Supporting Information

Peptide/Protein Stapling and Unstapling: Introduction of *s*-Tetrazine, Photochemical Release, and Regeneration of the Peptide/Protein

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General Methods

Chemicals. Organic solvents used for reactions and washes were of reagent grade and degassed by purging with nitrogen prior to use. Di-*tert*-butyldicarbonate (Boc_2O), diisopropylethylamine (DIPEA), piperidine, Thioredoxin (Trx), were obtained from Aldrich Chemical Co. and used as recieved. Fmoc-protected amino acids, *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium (HBTU), were purchased from Chem-Impex International and used as received. Dichloromethane (CH_2Cl_2), dimethylformamide (DMF), methanol (MeOH), trifluoroacetic acid (TFA) were purchased from Fisher Scientific. Ethyl cyanoglyoxylate-2-oxime (oxyma), Rink Novagel® resin and 2-chloro-chlorotrityl resin were obtained from Novabiochem. Dichlorotetrazine was synthesized via procedure reported by Coburn, M. D.; Buntain, G. A.; Harris, B. W.; Hiskey, M. A.; Lee, K. Y.; Ott, D. G. *J. Heterocycl. Chem.* **1991**, *28*, 2049.

Reaction Equipment. Solid-phase syntheses were carried out in peptide synthesis reaction vessels (25 or 50 mL) with coarse porosity fritted glass support and Teflon stopcocks. Photolysis experiments were performed in a Rayonet™ Srinivasan-Griffin Photoreactor (The Southern New England Ultraviolet Company) using either UV-A lamps (part # LZC-UVA) or UV-B lamps (part # LZC-UVB) purchased from Luzchem.

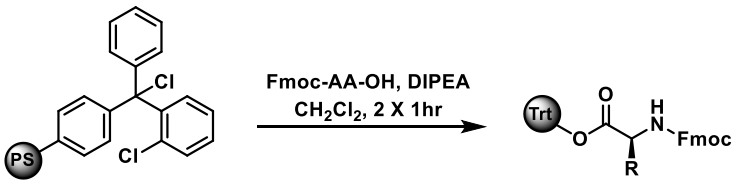
Resin Washing Procedures. Resin washing was conducted with the indicated solvent and was allowed to contact the resin for 30 seconds during each wash. The solvent was pushed through the frit using an "air push" apparatus made from a 15 mL disposable syringe and a 14/20 septum, or nitrogen gas was used in cases when an inert atmosphere is a requirement.

Chromatography. Prep-scale reverse-phase chromatography was conducted with a Gilson 215 liquid handler/injector fitted with Gilson 333/334 binary HPLC pumps and UV/vis dual wavelength detector (model 156) and Trilution software. The chromatographies were carried out on a Waters XBridge Prep BEH 130 C18 5µm OBD 19 × 100mm column (part # 186003587). The eluent was acetonitrile (HPLC grade) and Millipore water with 0.1% trifluoroacetic acid buffer unless otherwise noted and gradients specific to the compound.

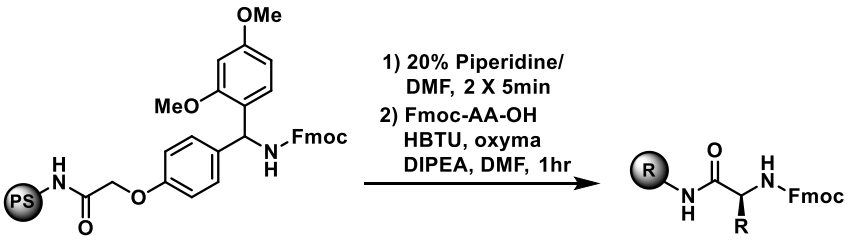
Instruments Used for Spectral Data. ^{1}H NMR, ^{13}C NMR and 2D NMR spectra were recorded on a Bruker Avance III equipped with either a 5 mm dual inverse probe or 5 mm DCH CryoProbe. The analytical LC-MS analyses were conducted using a Waters 2767 sample manager, consisting of a Waters 2525 binary gradient HPLC connected to a diode array detector and a Waters Micromass ZQ mass spectrometer with

electro-spray ionization. The LC-MS samples were analyzed as solutions in water or acetonitrile, prepared at 0.15 – 0.20 mg/mL concentration. The LC-MS chromatography was carried out on an Atlantis–C18 column (4.6 ×50 mm; 5 µm) with linear gradients of 0.05% formic acid in acetonitrile and 0.05% formic acid water. High resolution mass spectrometry was obtained on Waters LC-TOF mass spectrometer (model LCT-XE Premier) using electrospray ionization in positive or negative mode, depending upon the analyte. MALDI-MS spectra were collected with a Bruker Ultraflex III TOF/TOF matrix-assisted laser desorption/ionization mass spectrometer. All FTIR spectra were taken on a Nicolet 6700 FTIR spectrometer or PerkinElmer FTIR (model Spectrum BX).

General Procedures for the Resin Loading

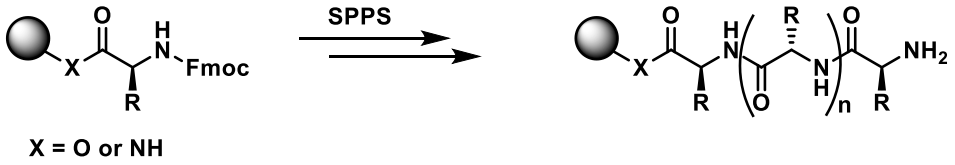


2-Chlorotrityl Chloride Resin Amino Acid Loading. 2-Chlorotrityl chloride resin (0.40 mmol) was placed in a peptide synthesis vessel and the resin was swelled in CH_2Cl_2 (10 mL) for 1 h. The solvent was drained and the resin was then treated with a pre-mixed solution of Fmoc-AA-OH (0.48 mmol, 1.2 equiv), and DIPEA (1.6 mmol, 4 equiv) dissolved in CH_2Cl_2 (5 mL) was added to the resin. The contents were rocked gently for 1 h, then drained and the resin washed with DMF (3 × 5 mL). The coupling procedure was repeated and the resin carried on to the next step.



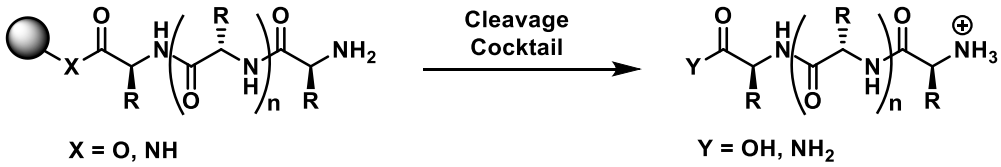
Rink Resin Amino Acid Loading. Rink Novagel resin (0.10 mmol) was placed in a peptide synthesis vessel and the resin was swelled in CH_2Cl_2 (10 mL) for 1 h. The solvent was drained and the resin was washed with DMF (3 × 6 mL) then treated with 20% piperidine/DMF (2 × 6 mL) allowing the solution to contact the resin for 10 minutes. The resin was washed with DMF (5 × 6 mL) and a pre-mixed solution of Fmoc-protected amino acid (0.50 mmol, 5 equiv), HBTU (190 mg, 0.5 mmol, 5 equiv), oxyma (71 mg, 0.5 mmol, 5 equiv) and DIPEA (174 µL, 1.0 mmol, 10 equiv) dissolved in DMF (4 mL) was added to the resin. The contents were rocked gently for 1 h, then drained and the resin washed with DMF (3 × 6 mL).

General Procedures for Manual Solid-Phase Peptide Synthesis



Solid-Phase Peptide Synthesis (SPPS). The resin-bound Fmoc-amino acid (0.1 mmol) was washed with DMF (3 × 5 mL) and then treated with a solution of 20% piperidine/DMF (2 × 6 mL) allowing each treatment to contact the resin for 5 minutes. The resin was washed with DMF (5 × 6 mL), then a pre-mixed solution of Fmoc-protected amino acid (0.5 mmol, 5.0 equiv), HBTU (190 mg, 0.5 mmol, 5.0 equiv), oxyma (71 mg, 0.5 mmol, 5.0 equiv) and DIPEA (174 μL, 1.0 mmol, 10.0 equiv) dissolved in DMF (4 mL) was added to the resin. The contents were rocked gently for 1 h, then drained and the resin washed with DMF (3 × 5 mL). The Fmoc deprotection procedure was repeated followed by the coupling of the next amino acid in the sequence to synthesize the desired peptide.

General Procedures for Peptide Cleavage and Global Deprotection

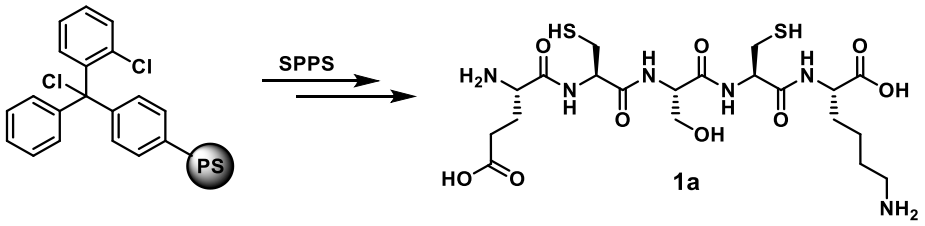


Cleavage Cocktail A: TFA/EDT/TIPSH/water (92.5: 2.5: 2.5: 2.5) Peptides with Cys

Cleavage Cocktail B: TFA/thioanisole/EDT/water (87.5: 5: 5: 2.5) Peptides with Cys & Trp, Arg

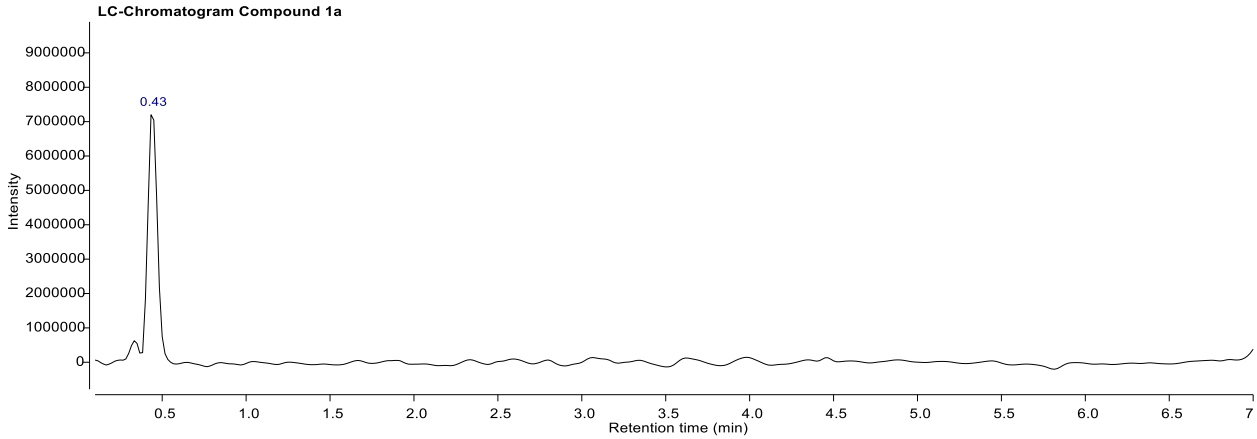
Cleavage Cocktail C: TFA/thioanisole/EDT/TIPSH/water (87.5:5:2.5:2.5:2.5) Peptides with Cys & Met

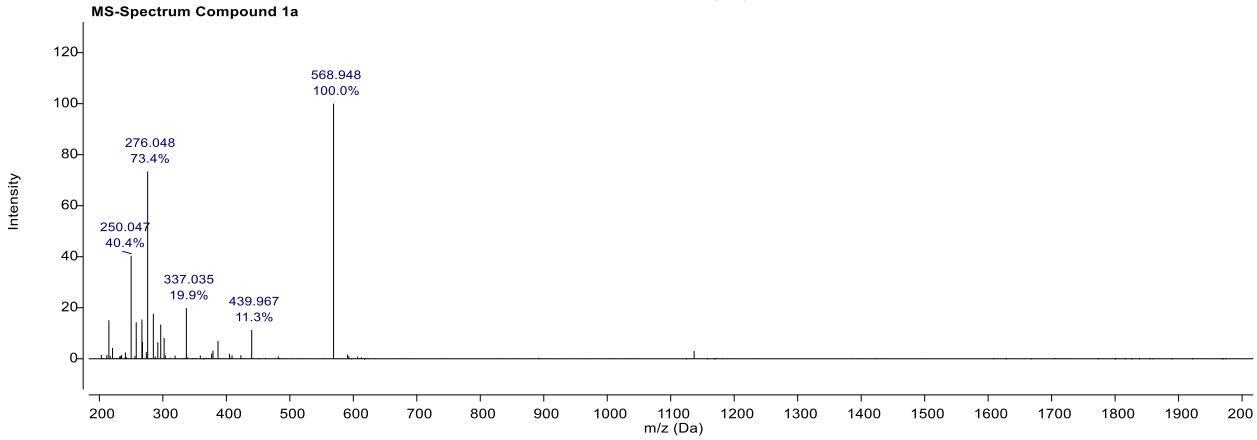
The resin-bound peptide (~0.1 mmol) was pre-swelled in CH_2Cl_2 for 30 minutes and then treated with cleavage cocktail A, B or C (7 mL) and stirred under a nitrogen atmosphere for 4 hours. The filtrate was collected and additional cleavage cocktail (3 × 1 mL) was used to wash the resin. The pooled filtrates were condensed (ca. 1 mL) and Et_2O (15 mL) was added to precipitate the peptide. The white precipitate was collected by vacuum filtration and the solids wash with additional Et_2O. The crude peptide was dried *in vacuo* overnight and purified by reverse-phase high-pressure liquid chromatography (HPLC).

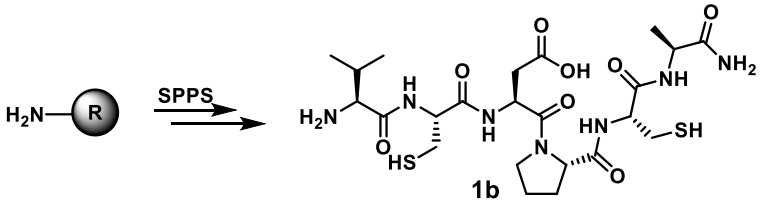


Peptide 1a, was constructed by SPPS from 1.0 mmol loaded 2-chloro-chlorotrityl resin. Removal of the peptide from resin was conducted with cocktail A (20 mL) following the general cleavage method. The peptide was purified by reverse-phase HPLC (5 - 15% organic over 5 min) to give 360 mg (45% •2 TFA salt form) of a white amorphous powder after lyophilization: HRMS (ES) found *m/z* 569.2067 [$(M+H)^+$; calcd for $C_{28}H_{37}N_6O_9S_2$: 569.2063]; ^{1}H NMR (500 MHz, D_2O) δ ppm 1.38 - 1.48 (m, 2 H) 1.68 (quin, *J* = 7.3 Hz, 2 H) 1.72 - 1.81 (m, 1 H) 1.85 - 1.94 (m, 1 H) 2.19 (q, *J* = 7.3 Hz, 2 H) 2.55 (t, *J* = 7.3 Hz, 2 H) 2.90 - 3.02 (m, 6 H) 3.89 (t, *J* = 4.9 Hz, 2 H) 4.14 (t, *J* = 6.5 Hz, 1 H) 4.32 (dd, *J* = 8.9, 5.2 Hz, 1 H) 4.52 (t, *J* = 5.4 Hz, 1 H) 4.58 (t, *J* = 6.2 Hz, 1 H) 4.61 (t, *J* = 6.30 Hz, 1 H); ^{13}C NMR (126MHz, D_2O) δ 176.6, 176.2, 171.6, 171.5, 171.4, 169.4, 61.1, 55.8, 55.7, 55.6, 53.4, 52.4, 39.3, 30.3, 29.4, 26.3, 26.1, 25.5, 25.4, 22.2**;** IR (KBr, cm^{-1}) 3425 (br), 3286 (br), 3077 (br), 2950 (br), 1682 (s), 1628(s), 1535 (m), 1429 (m), 1207 (s), 1132 (s).

