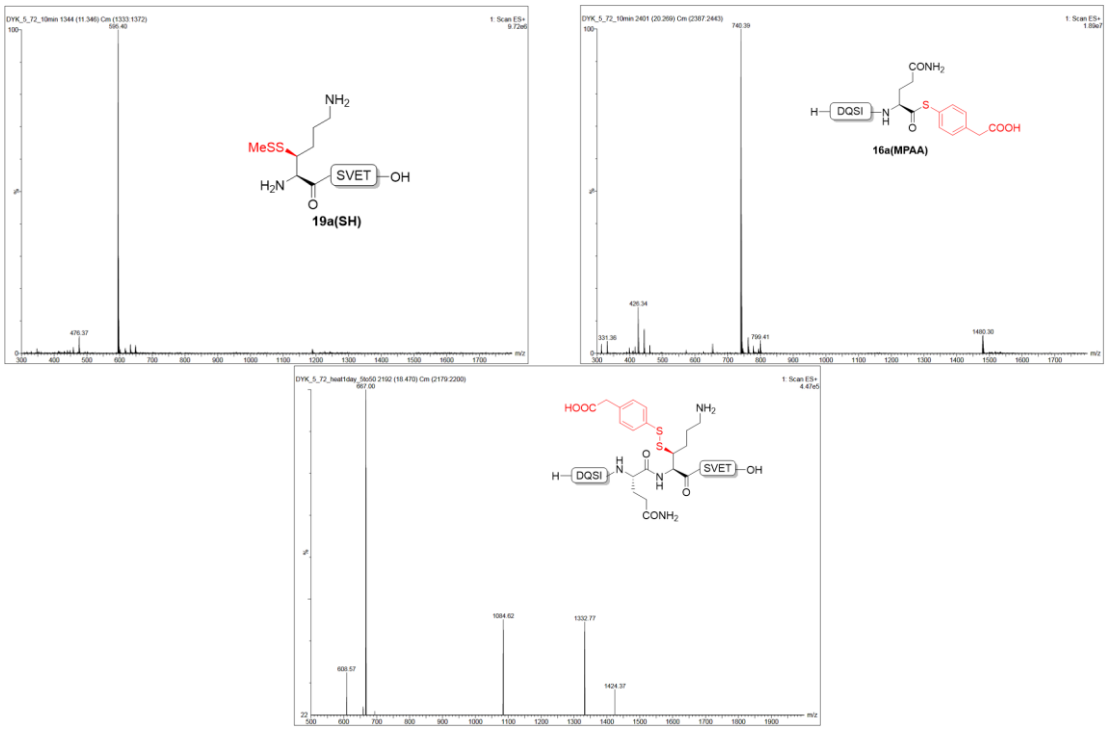
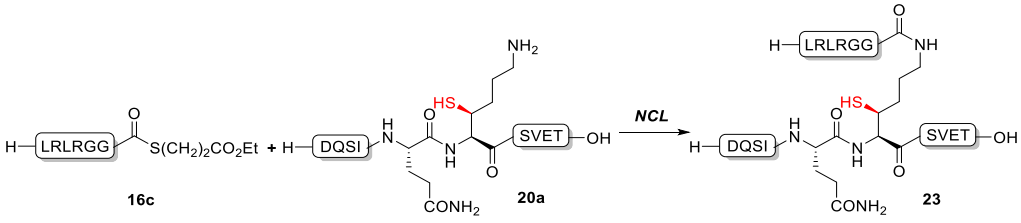


Figure S29. MS traces and ESI-MS data from HPLC-MS analysis of the ligation between excess **16a** and **19a**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column. ESI-MS of **19a(SH)**: calcd for $C_{23}H_{42}N_6O_{10}S$: 594.68 Da (average isotopes), (*m/z*) $[M+H]^+$: 595.28, found: $[M+H]^+$: 595.40; ESI-MS of **16a(MPAA)**: calcd for $C_{31}H_{45}N_7O_{12}S$: 739.80 Da (average isotopes), (*m/z*) $[M+H]^+$: 740.29, found: $[M+H]^+$: 740.39; ESI-MS of **20a(+MPAA)**: calcd for $C_{54}H_{85}N_{13}O_{22}S_2$: 1332.46 Da (average isotopes), (*m/z*) $[M+H]^+$: 1332.54, [$M+2H]^{2+}$: 666.78; found: $[M+H]^+$: 1332.77, $[M+2H]^{2+}$: 667.10.

Attempted ε-Peptide ligation between 16c and 20a



0.72 mg of peptidyl thioester **16c** (1.5 equiv) and 0.72 mg of peptide **20a** (1.0 equiv) were subjected to the ligation conditions following General Procedure **3.5** as described previously. The reaction was stirred at 37 °C under an argon atmosphere and monitored by LC-MS (5 to 50% solvent B over 30 min, Agilent C18 column).

As shown in Figure S30, the reaction at 37 °C for 21 hours produced small amount of ligated peptide **23** (t_R=16.95 min, 17.45 min), along with peptidyl acid **16c(OH)** (t_R=14.97 min, 15.63 min)that derived from the hydrolysis of **16c**, as well as the mostly unreacted starting material **20a**. There were two sets of peaks observed for both

16c(OH) and **23**, which shared the same mass. The reason for this observation has not been identified at this stage. Nevertheless, the estimated conversion based on calculations of the UV traces integrates of peptides **20a** and **23** was about 13%, indicating the low efficiency of the ε-ligation even with less sterically demanding glycine residue at forced heating conditions.

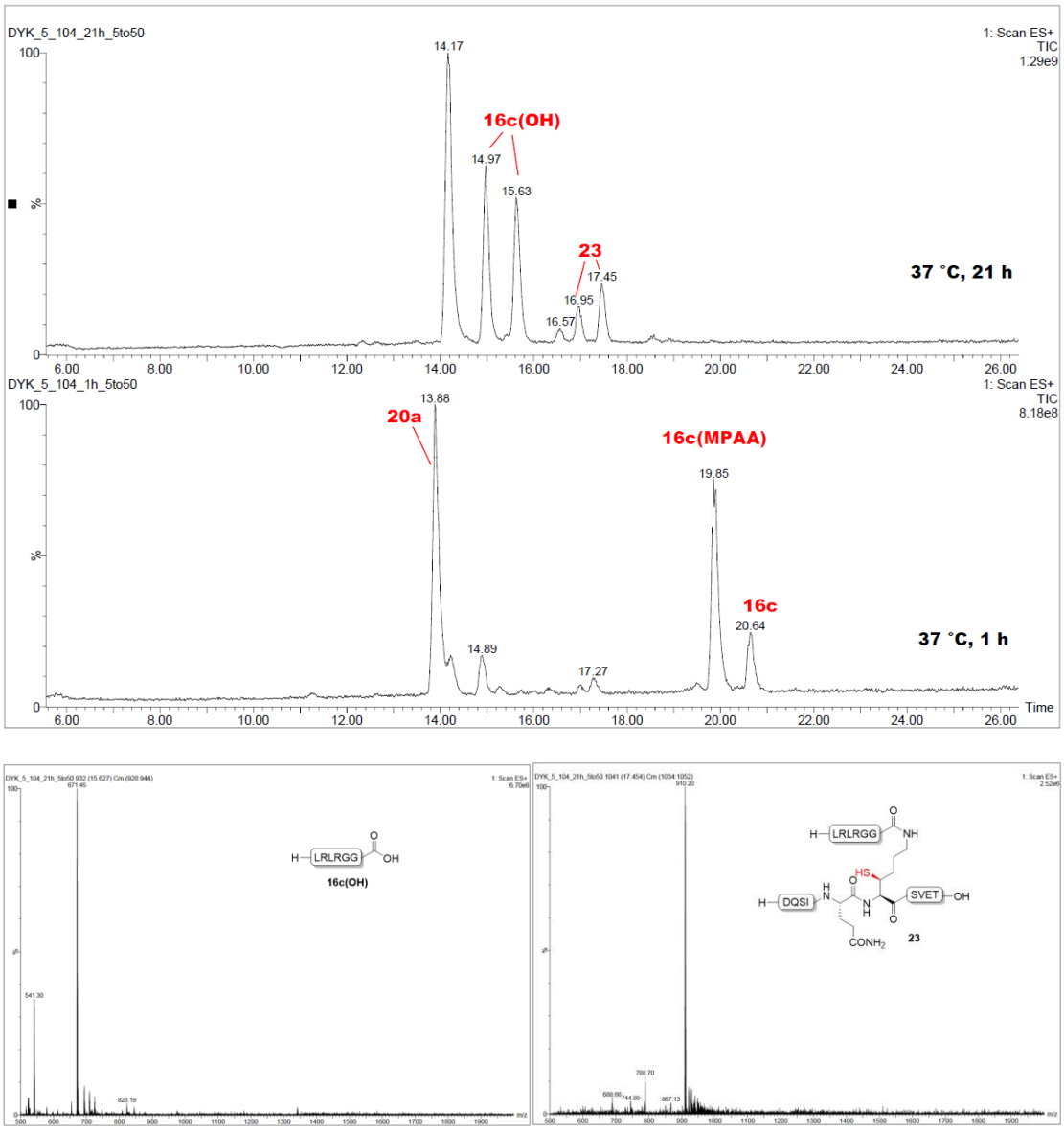
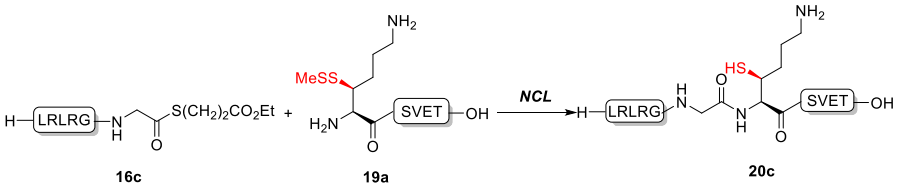


Figure S30. MS traces and ESI-MS data from HPLC-MS analysis of ligation between **16c** and **20a**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 16.2 min. ESI-MS of **16c(OH)**: calcd for $C_{28}H_{54}N_{12}O_7$: 670.82 Da (average isotopes), (*m/z*) $[M+H]^+$:671.43, found: $[M+H]^+$: 671.45; ESI-MS of **23**: calcd for $C_{74}H_{131}N_{25}O_{26}S$: 1819.07 Da (average isotopes), (*m/z*), $[M+2H]^{2+}$: 909.98; found: $[M+2H]^{2+}$: 910.20.

VI. LC-MS Data of Ligation and Desulfurization Reactions

Ligation reaction between 16c and 19a



1.09 mg of peptidyl thioester **16c** (1.1 equiv) and 0.79 mg of thiolysine peptide **19a** (1.0 equiv) were subjected to the ligation conditions following General Procedure **3.5** as described previously. The reaction was stirred for 8 h at room temperature under an argon atmosphere, and quenched with 1.0 mL of $CH_3CN/H_2O/AcOH$ (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **20c** (1.46 mg, 95%) as a white solid.

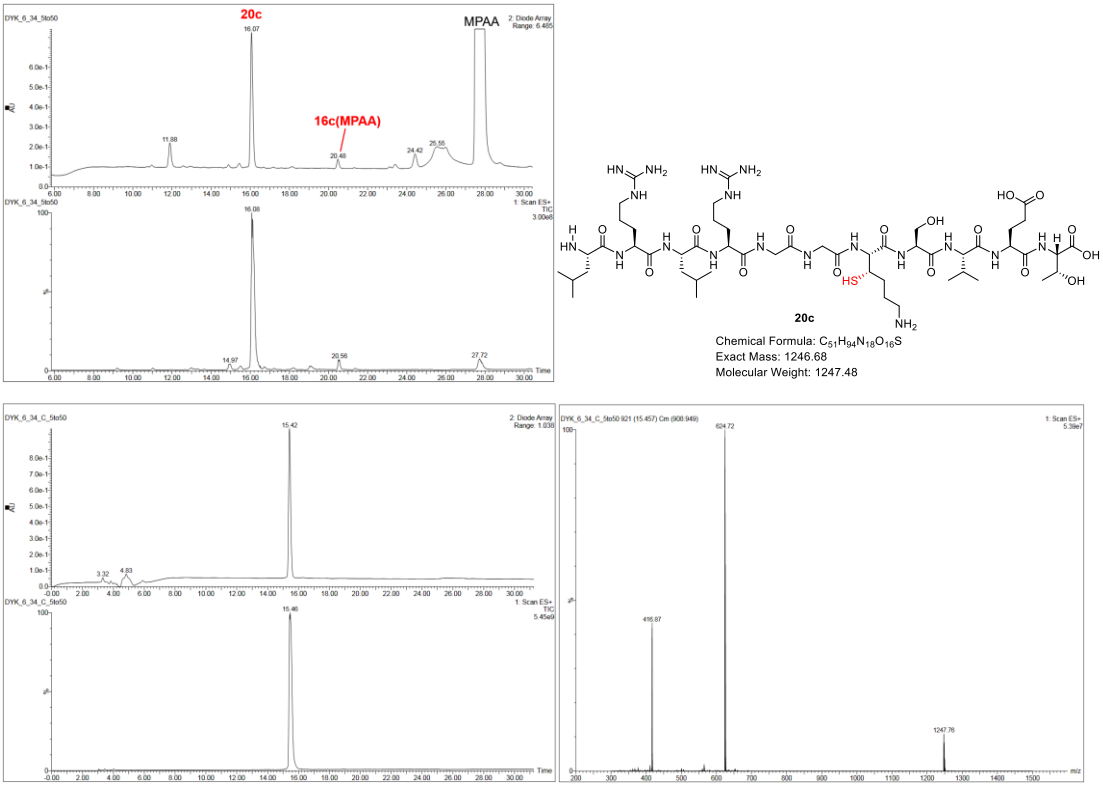
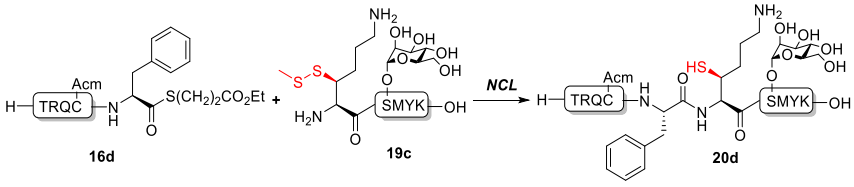


Figure S31. Top left: UV and MS traces of the ligation reaction between peptides **16c** and **19a** after 8 h; Top right: Structure and calculated data of **20c**; Bottom left: UV and MS traces of the purified product **20c**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 15.4 min; Bottom right: ESI-MS data of the purified product **20c**. Calcd for $C_{51}H_{94}N_{18}O_{16}S$: 1247.48 Da

(average isotopes), (*m/z*) $[M+H]^+$: 1246.69, $[M+2H]^{2+}$: 624.35, $[M+3H]^{3+}$: 416.57; found: $[M+H]^+$: 1247.76, $[M+2H]^{2+}$: 624.72, $[M+3H]^{3+}$: 416.87.

Ligation reaction between 16d and 19c



0.94 mg of peptidyl thioester **16d** (1.1 equiv) and 0.94 mg of thiolysine peptide **19c** (1.0 equiv) were subjected to the ligation conditions following General Procedure **3.5** as described previously. The reaction was stirred for 8 h at room temperature under an argon atmosphere, and quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **20d** (1.19 mg, 73%) as a white solid.

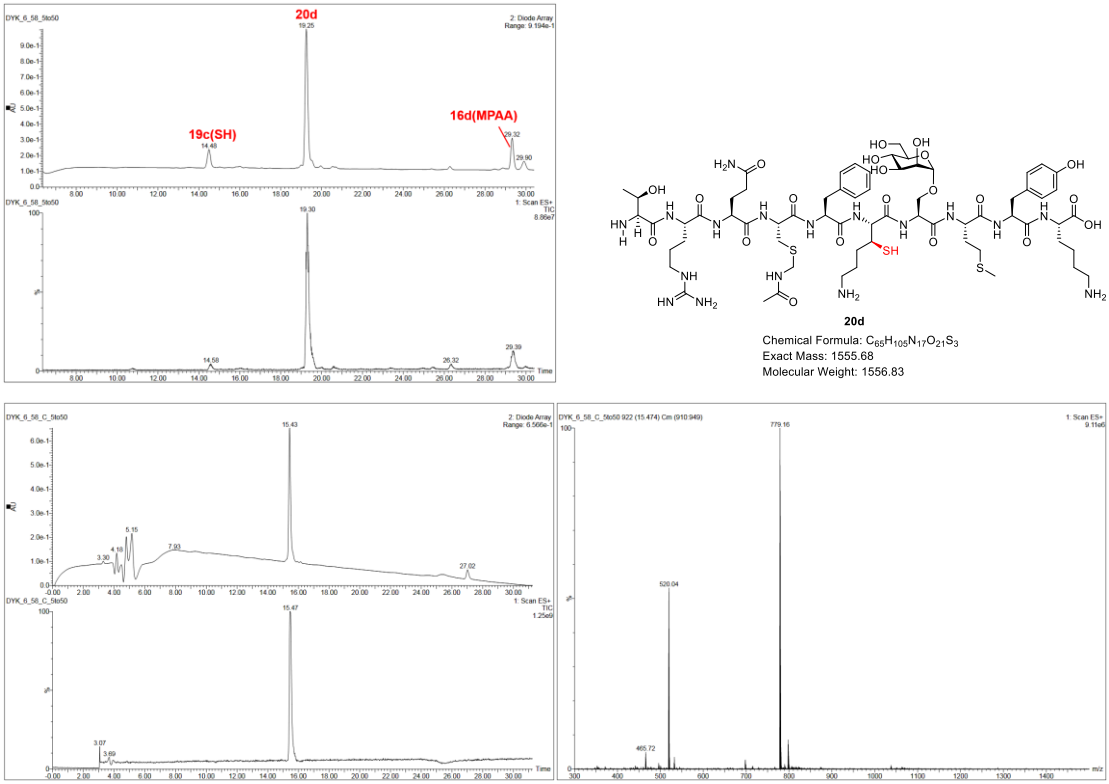
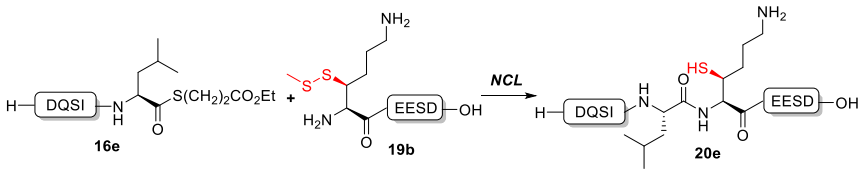


Figure S32. Top left: UV and MS traces of the ligation reaction between peptides **16d** and **19c** after 8 h; Top right: Structure and calculated data of **20d**; Bottom left: UV and MS traces of the purified product **20d**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 15.4 min; Bottom right: ESI-MS data of the purified product **20d**. Calcd for $C_{65}H_{105}N_{17}O_{21}S_3$: 1556.83 Da

(average isotopes), (*m/z*) $[M+2H]^{2+}$: 778.85, $[M+3H]^{3+}$: 519.57; found: $[M+2H]^{2+}$: 779.16, $[M+3H]^{3+}$: 520.04.

Ligation reaction between 16e and 19b



1.10 mg of peptidyl thioester **16e** (1. equiv) and 0.96 mg of thiolysine peptide **19b** (1.0 equiv) were subjected to the ligation conditions following General Procedure **3.5** as described previously. Notably, the reaction was stirred for 8 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of $CH_3CN/H_2O/AcOH$ (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **20e** (1.19 mg, 71%) as a white solid.

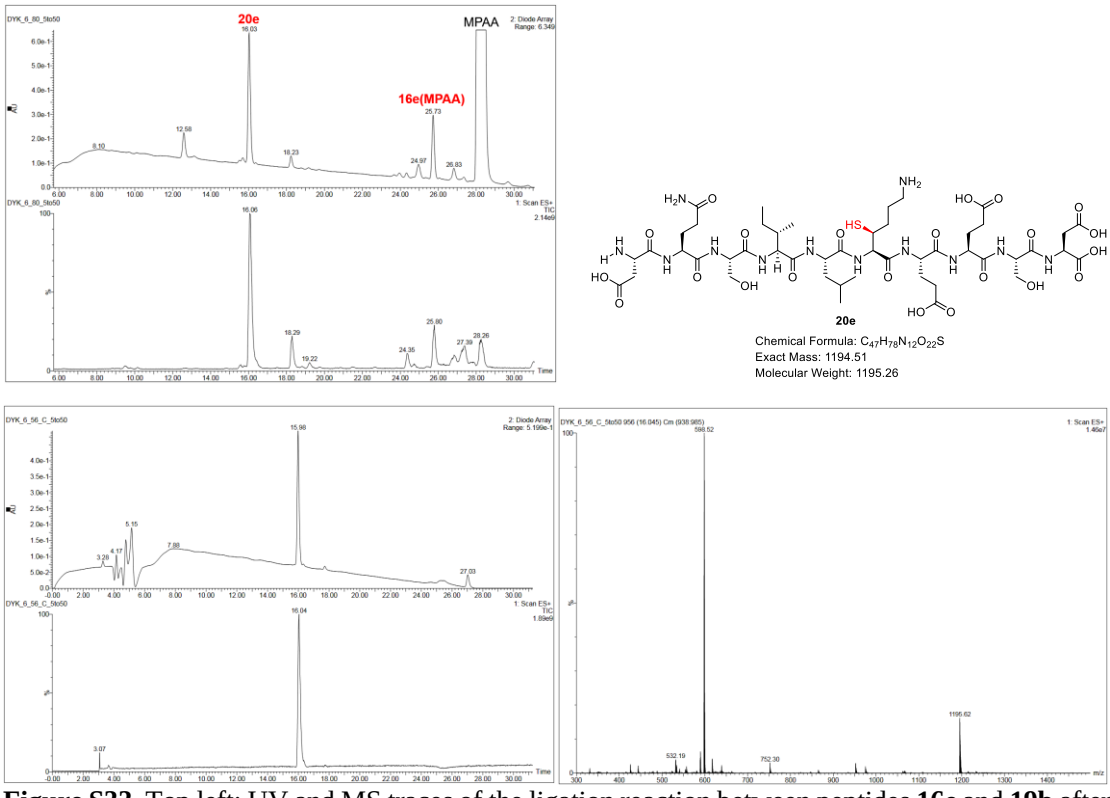
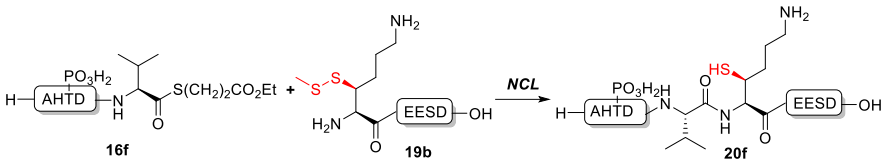


Figure S33. Top left: UV and MS traces of the ligation reaction between peptides **16e** and **19b** after 8 h; Top right: Structure and calculated data of **20e**; Bottom left: UV and MS traces of the purified product **20e**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 16.0 min; Bottom right: ESI-MS data of the purified product **20e**. Calcd for $C_{47}H_{78}N_{12}O_{22}S$: 1195.26 Da

(average isotopes), (*m/z*) $[M+H]^+$: 1195.51, $[M+2H]^{2+}$: 598.26; found: $[M+H]^+$: 1195.62, $[M+2H]^{2+}$: 598.52.

Ligation reaction between 16f and 19b



1.02 mg of peptidyl thioester **16f** (1.1 equiv) and 0.84 mg of thiolysine peptide **19b** (1.0 equiv) were subjected to the ligation conditions following General Procedure **3.5** as described previously. The reaction was stirred for 56 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **20f** (0.67 mg, 44%) as a white solid.

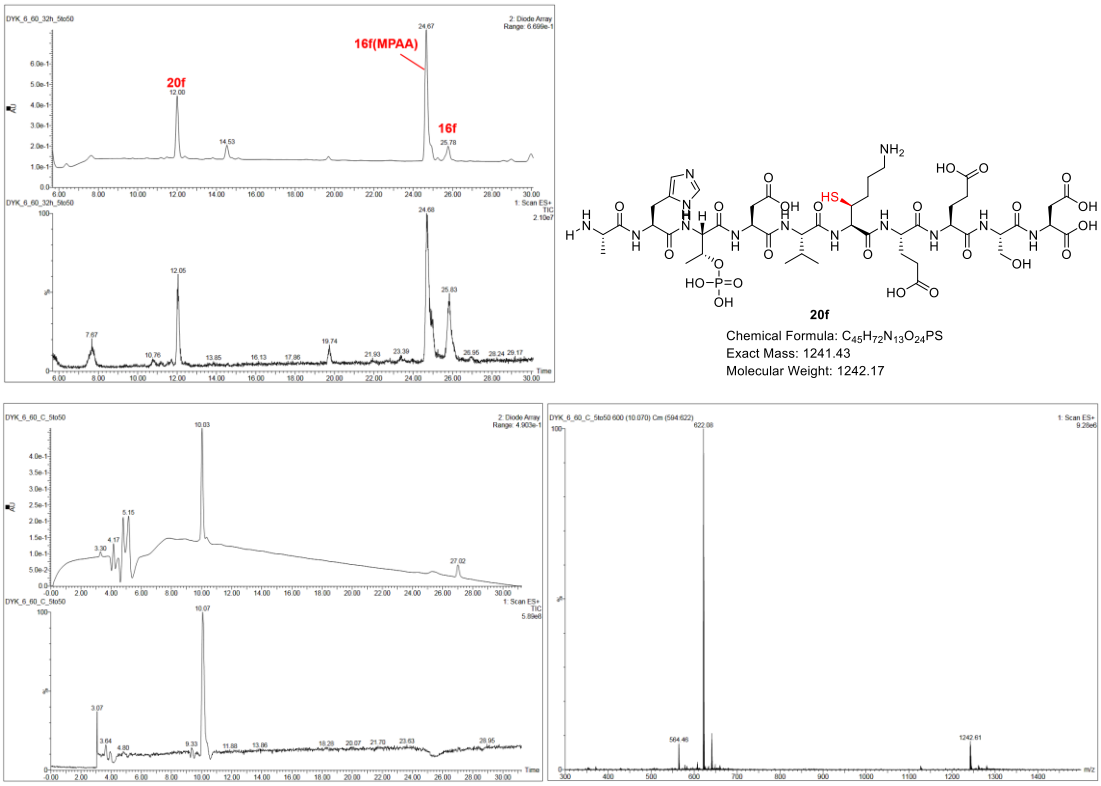
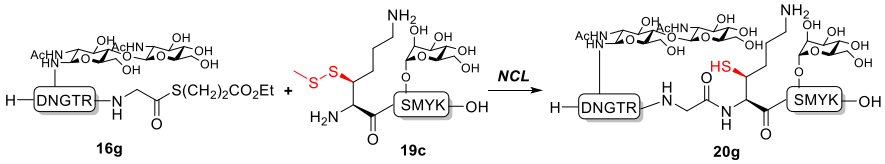


Figure S34. Top left: UV and MS traces of the ligation reaction between peptides **16f** and **19b** after 56 h; Top right: Structure and calculated data of **20f**; Bottom left: UV and MS traces of the purified product **20f**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 10.0 min; Bottom right: ESI-MS data of the purified product **20f**. Calcd for $C_{45}H_{72}N_{13}O_{24}PS$: 1242.17 Da

(average isotopes), (*m/z*) $[M+H]^+$: 1242.43, $[M+2H]^{2+}$: 621.72; found: $[M+H]^+$: 1242.61, $[M+2H]^{2+}$: 622.08.

Ligation between 16g and 19c



0.66 mg of peptidyl thioester **16g** (1.2 equiv) and 0.43 mg (1.0 equiv) of thiolysine peptide **19c** were subjected to the ligation conditions following General Procedure **3.5** as described previously. The reaction was stirred for 8 h at room temperature under an argon atmosphere, and quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **20g** (0.76 mg, 85%) as a white solid.

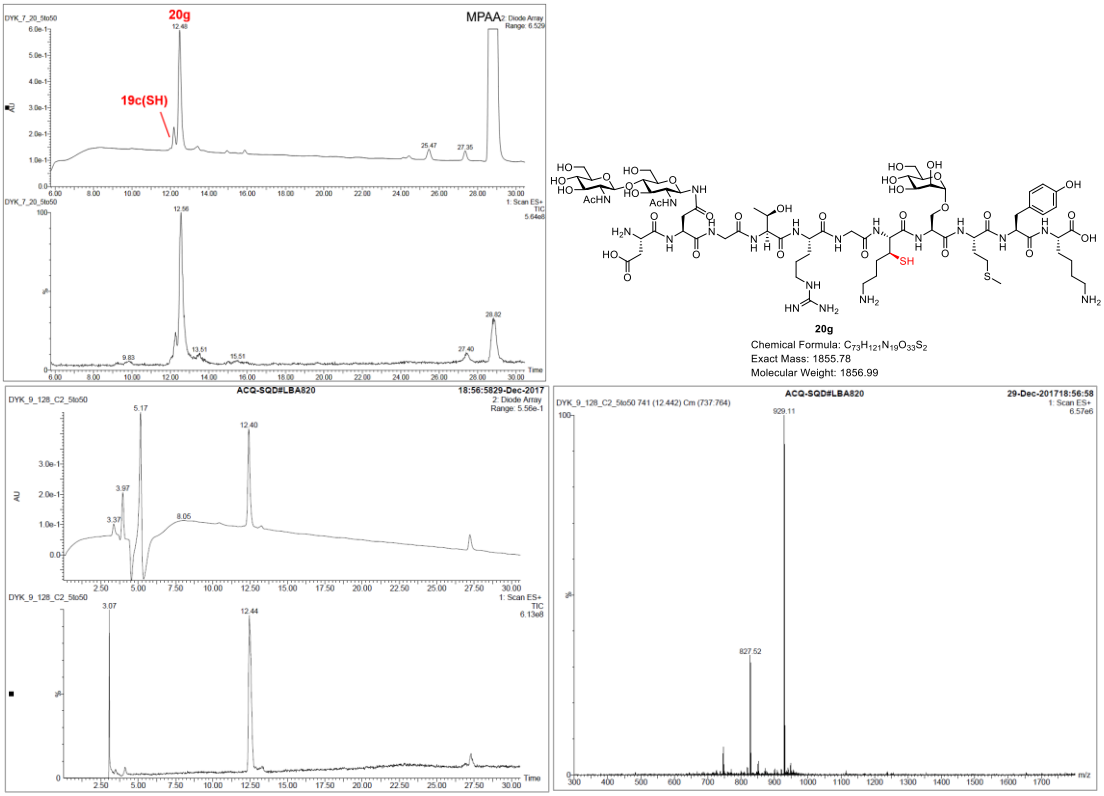
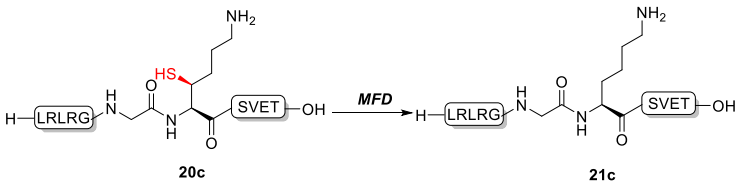


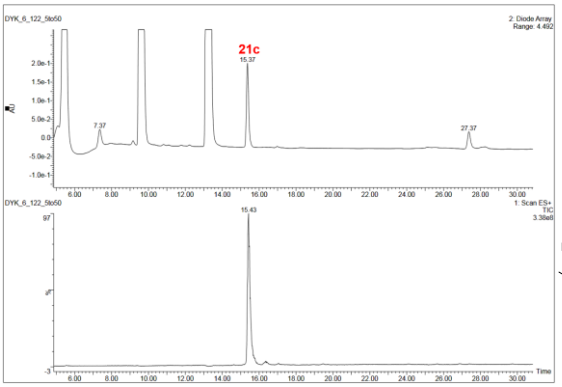
Figure S35. Top left: UV and MS traces of the ligation reaction between peptides **16g** and **19c** after 8 h; Top right: Structure and calculated data of **20g**; Bottom left: UV and MS traces of the purified product **20g**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 12.4 min;

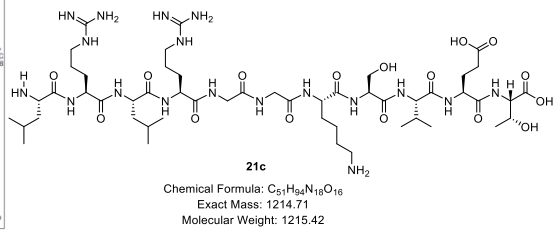
Bottom right: ESI-MS data of the purified product **20g**. Calcd for $C_{73}H_{121}N_{19}O_{33}S_2$: 1856.99 Da (average isotopes), (*m/z*) $[M+H]^+$: $[M+2H]^{2+}$: 928.90; found: $[M+H]^+$: $[M+2H]^{2+}$: 929.11.