Gradient 5-60% MeCN, 7 min, 2mL/min

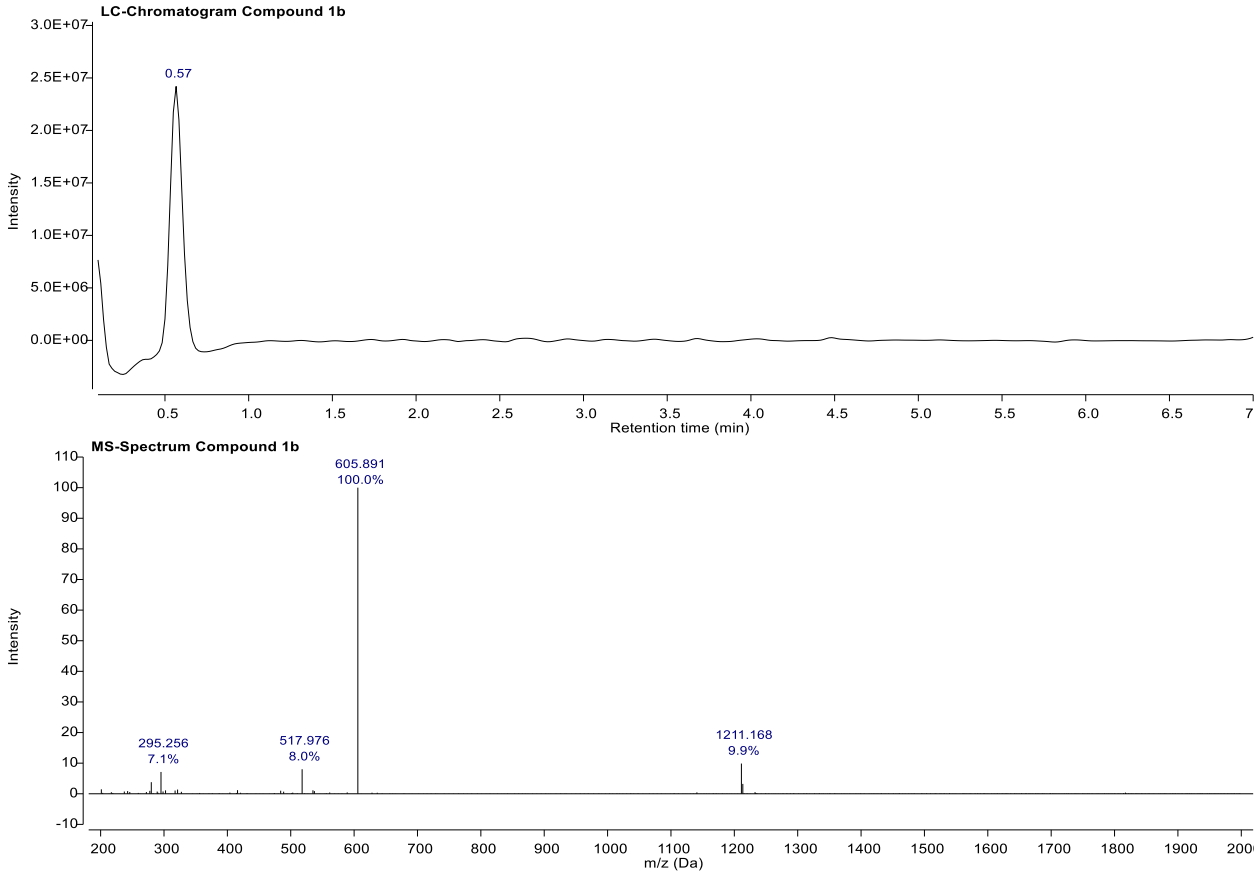


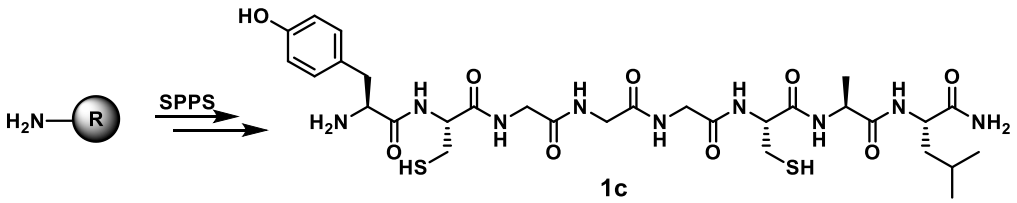




Peptide 1b was constructed by SPPS from 0.10 mmol loaded Rink amide resin. Removal of the peptide from resin was conducted with cocktail A following the general cleavage method. The peptide was purified by reverse-phase HPLC (5 - 35% organic over 12 min) to give 46.4 mg (65% • TFA salt form) of a white amorphous powder after lyophilization: HRMS (ES) Found *m/z* 606.2378 [$(M+H)^+$; calcd for $C_{23}H_{40}N_7O_8S_2$: 606.2380]; ^{1}H NMR (500MHz ,DMSO-d_6) δ 8.55 (br. s., 1 H), 8.52 (d, *J* = 7.3 Hz, 2 H), 8.08 (d, *J* = 7.3 Hz, 1 H), 7.74 (d, *J* = 7.5 Hz, 1 H), 7.06 (s, 2 H), 7.02 (s, 2 H), 4.78 (q, *J* = 6.9 Hz, 1 H), 4.44 (dd, *J* = 5.4, 11.3 Hz, 1 H), 4.27 (dd, *J* = 3.8, 8.5 Hz, 1 H), 4.23 (dd, *J* = 4.8, 8.1 Hz, 1 H), 4.21 (dd, *J* = 4.8, 8.3 Hz, 1 H), 4.11 (quin, *J* = 7.2 Hz, 1 H), 3.71 (t, *J* = 6.3 Hz, 2 H), 3.55 (d, *J* = 5.5 Hz, 1 H), 2.82 (d, *J* = 4.6 Hz, 1 H), 2.83 (dd, *J* = 4.8, 13.7 Hz, 1 H), 2.76 (br. s., 1 H), 2.77 (dd, *J* = 8.5, 15.0 Hz, 1 H), 2.75 (br. s., 1 H), 2.71 (dd, *J* = 7.1, 16.4 Hz, 1 H), 2.42 (dd, *J* = 6.9, 16.2 Hz, 1 H), 2.11 - 2.00 (m, 2 H), 1.99 - 1.92 (m, 1 H), 1.92 - 1.84 (m, 2 H), 1.23 (d, *J* = 7.1 Hz, 3 H), 0.92 (d, *J* = 6.7 Hz, 3 H), 0.89 (d, *J* = 6.7 Hz, 3 H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.1, 171.9, 171.7, 169.2, 168.9, 167.9, 60.2, 57.2, 55.5, 54.9, 48.3, 47.8, 47.0, 35.6, 29.9, 29.1, 26.3, 25.8, 24.5, 18.4, 18.0, 17.7; IR (KBr, cm^{-1}) 3332(br), 3067(m), 2974(m), 1664(vs), 1525(m), 1451(w), 1200(m), 1132(w).

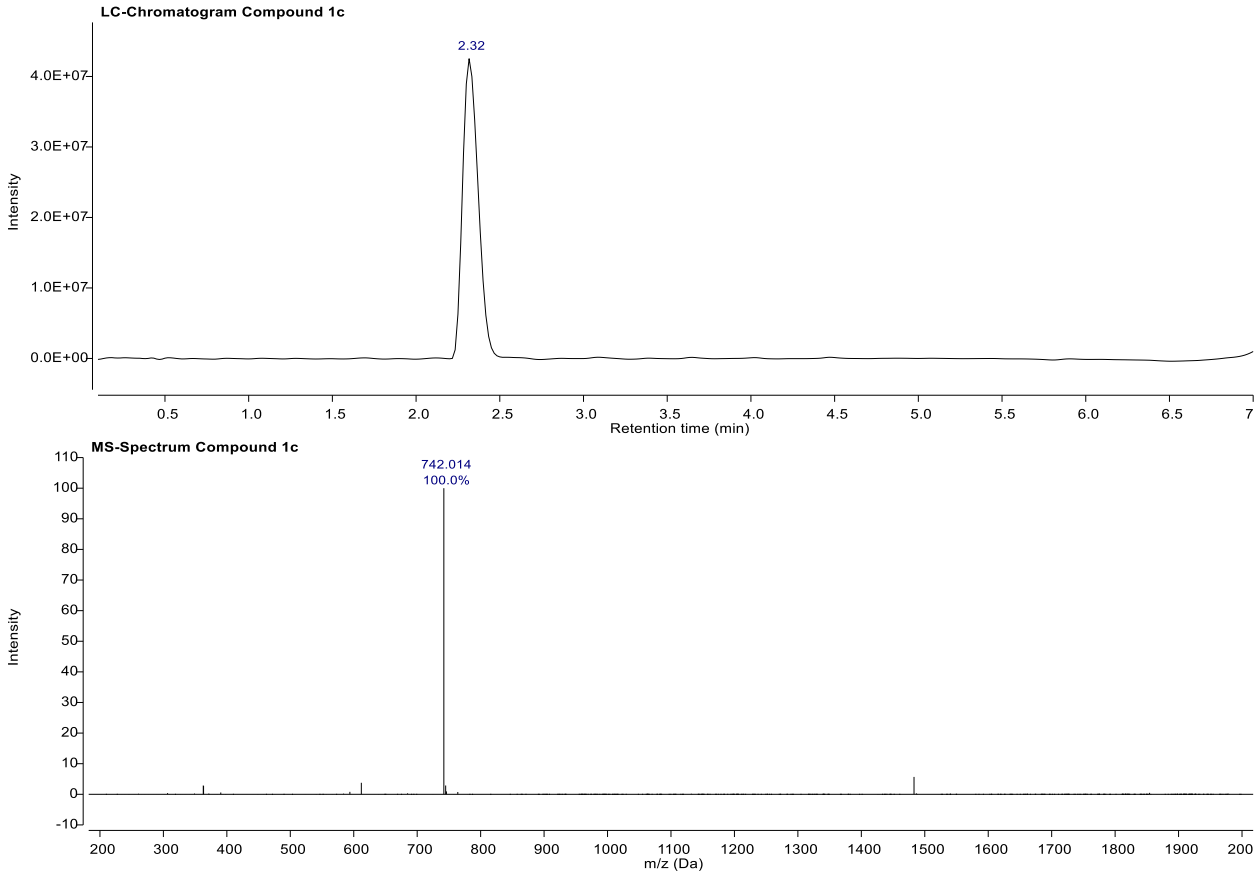
Gradient 5-60% MeCN, 7 min, 2mL/min

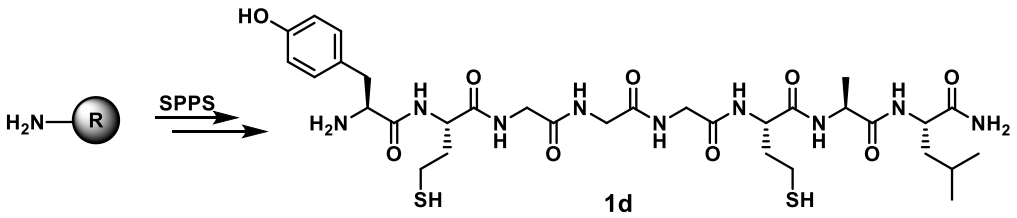




Peptide 1c was constructed by SPPS from 0.10 mmol loaded Rink amide resin. Removal of the peptide from resin was conducted with cocktail A following the general cleavage method. The peptide was purified by reverse-phase HPLC (5 - 35% organic over 15 min) to give 39.2 mg (46% • TFA salt form) of a white amorphous powder after lyophilization: HRMS (ES) Found *m/z* 742.3011 $[(M+H)^+$; calcd for $C_{30}H_{48}N_9O_9S_2$: 742.3016]; ^{1}H NMR (500MHz ,DMSO-d_6) δ = 9.37 (br. s., 1 H), 8.82 (d, *J* = 7.7 Hz, 1 H), 8.31 (br. t, *J* = 5.6 Hz, 2 H), 8.22 (d, *J* = 7.1 Hz, 1 H), 8.18 (t, *J* = 5.0 Hz, 1 H), 8.10 (d, *J* = 7.1 Hz, 2 H), 7.73 (d, *J* = 8.1 Hz, 1 H), 7.23 (s, 1 H), 7.05 (d, *J* = 8.1 Hz, 2 H), 6.97 (s, 1 H), 6.71 - 6.67 (m, *J* = 8.1 Hz, 2 H), 4.49 (dd, *J* = 6.7, 13.7 Hz, 1 H), 4.41 (dd, *J* = 7.3, 13.1 Hz, 1 H), 4.23 (dd, *J* = 7.1, 14.3 Hz, 1 H), 4.19 (dd, *J* = 8.1, 15.7 Hz, 1 H), 4.04 (t, *J* = 5.9 Hz, 1 H), 3.85 (dd, *J* = 5.7, 16.6 Hz, 1 H), 3.77 (d, *J* = 5.4 Hz, 6 H), 3.75 (dd, *J* = 5.5, 17.0 Hz, 1 H), 3.01 (dd, *J* = 5.2, 14.3 Hz, 1 H), 2.85 - 2.74 (m, 4 H), 2.75 - 2.65 (m, 1 H), 2.40 (t, *J* = 8.4 Hz, 1 H), 1.58 (quind, *J* = 6.6, 13.3 Hz, 1 H), 1.45 (t, *J* = 7.2 Hz, 2 H), 1.22 (d, *J* = 7.1 Hz, 3 H), 0.87 (d, *J* = 6.7 Hz, 3 H), 0.82 (d, *J* = 6.5 Hz, 3 H); ^{13}C NMR (126MHz ,DMSO-d_6) δ 174.0, 171.7, 169.5, 169.3, 169.2, 169.0, 168.9, 168.3, 156.6, 130.5, 124.7, 115.4 (2C), 55.1, 54.9, 53.6, 50.9, 48.7, 42.0, 42.0, 42.0, 41.0, 36.2, 26.2, 26.2, 24.2, 23.1, 21.6, 17.6; IR (KBr, cm^{-1}) 3306(br), 2957(m), 1661(vs), 1518(s), 1202(m), 1136(w).

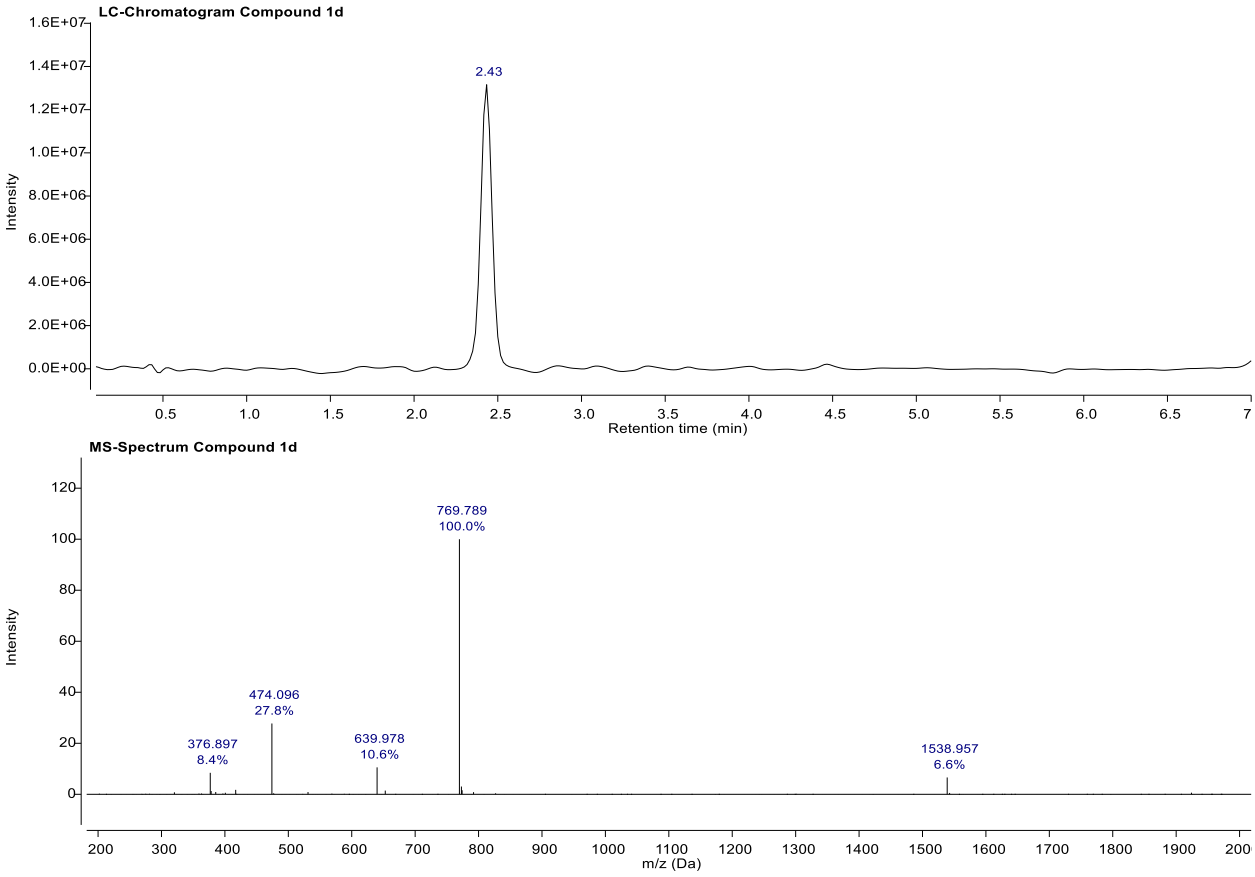
Gradient 5-60% MeCN, 7 min, 2mL/min

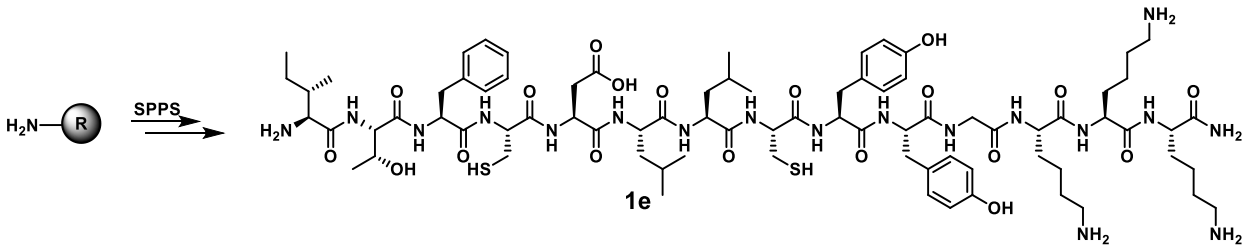




Peptide 1d was constructed by SPPS from 0.10 mmol loaded Rink amide resin. Removal of the peptide from resin was conducted with cocktail A following the general cleavage method. The peptide was purified by reverse-phase HPLC (5 - 35% organic over 15 min) to give 43.8 mg (50% • TFA salt form) of a white amorphous powder after lyophilization: HRMS (ES) Found *m/z* 770.3336 [$(M+H)^+$; calcd for $C_{40}H_{487}N_7O_7S_2$: 770.3336]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.33 (s, 1H), 8.67 (d, *J* = 8.0 Hz, 1H), 8.18 (t, *J* = 5.8 Hz, 1H), 8.14 (q, *J* = 5.5 Hz, 2H), 8.09 (d, *J* = 7.2 Hz, 1H), 8.06 – 7.98 (m, 3H), 7.69 (d, *J* = 8.4 Hz, 1H), 7.22 (s, 1H), 7.03 (d, *J* = 8.5 Hz, 2H), 6.94 (s, 1H), 6.69 (d, *J* = 8.5 Hz, 2H), 4.47 (ddd, *J* = 8.2, 5.3 Hz, 1H), 4.38 (ddd, *J* = 8.3, 5.1 Hz, 1H), 4.28 – 4.13 (m, 2H), 4.02 – 3.93 (m, 1H), 3.83 (dd, *J* = 16.6, 5.8 Hz, 1H), 3.79 – 3.68 (m, 4H), 2.99 (dd, *J* = 14.2, 5.4 Hz, 1H), 2.79 (dd, *J* = 13.8, 8.5 Hz, 1H), 2.68 (s, 0H), 2.48 – 2.40 (m, 2H), 2.37 (t, *J* = 8.0 Hz, 1H), 2.29 (t, *J* = 8.0 Hz, 1H), 2.08 – 1.74 (m, 4H), 1.58 (dt, *J* = 13.4, 6.7 Hz, 1H), 1.45 (dd, *J* = 8.4, 6.2 Hz, 2H), 1.21 (d, *J* = 7.1 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 3H), 0.83 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (126 MHz, DMSO) δ 173.9, 171.7, 170.7, 170.5, 169.1, 168.9, 168.8, 168.1, 156.5, 130.4, 124.7, 115.3, 53.6, 51.7, 51.5, 50.8, 48.4, 42.0, 41.9, 41.0, 36.8, 36.5, 36.1, 24.2, 23.0, 21.6, 20.3, 20.2, 17.6; IR (KBr, cm^{-1}) 3309(br), 2956(m), 1662(vs), 1515(s), 1202(m), 1136(w).