Desulfurization reaction of 20c



0.78 mg of peptide **20c** obtained from the ligation reaction was subjected to the desulfurization conditions following General Procedure **3.6** as described previously. The reaction was stirred at 37 °C under an argon atmosphere for 1 h, and quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **21c** (0.53 mg, 70%) as a white solid.





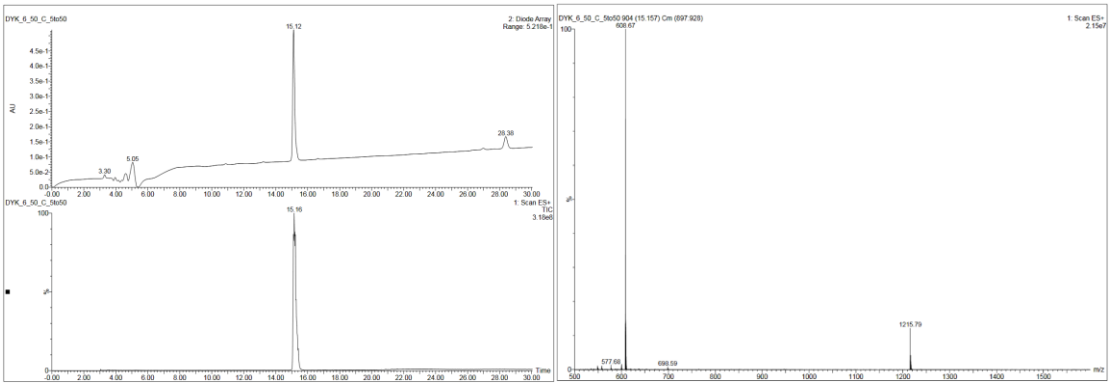
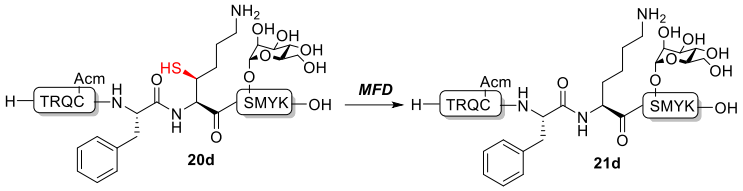


Figure S36. Top left: UV and MS traces of the desulfurization reaction of **20c**; Top right: Structure and calculated data of **21c**; Bottom left: UV and MS traces of the purified product **21c**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 15.1 min; Bottom right: ESI-

MS data of the purified product **21c**. Calcd for $C_{51}H_{94}N_{18}O_{16}$: 1215.42 Da (average isotopes), (*m/z*) $[M+H]^+$: 1215.72, $[M+2H]^{2+}$: 608.36; found: $[M+H]^+$: 1215.79, $[M+2H]^{2+}$: 608.67.

Desulfurization reaction of 20d



1.10 mg of peptide **20d** obtained from the ligation reaction was subjected to the desulfurization conditions following General Procedure **3.6** as described previously. The reaction was stirred at 37 °C under an argon atmosphere for 1 h, and quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **21d** (0.95 mg, 88%) as a white solid.

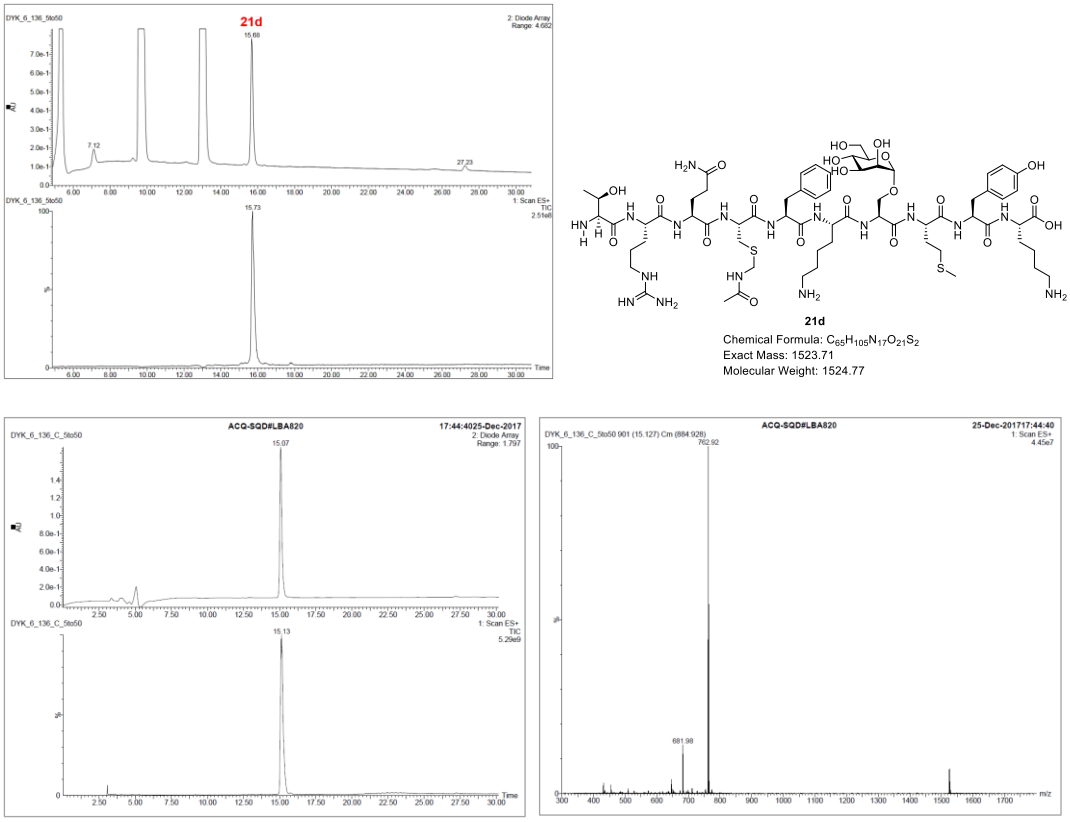
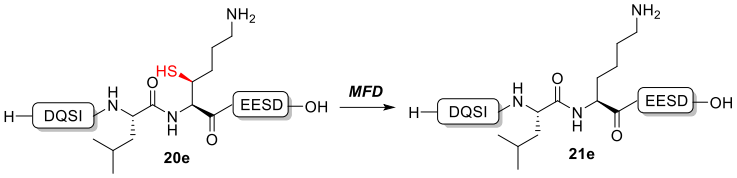


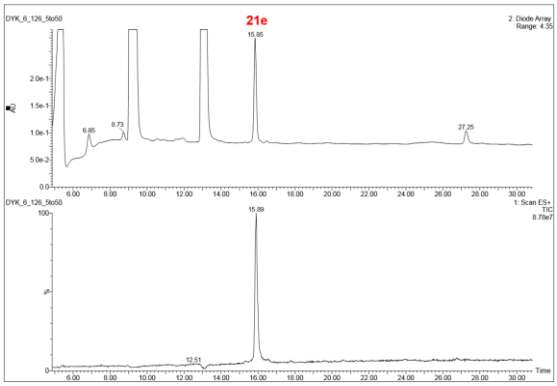
Figure S37. Top left: UV and MS traces of the desulfurization reaction of **20d**; Top right: Structure and calculated data of **21d**; Bottom left: UV and MS traces of the purified product **21d**. Linear

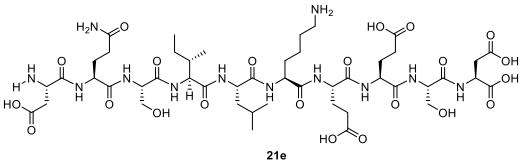
gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 15.1 min; Bottom right: ESI-MS data of the purified product **21d**. Calcd for $C_{65}H_{105}N_{17}O_{21}S_2$: 1524.77 Da (average isotopes), (*m/z*) $[M+H]^+$: 1524.72, $[M+2H]^{2+}$: 762.86; found: $[M+H]^+$: 1524.59, $[M+2H]^{2+}$: 762.92.

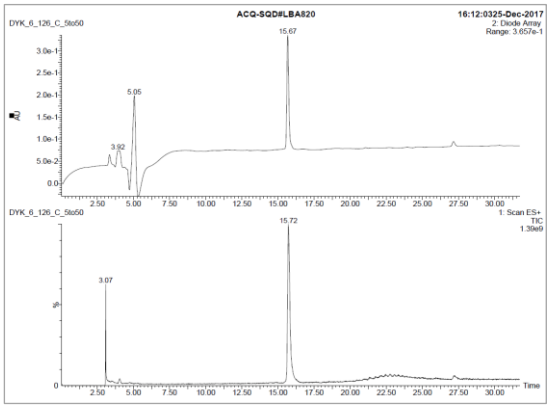
Desulfurization reaction of 20e



0.66 mg of peptide **20e** obtained from the ligation reaction was subjected to the desulfurization conditions following General Procedure **3.6** as described previously. The reaction was stirred at 37 °C under an argon atmosphere for 1 h, and quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **21e** (0.50 mg, 78%) as a white solid.







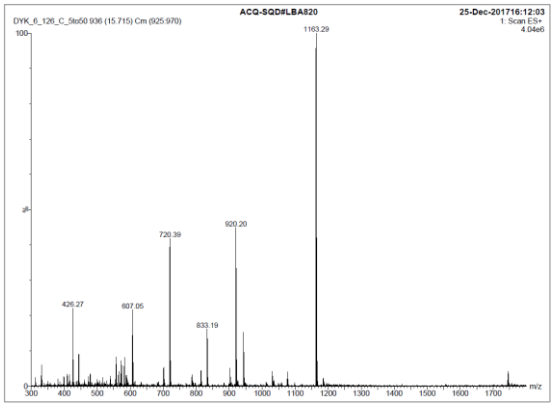
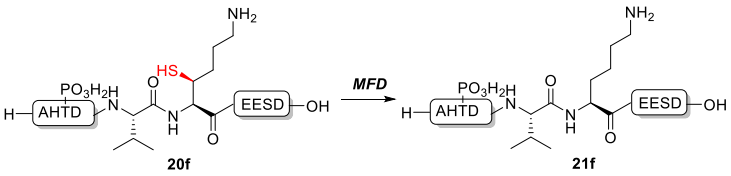
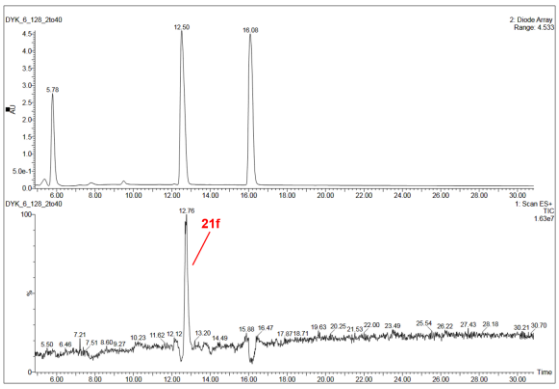


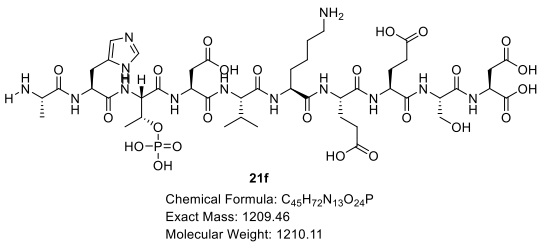
Figure S38. Top left: UV and MS traces of the desulfurization reaction of **20e**; Top right: Structure and calculated data of **21e**; Bottom left: UV and MS traces of the purified product **21e**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 15.7 min; Bottom right: ESI-MS data of the purified product **21e**. Calcd for $C_{47}H_{78}N_{12}O_{22}$: 1163.20 Da (average isotopes), (*m/z*) $[M+H]^+$: 1163.54; found: $[M+H]^+$: 1163.29.

Desulfurization reaction of 20f



0.61mg of peptide **20f** obtained from the ligation reaction was subjected to the desulfurization conditions following General Procedure **3.6** as described previously. The reaction was stirred at 37 °C under an argon atmosphere for 1 h, and quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 15%solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **21f** (0.45 mg, 76%) as a white solid.





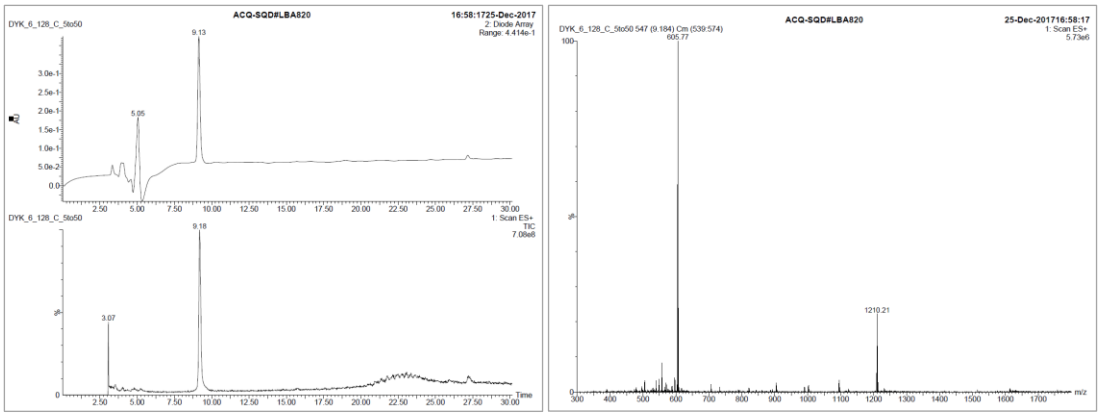
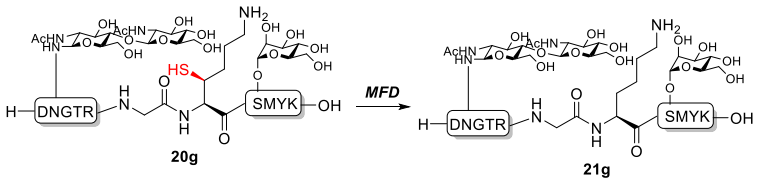
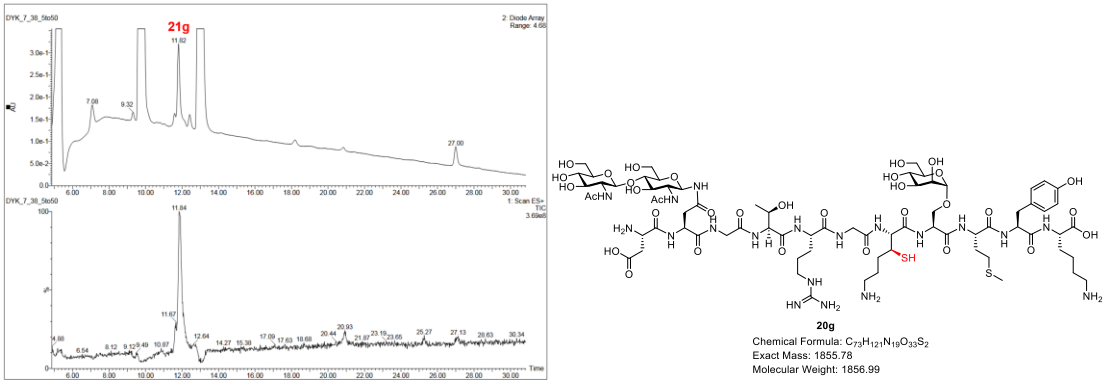


Figure S39. Top left: UV and MS traces of the desulfurization reaction of **20f**; Top right: Structure and calculated data of **21f**; Bottom left: UV and MS trace of the purified product **21f**. Linear gradient: 5 to 50% solvent B over 30 min, Agilent C18 column, t_R = 9.1 min; Bottom right: ESI-MS data of the purified product **21f**. Calcd for $C_{45}H_{72}N_{13}O_{24}P$: 1210.11 Da (average isotopes), (*m/z*) $[M+H]^+$: 1210.46, $[M+2H]^{2+}$: 605.73; found: $[M+H]^+$: 1210.21, $[M+2H]^{2+}$: 605.77.