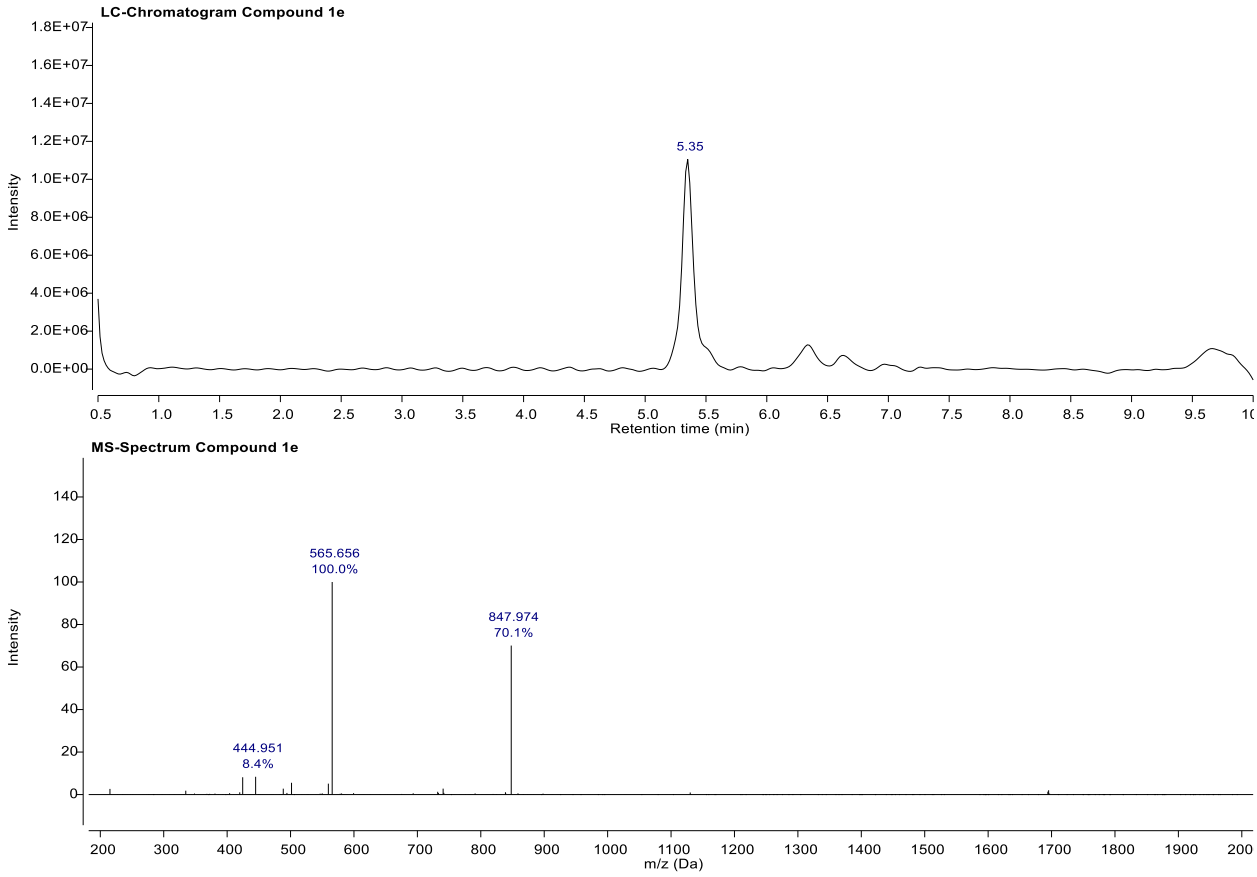
Gradient 5-60% MeCN, 7 min, 2mL/min

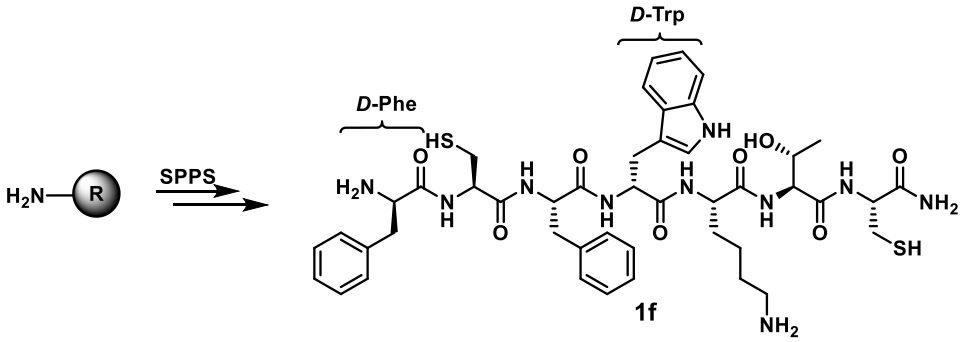




Peptide 1e was constructed by SPPS from 0.10 mmol loaded Rink amide resin. Removal of the peptide from resin was conducted with cocktail A following the general cleavage method. The peptide was purified by reverse-phase HPLC [eluent water/MeCN/AcOH (85:10:5) and MeCN (organic) buffered with 0.1%TFA] (5 - 40% organic over 15 min) to give 49.5 mg (23% • 4 TFA salt form) of a white amorphous powder after lyophilization: MALDI-TOF Found *m/z* 1694.004 [$(M+H)^+$; calcd for $C_{79}H_{125}N_{18}O_{19}S_2$: 1693.880]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.45 (s, 1H), 9.21 (d, *J* = 15.0 Hz, 2H), 8.43 (d, *J* = 7.5 Hz, 1H), 8.31 (dd, *J* = 8.2, 3.8 Hz, 2H), 8.16 – 7.87 (m, 13H), 7.85 (d, *J* = 7.9 Hz, 2H), 7.81 – 7.62 (m, 13H), 7.39 (s, 1H), 7.25 – 7.18 (m, 4H), 7.18 – 7.12 (m, 1H), 7.06 (s, 1H), 7.01 (d, *J* = 8.5 Hz, 2H), 6.93 (d, *J* = 8.3 Hz, 2H), 6.63 (d, *J* = 8.3 Hz, 2H), 6.60 (d, *J* = 8.5 Hz, 2H), 5.00 (d, *J* = 4.4 Hz, 1H), 4.62 (dq, *J* = 7.9, 4.7 Hz, 1H), 4.57 (q, *J* = 7.1 Hz, 1H), 4.41 (q, *J* = 7.2 Hz, 2H), 4.38 – 4.23 (m, 6H), 4.20 (q, *J* = 7.7 Hz, 1H), 4.18 – 4.08 (m, 1H), 3.93 (q, *J* = 4.9 Hz, 1H), 3.82 – 3.64 (m, 3H), 3.05 (dd, *J* = 14.2, 3.9 Hz, 1H), 2.90 (dd, *J* = 14.0, 3.2 Hz, 1H), 2.82 (t, *J* = 7.1 Hz, 2H), 2.79 – 2.56 (m, 15H), 2.38 (t, *J* = 8.6 Hz, 1H), 2.21 (t, *J* = 8.5 Hz, 1H), 1.79 – 1.55 (m, 7H), 1.56 – 1.47 (m, 11H), 1.47 – 1.37 (m, 5H), 1.37 – 1.23 (m, 3H), 1.24 – 1.19 (m, 1H), 1.05 (d, *J* = 6.3 Hz, 4H), 0.86 (t, *J* = 6.7 Hz, 7H), 0.83 – 0.74 (m, 13H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.4 , 172.1 , 171.7 , 171.6 , 171.4 , 171.3 , 170.9 , 170.9 , 170.4 , 169.6 , 168.7 , 167.9 , 155.9 , 155.8 , 137.5 , 130.1 , 130.0 , 129.1 , 128.0 , 126.2 , 118.5 , 114.9 , 114.9 , 66.9 , 57.9 , 56.3 , 55.0 , 54.5 , 54.3 , 53.6 , 52.5 , 52.2 , 52.1 , 51.2 , 49.7 , 40.7 , 40.3 , 39.3 , 39.1 , 38.8 , 38.7 , 38.7 , 36.3 , 31.5 , 31.2 , 26.7 , 26.6 , 26.4 , 24.1 , 24.0 , 23.8 , 23.2 , 23.1 , 22.3 , 22.3 , 21.6 , 21.5 , 19.3 , 14.5 , 11.1; IR (KBr, cm^{-1}) 3285(br), 3080(br), 2960(m), 1676(s), 1634(s), 1516(m), 1203(m), 1137(m).

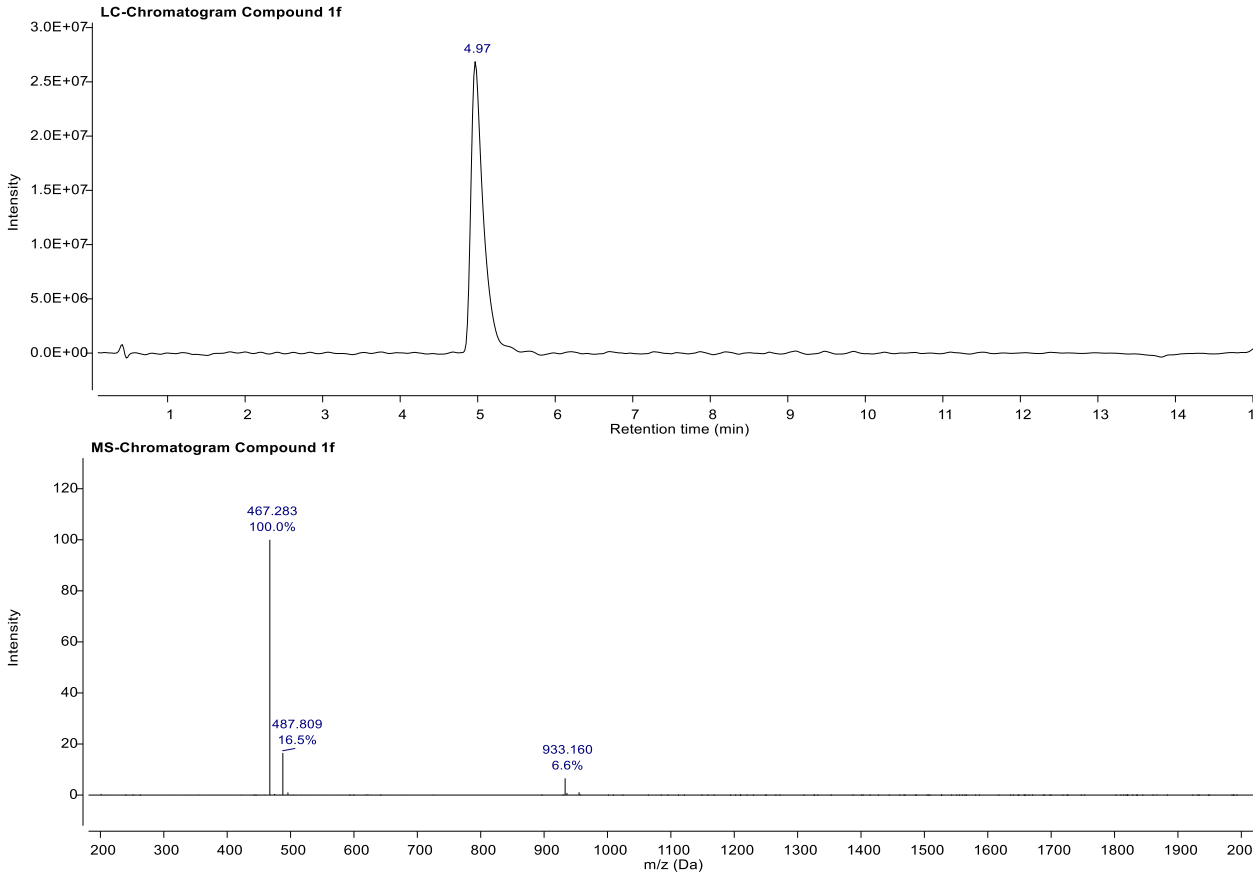
Gradient 5-60% MeCN, 10 min, 2mL/min

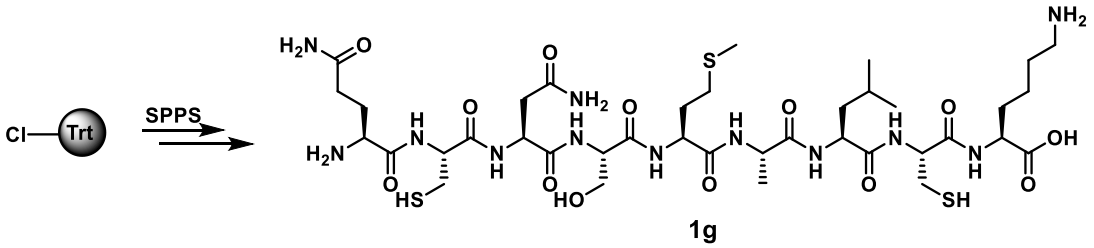




Peptide 1f was constructed by SPPS from 0.10 mmol loaded Rink amide resin. Removal of the peptide from resin was conducted with cocktail B following the general cleavage method. The peptide was purified by reverse-phase HPLC (5 - 60% organic over 12 min) to give 45.6 mg (44% • TFA salt form) of a white amorphous powder after lyophilization: HRMS (ES) Found *m/z* 933.4111 $[(M+H)^+$; calcd for $C_{45}H_{61}N_{10}O_8S_2$: 933.4115]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.81 (s, 1H), 8.63 (d, *J* = 8.0 Hz, 1H), 8.33 (d, *J* = 8.3 Hz, 1H), 8.28 (d, *J* = 8.1 Hz, 1H), 8.17 (d, *J* = 8.0 Hz, 1H), 8.15 (s, 2H), 7.99 (d, *J* = 8.0 Hz, 1H), 7.85 (d, *J* = 8.0 Hz, 1H), 7.71 (s, 2H), 7.66 (d, *J* = 7.9 Hz, 1H), 7.39 (s, 1H), 7.36 – 7.23 (m, 5H), 7.15 (d, *J* = 2.4 Hz, 1H), 7.11 (dt, *J* = 4.9, 1.7 Hz, 2H), 7.06 – 7.01 (m, 3H), 7.01 – 6.94 (m, 1H), 4.68 (ddd, *J* = 8.4, 5.8 Hz, 1H), 4.54 (ddd, *J* = 8.6, 4.3 Hz, 1H), 4.42 (q, *J* = 6.5 Hz, 1H), 4.36 (dp, *J* = 10.5, 5.6, 4.9 Hz, 2H), 4.23 (dd, *J* = 7.9, 4.3 Hz, 1H), 4.17 – 4.10 (m, 1H), 4.03 (dd, *J* = 6.5, 4.5 Hz, 1H), 3.11 – 3.02 (m, 2H), 2.96 (dd, *J* = 14.0, 7.8 Hz, 1H), 2.92 – 2.81 (m, 2H), 2.81 – 2.72 (m, 2H), 2.72 – 2.64 (m, 2H), 2.54 (t, *J* = 7.9 Hz, 1H), 2.29 (t, *J* = 8.5 Hz, 1H), 2.02 (t, *J* = 8.6 Hz, 1H), 1.63 (td, *J* = 13.3, 6.9 Hz, 1H), 1.53 – 1.36 (m, 3H), 1.13 (p, *J* = 7.4 Hz, 2H), 1.05 (d, *J* = 6.3 Hz, 3H); ^{13}C NMR (126 MHz, DMSO) δ 171.8, 171.4, 171.3, 170.3, 169.8, 168.8, 168.0, 137.5, 136.1, 134.8, 129.5, 129.5, 129.2, 128.5, 127.9, 127.2, 127.2, 126.1, 123.9, 120.8, 118.6, 118.1, 111.2, 109.7, 66.3, 58.3, 54.6, 54.5, 54.0, 53.3, 53.2, 52.1, 38.7, 37.3, 37.3, 31.3, 28.7, 26.7, 26.6, 26.1, 22.0, 19.5; IR (KBr, cm^{-1}) 3281(br), 2928(w), 1671(s), 1523(m), 1202(m).