Desulfurization reaction of 20g



0.76mg of peptide **20g** obtained from the ligation reaction was subjected to the desulfurization conditions following General Procedure **3.6** as described previously. The reaction was stirred at 37 °C under an argon atmosphere for 1 h, and quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **21g** (0.54 mg, 72%) as a white solid.



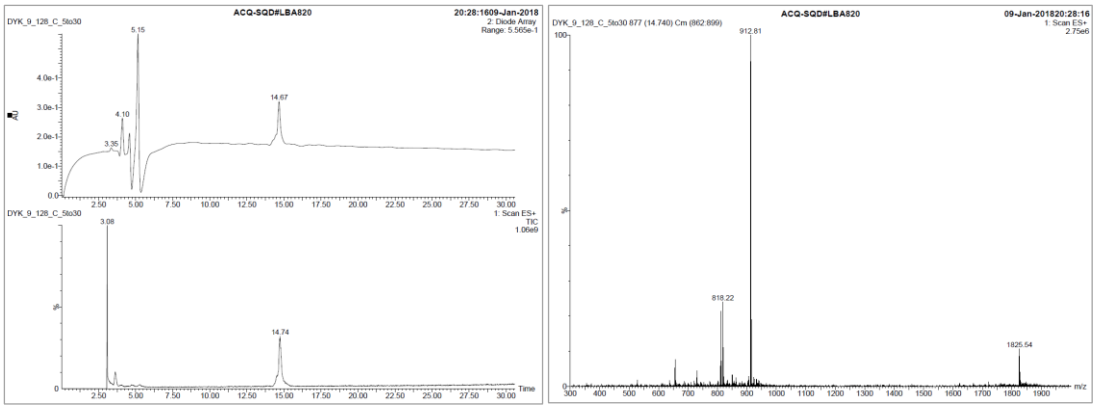
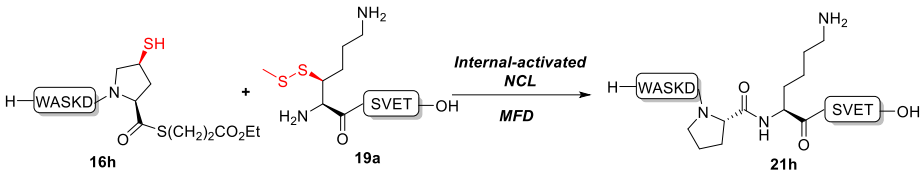
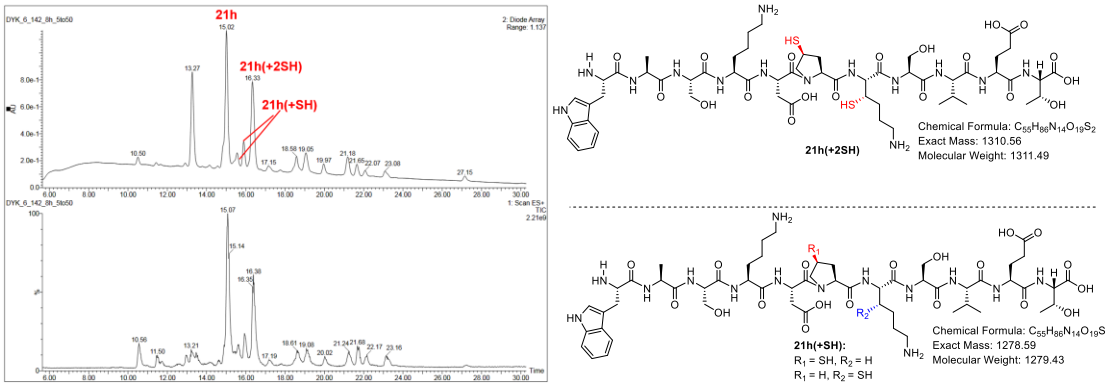


Figure S40. Top left: UV and MS traces of the desulfurization reaction of **20g**; Top right: Structure and calculated data of **21g**; Bottom left: UV and MS trace of the purified product **21g**. Linear gradient: 5 to 30% solvent B over 30 min, Agilent C18 column, t_R = 14.7 min; Bottom right: ESI-MS data of the purified product **21g**. Calcd for $C_{73}H_{121}N_{19}O_{33}S$: 1824.93 Da (average isotopes), (*m/z*) $[M+H]^+$: 1824.82, $[M+2H]^{2+}$: 912.91; found: $[M+H]^+$: 1825.54, $[M+2H]^{2+}$: 912.81.

One-pot ligation desulfurization reactions between 16h and 19a



1.24 mg of peptidyl thioester **16h** (1.1 equiv) and 0.85 mg of thiolysine peptide **19a** (1.0 equiv) were subjected to the ligation conditions following General Procedure **3.7** as described previously. The reaction was stirred at room temperature and monitored by LC-MS. Upon completed consumption of **19a** after 8 h, formation of desulfurized peptide **21h**, mono-desulfurized **21h(+SH),** and non-desulfurized peptide **21h(+2SH)** were observed (Figure S41).



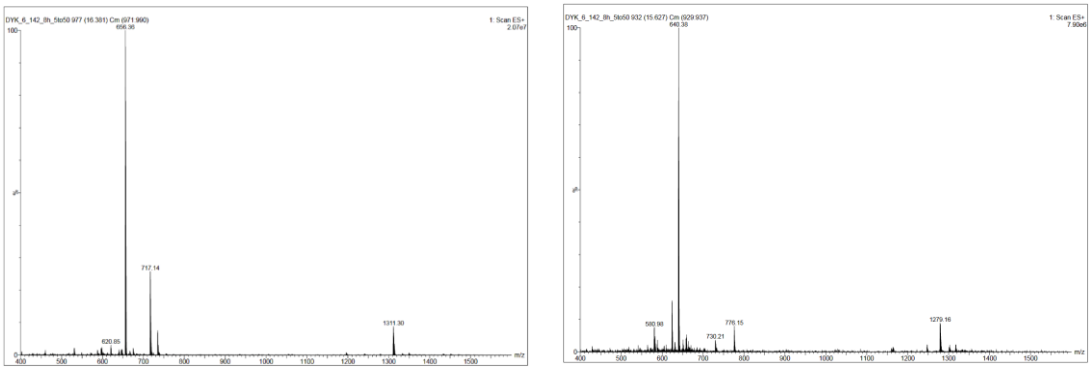
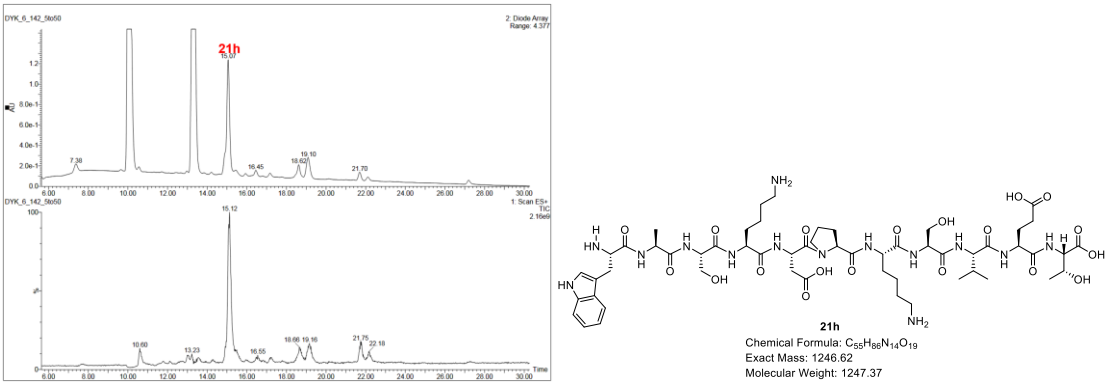


Figure S41. Top left: UV and MS traces of the ligation reaction between **16h** and **19a** after 8 h; Top right: Structure and calculated data of **21h(+2SH)** and **21h(+SH)**; Bottom left: ESI-MS data of the non-desulfurized peptide **21h(+2SH)**, calcd for $C_{55}H_{86}N_{14}O_{19}S_2$: 1311.49 Da (average isotopes), (*m/z*) $[M+H]^+$:1311.57, $[M+2H]^{2+}$:656.29; found: $[M+H]^+$: 1311.30, $[M+2H]^{2+}$:656.36; Bottom right: ESI-MS data of the mono-desulfurized peptide (**21h+SH)**, calcd for $C_{55}H_{86}N_{14}O_{19}S$: 1279.43 Da (average isotopes), (*m/z*) $[M+H]^+$:1279.60, $[M+2H]^{2+}$:640.30; found: $[M+H]^+$: 1279.16, $[M+2H]^{2+}$:640.38.

200 μL of Bond-breaker® TCEP solution, 100 μL of VA-044 (0.1 M), and 20 μL of ^{t}BuSH were added sequentially to the above reaction mixture carefully under an argon atmosphere following General Procedure **3.7**. The reaction was stirred for another 1 h at 37 °C under an argon atmosphere, and quenched with 1.0 mL of CH_3CN/H_2O/AcOH (5:90:5, *v/v/v*) solution. The resulting mixture was subjected to LC-MS analysis, and purified using preparative HPLC (5 to 50% solvent B over 30 min, Agilent Eclipse XDB-C18 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford **21h** (0.97mg, 59%) as a white solid.



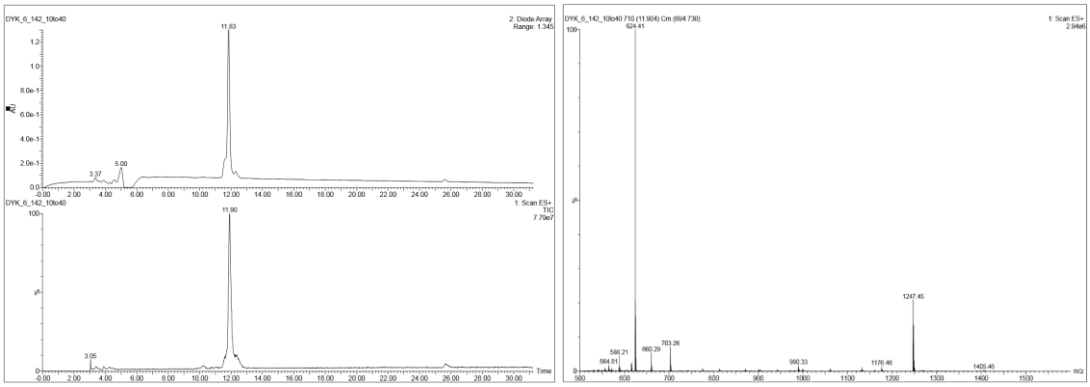
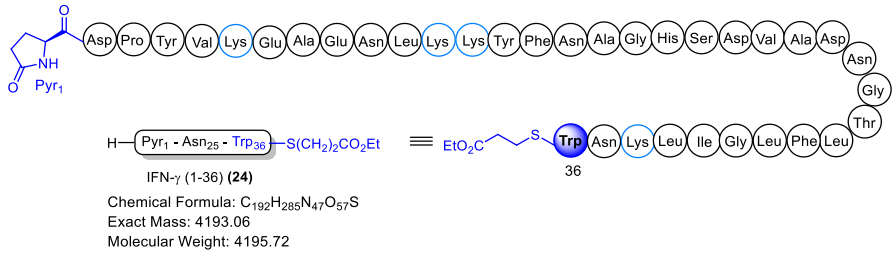


Figure S42. Top left: UV and MS traces of the one-pot desulfurization reaction following ligation; Top right: Structure and calculated data of **21h**; Bottom left: UV and MS trace of the purified product **21h**. Linear gradient: 10 to 40% solvent B over 30 min, Agilent C18 column, t_R = 11.8 min; Bottom right: ESI-MS data of the purified product **21h**. Calcd for $C_{55}H_{86}N_{14}O_{19}$: 1247.37 Da (average isotopes), (*m/z*) $[M+H]^+$: 1247.63, $[M+2H]^{2+}$: 624.32; found: $[M+H]^+$: 1247.45, $[M+2H]^{2+}$: 624.41.

VII. Chemical Synthesis of Interferon Gamma (IFN-γ)

Synthesis of IFN-γ(1-36) (24)



IFN-γ (1-36) **(24)**

Chemical Formula: $C_{192}H_{285}N_{47}O_{57}S$

Exact Mass: 4193.06

Molecular Weight: 4195.72

The fully protected peptidyl acid (1-35) was prepared *via* SPPS according to General Procedure **3.2.1** using CS Bio synthesizer on a 0.05 mmol scale, where Boc-Pyr-OH was used to incorporate the *N*-terminal residue. After cleavage using DCM/TFE/AcOH (3:1:1, *v/v/v*), the solvent was blown off under a nitrogen atmosphere. The resulting residue was dissolved in a solution of 20% CH_3CN in water, which was lyophilized to afford approx. 210 mg of crude peptidyl acid.

On the other hand, 640 mg of thioester Boc-Trp(Boc)-S$(CH_2)_2CO_2$Et (**S11**) was treated with 4.5 mL of 4 M HCl in 1,4-dioxane, and the resulting solution was stirred at room temperature for 1 h. The solvent was blown off under a nitrogen atmosphere, and the resulting residue was dissolved in a solution of 20% CH_3CN in water, which was lyophilized to generate HCl·H-Trp-S$(CH_2)_2CO_2$Et (**S12**) as solid powder, which was used directly in the next step without further purification.

The fully protected peptidyl acid (1.0 equiv) and HCl·H-Trp-S$(CH_2)_2CO_2$Et (**S12**) (3.0 equiv) in $CHCl_3$/TFE (3:1, *v/v*) was cooled to -15°C. HOOBt (3.0 equiv) and EDCI (3.0 equiv) were added sequentially. The resulting reaction was warmed to room temperature and continued to stir for 3 h. The solvent was then blown off under a nitrogen atmosphere, and the resulting residue was treated with 10 mL of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) according to General Procedure **3.4.2**.

After LC-MS analysis, purification of the crude peptide using preparative HPLC (37 to 47% solvent B over 30 min, Proto-300 C4 column) afforded IFN-γ(1-36) (**24**) as a white solid after lyophilization (55.7mg, 27%).

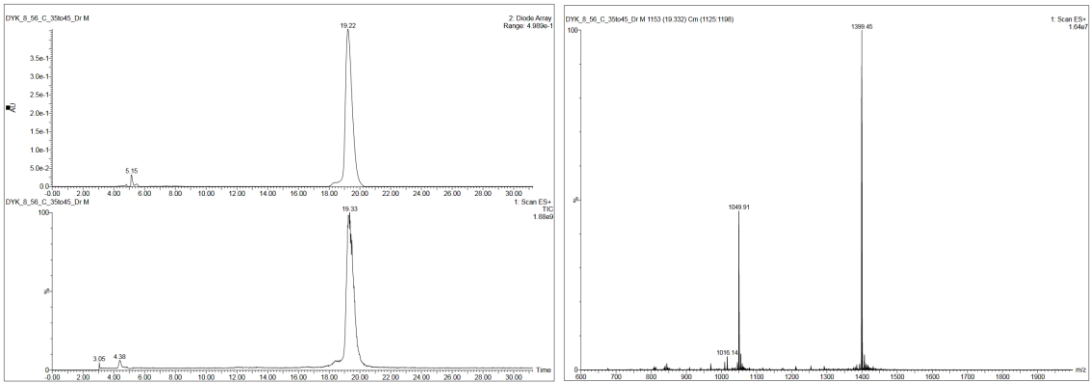
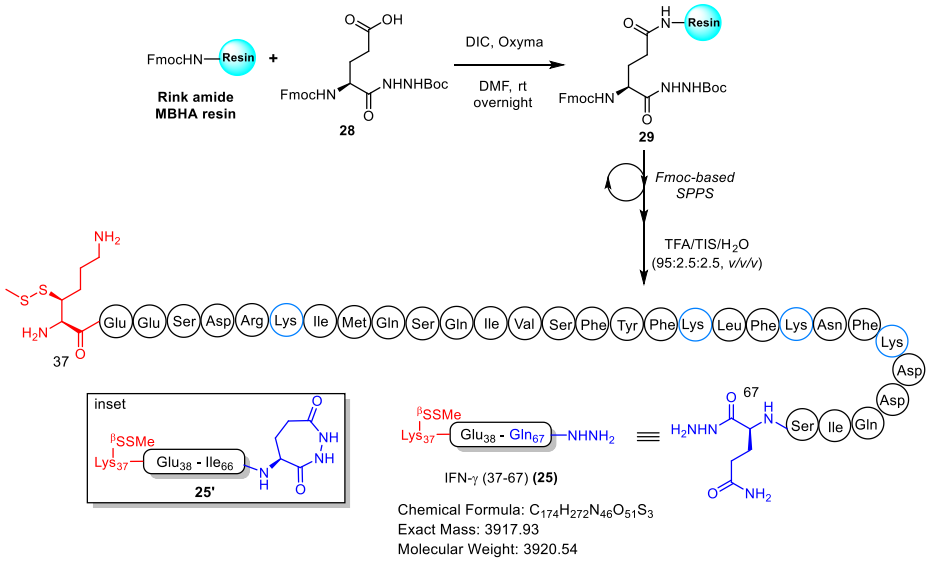


Figure S43. Left: UV and MS traces of the purified IFN-γ(1-36) (**24**) . Linear gradient: 35 to 45% solvent B over 30 min, Proto-300 C4 column, t_R = 19.2 min; Right: ESI-MS data of the purified IFN-γ(1-36) (**24**)**.** Calcd for $C_{192}H_{285}N_{47}O_{57}S$: 4195.72 Da (average isotopes), (*m/z*) $[M+3H]^{3+}$: 1399.36, $[M+4H]^{4+}$: 1049.77; found $[M+3H]^{3+}$: 1399.45, $[M+4H]^{4+}$: 1049.91.

Side-chain anchoring strategy in the synthesis of IFN-γ(37-67) (25)



The fisrt amino acid residue Fmoc-Glu-NHNHBoc (**28**) was loaded to Rink amide MBHA resin (loading= 0.35 mmol/g) according to the General Procedure **3.1**. Specifically, Fmoc-Rink amide resin was treated with 20% piperidine in DMF (5 min × 2) to remove the Fmoc group, and washed with DMF (× 3), DCM (× 3) and DMF (× 3). The Fmoc-Glu-NHNHBoc (**28**) (2.0 equiv), DIC (2.0 equiv) and Oxyma (2.0 equiv) was dissolved in DMF (approx. 10 mL per gram of resin), and the resulting solution was added to the resin. The reaction was agitated overnight. Then the resin was collected and washed with 8:1:1 (*v*/*v*/*v*) of DMF/Ac_2O/DIEA (× 3), DCM (× 3), DMF (× 2), DCM (× 3), and dried *in vacuo* for 12 hours, and test the loading is 0.22 mmol/g.

The fully protected peptidyl acid (37-66) was prepared *via* SPPS according to the General Procedure **3.2.1** using CS Bio synthesizer on a 0.05 mmol scale, where Boc-Lys$^{\beta\text{-SSMe}}$(Boc)-OH (**13a**) was coupled manually (1.0 equiv × 2) at the *N*-terminus. Peptide was cleaved from resin and deprotection using 10 mL of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) for 3 h, the solvent was then blown off under a nitrogen atmosphere, and the resulting residue was treated with 10 mL of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) according to General Procedure **3.3**.

After LC-MS analysis, there was small amounts of cyclic byproduct **25'** detected, purification of the crude peptide using preparative HPLC (29 to 39% solvent B over 30 min, Proto-300 C4 column) afforded IFN-γ(37-67) (**25**) as a white solid after lyophilization (53.0mg, 27%).

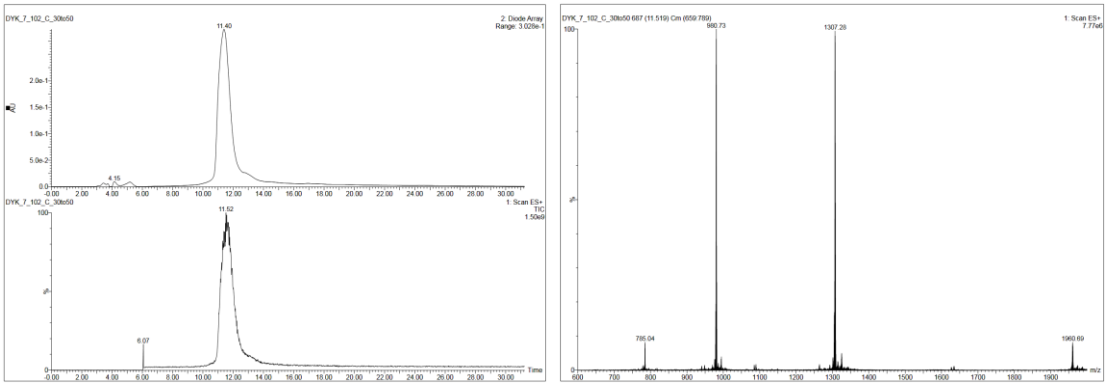
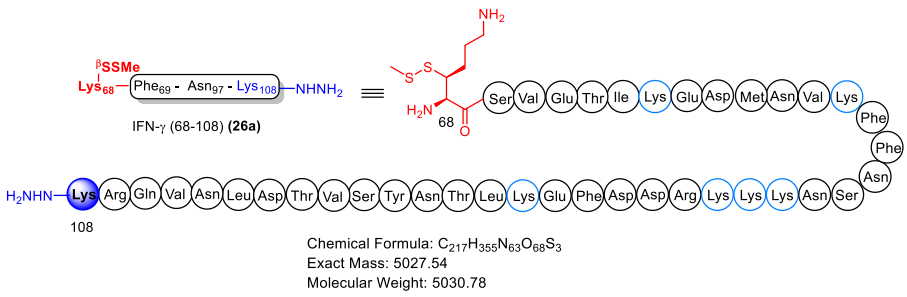


Figure S44. Left: UV and MS traces of the purified IFN-γ(37-67) (**25**). Linear gradient: 30 to 50% solvent B over 30 min, Proto-300 C4 column, t_R = 11.4 min; Right: ESI-MS data of the purified IFN-γ(37-67) (**25**). Calcd for $C_{174}H_{272}N_{46}O_{51}S_3$: 3920.54 Da (average isotopes), (*m/z*) $[M+2H]^{2+}$: 1960.47, $[M+3H]^{3+}$: 1307.32, $[M+4H]^{4+}$: 980.74, $[M+5H]^{5+}$: 784.79; found $[M+2H]^{2+}$: 1960.69, $[M+3H]^{3+}$: 1307.28, $[M+4H]^{4+}$: 980.73, $[M+5H]^{5+}$: 785.04.

Synthesis of IFN-γ(68-108) (26a)



The fully protected peptidyl acid (68-107) was prepared *via* SPPS according to the General Procedure **3.2.1** using CS Bio synthesizer on a 0.05 mmol scale, where Boc-Lys$^{\beta\text{-SSMe}}$(Boc)-OH (**13a**) was coupled manually (1.0 equiv × 2) at the *N*-terminus.

Peptide was cleaved from resin and deprotection using 10 mL of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) for 3 h, the solvent was then blown off under a nitrogen atmosphere, and the resulting residue was treated with 10 mL of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) according to General Procedure **3.3**.

After LC-MS analysis, purification of the crude peptide using preparative HPLC (22 to 35% solvent B over 30 min, Proto-300 C4 column) afforded IFN-γ(68-108) (**26a**) as a white solid after lyophilization (61.3mg, 24%).

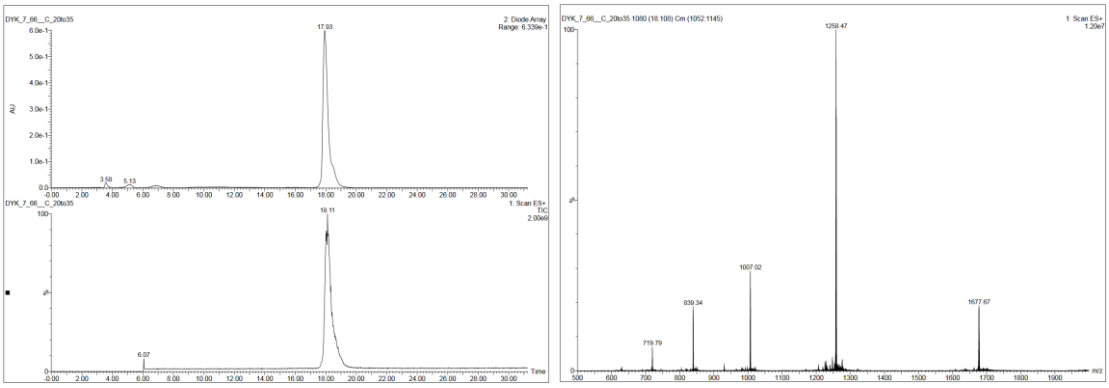
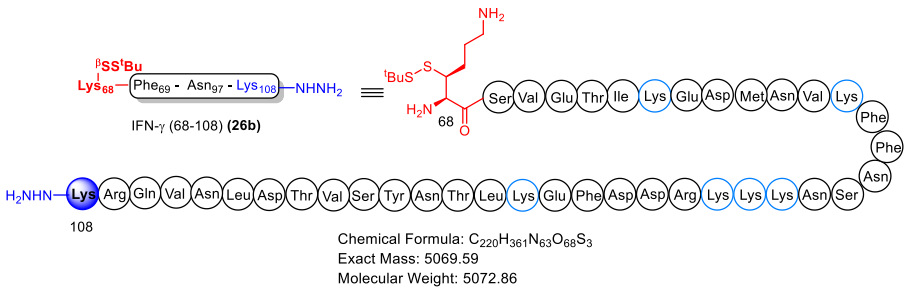


Figure S45. Left: UV and MS traces of the purified IFN-γ(68-108) (**26a**). Linear gradient: 20 to 35% solvent B over 30 min, Proto-300 C4 column, t_R = 17.9 min; Right: ESI-MS data of the purified IFN-γ(68-108) (**26a**). Calcd for $C_{217}H_{355}N_{63}O_{68}S_3$: 5030.78 Da (average isotopes), (*m/z*) $[M+3H]^{3+}$: 1677.52, $[M+4H]^{4+}$: 1258.39, $[M+5H]^{5+}$: 1006.92, $[M+6H]^{6+}$: 839.27, $[M+7H]^{7+}$: 719.51; found $[M+3H]^{3+}$: 1677.67, $[M+4H]^{4+}$: 1258.47, $[M+5H]^{5+}$: 1007.02, $[M+6H]^{6+}$: 839.34, $[M+7H]^{7+}$: 719.79.

Synthesis of IFN-γ(68-108) (26b)



The fully protected peptidyl acid (68-107) was prepared *via* SPPS according to the General Procedure **3.2.1** using CS Bio synthesizer on a 0.05 mmol scale, where Boc-Lys$^{β\text{-}SStBu}$(Boc)-OH (**13b**) was coupled manually (1.0 equiv × 2) at the *N*-terminus. Peptide was cleaved from resin and deprotection using 10 mL of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) for 3 h, the solvent was then blown off under a nitrogen atmosphere,

and the resulting residue was treated with 10 mL of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) according to General Procedure **3.3**.

After LC-MS analysis, purification of the crude peptide using preparative HPLC (23 to 50% solvent B over 30 min, Proto-300 C4 column) afforded IFN-γ(68-108) (**26b**) as a white solid after lyophilization (59.7mg, 25%).

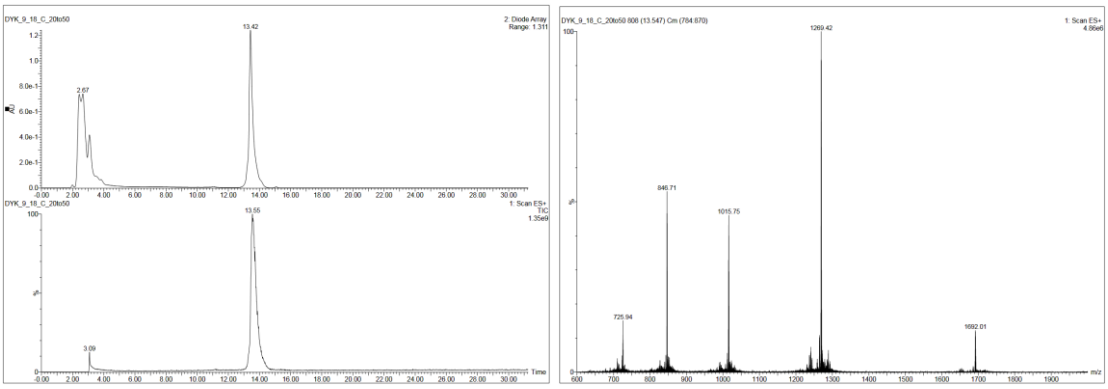
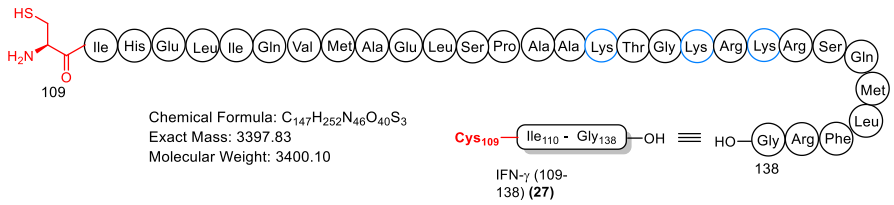


Figure S46. Left: UV and MS traces of the purified IFN-γ(68-108) (**26b**). Linear gradient: 20 to 50% solvent B over 30 min, Proto-300 C4 column, t_R = 13.4 min; Right: ESI-MS data of the purified IFN-γ(68-108) (**26b**). Calcd for $C_{220}H_{361}N_{63}O_{68}S_3$: 5072.86 Da (average isotopes), (*m/z*) $[M+3H]^{3+}$: 1691.54, $[M+4H]^{4+}$: 1268.91, $[M+5H]^{5+}$: 1015.33, $[M+6H]^{6+}$: 846.27, $[M+7H]^{7+}$: 725.52; found $[M+3H]^{3+}$: 1692.01, $[M+4H]^{4+}$: 1269.42, $[M+5H]^{5+}$: 1015.75, $[M+6H]^{6+}$: 846.71, $[M+7H]^{7+}$: 725.94.

Synthesis of IFN-γ(109-138) (27)



The fully protected peptidyl acid (109-138) was prepared *via* SPPS according to the General Procedure **3.2.1** using CS Bio synthesizer on a 0.05 mmol scale. Peptide was cleaved from resin and deprotection using 10 mL of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) for 3 h, the solvent was then blown off under a nitrogen atmosphere, and the resulting residue was treated with 10 mL of TFA/H_2O/TIS (95:2.5:2.5, *v/v/v*) according to General Procedure **3.3**.

After LC-MS analysis, purification of the crude peptide using preparative HPLC (28 to 38% solvent B over 30 min, Proto-300 C4 column) afforded IFN-γ(109-138) (**27**) as a white solid after lyophilization (30.0 mg, 17%).