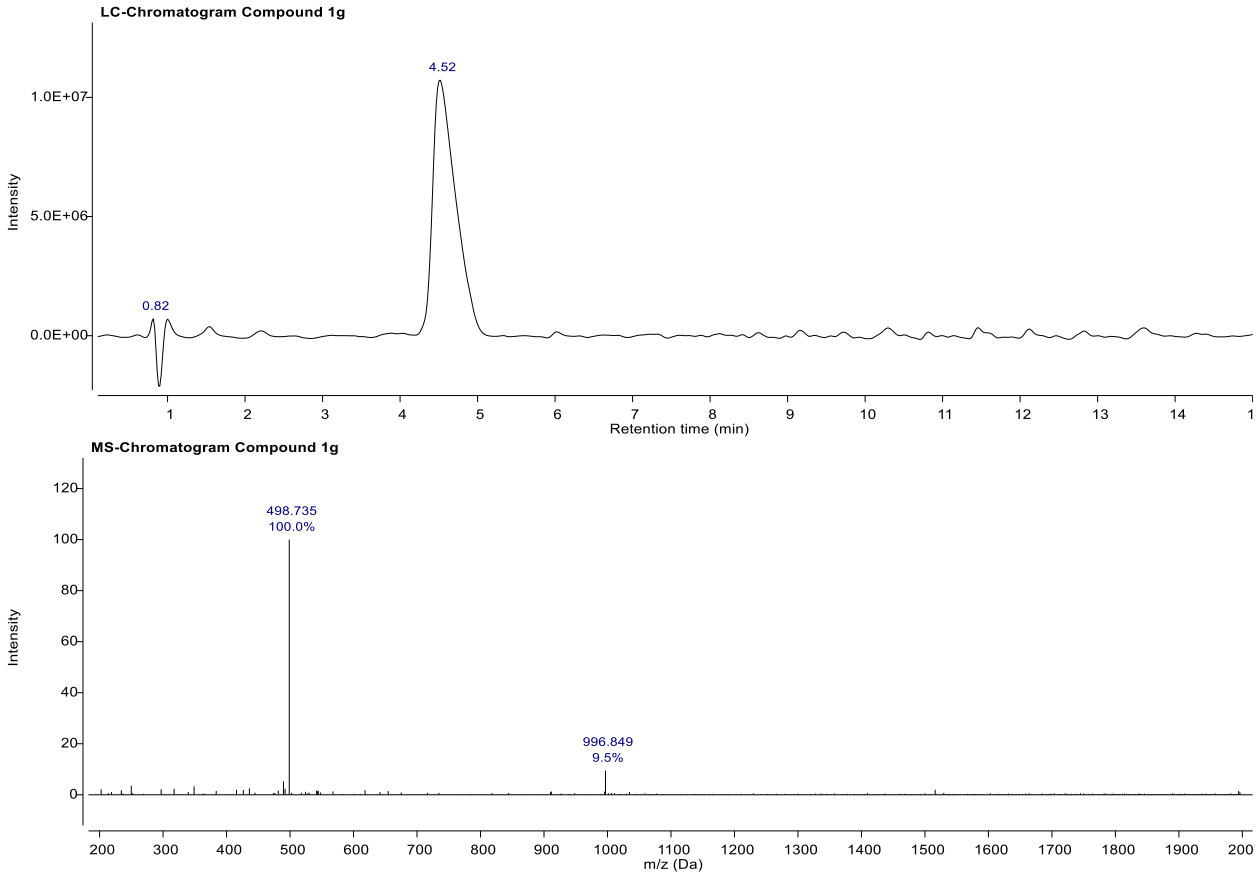
Gradient 5-60% MeCN, 15 min, 2mL/min

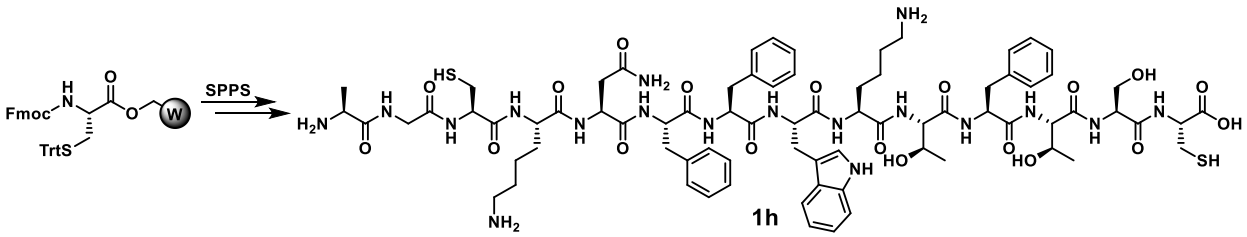




Peptide 1g was constructed by SPPS from 0.20 mmol loaded 2-chloro-chlorotirtyl resin. Removal of the peptide from resin was conducted with cocktail C following the general cleavage method. The peptide was purified by reverse-phase HPLC (5 - 60% organic over 15 min) to give 124 mg (51% • 2 TFA salt form) of a white amorphous powder after lyophilization: HRMS (ES) Found *m/z* 997.4268 [$(M+H)^+$; calcd for $C_{38}H_{69}N_{12}O_{13}S_3$: 997.4269]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.8 (d, *J* = 7.9 Hz, 1H), 8.5 (d, *J* = 7.4 Hz, 1H), 8.2 (s, 3H), 8.2 (d, *J* = 7.6 Hz, 1H), 8.1 (d, *J* = 7.7 Hz, 1H), 8.1 (d, *J* = 6.9 Hz, 1H), 7.9 (d, *J* = 7.1 Hz, 1H), 7.9 (dd, *J* = 8.0, 2.4 Hz, 2H), 7.8 (s, 3H), 7.5 (d, *J* = 26.5 Hz, 2H), 7.0 (d, *J* = 30.5 Hz, 2H), 5.1 (s, 1H), 4.6 (q, *J* = 7.1 Hz, 1H), 4.5 (td, *J* = 7.5, 5.3 Hz, 1H), 4.4 (td, *J* = 7.7, 5.0 Hz, 1H), 4.3 (ddd, *J* = 13.6, 9.1, 4.8 Hz, 1H), 4.3 (d, *J* = 7.6 Hz, 1H), 4.2 – 4.2 (m, 2H), 4.1 (td, *J* = 8.5, 4.7 Hz, 1H), 3.9 (s, 1H), 3.6 (s, 1H), 3.6 (s, 1H), 2.9 – 2.7 (m, 7H), 2.6 (dd, *J* = 15.5, 6.1 Hz, 1H), 2.5 (d, *J* = 1.8 Hz, 10H), 2.5 – 2.4 (m, 3H), 2.3 (t, *J* = 8.6 Hz, 1H), 2.2 (dd, *J* = 9.1, 6.5 Hz, 2H), 2.0 (s, 3H), 2.0 – 1.9 (m, 3H), 1.9 – 1.8 (m, 1H), 1.8 – 1.7 (m, 1H), 1.6 (dt, *J* = 11.3, 5.5 Hz, 2H), 1.6 – 1.5 (m, 2H), 1.5 (t, *J* = 7.3 Hz, 2H), 1.3 (q, *J* = 8.2 Hz, 2H), 1.2 (d, *J* = 7.1 Hz, 3H), 0.9 (d, *J* = 6.5 Hz, 3H), 0.8 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.5, 173.4, 172.1, 172.0, 171.6, 171.3, 171.0, 170.3, 169.7, 169.2, 168.5, 61.4, 55.7, 55.0, 54.7, 52.1, 51.8, 51.8, 51.1, 49.9, 48.4, 40.5, 38.6, 37.0, 31.3, 30.3, 30.3, 29.6, 27.0, 26.6, 26.4, 26.4, 24.1, 23.2, 22.4, 21.5, 17.6, 14.7; IR (KBr, cm^{-1}) 3286(br), 3078(w), 2960(w), 1664(s), 1635(s), 1541(m), 1202(m), 1137(w), 1033(m), 1008(m).

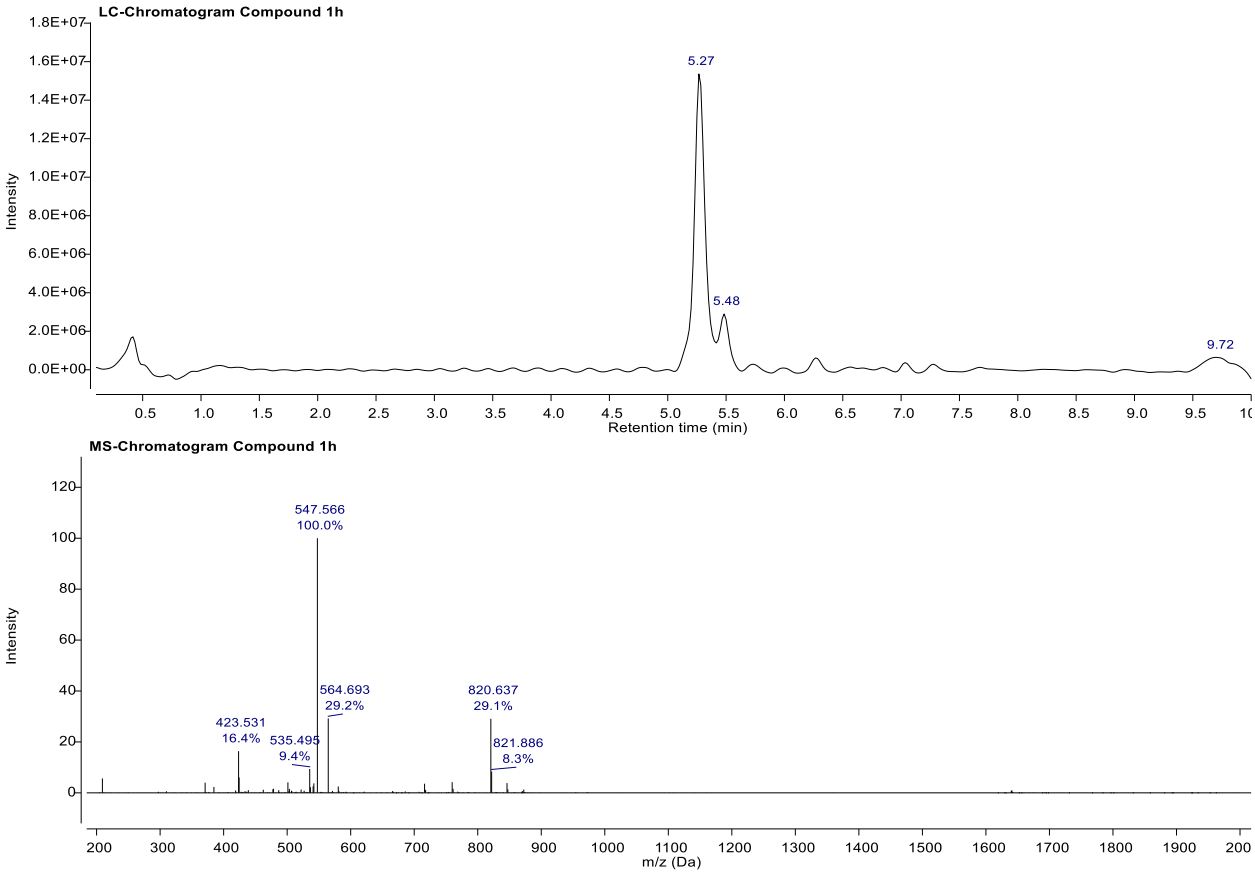
Gradient 5-60% MeCN, 15 min, 2mL/min

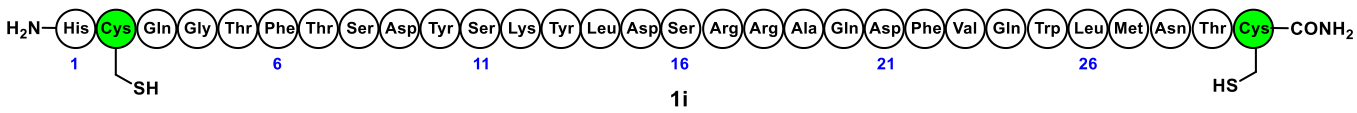




Peptide 1h was constructed by automated SPPS from 0.10 mmol pre-loaded Fmoc-Cys(Trt)-Wang resin. Removal of the peptide from resin was conducted with cocktail B following the general cleavage method. The peptide was purified by reverse-phase HPLC (10 - 60% organic over 15 min) to give 38 mg (19% • 3 TFA salt form) of a white amorphous powder after lyophilization: MALDI-TOF Found *m/z* 1639.125 [$(M+H)^+$; calcd for $C_{76}H_{107}N_{18}O_{19}S_2$: 1639.740]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.79 (s, 1H), 8.67 (t, *J* = 5.8 Hz, 1H), 8.28 – 8.01 (m, 8H), 8.01 – 7.87 (m, 4H), 7.84 – 7.64 (m, 7H), 7.61 (d, *J* = 7.9 Hz, 1H), 7.47 (s, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.28 – 7.13 (m, 13H), 7.13 – 7.08 (m, 5H), 7.08 – 7.01 (m, 2H), 6.98 (t, *J* = 7.4 Hz, 1H), 6.60 (s, 1H), 5.17 – 5.07 (m, 1H), 4.90 (d, *J* = 5.0 Hz, 1H), 4.71 – 4.62 (m, 1H), 4.57 (q, *J* = 7.4 Hz, 1H), 4.51 (q, *J* = 6.8 Hz, 2H), 4.48 – 4.41 (m, 3H), 4.41 – 4.30 (m, 5H), 4.25 – 4.15 (m, 2H), 4.05 – 3.93 (m, 3H), 3.94 – 3.86 (m, 2H), 3.82 (dd, *J* = 16.8, 5.6 Hz, 1H), 3.72 – 3.54 (m, 4H), 3.50 (s, 1H), 3.18 – 3.11 (m, 2H), 3.11 – 3.05 (m, 2H), 3.03 – 2.93 (m, 3H), 2.93 – 2.75 (m, 7H), 2.75 – 2.62 (m, 7H), 2.45 – 2.39 (m, 1H), 2.36 (dd, *J* = 15.5, 6.3 Hz, 1H), 1.71 – 1.56 (m, 3H), 1.56 – 1.39 (m, 8H), 1.35 (d, *J* = 7.0 Hz, 3H), 1.32 – 1.16 (m, 5H), 1.14 (d, *J* = 6.8 Hz, 1H), 1.04 (d, *J* = 6.3 Hz, 3H), 0.99 (d, *J* = 6.2 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.8, 171.4, 171.4, 171.2, 171.1, 171.0, 170.9, 170.7, 170.5, 169.9, 169.8, 169.7, 169.7, 169.7, 168.4, 137.8, 137.6, 137.6, 136.1, 129.3, 129.3, 129.1, 128.1, 128.0, 128.0, 127.4, 126.2, 126.2, 123.7, 120.9, 118.3, 109.9, 72.5, 70.6, 70.5, 69.8, 66.8, 66.5, 63.1, 61.8, 61.6, 57.9, 57.9, 57.8, 57.8, 55.0, 54.6, 54.1, 53.9, 53.7, 53.4, 52.4, 52.3, 49.6, 48.2, 41.8, 37.4, 37.2, 37.1, 31.3, 31.2, 27.7, 26.7, 26.7, 26.6, 25.6, 22.3, 22.2, 19.4, 19.3, 19.3, 17.3; IR (KBr, cm^{-1}) 3399(br), 3298(br), 3063(w), 2929(w), 1663(s), 1553(m), 1202(w), 1134(w), 1032(w), 1008(w).

Gradient 5-60% MeCN, 10 min, 2mL/min

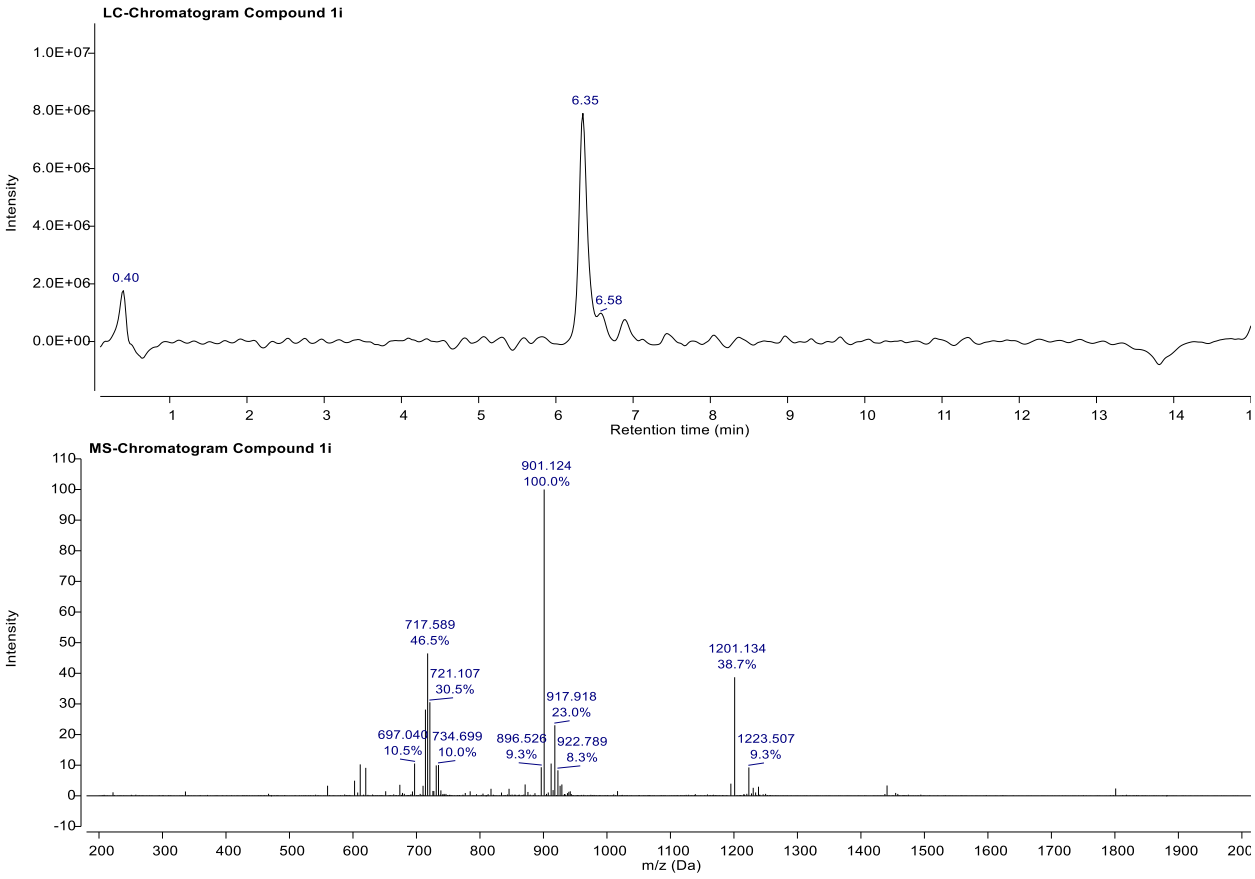


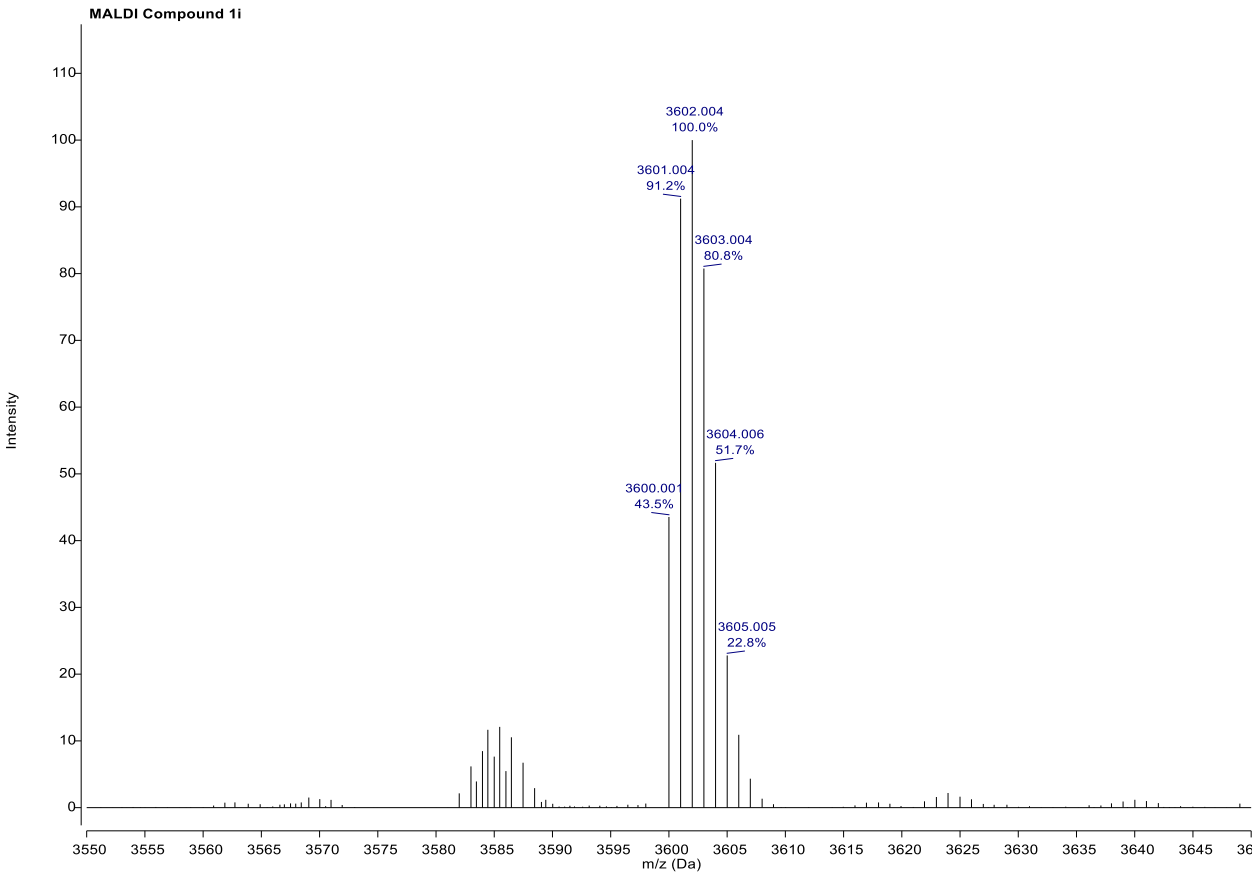


1i

Peptide 1i was constructed by automated SPPS from 0.10 mmol loaded Rink amide resin. Removal of the peptide from resin was conducted with cocktail B following the general cleavage method. The peptide was purified by reverse-phase HPLC (10 - 60% organic over 20 min) to give 39.6 mg (11% • 4 TFA salt form) of a white amorphous powder after lyophilization: MALDI-TOF Found *m/z* 3600.001 [$(M+H)^+$; calcd for $C_{156}H_{231}N_{45}O_{48}S_3$: 3599.625]; IR (KBr, cm^{-1}) 3306(br), 2933(w), 1655(s), 1541(m), 1202(m).