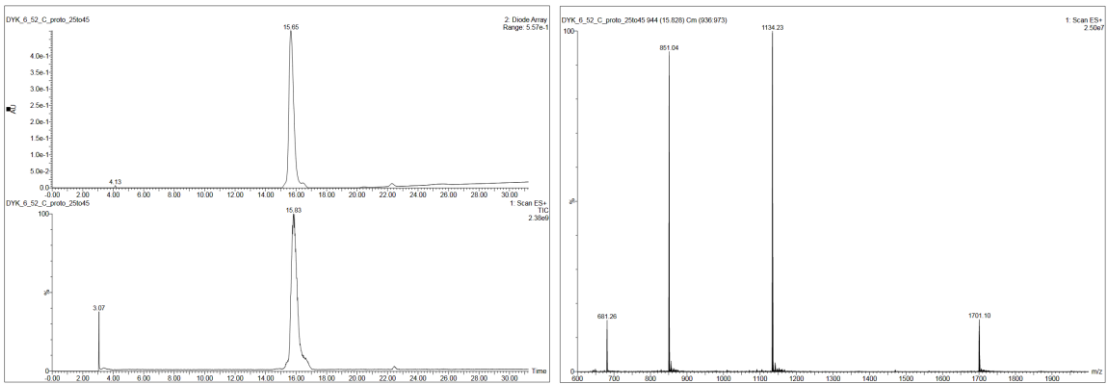
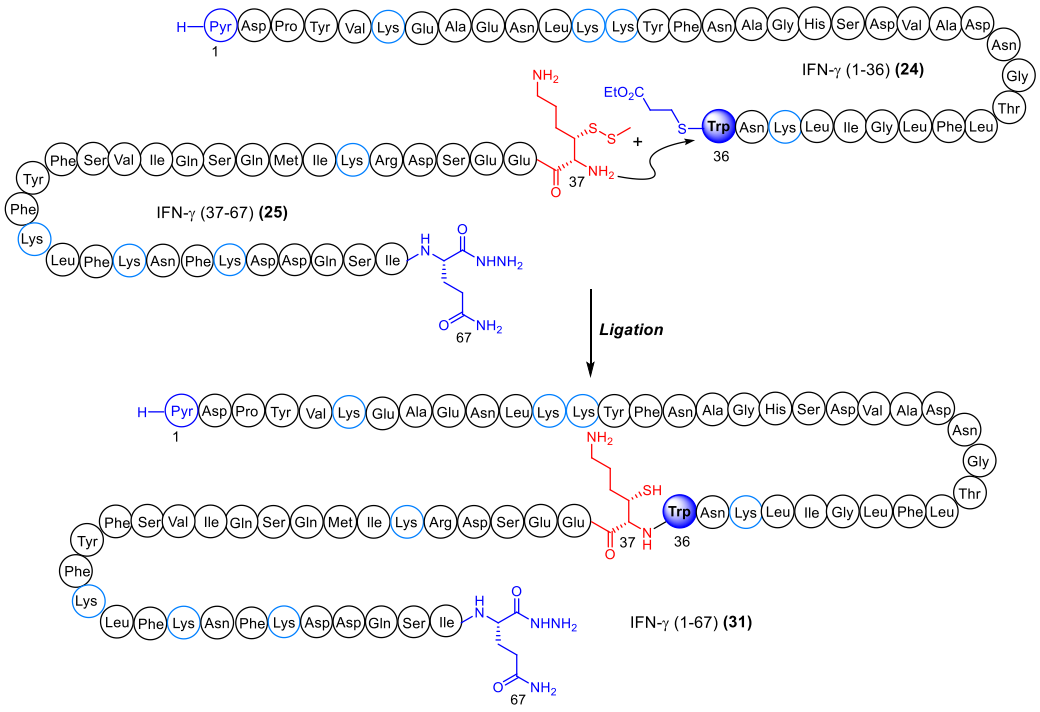


Figure S47. Left: UV and MS traces of the purified IFN-γ(109-138) (**27**). Linear gradient: 25 to 45% solvent B over 30 min, Proto-300 C4 column, t_R = 15.7 min; Right: ESI-MS data of the purified IFN-γ(109-138) (**27**). Calcd for $C_{147}H_{252}N_{46}O_{40}S_3$: 3400.10 Da (average isotopes), (*m/z*) $[M+2H]^{2+}$: 1700.42, $[M+3H]^{3+}$: 1133.95, $[M+4H]^{4+}$: 850.71, $[M+5H]^{5+}$: 680.77; found $[M+2H]^{2+}$: 1701.10, $[M+3H]^{3+}$: 1134.23, $[M+4H]^{4+}$: 851.04, $[M+5H]^{5+}$: 681.26.

Ligation reaction between of IFN-γ(1-36) (24) and IFN-γ(37-67) (25)



3.54 mg of IFN-γ(1-36) (**24** 1.0 equiv) and 3.72 mg of IFN-γ(37-67) (**25** 1.1 equiv) were dissolved in 420 μL of ligation buffer, and subjected to the ligation conditions following General Procedure **3.5** as described previously. The reaction was stirred at room temperature under an argon atmosphere and monitored by LC-MS.

After reacted for 20 h, the reaction was quenched with 2.0 mL of CH_3CN/H_2O/AcOH (47.5/47.5/5, *v/v/v*) solution. The crude mixture was purified using preparative

HPLC (32 to 50% solvent B over 30 min, Proto-300 C4 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford IFN-γ(1-67) (**31**) (3.00 mg, 45%) as a white solid.

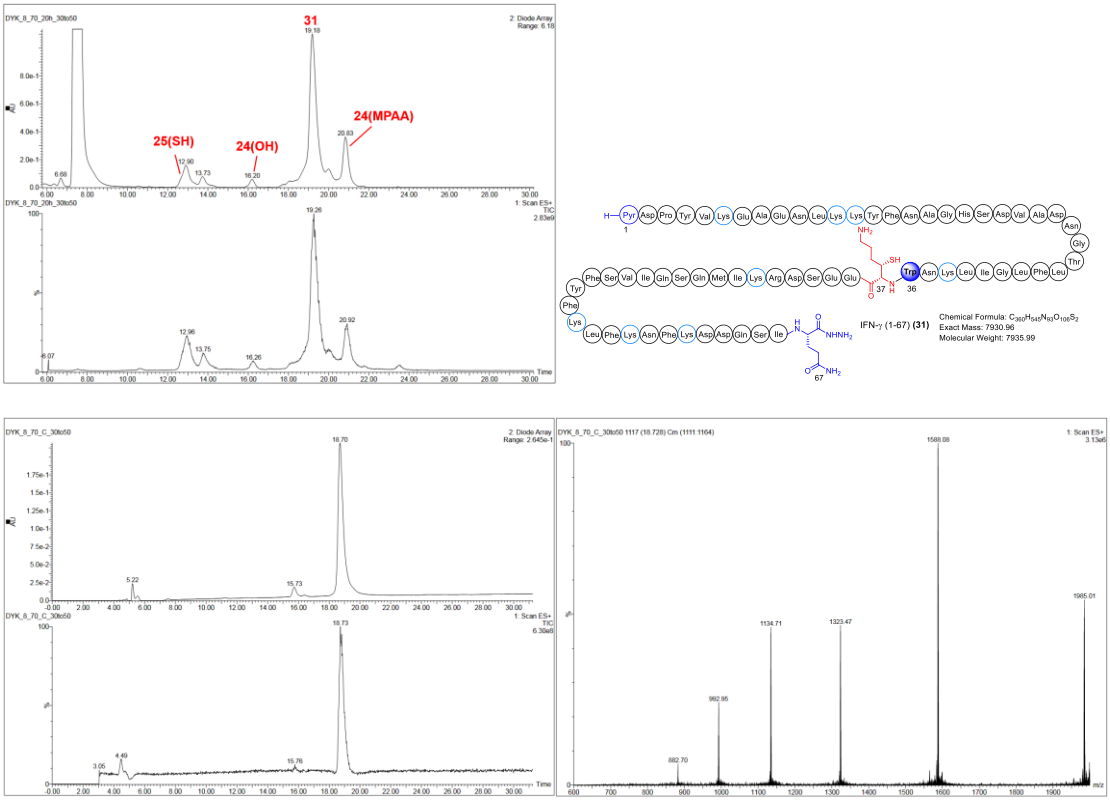
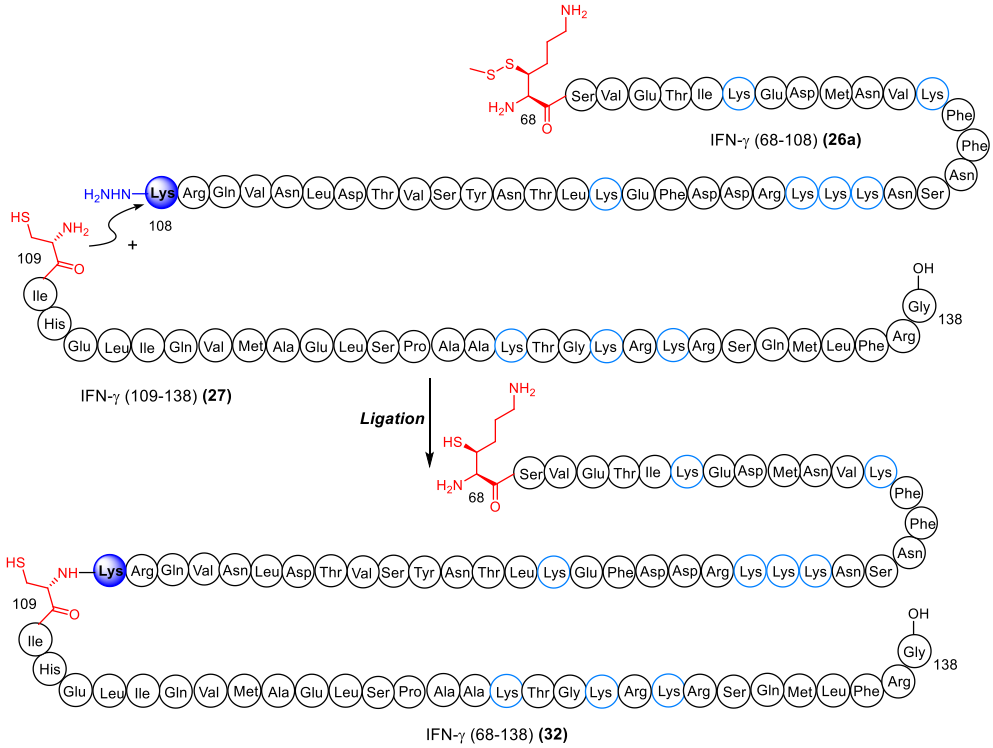
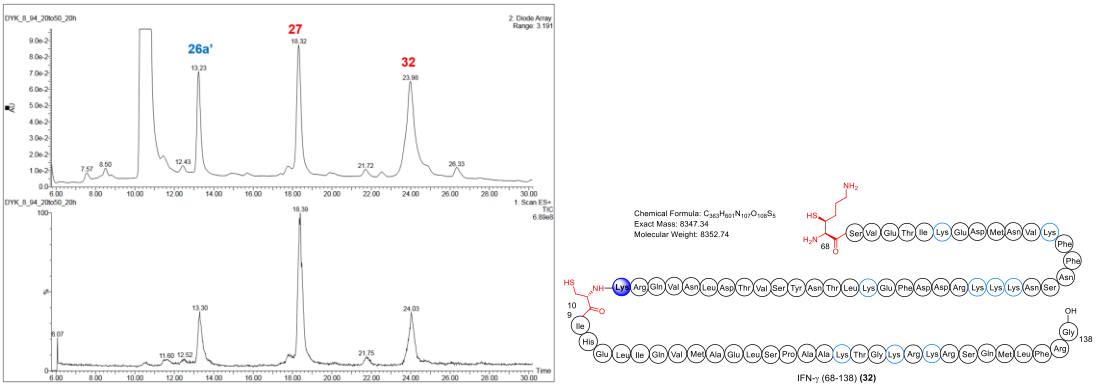


Figure S48. Top left: UV and MS traces of the ligation reaction between IFN-γ(1-36) (**24**) and IFN-γ(37-67) (**25**) after 20 h; Top right: Sequence and calculated data of IFN-γ(1-67) (**31**); Bottom left: UV and MS traces of the purified product IFN-γ(1-67) (**31**). Linear gradient: 30 to 50% solvent B over 30 min, Proto-300 C4 column, t_R = 18.7 min; Bottom right: ESI-MS data of the purified product IFN-γ(1-67) (**31**). Calcd for $C_{360}H_{545}N_{93}O_{106}S_2$: 7935.99 Da (average isotopes), (*m/z*) $[M+4H]^{4+}$: 1984.75, $[M+5H]^{5+}$: 1588.00, $[M+6H]^{6+}$: 1323.50, $[M+7H]^{7+}$: 1134.57, $[M+8H]^{8+}$: 992.88, $[M+9H]^{9+}$: 882.67; found: $[M+4H]^{4+}$: 1985.01, $[M+5H]^{5+}$: 1588.08, $[M+6H]^{6+}$: 1323.47, $[M+7H]^{7+}$: 1134.71, $[M+8H]^{8+}$: 992.95, $[M+9H]^{9+}$: 882.70.

Ligation reaction between of IFN-γ(68-108) (26a) and IFN-γ(109-138) (27)



According to General Procedure **3.8** described previously, 4.08 mg of IFN-γ(68-108) (**26a** 1.0 equiv) was dissolved in 200 μL of buffer A, the resulting solution was transferred into -15 °C bath, and 28 μL of 200 mM $NaNO_2$ (7.0 equiv) was added dropwise. After oxidized for 20 min, 4.34 mg of IFN-γ(109-138) (**27** 1.6 equiv) dissolved in 400 μL of buffer B was added, and adjusted pH to 7.0 with 2.0 M NaOH. The resulting reaction was warmed to room temperature and monitored by LC-MS. Upon completed consumption of **26a** after 20h, 400 μL of buffer C was added and reacted for 20 min. The reaction was quenched with 2.0 mL of CH_3CN/H_2O/AcOH (47.5/47.5/5, *v/v/v*) solution. There was cyclized peptide byproduct detected through LC-MS analysis, which was temporarily assigned as cyclized IFN-γ(68-108) (**26a'**) generated from self-ligation (Figure S49).



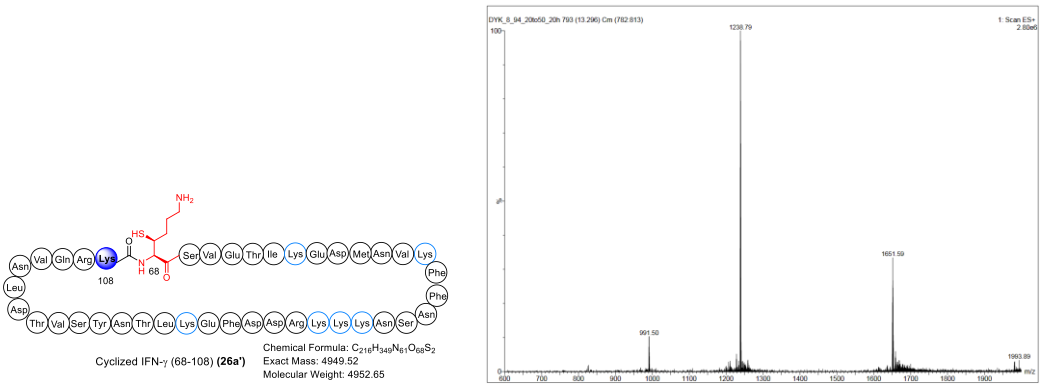


Figure S49. Top left: UV and MS traces of the ligation reaction between IFN-γ(38-108) (**26a**) and IFN-γ(109-138) (**27**) after 20 h. Linear gradient: 30 to 50% solvent B over 30 min, Proto-300 C4 column; Top right: Sequence and calculated data of IFN-γ(68-138) (**32**); Bottom left: Sequence and calculated data of cyclized IFN-γ(68-108) (**26a'**); Bottom right: ESI-MS data of cyclized IFN-γ(68-108) (**26a'**), t_R = 13.3 min (20 to 50% solvent B over 30 min, Proto-300 C4 column); ESI-MS: calcd for $C_{216}H_{349}N_{61}O_{68}S_2$: 4952.65 Da (average isotopes), (*m/z*) $[M+3H]^{3+}$: 1651.62, $[M+4H]^{4+}$: 1238.89, $[M+5H]^{5+}$: 991.31; found: $[M+3H]^{3+}$: 1651.59, $[M+4H]^{4+}$: 1238.79, $[M+5H]^{5+}$: 991.50.

The crude mixture was purified using preparative HPLC (30 to 50% solvent B over 30 min, Proto-300 C4 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford IFN-γ(68-138) (**32**) (1.89 mg, 28%) as a white solid.

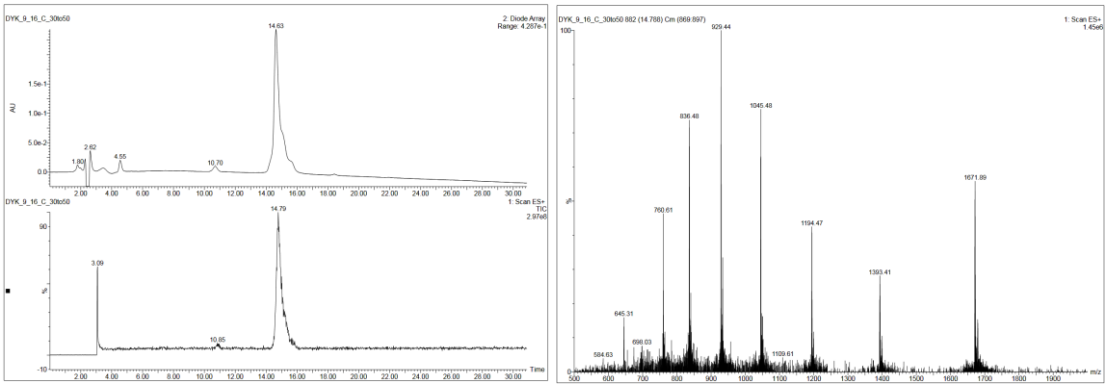
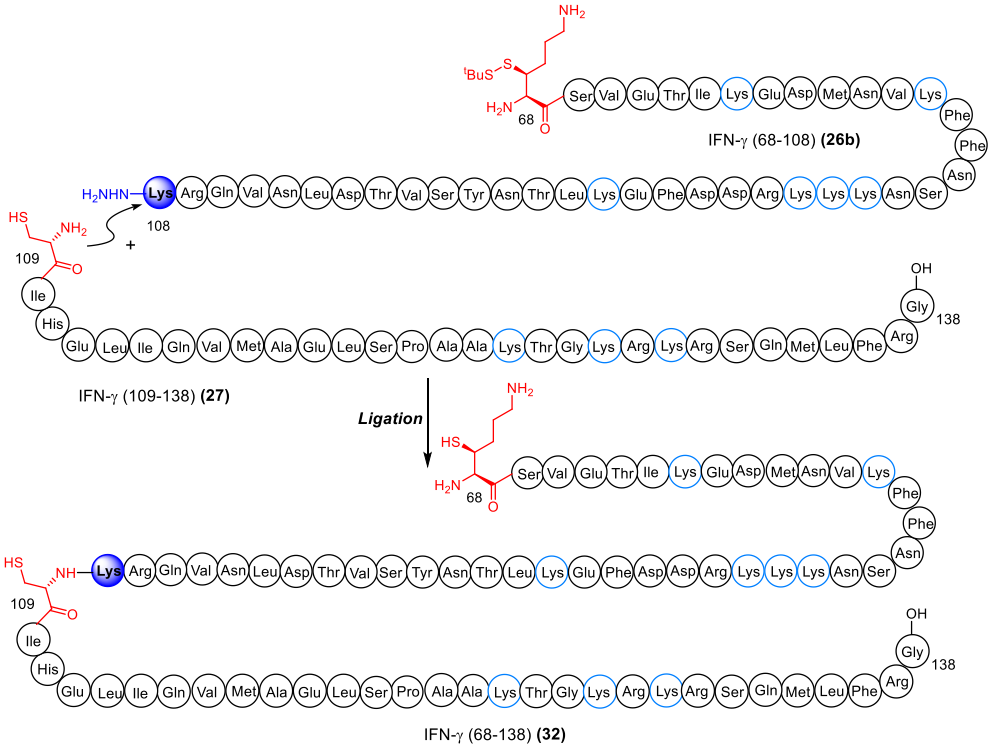


Figure S50. Left: UV and MS traces of the ligated peptide IFN-γ(68-138) (**32**). Linear gradient: 30 to 50% solvent B over 30 min, Proto-300 C4 column, t_R = 14.6 min; Right: ESI-MS data of the ligated peptide IFN-γ(68-138) (**32**). Calcd for $C_{363}H_{601}N_{107}O_{108}S_5$: 8352.74 Da (average isotopes), (*m/z*) $[M+5H]^{5+}$: 1671.28, $[M+6H]^{6+}$: 1392.90, $[M+7H]^{7+}$: 1194.06, $[M+8H]^{8+}$: 1044.93, $[M+9H]^{9+}$: 928.94, $[M+10H]^{10+}$: 836.14, $[M+11H]^{11+}$: 760.22; found: $[M+5H]^{5+}$: 1671.89, $[M+6H]^{6+}$: 1393.41, $[M+7H]^{7+}$: 1194.47, $[M+8H]^{8+}$: 1045.48, $[M+9H]^{9+}$: 929.44, $[M+10H]^{10+}$: 836.48, $[M+11H]^{11+}$: 760.61.

Ligation reaction between of IFN-γ(68-108) (26b) and IFN-γ(109-138) (27)



According to General Procedure **3.8** described previously, 8.27 mg of IFN-γ(68-108) (**26b**) (1.0 equiv) and 8.38mg of IFN-γ(109-138) (**27)** (1.5 equiv) were dissolved in 400 μL of buffer A, the resulting solution was transferred into -15 °C bath, and 57 μL of 200 mM $NaNO_2$ (7.0 equiv) was added dropwise. After oxidized for 20 min, 400 μL of buffer B was added, and adjusted pH to 7.0 with 2.0 M NaOH. The resulting reaction was warmed to room temperature and monitored by LC-MS. Upon completed consumption of **26b** after 21h, 300 μL of 0.5 M TCEP·HCl (pH 7.2) solution was added and reacted for 20 min. The reaction was quenched with 2.0 mL of CH_3CN/H_2O/AcOH (47.5/47.5/5, *v/v/v*) solution, and the cyclized IFN-γ(68-108) (**26b'**) was largely suppressed through LC-MS analysis (Figure S51). The crude mixture was purified using preparative HPLC (30 to 50% solvent B over 30 min, Proto-300 C4 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford IFN-γ(68-138) (**32**) (6.20 mg, 46%) as a white solid.

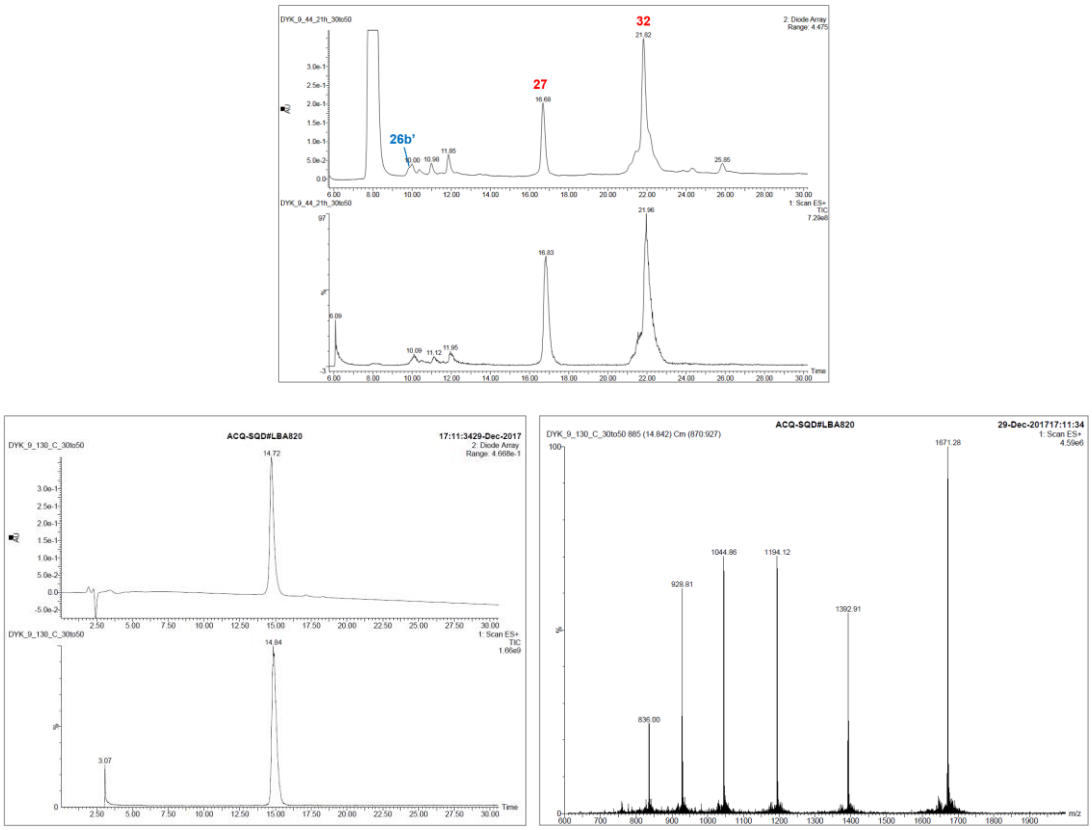
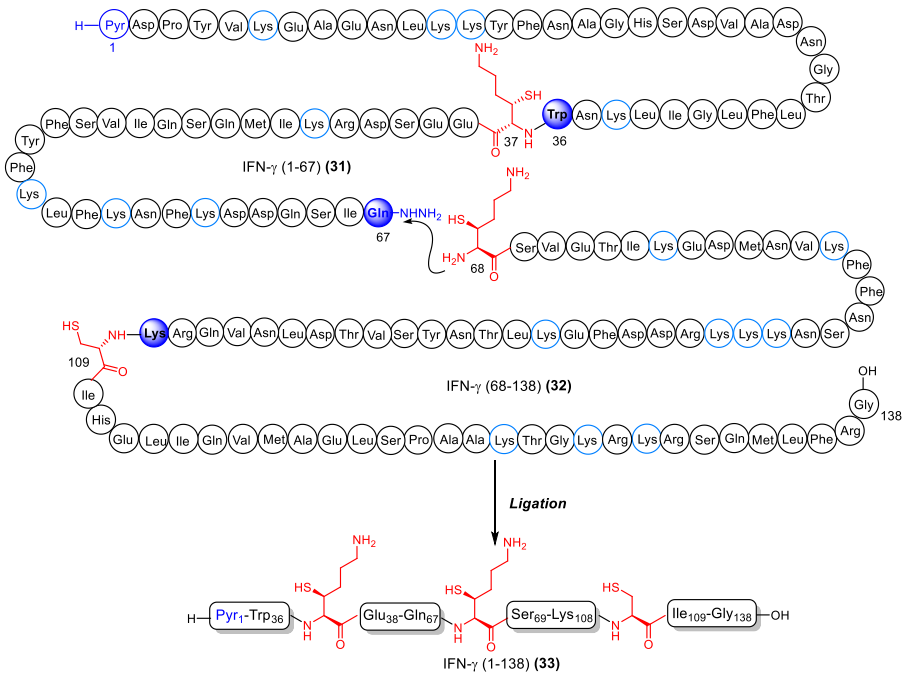


Figure S51. Top: UV and MS traces of the ligation reaction between IFN-γ(68-108) (**26b**) and IFN-γ(109-138) (**27**) after 21 h; Bottom left: UV and MS traces of the ligated peptide IFN-γ(68-138) (**32**). Linear gradient: 30 to 50% solvent B over 30 min, Proto-300 C4 column, t_R = 14.7 min; Right: ESI-MS data of the ligated peptide IFN-γ(68-138) (**32**); Bottom right: ESI-MS data of the ligated peptide IFN-γ(68-138) (**32**). Calcd for $C_{363}H_{601}N_{107}O_{108}S_5$: 8352.74 Da (average isotopes), (*m/z*) $[M+5H]^{5+}$: 1671.28, $[M+6H]^{6+}$: 1392.90, $[M+7H]^{7+}$: 1194.06, $[M+8H]^{8+}$: 1044.93, $[M+9H]^{9+}$: 928.94, $[M+10H]^{10+}$: 836.14, $[M+11H]^{11+}$: 760.22; found: $[M+5H]^{5+}$: 1671.28, $[M+6H]^{6+}$: 1392.91, $[M+7H]^{7+}$: 1194.12, $[M+8H]^{8+}$: 1045.86, $[M+9H]^{9+}$: 928.81, $[M+10H]^{10+}$: 836.00, $[M+11H]^{11+}$: 760.61.

Ligation reaction between of IFN-γ(1-67) (31) and IFN-γ(68-138) (32)



According to General Procedure **3.8** described previously, 4.52 mg of IFN-γ(1-67) (**31**) (1.0 equiv) and 5.19 mg of IFN-γ(68-138) (**32)** (1.1 equiv) were dissolved in 400 μL of buffer A. The resulting solution was transferred into -15 °C bath, and 20 μL of 200 mM $NaNO_2$ (7.0 equiv) was added dropwise. After oxidized for 20 min, 400 μL of buffer B was added, and adjusted pH to 7.0 with 2.0 M NaOH, the resulting reaction was warmed to room temperature and and monitored by LC-MS. After reacted for 30 h, 300 μL solution of buffer C was added and reacted for 20 min. The reaction was quenched with 2.0 mL of CH_3CN/H_2O/AcOH (47.5/47.5/5, *v/v/v*) solution, and analyzed with LC-MS (Figure S52).

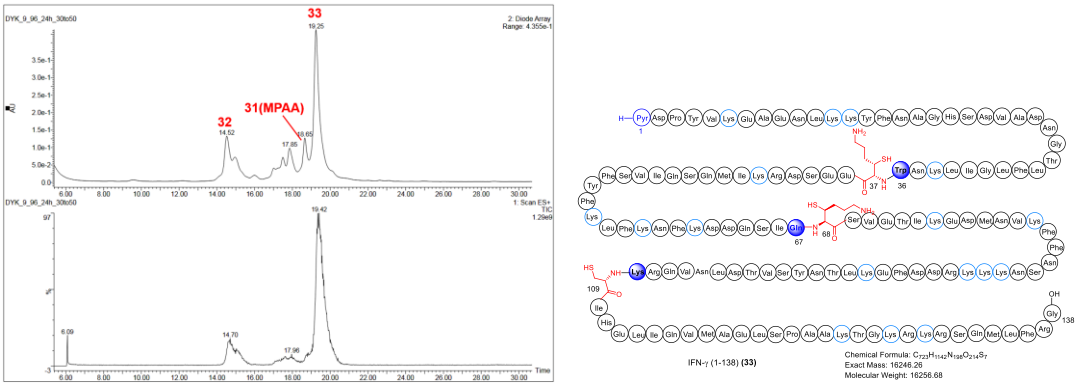


Figure S52. Left: UV and MS traces of the ligation reaction between IFN-γ(1-67) (**31**) and IFN-γ(68-138) (**32**) after 30 h. Linear gradient: 30 to 50% solvent B over 30 min, Proto-300 C4 column,; Right: Sequence and calculated data of IFN-γ(1-138) (**33**).

The crude mixture was purified using preparative HPLC (33 to 43% solvent B over 30 min, Proto-300 C4 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford protein **33** (4.33 mg, 47%) as a white solid.