Gradient 5-60% MeCN, 15 min, 2mL/min

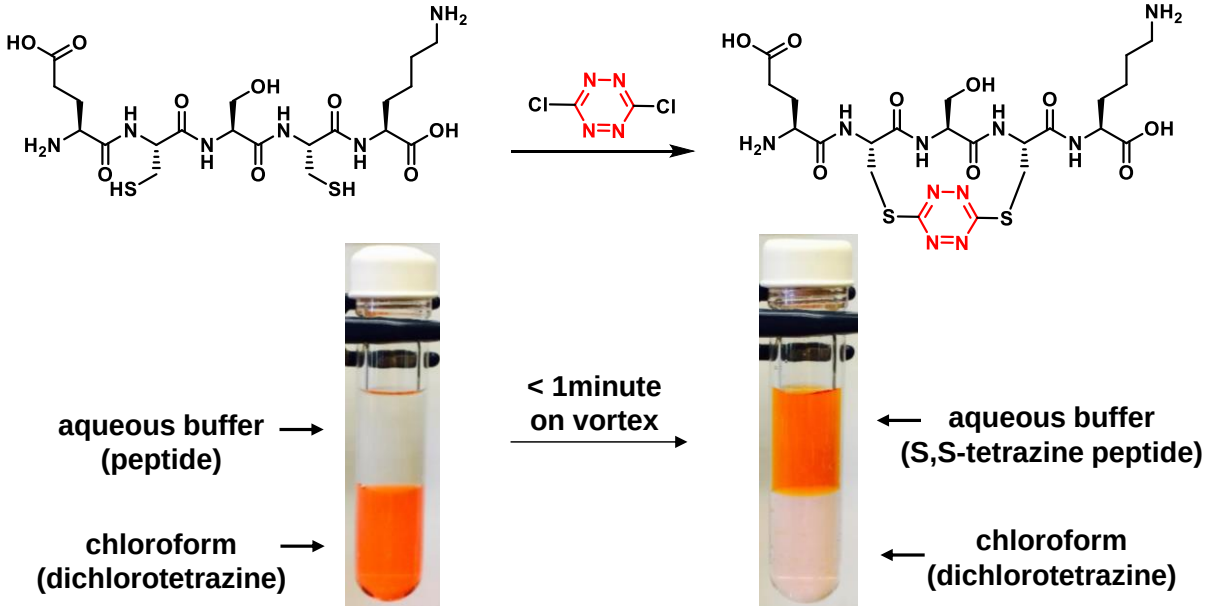



MALDI Compound 1i
Intensity
m/z (Da)
3602.004
100.0%
3601.004
91.2%
3603.004
80.8%
3604.006
51.7%
3600.001
43.5%
3605.005
22.8%

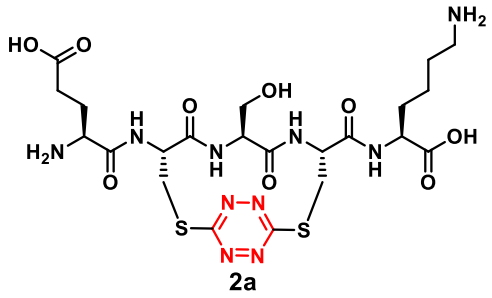
General Phase-Transfer Protocol for Tetrazine Insertion

A round bottom flask, was charged with unprotected peptide **1** (1 - 1000 µmol) then sealed with a septum and purged with argon. Next, a degassed solution of 50mM (pH ~5) monosodium phosphate was added (1 -2 mM concentration of peptide in solution) followed by a solution of dichlorotetrazine (3 equiv) in $CHCl_3$ (equal volume to peptide). The two-phases were stirred vigorously for 1 minute. The mixture was divide between Falcon tubes then transferred to a benchtop centrifuge and further separated at 2500 RPM for 1 minute. The aqueous phase, now orange in color, was collected and each organic layer was extracted with an additional portion of water then transferred to a benchtop centrifuge and separated again at 2500 RPM for 1 minute. All of the aqueous fractions were combined and lyophilized. The crude mixture was then purified by reverse-phase high-pressure liquid chromatography (HPLC) to yield an orange powder after lyophilization. **Note:** peptide masses were calculated as the salt free form; quantities of starting material and yields may vary slightly due to the TFA counterion(s).

Colormetric Change that Occurs After Mixing the Biphasic Mixture for 1 Minute.



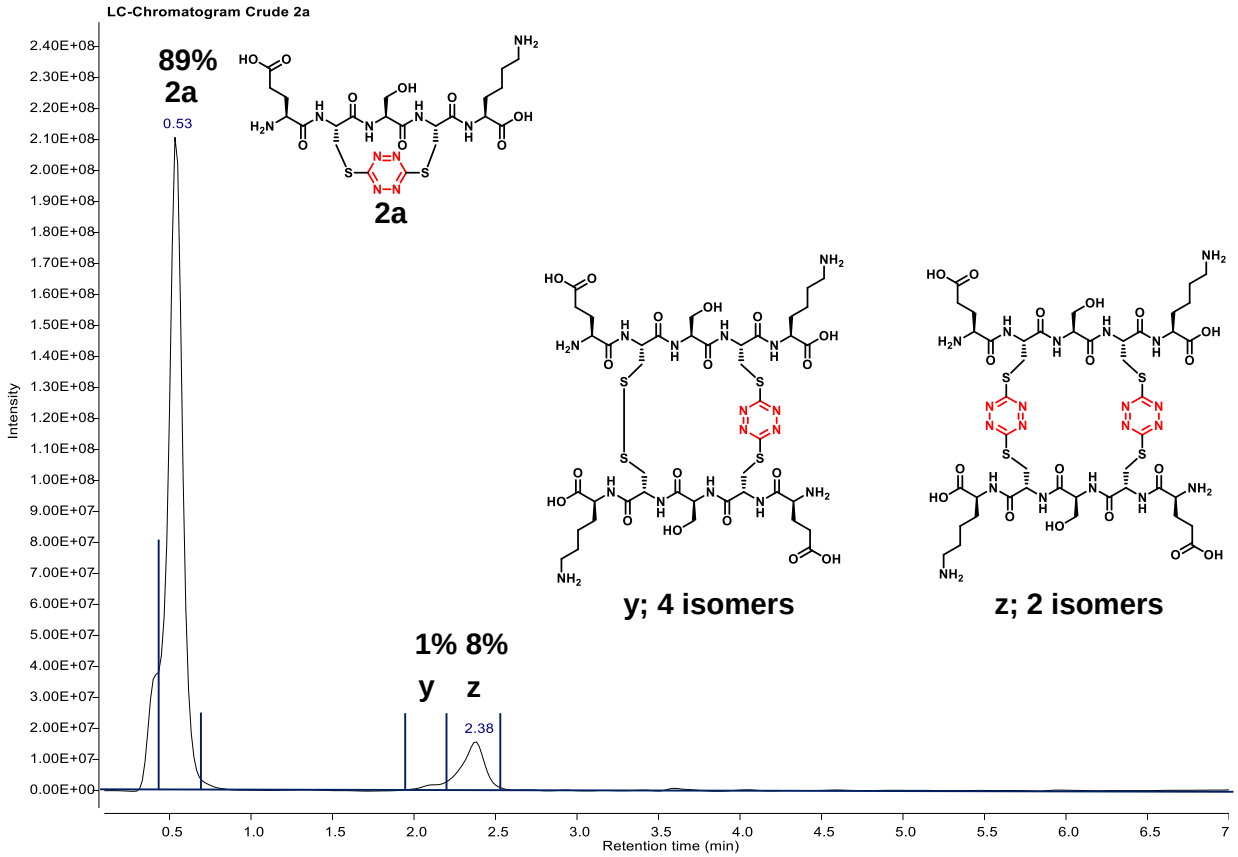
Conditions for the above reaction are different from the general phase-transfer protocol and employ only 1.1 equivalents of dichlorotetrazine to illustrate the colorimetric transfer of tetrazine into the peptide.



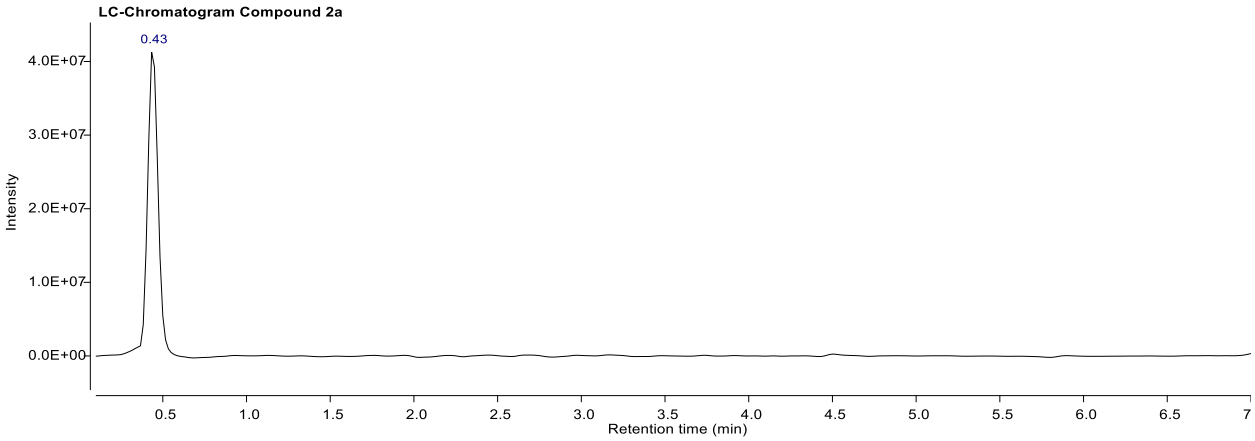
2a

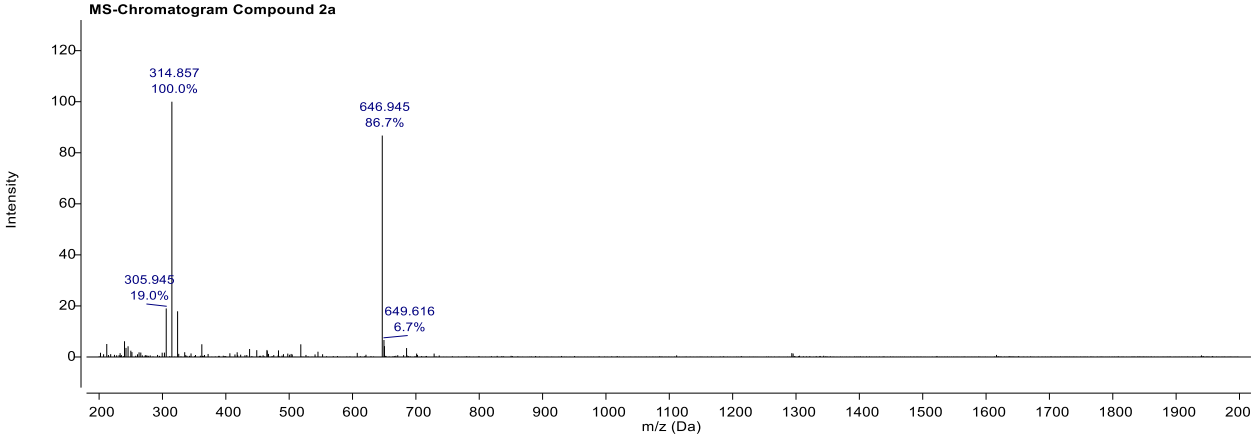
Peptide 2a. Peptide **1a** (5.7 mg, 10 µmol) was subjected to the general phase-transfer protocol to construct **2a**. The crude reaction mixture was purified by reverse-phase HPLC (gradient 5-15% organic over 5 min) to give (5.1 mg, 78%) of an orange powder after lyophilization. HRMS (ES) Found *m/z* 647.2028 [$(M+H)^+$; calcd for $C_{22}H_{35}N_{10}O_9S_2$: 647.2030]; ^{1}H NMR (500 MHz, D_2O) δ ppm 1.36 (quin, *J*=7.80 Hz, 2 H) 1.58 - 1.66 (m, 3 H) 1.68 (q, *J*=7.90 Hz, 2 H) 1.79 (ddd, *J*=13.90, 8.30, 5.50 Hz, 1 H) 1.78 (ddd, *J*=13.25, 8.10, 5.30 Hz, 1 H) 2.07 (q, *J*=7.34 Hz, 2 H) 2.39 (dd, *J*=7.30, 5.10 Hz, 1 H) 2.39 (dd, *J*=9.80, 7.40 Hz, 1 H) 2.93 (t, *J*=7.48 Hz, 9 H) 3.50 (dd, *J*=15.60, 4.06 Hz, 3 H) 3.54 (dd, *J*=18.20, 6.20 Hz, 1 H) 3.57 (dd, *J*=11.30, 7.70 Hz, 1 H) 4.10 (t, *J*=6.52 Hz, 1 H) 4.14 (dd, *J*=8.44, 5.24 Hz, 1 H) 4.20 (dd, *J*=7.10, 6.40 Hz, 1 H) 4.53 (dd, *J*=15.39, 2.99 Hz, 1 H) 4.56 (dd, *J*=15.60, 4.92 Hz, 1 H) 4.80 (t, *J*=3.42 Hz, 1 H) 4.99 (dd, *J*=4.90, 1.90 Hz, 1 H); ^{13}C NMR (126 MHz, Deuterium Oxide) δ 178.6, 178.1, 171.7, 170.6, 170.4, 169.9, 169.1, 169.1, 61.8, 55.0, 53.9, 52.4, 52.2, 51.5, 39.1, 31.4, 31.1, 30.7, 30.2, 26.7, 26.2, 22.1; IR (KBr, cm^{-1}) 3430(br), 3291(br), 3074(br), 2945(br), 1658(s), 1525(m), 1197(s), 1139(m); UV-vis λ_{Max} 278 nm, 419 nm, 507 nm.

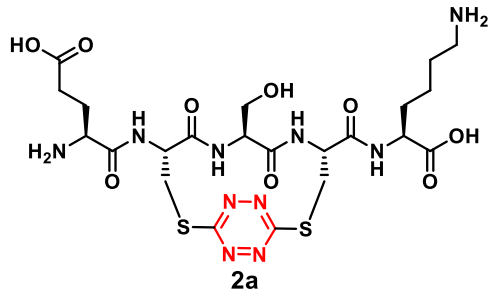
Crude LC Trace for 2a: Gradient 5-60% MeCN, 7 min, 2mL/min



Purified LC-MS of 2a: Gradient 5-60% MeCN, 7 min, 2mL/min

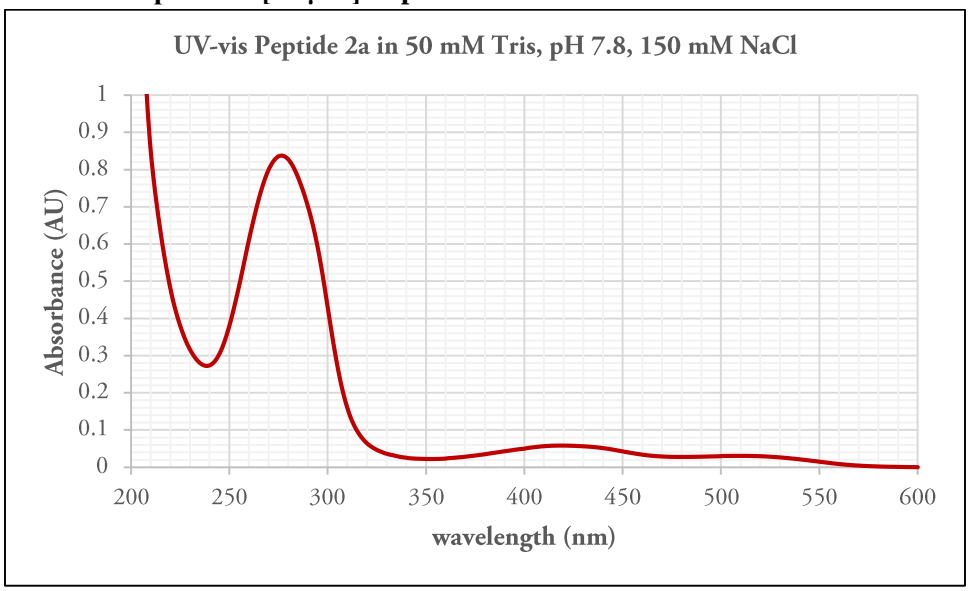




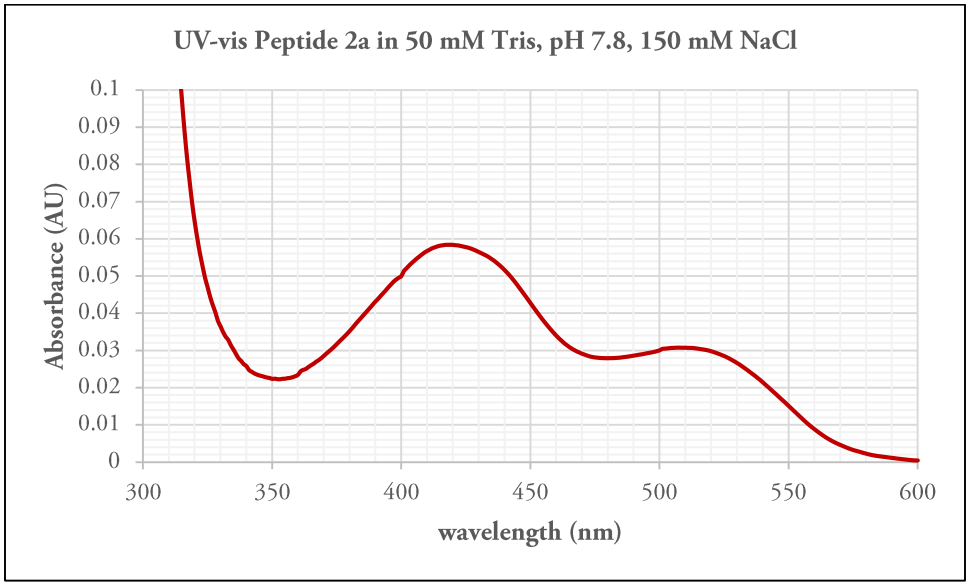


(Large Scale) Peptide 2a. Peptide **1a** (570 mg, 1000 µmol) was subjected to the general phase-transfer protocol to construct **2a**. The crude reaction mixture was purified by reverse-phase HPLC (gradient 5-15% organic over 5 min) to give (465 mg, 72 %) of an orange powder after lyophilization. Spectral data was identical to compound **2a**.