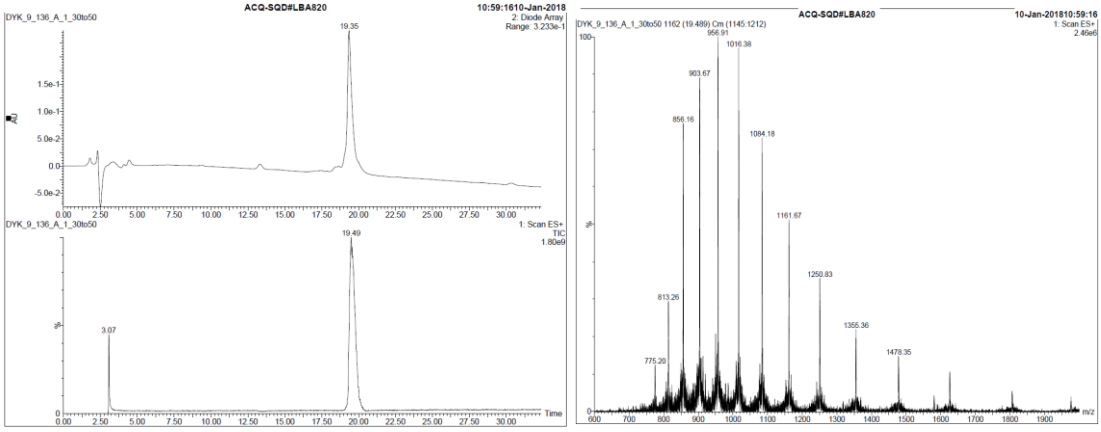
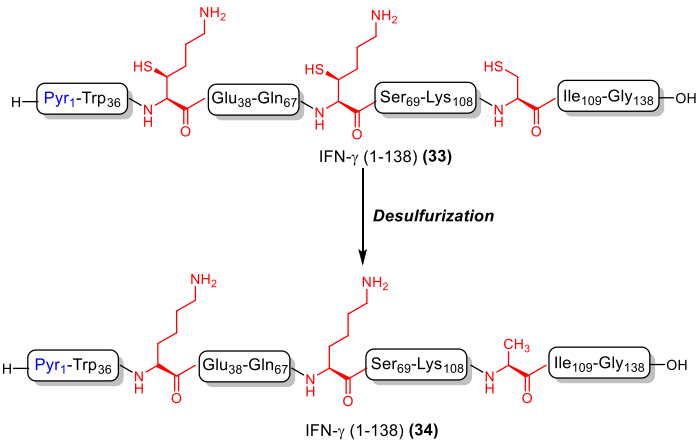


Figure S53. Left: UV and MS traces of the ligated protein **33**. Linear gradient: 30 to 50% solvent B over 30 min, Proto-300 C4 column, t_R = 19.4 min; Right: ESI-MS data of the purified peptide protein **33**. Calcd for $C_{723}H_{1142}N_{198}O_{214}S_7$: 16256.68 Da (average isotopes), (*m/z*) $[M+9H]^{9+}$: 1807.30, $[M+10H]^{10+}$: 1626.67, $[M+11H]^{11+}$: 1478.88, $[M+12H]^{12+}$: 1355.72, $[M+13H]^{13+}$: 1251.51, $[M+14H]^{14+}$: 1162.19, $[M+15H]^{15+}$: 1084.78, $[M+16H]^{16+}$: 1017.04, $[M+17H]^{17+}$: 957.28, $[M+18H]^{18+}$: 904.15, $[M+19H]^{19+}$: 856.61, $[M+20H]^{20+}$: 813.83, $[M+21H]^{21+}$: 775.13; found: $[M+9H]^{9+}$: 1807.44, $[M+10H]^{10+}$: 1626.67, $[M+11H]^{11+}$: 1478.35, $[M+12H]^{12+}$: 1355.36, $[M+13H]^{13+}$: 1250.83, $[M+14H]^{14+}$: 1161.67, $[M+15H]^{15+}$: 1084.18, $[M+16H]^{16+}$: 1016.38, $[M+17H]^{17+}$: 956.91, $[M+18H]^{18+}$: 903.67, $[M+19H]^{19+}$: 856.16, $[M+20H]^{20+}$: 813.26, $[M+21H]^{21+}$: 775.20.

Global desulfurization of protein 33



According to General Procedure **3.6**, 4.33 mg of protein **33** was dissolved in 200 µL of buffer (6 M Gn•HCl, 200 mM Na_2HPO_4, pH 7.2), 200 µL of 0.5 M Bond-breaker® TCEP solution (Pierce), 20 µL of 2-methyl-2-propanethiol and 100 µL of radical initiator VA-044 (0.1 M in degassed water) were added sequentially. The reaction mixture was stirred at 37 °C and monitored by LC-MS (Figure S54).

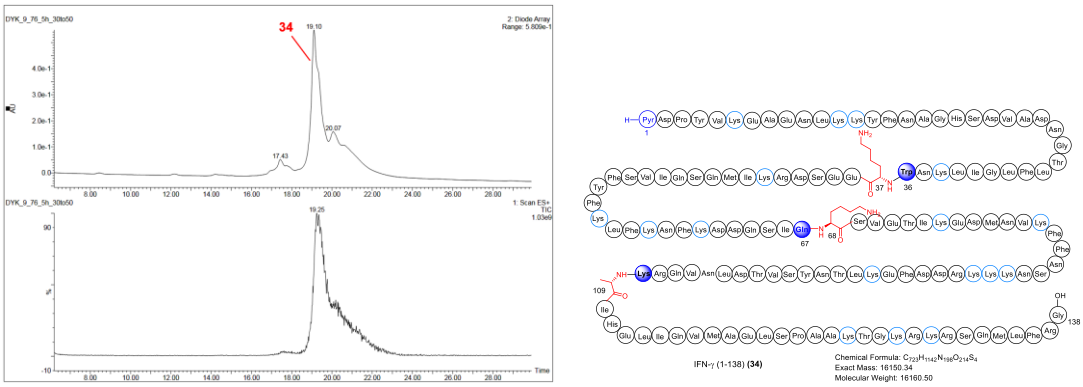


Figure S52. Top left: UV and MS traces of the desulfurization reaction of protein **33** after 5 h. Linear gradient: 30 to 50% solvent B over 30 min, Proto-300 C4 column; Top right: Sequence and calculated data of IFN-γ(1-138) (**34**).

Upon completion after 5 h, the reaction was quenched with 2.0 mL of H_2O/MeCN/AcOH (47.5:47.5:5, *v/v/v*) solution. The crude mixture was purified using preparative HPLC (33 to 43% solvent B over 30 min, Proto-300 C4 column). The fractions containing pure peptide were collected, and concentrated *via* lyophilization to afford IFN-γ(1-138) (**34**) (2.00 mg, 47%) as a white solid.

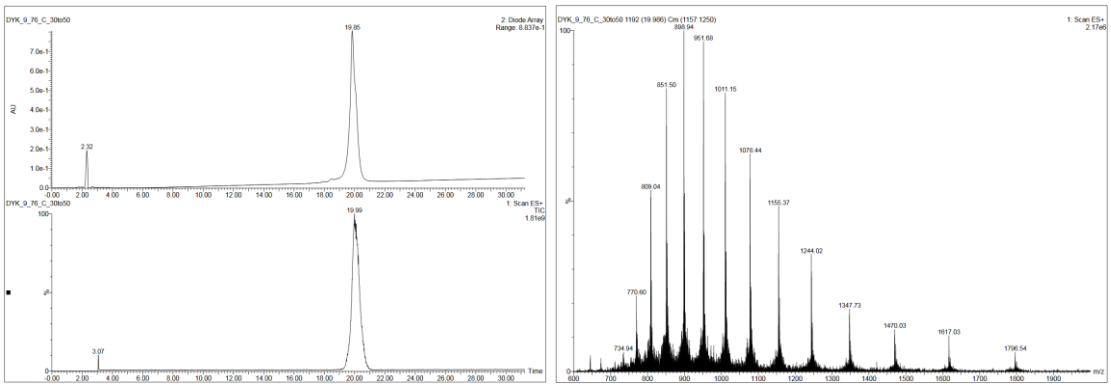
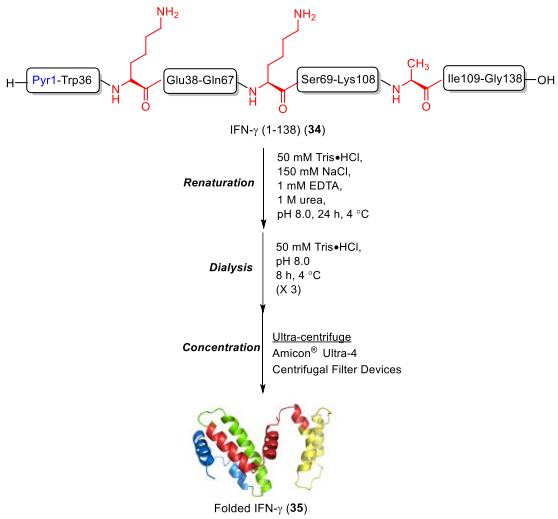


Figure S55. Left: UV and MS traces of the purified product IFN-γ(1-138) (**34**). Linear gradient: 30 to 50% solvent B over 30 min, Proto-300 C4 column, t_R = 19.9 min; Right: ESI-MS data of the purified IFN-γ(1-138) (**34**). Calcd for $C_{723}H_{1142}N_{198}O_{214}S_7$: 16256.68 Da (average isotopes), (*m/z*) $[M+9H]^{9+}$: 1796.05, $[M+10H]^{10+}$: 1616.54, $[M+11H]^{11+}$: 1469.68, $[M+12H]^{12+}$: 1347.29, $[M+13H]^{13+}$: 1243.93, $[M+14H]^{14+}$: 1154.96 $[M+15H]^{15+}$: 1078.03, $[M+16H]^{16+}$: 1010.72,

[M+17H]$^{17+}$: 951.32, [M+18H]$^{18+}$: 898.53, [M+19H]$^{19+}$: 851.29, [M+20H]$^{20+}$: 808.78, [M+21H]$^{21+}$: 770.31; found: [M+9H]$^{9+}$: 1796.54, [M+10H]$^{10+}$: 1617.03, [M+11H]$^{11+}$: 1470.03, [M+12H]$^{12+}$: 1347.73, [M+13H]$^{13+}$: 1244.02, [M+14H]$^{14+}$: 1155.37, [M+15H]$^{15+}$: 1078.44, [M+16H]$^{16+}$: 1011.15, [M+17H]$^{17+}$: 951.68, [M+18H]$^{18+}$: 898.94, [M+19H]$^{19+}$: 851.50, [M+20H]$^{20+}$: 809.04, [M+21H]$^{21+}$: 770.60.

Folding of IFN-γ(1-138) (34)



0.45 mg of IFN-γ(1-138) (**34**, 0.0278 μmol) was dissolved in degassed buffer (50 mM Tris•HCl, 150 mM NaCl, 1 mM EDTA, 1 M urea, pH 8.0). The solution (0.1 mg/mL) was gently stirred at 4 °C for 24 h, followed by dialysis against a Tris buffer (50 mM Tris•HCl, pH 8.0) at 4 °C for 8 h using a folding tube (MWCO 3,000). The buffer was then replaced with freshly prepared, and replaced again after dialysis for another 8 h. After dialysis for 24 h, the solution containing protein was concentrated by using centrifugal ultra filtration (WMCO 3,000). The concentration of folded IFN-γ (**35**) was 0.12 mg/mL as determined by a Bradford Protein Assay Kit (Solarbio). The folded IFN-γ (**35**) was further confirmed with LC-MS (Figure **S56**).

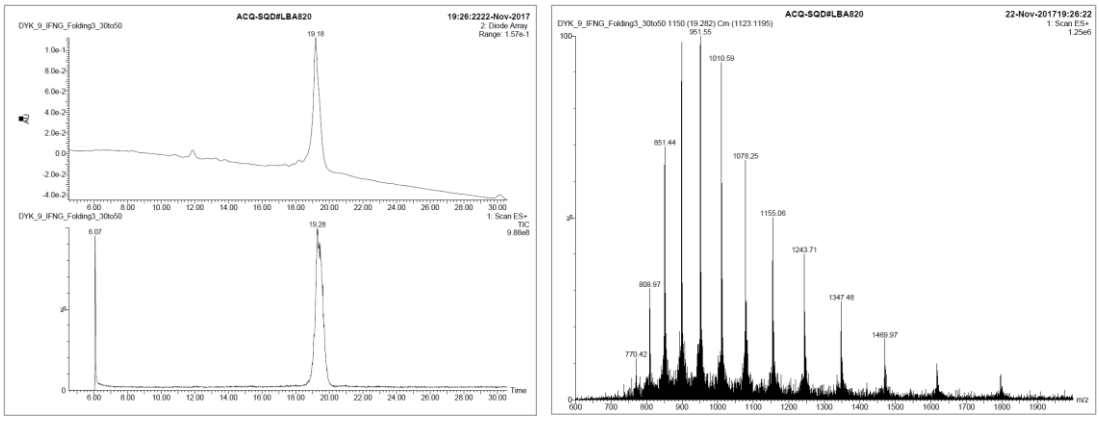
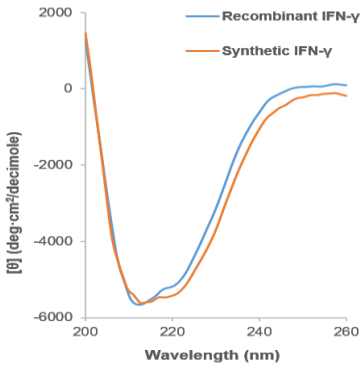


Figure S56. Left: UV and MS traces of the folded IFN-γ (**35**). Linear gradient: 30 to 50% solvent B over 30 min, Proto-300 C4 column, t_R = 19.2 min; Right: ESI-MS data of the folded IFN-γ (**35**). calcd for $C_{723}H_{1142}N_{198}O_{214}S_7$: 16256.68 Da (average isotopes), (*m/z*) $[M+9H]^{9+}$: 1796.05, $[M+10H]^{10+}$: 1616.54, $[M+11H]^{11+}$: 1469.68, $[M+12H]^{12+}$: 1347.29, $[M+13H]^{13+}$: 1243.93, $[M+14H]^{14+}$: 1154.96 $[M+15H]^{15+}$: 1078.03, $[M+16H]^{16+}$: 1010.72, $[M+17H]^{17+}$: 951.32, $[M+18H]^{18+}$: 898.53, $[M+19H]^{19+}$: 851.29, $[M+20H]^{20+}$: 808.78, $[M+21H]^{21+}$: 770.31; found: $[M+9H]^{9+}$: 1796.10, $[M+10H]^{10+}$: 1616.66, $[M+11H]^{11+}$: 1469.97, $[M+12H]^{12+}$: 1347.48, $[M+13H]^{13+}$: 1243.71, $[M+14H]^{14+}$: 1155.06, $[M+15H]^{15+}$: 1078.25, $[M+16H]^{16+}$: 1010.59, $[M+17H]^{17+}$: 951.55, $[M+18H]^{18+}$: 898.82, $[M+19H]^{19+}$: 851.44, $[M+20H]^{20+}$: 808.97, $[M+21H]^{21+}$: 770.42.

CD spectra analysis of IFN-γ(1-138) (35)

CD spectra were obtained on a JASCO J-810 circular dichroism spectrometer (Japan). The filtrate after centrifugal ultra filtration mentioned above was used as the reference solution, and for the dissolving test samples. A standard sample of recombinant IFN-γ (0.12 mg/mL, Genscript) and a sample of synthetic IFN-γ (**35**) (0.12 mg/mL) were detected seperately. The spectra were collected using a 1 mm pathlength cuvette, the wavelength range was from 200 nm to 260 nm, and each spectrum was scanned for 3 times, and the spectra were plotted as the average of three runs.



M-QDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKN
0 1
WKEESDRKIMQSQIVSFYFKLFKNFKDDQSIQKSVE
TIKEDMNVKFFNSNKKKRDDFEKLTNYSVTDLNVQR
KAIHELIQVMAELSPAAKTGKRKRSQMLFRG-RRASQ
138 143
Primary sequence of recombinant IFN-γ

Figure S56. Left: CD spectra of the recombinant IFN-γ and the synthetic IFN-γ (**35**). Right: Primary sequence of recombinant IFN-γ purchased from Genscript bearing one Met_0 at *N*-termini and five amino acid residues (Arg_{139}-Gln_{143}) at *C*-termini.

SDS-PAGE analysis of IFN-γ(1-138) (35)

The recombinant IFN-γ (Genscript) and synthetic IFN-γ (**35**) were analyzed by reducing SDS-PAGE using 4-12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsdad, CA). Sample were combined in a 3:1 ratio with NuPAGE sample buffer and electrophoresed at 200 V (constant voltage) in MES buffer(Invitrogen) for 35 min. Gels were stained with Coomassie Blue.

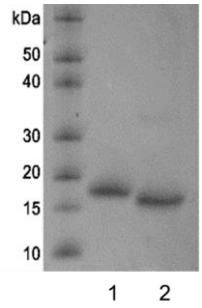


Figure S57. Left: SDS-PAGE of the recombinant IFN-γ (lane 1) and the synthetic IFN-γ (**35**) (lane 2).

VIII. Anti-proliferation Assay

Human HeLa cell line was obtained from Prof. Mo Li's group (Peking University), and maintained in DMEM media (Corning) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 unit/mL penicillin. HeLa cells were diluted into 2 × 10^5 cells/mL and 100 μL media was added to each well (2 ×10^4 cells). For measurement, 5 dilutions of samples ranging from 16 to 10000 ng/mL were prepared in medium and were added in triplicate into a 96-well microplate with 100 μL per well, PBS buffer as control. After incubation for 48 hours at 37 °C, the CCK-8 method was employed to measure the cell viability. The absorbance was measured by Multiskan™ GO (Thermo Scientific™).