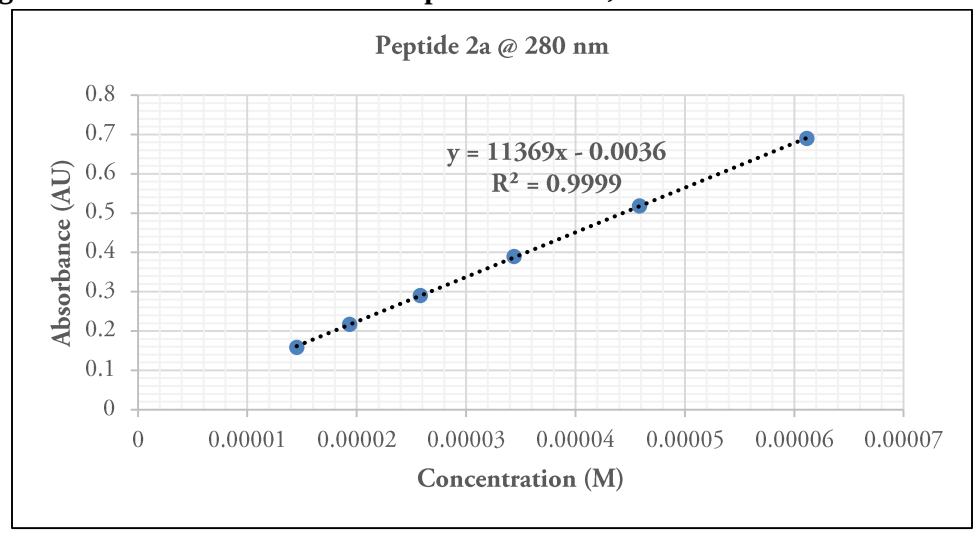
UV-Vis Spectrum of Peptide 2a [73 µM] in pH 7.8 buffer

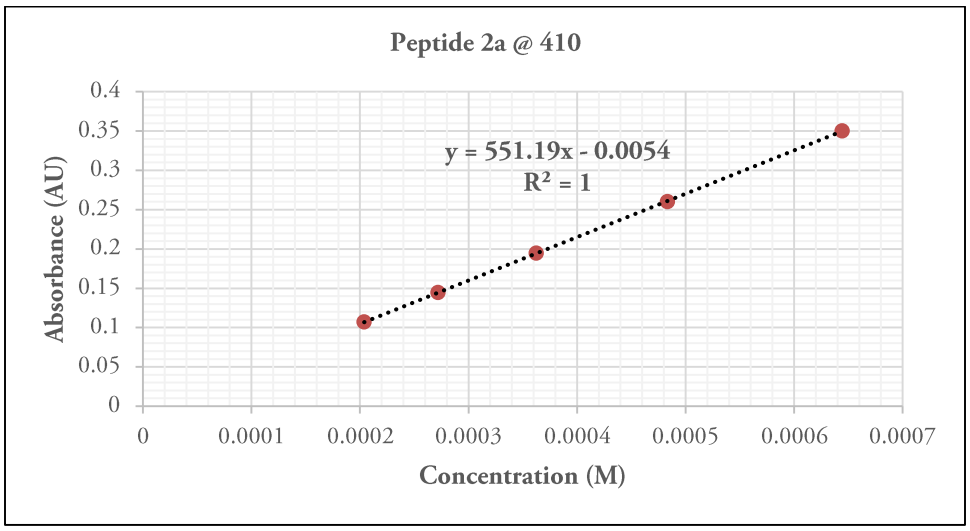


Zoom Region 300 – 600 nm: UV-Vis Spectrum of Peptide 2a [73 µM] in pH 7.8 buffer



Calculating the Extinction Coefficient for Peptide 2a at 280, 410 and 532 nm





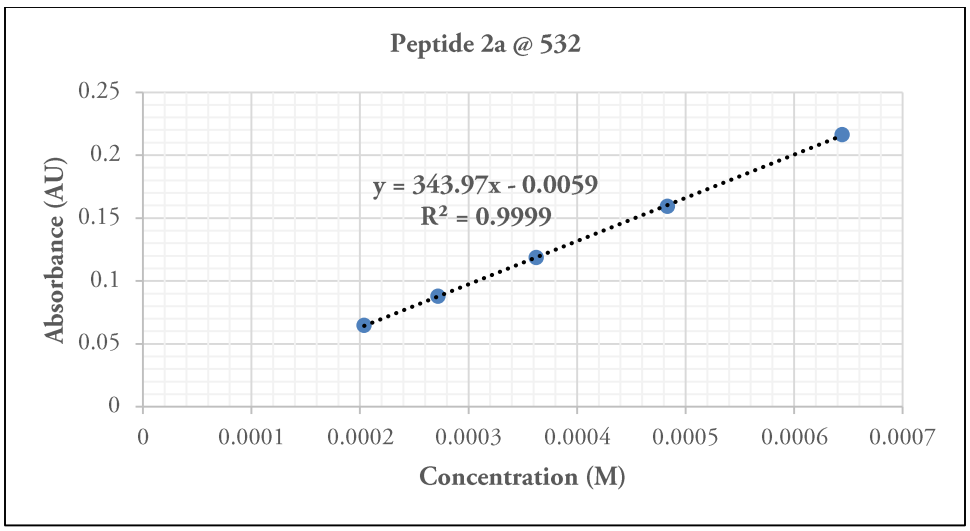
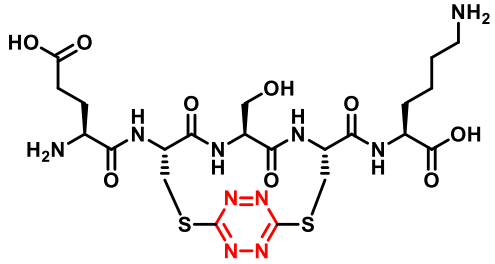
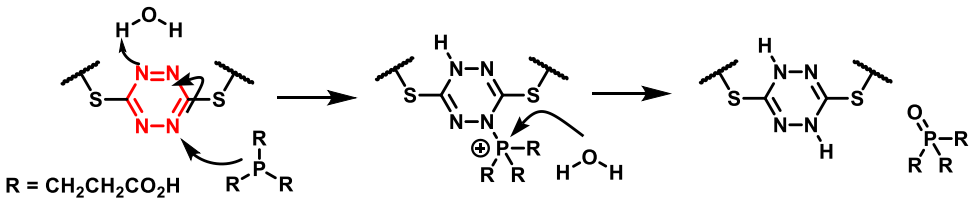


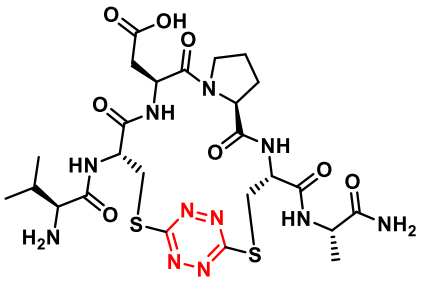
Table of Stabilty Data for the S,S-Tetrazine Peptide 2a



Buffer	Stability
100 mM phosphate buffer; pH 5	stable for > 1 week
100 mM phosphate buffer; pH 7	stable for > 1 week
100 mM phosphate buffer; pH 8	stable for > 1 week
100 mM phosphate buffer; pH 10	slowly decomposes half-life ≤ 4 days
100 mM acetate buffer, pH 5	stable for > 1 week
100 mM Tris buffer, pH 7	stable for > 1 week
100 mM Tris buffer pH 9	slowly decomposes half-life ≥ 1 week
100 mM ammonium bicarbonate	stable for > 1 week
citric acid buffer, pH 3	stable for > 1 week
6 M Guanidine HCl, pH 7 PBS	stable for > 1 week
8 M Urea, pH 7 PBS	stable for > 1 week
Storage Conditions	**Storage Period**
ambient temperature	stable for months as a lyophilized powder
elevated temperature	stable after reflux in water (100ºC) for 24 hours
freeze-thaw cycles	stable for 5 cycles of freeze-thaw in buffer
light exposure	stable for >1 week in buffer under fluorescent lights, stable for months as a lyophilized powder
refrigerated temperature	stable for > 1 year when stored as a lyophilized powder in a refrigerator at 10ºC
Organic solvents/Reagents	**Stability**
Methanol	stable for > 1 week
dimethyl sulfoxide	stable for > 1 week
acetonitrile/water (4:1)	stable for > 1 week
glycerol/water (1:1)	stable for > 1 week
trifluoroethanol	stable for > 1 week
trifluoroacetic acid	stable for > 2 days
dimethyl sulfoxide/trimethylamine (9:1)	decomposes
1 mM cysteine in 100 mM Tris buffer, pH 7	turns colorless, mass of **2a** increases by 2
1 mM TCEP in 100 mM Tris buffer, pH 7	turns colorless, mass of **2a** increases by 2

Proposed Mechanism for the reduction of s-tetrazine by TCEP

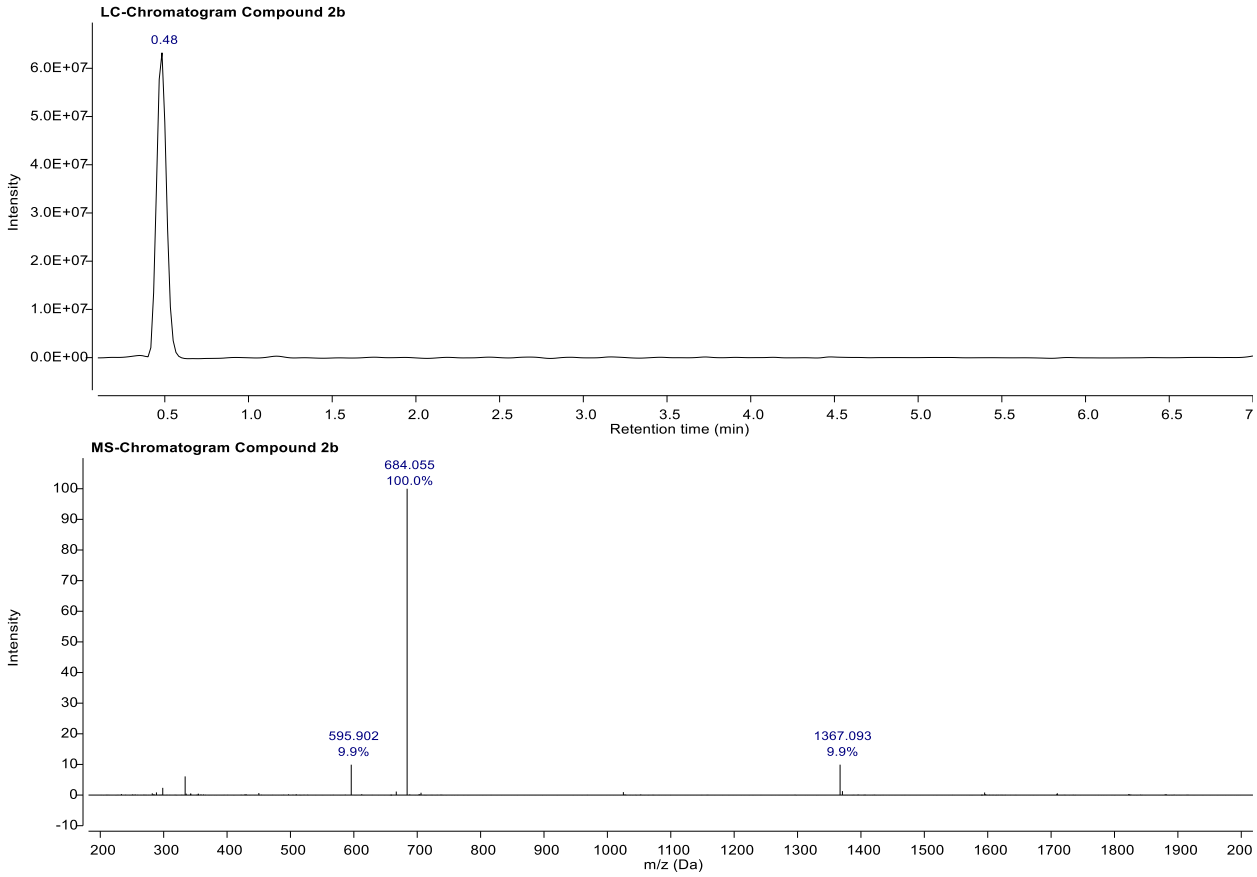


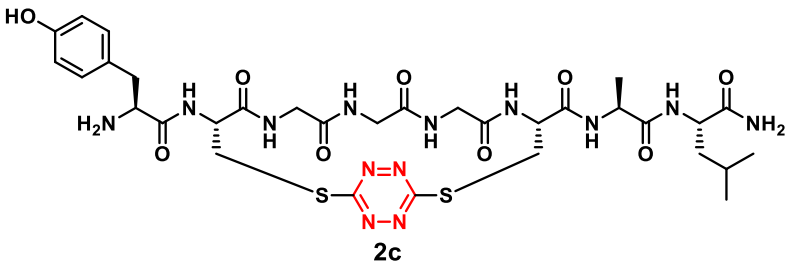


2b

Peptide 2b. Peptide **1b** (14.1 mg, 23µmol) was subjected to the general phase-transfer protocol to construct **2b**. The crude reaction mixture was purified by reverse-phase HPLC (gradient 5-30% organic over 10 min) to give (10.5 mg, 67%) of an orange powder after lyophilization.: HRMS (ES) Found *m/z* 684.2341 [$(M+H)^+$; calcd for $C_{25}H_{38}N_{11}O_8S_2$: 684.2346]; ^{1}H NMR (500MHz ,DMSO-d_6) δ = 8.71 (d, *J* = 6.8 Hz, 1 H), 8.46 (d, *J* = 4.5 Hz, 1 H), 8.00 (d, *J* = 7.7 Hz, 1 H), 7.91 (d, *J* = 9.6 Hz, 1 H), 7.43 (s, 1 H), 7.15 (br. s., 3 H), 7.04 (s, 1 H), 4.77 (dd, *J* = 3.4, 6.8 Hz, 1 H), 4.74 (dd, *J* = 2.6, 15.8 Hz, 1 H), 4.69 (dd, *J* = 1.9, 9.0 Hz, 1 H), 4.67 (dd, *J* = 1.9, 9.6 Hz, 1 H), 4.42 - 4.34 (m, 1 H), 4.20 (quin, *J* = 7.2 Hz, 1 H), 4.06 (dd, *J* = 11.2, 14.6 Hz, 1 H), 3.77 (dd, *J* = 5.9, 7.9 Hz, 1 H), 3.66 (d, *J* = 5.1 Hz, 1 H), 3.54 - 3.47 (m, 2 H), 3.44 (dd, *J* = 2.6, 15.4 Hz, 1 H), 3.42 (dd, *J* = 2.8, 10.0 Hz, 1 H), 2.37 (dd, *J* = 2.4, 17.1 Hz, 1 H), 2.19 (dd, *J* = 10.5, 17.3 Hz, 1 H), 2.06 (sxt, *J* = 6.8 Hz, 1 H), 1.99 - 1.88 (m, 2 H), 1.77 (quind, *J* = 6.6, 12.0 Hz, 1 H), 1.71 - 1.62 (m, 1 H), 1.24 (d, *J* = 7.1 Hz, 3 H), 0.93 (d, *J* = 7.1 Hz, 3 H), 0.91 (d, *J* = 6.8 Hz, 3 H); ^{13}C NMR (126 MHz, DMSO) δ 173.8, 171.4, 171.1, 171.1, 171.1, 168.7, 168.4, 167.4, 158.3, 59.6, 57.6, 54.3, 51.2, 48.4, 48.1, 46.4, 34.6, 32.0, 31.0, 30.1, 28.6, 25.0, 18.6, 18.5, 17.6; IR (KBr, cm^{-1}) 3285(br), 3069(br), 1671(s), 1639(s), 1523(m), 1406(w), 1240(m), 1201(m), 1137(m).

Gradient 5-60% MeCN, 7 min, 2mL/min

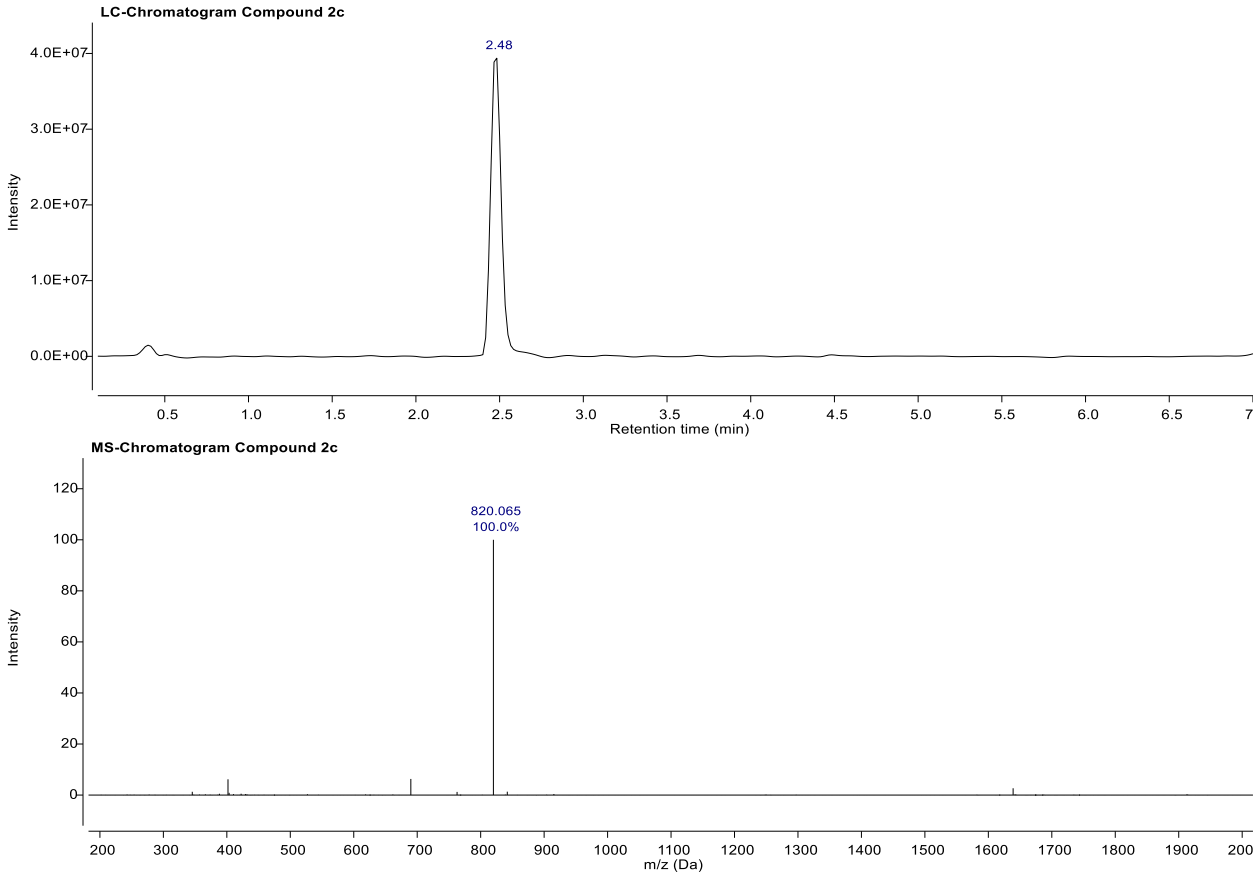


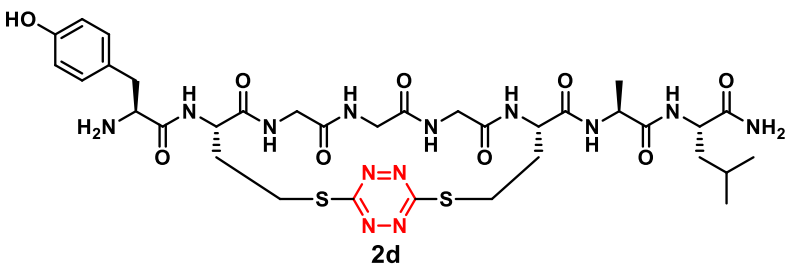


2c

Peptide 2c. Peptide **1c** (15.3 mg, 21µmol) was subjected to the general phase-transfer protocol to construct **2c**. The crude reaction mixture was purified by reverse-phase HPLC (gradient 5-40% organic over 15 min) to give (12.9 mg, 76%) of an orange powder after lyophilization: HRMS (ES) Found *m/z* 820.2981 [$(M+H)^+$; calcd for $C_{32}H_{46}N_{13}O_9S_2$: 820.2983]; ^{1}H NMR (500MHz, DMSO-d_6) δ = 9.36 (s, 1 H), 9.16 (d, *J* = 7.3 Hz, 1 H), 8.72 (dd, *J* = 4.1, 7.0 Hz, 1 H), 8.22 (d, *J* = 7.3 Hz, 1 H), 8.19 - 8.15 (m, 3 H), 7.88 (d, *J* = 8.1 Hz, 1 H), 7.84 (d, *J* = 8.3 Hz, 1 H), 7.73 (t, *J* = 5.7 Hz, 1 H), 7.24 (s, 1 H), 7.05 (br. s., 1 H), 7.03 (d, *J* = 8.5 Hz, 2 H), 6.96 (br. s., 1 H), 6.69 (d, *J* = 8.3 Hz, 2 H), 6.51 (s, 1 H), 4.81 - 4.72 (m, 2 H), 4.28 (quin, *J* = 7.2 Hz, 1 H), 4.21 (dt, *J* = 6.0, 8.5 Hz, 1 H), 4.03 (t, *J* = 6.0 Hz, 1 H), 4.00 (dd, *J* = 7.5, 16.2 Hz, 1 H), 3.80 (dd, *J* = 4.1, 10.3 Hz, 1 H), 3.77 (dd, *J* = 6.0, 16.5 Hz, 1 H), 3.71 - 3.55 (m, 4 H), 3.49 (dd, *J* = 3.8, 16.0 Hz, 1 H), 3.02 (dd, *J* = 5.6, 14.3 Hz, 1 H), 2.85 (dd, *J* = 7.5, 13.9 Hz, 1 H), 1.65 - 1.55 (m, 1 H), 1.51 - 1.42 (m, 2 H), 1.25 (d, *J* = 7.1 Hz, 3 H), 0.88 (d, *J* = 6.6 Hz, 3 H), 0.84 (d, *J* = 6.4 Hz, 3 H); ^{13}C NMR (126MHz ,DMSO-d_6) δ = 174.0, 172.2, 171.6, 171.0, 169.2, 169.1, 169.0, 169.0, 168.8, 168.2, 158.1, 156.5, 130.5, 124.6, 115.3, 53.6, 52.6, 52.1, 50.9, 48.6, 42.5, 42.2, 42.1, 40.9, 36.0, 32.0, 31.3, 24.2, 23.0, 21.6, 17.8; IR (KBr, cm^{-1}) 3293(br), 2928(w), 1670(s), 1517(m), 1238(m).

Gradient 5-60% MeCN, 7 min, 2mL/min

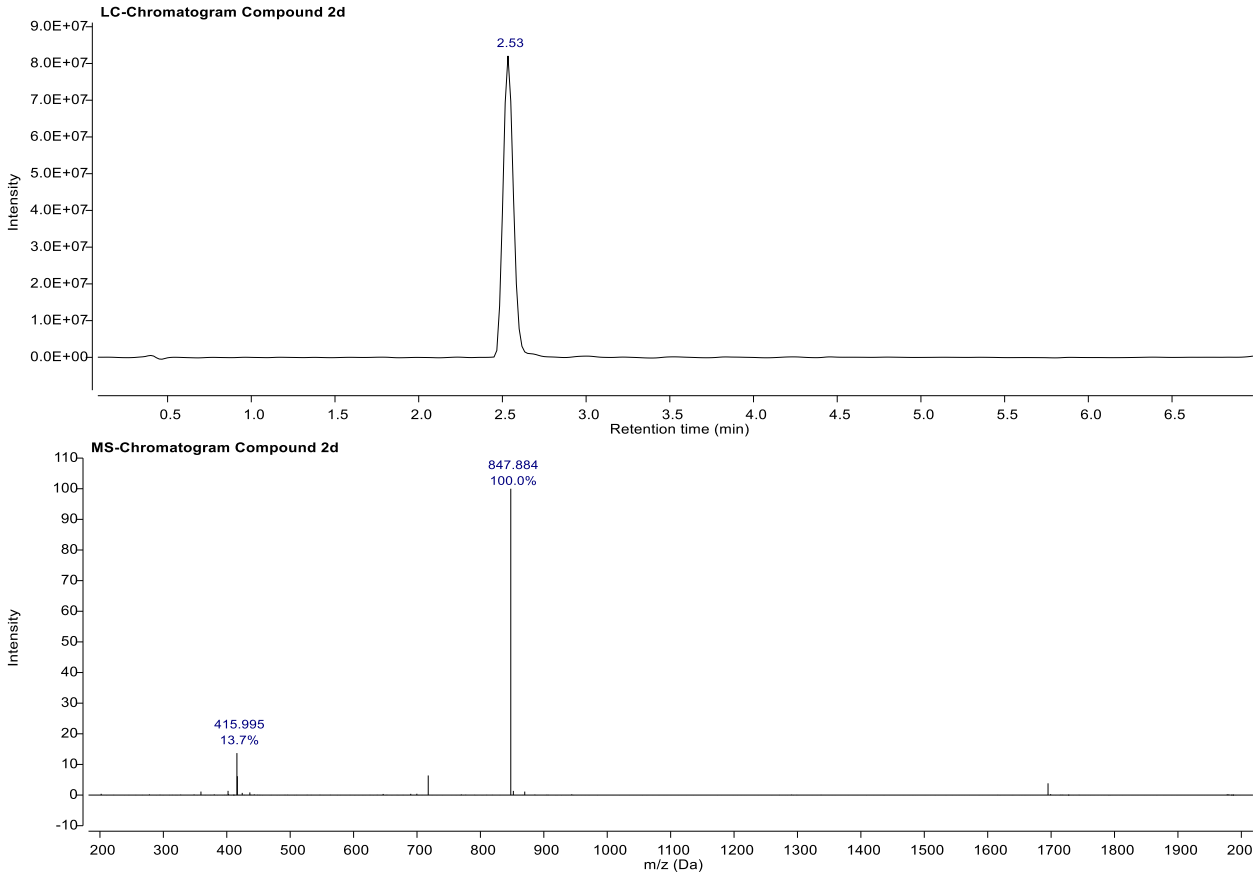


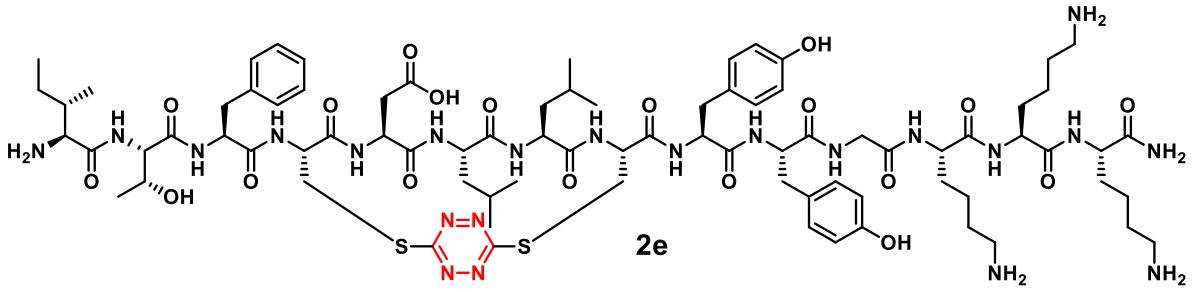


2d

Peptide 2d. Peptide **1d** (16.0 mg, 21 µmol) was subjected to the general phase-transfer protocol to construct **2d**. The crude reaction mixture was purified by reverse-phase HPLC (gradient 5-40% organic over 15 min) to give (11.2 mg, 64%) of an orange powder after lyophilization: HRMS (ES) Found *m/z* 848.3286 [$(M+H)^+$; calcd for $C_{34}H_{50}N_{13}O_9S_2$: 848.3296]; ^{1}H NMR (500MHz ,DMSO-d_6) δ = 9.36 (br. s., 1 H), 8.76 (d, *J* = 7.5 Hz, 1 H), 8.31 (t, *J* = 5.4 Hz, 1 H), 8.21 (d, *J* = 6.3 Hz, 1 H), 8.16 - 8.06 (m, 3 H), 8.02 (t, *J* = 5.9 Hz, 1 H), 7.96 (d, *J* = 8.1 Hz, 1 H), 7.77 (d, *J* = 7.7 Hz, 1 H), 7.23 (br. s., 1 H), 7.04 (d, *J* = 7.1 Hz, 2 H), 6.96 (br. s., 1 H), 6.70 (d, *J* = 7.1 Hz, 2 H), 4.59 (t, *J* = 9.0 Hz, 1 H), 4.45 (dd, *J* = 6.9, 15.0 Hz, 1 H), 4.26 (ddd, *J* = 6.5, 7.3, 14.9 Hz, 1 H), 4.20 (dd, *J* = 7.5, 15.9 Hz, 1 H), 4.02 (br. s., 1 H), 3.90 (dd, *J* = 5.9, 16.1 Hz, 1 H), 3.77 (dd, *J* = 5.2, 16.4 Hz, 1 H), 3.74 (dd, *J* = 4.8, 14.9 Hz, 1 H), 3.69 - 3.53 (m, 4 H), 3.41 - 3.33 (m, 2 H), 3.24 (quin, *J* = 7.4 Hz, 2 H), 2.99 (dd, *J* = 5.0, 13.7 Hz, 1 H), 2.83 (dd, *J* = 7.5, 14.1 Hz, 1 H), 2.30 (d, *J* = 7.7 Hz, 1 H), 2.08 (ddd, *J* = 5.4, 8.7, 14.3 Hz, 1 H), 2.04 - 1.91 (m, 2 H), 1.59 (dt, *J* = 6.4, 13.0 Hz, 1 H), 1.51 - 1.38 (m, 1 H), 1.23 (d, *J* = 7.1 Hz, 3 H), 0.87 (d, *J* = 5.4 Hz, 3 H), 0.83 (d, *J* = 5.7 Hz, 3 H); ^{13}C NMR (126MHz ,DMSO-d_6) δ = 174.1, 171.8, 171.7, 171.7, 170.8, 170.5, 169.2, 169.1, 168.8, 168.3, 156.6, 130.5, 124.8, 115.4, 53.7, 51.4, 51.2, 50.8, 48.5, 42.6, 42.3, 42.1, 41.0, 36.1, 31.5, 31.2, 26.4, 26.2, 24.2, 23.1, 21.6, 17.7; IR (KBr, cm^{-1}) 3290(br), 2927(w), 1672(s), 1516(m), 1240(m).

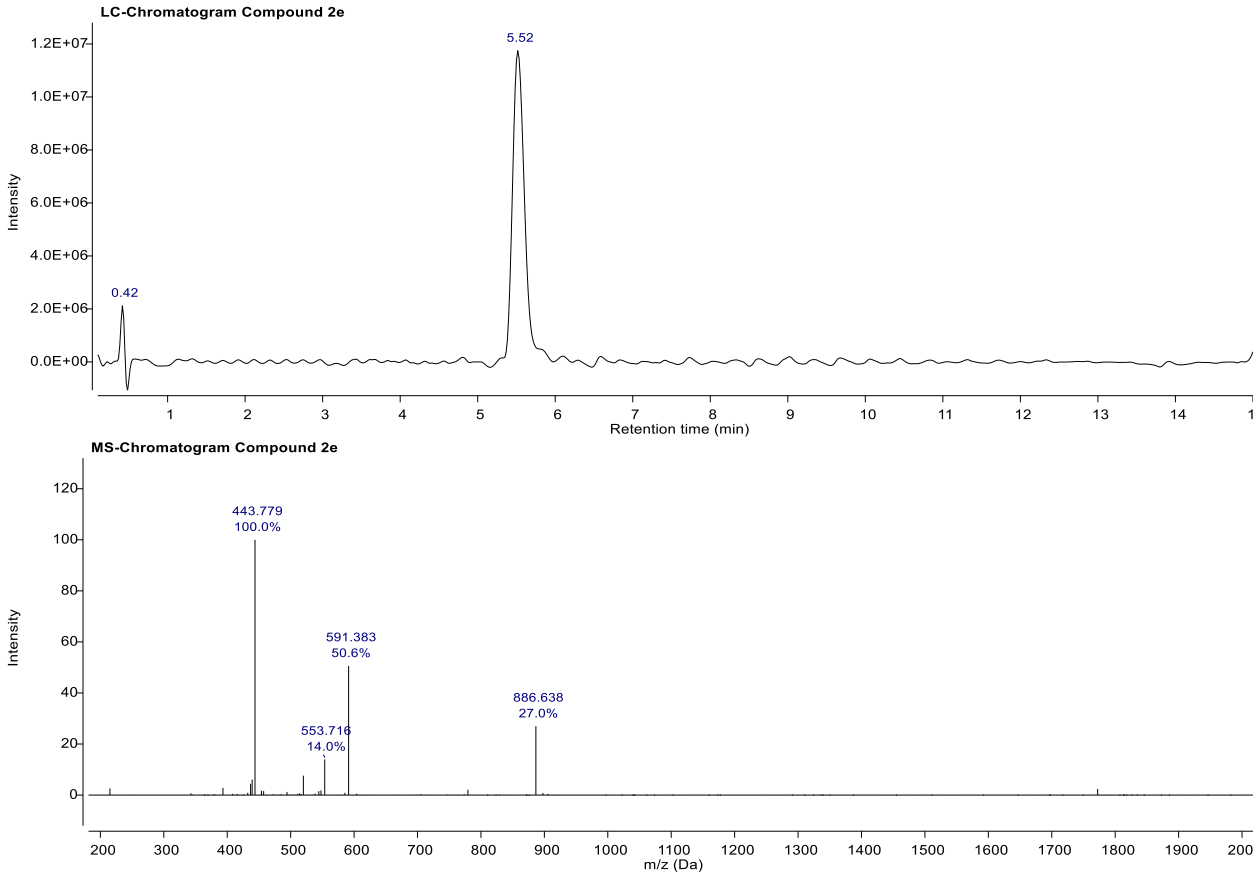
Gradient 5-60% MeCN, 7 min, 2mL/min

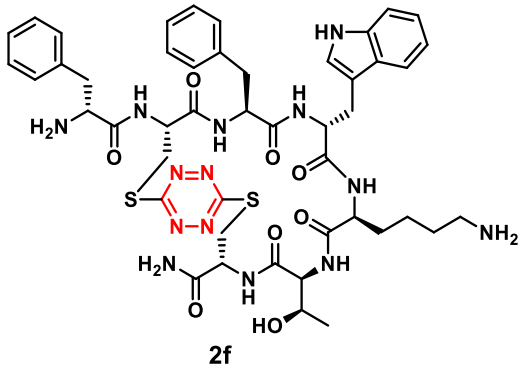




Peptide 2e. Peptide **1e** (17.0 mg, 10µmol) was subjected to the general phase-transfer protocol to construct **2e**. The crude reaction mixture was purified by reverse-phase HPLC [eluent water/MeCN/AcOH (85:10:5) and MeCN (organic) buffered with 0.1%TFA] (gradient 0-60% organic over 20 min) to give (11.8 mg, 67%) of an orange powder after lyophilization: MALDI-TOF Found *m/z* 1793.153 [$(M+Na)^+$; calcd for $C_{81}H_{122}N_{22}NaO_{19}S_2$: 1793.860]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.93 (s, 1H), 8.63 (d, *J* = 7.2 Hz, 1H), 8.41 (d, *J* = 8.3 Hz, 1H), 8.27 (d, *J* = 7.6 Hz, 1H), 8.23 – 8.12 (m, 6H), 8.12 – 8.05 (m, 1H), 8.01 (s, 7H), 7.95 (s, 3H), 7.89 (d, *J* = 8.0 Hz, 2H), 7.43 (s, 1H), 7.29 – 7.23 (m, 1H), 7.22 (s, 1H), 7.21 (s, 2H), 7.18 – 7.12 (m, 1H), 7.10 (s, 1H), 7.04 (d, *J* = 8.3 Hz, 2H), 6.97 (d, *J* = 8.3 Hz, 2H), 6.65 (d, *J* = 8.0 Hz, 2H), 6.62 (d, *J* = 8.2 Hz, 2H), 4.84 (q, *J* = 6.5 Hz, 1H), 4.73 – 4.63 (m, 2H), 4.59 (dd, *J* = 13.9, 5.9 Hz, 1H), 4.42 (dq, *J* = 15.0, 7.6 Hz, 2H), 4.34 – 4.22 (m, 3H), 4.22 – 4.09 (m, 3H), 3.92 (dd, *J* = 12.5, 6.8 Hz, 3H), 3.81 – 3.68 (m, 3H), 3.02 (dd, *J* = 14.2, 4.3 Hz, 1H), 2.89 (dd, *J* = 34.1, 11.3 Hz, 3H), 2.85 – 2.79 (m, 1H), 2.74 (h, *J* = 6.1 Hz, 8H), 2.66 (dd, *J* = 16.2, 4.8 Hz, 2H), 1.73 – 1.61 (m, 2H), 1.59 – 1.51 (m, 10H), 1.50 – 1.42 (m, 3H), 1.32 (dt, *J* = 15.7, 7.9 Hz, 9H), 1.22 (d, *J* = 6.9 Hz, 1H), 1.05 (d, *J* = 6.1 Hz, 4H), 0.88 (d, *J* = 6.5 Hz, 3H), 0.85 (d, *J* = 6.3 Hz, 3H), 0.83 (d, *J* = 6.5 Hz, 3H), 0.81 (d, *J* = 4.0 Hz, 3H), 0.80 (d, *J* = 3.7 Hz, 4H), 0.78 (d, 3H), 0.75 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.5, 172.1, 171.7, 171.6, 171.4, 171.4, 171.1, 170.9, 170.8, 170.7, 169.2, 169.2, 169.0, 168.7, 167.8, 155.9, 155.8, 137.4, 130.2, 129.2, 128.0, 127.7, 127.5, 126.3, 66.9, 65.8, 58.3, 56.3, 54.5, 54.4 , 53.5 , 52.8 , 52.4 , 52.2 , 51.1 , 50.8 , 49.6 , 42.0 , 38.5 , 37.4 , 36.7 , 36.3 , 36.2 , 31.4 , 30.8, 26.5, 26.5, 26.5, 24.0, 23.9, 23.5, 23.1, 22.3, 22.2, 21.8, 21.5, 21.2, 19.4, 14.5, 11.2; IR (KBr, cm^{-1}) 3412(br), 2963(w), 1668(s), 1517(m), 1238(w), 1203(m), 1134(m), 1033(w), 1009(w).

Gradient 5-60% MeCN, 15 min, 2mL/min

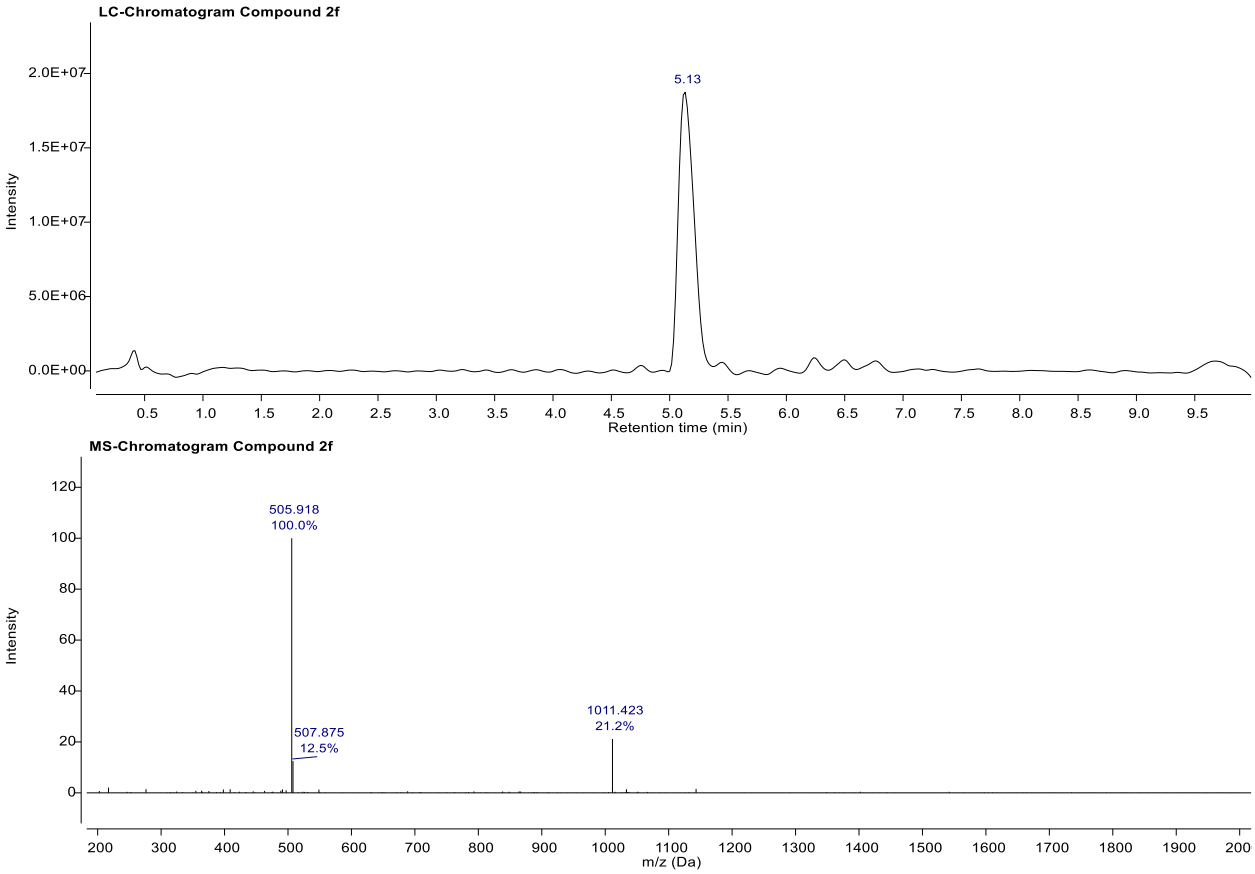


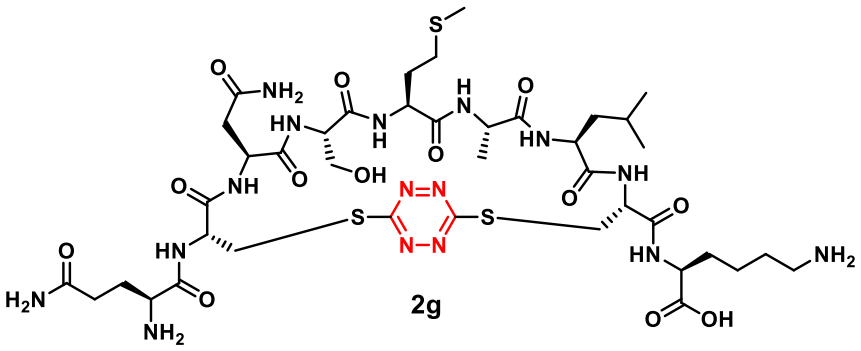


2f

Peptide 2f. Peptide **1f** (11.0 mg, 11.8 µmol) was subjected to the general phase-transfer protocol to construct **2f**. The crude reaction mixture was purified by reverse-phase HPLC (gradient 10-60% organic over 15 min) to give 6.2 mg (52%) of an orange powder after lyophilization: HRMS (ES) Found *m/z* 1011.4095 $[(M+H)^+$; calcd for $C_{47}H_{59}N_{14}O_8S_2$: 1011.4082]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.87 (s, 1H), 9.07 (d, *J* = 8.3 Hz, 1H), 8.57 (d, *J* = 8.2 Hz, 1H), 8.36 (d, *J* = 8.6 Hz, 1H), 8.30 (d, *J* = 8.6 Hz, 1H), 8.23 (d, *J* = 8.4 Hz, 1H), 8.14 (s, 3H), 7.73 (s, 3H), 7.66 (d, *J* = 7.9 Hz, 1H), 7.50 (d, *J* = 8.1 Hz, 1H), 7.38 (d, *J* = 30.6 Hz, 2H), 7.31 (d, *J* = 8.0 Hz, 1H), 7.30 – 7.28 (m, 4H), 7.28 – 7.21 (m, 2H), 7.17 (d, *J* = 2.4 Hz, 1H), 7.12 – 6.97 (m, 5H), 6.88 (d, *J* = 6.8 Hz, 2H), 6.59 (s, 1H), 4.97 (d, *J* = 4.8 Hz, 1H), 4.84 (td, *J* = 8.8, 4.5 Hz, 1H), 4.70 (dd, *J* = 8.3, 3.4 Hz, 1H), 4.68 – 4.64 (m, 1H), 4.57 (ddd, *J* = 14.2, 8.6, 5.8 Hz, 1H), 4.28 (dt, *J* = 14.3, 8.6, 6.1 Hz, 2H), 4.20 (dd, *J* = 8.2, 4.5 Hz, 1H), 4.15 – 4.07 (m, 1H), 3.86 (q, *J* = 5.3 Hz, 1H), 3.67 – 3.53 (m, 2H), 3.45 (dd, *J* = 14.0, 8.2 Hz, 1H), 3.09 (dt, *J* = 13.9, 5.3 Hz, 3H), 2.88 (ddd, *J* = 19.7, 14.2, 9.2 Hz, 2H), 2.75 – 2.66 (m, 5H), 2.63 (dd, *J* = 13.4, 4.8 Hz, 1H), 1.61 (dq, *J* = 17.3, 5.4 Hz, 1H), 1.54 – 1.38 (m, 3H), 1.27 – 1.07 (m, 3H), 0.96 (d, *J* = 6.3 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.6, 171.4, 171.3, 171.0, 170.0, 169.0, 168.5, 168.4, 157.8, 137.2, 136.2, 134.8, 129.6, 129.3, 128.6, 127.9, 127.2, 127.1, 126.2, 124.1, 121.0, 118.6, 118.3, 111.4, 109.8, 66.9, 57.4, 53.6, 53.5, 53.4, 52.9, 51.4, 50.4, 38.7, 37.9, 37.3, 33.8, 32.0, 30.7, 28.7, 26.6, 22.1, 19.5.

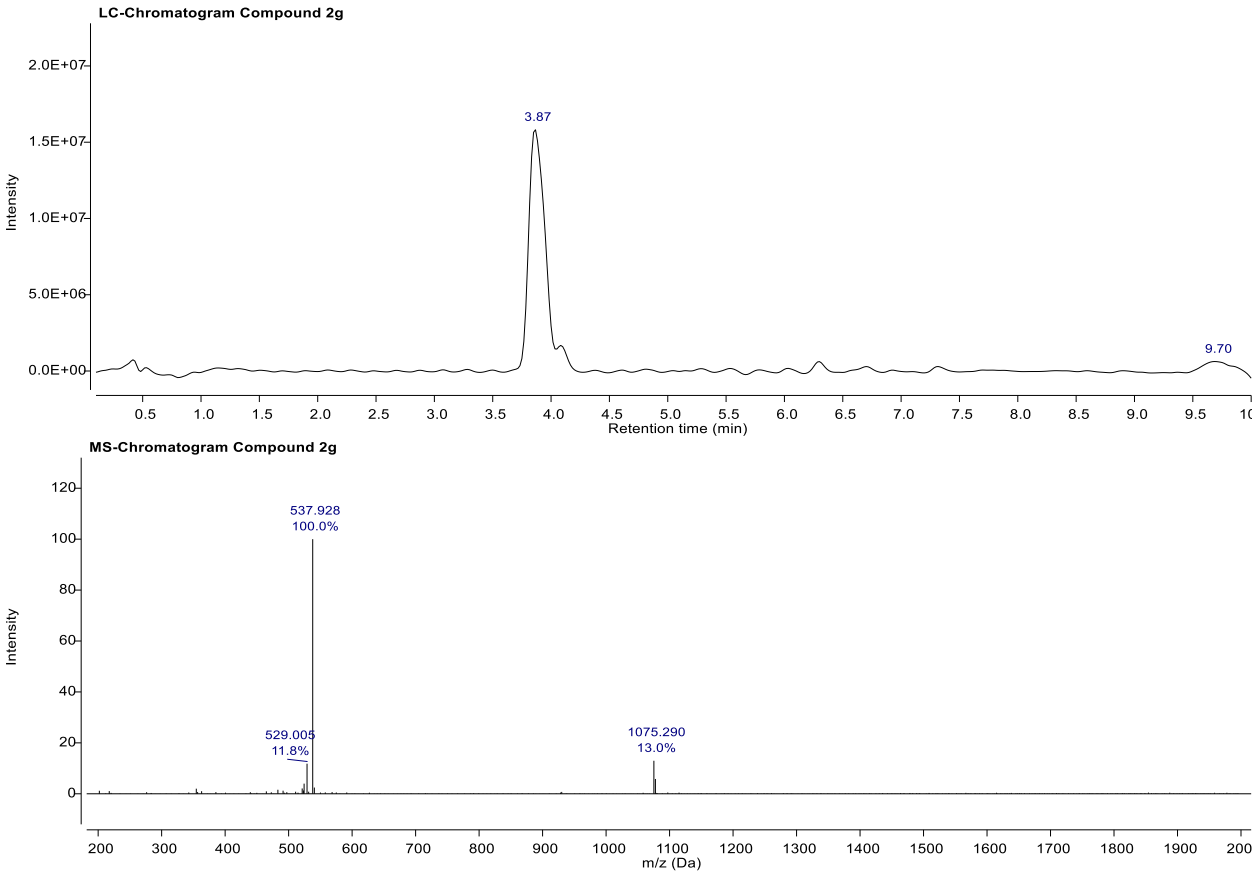
Gradient 5-60% MeCN, 10 min, 2mL/min

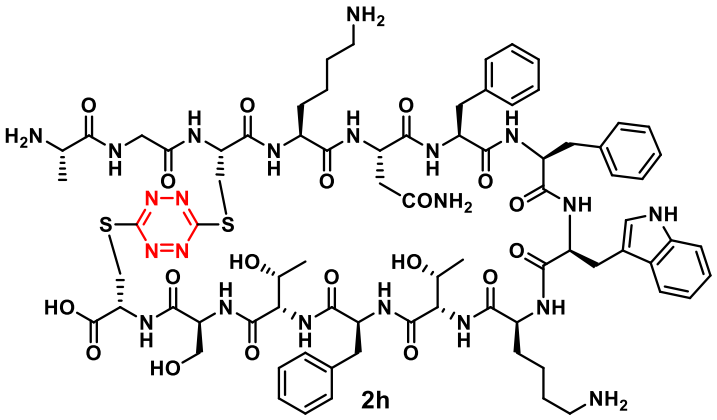




Peptide 2g. Peptide **1g** (10.0 mg, 10 µmol) was subjected to the general phase-transfer protocol to construct **2e**. The crude reaction mixture was purified by reverse-phase HPLC [elutant water/MeCN/AcOH (85:10:5) and MeCN (organic) buffered with 0.1%TFA] (gradient 0-60% organic over 15 min) to give (4.7 mg, 44 %) of an orange powder after lyophilization: HRMS (ES) Found *m/z* 1075.4239 $[(M+H)^+$; calcd for $C_{34}H_{50}N_{13}O_9S_2$: 1075.4236]^{1}H NMR (500 MHz, DMSO-d_6) δ 9.19 (d, *J* = 8.1 Hz, 1H), 8.47 (d, *J* = 7.3 Hz, 1H), 8.41 – 8.28 (m, 5H), 8.22 (d, *J* = 8.1 Hz, 1H), 8.07 (d, *J* = 7.8 Hz, 1H), 8.01 (d, *J* = 5.2 Hz, 1H), 7.99 – 7.89 (m, 3H), 7.66 (s, 2H), 7.59 – 7.48 (m, 2H), 7.08 (s, 1H), 6.98 (s, 1H), 4.82 (ddd, *J* = 13.6, 8.2, 5.0 Hz, 1H), 4.65 (dd, *J* = 14.7, 6.9 Hz, 1H), 4.53 (dd, *J* = 13.7, 6.2 Hz, 1H), 4.28 – 4.05 (m, 6H), 3.86 (dd, *J* = 11.3, 5.8 Hz, 1H), 3.79 – 3.69 (m, 3H), 3.64 (dd, *J* = 14.0, 6.9 Hz, 1H), 2.73 (q, *J* = 6.8 Hz, 2H), 2.67 (dd, *J* = 15.6, 6.5 Hz, 1H), 2.58 (dd, *J* = 15.3, 6.1 Hz, 1H), 2.42 – 2.34 (m, 1H), 2.23 (dd, *J* = 9.2, 6.7 Hz, 2H), 2.01 (s, 4H), 1.94 (q, *J* = 7.4 Hz, 2H), 1.86 – 1.77 (m, 1H), 1.77 – 1.68 (m, 1H), 1.65 – 1.46 (m, 5H), 1.44 – 1.30 (m, 5H), 1.16 (d, *J* = 7.1 Hz, 3H), 0.88 (d, *J* = 6.5 Hz, 3H), 0.83 (d, *J* = 6.5 Hz, 4H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.5, 173.1, 172.2, 172.1, 172.0, 171.7, 171.3, 170.9, 170.2, 169.4, 168.8, 168.7, 61.1, 56.0, 52.1, 51.8, 51.8, 51.6, 51.2, 50.2, 48.6, 38.5, 36.8, 32.3, 30.9, 30.4, 30.4, 30.2, 29.9, 27.0, 26.5, 24.2, 23.2, 22.2, 21.5, 17.8, 14.6; IR (KBr, cm^{-1}) 3413(br), 2923(m), 1655(s), 1541(m), 1236(m).

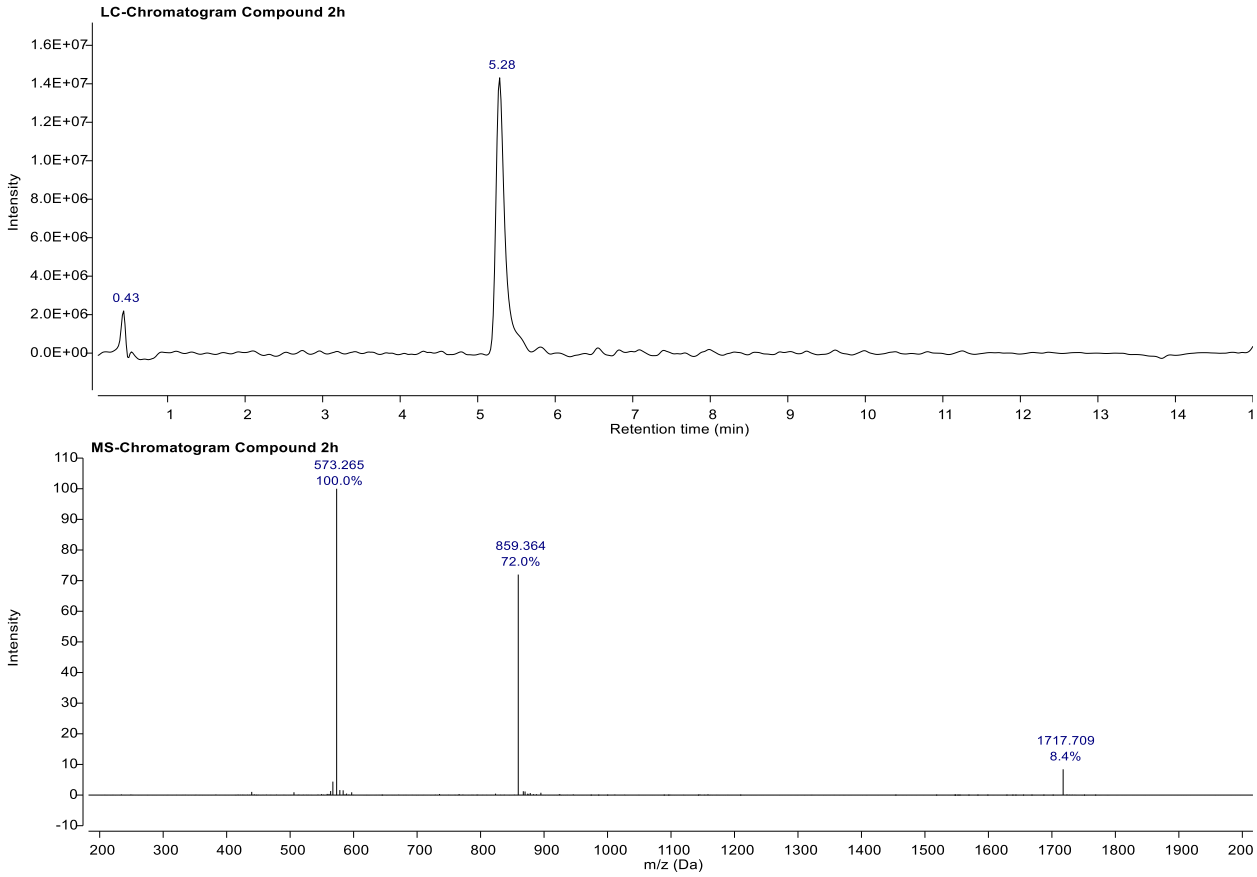
Gradient 5-60% MeCN, 10 min, 2mL/min

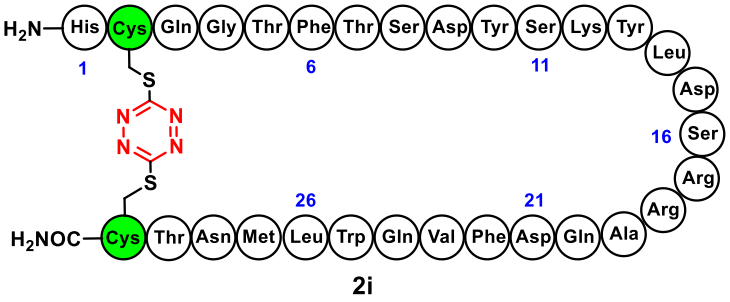




Peptide 2h. Peptide **1h** (8.3 mg, 5.1 µmol) was subjected to the general phase-transfer protocol to construct **2h**. The crude reaction mixture was purified by reverse-phase HPLC (gradient 10-60% organic over 15 min) to give (5.5 mg, 63%) of an orange powder after lyophilization: MALDI-TOF Found *m/z* 1717.968 [$(M+H)^+$; calcd for $C_{78}H_{105}N_{22}O_{19}S_2$: 1717.736]. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.79 (s, 1H), 8.66 (t, *J* = 4.9 Hz, 2H), 8.50 (d, *J* = 6.4 Hz, 2H), 8.43 – 8.37 (m, 1H), 8.33 – 8.26 (m, 1H), 8.20 – 8.16 (m, 5H), 8.12 – 8.06 (m, 1H), 8.05 – 8.01 (m, 5H), 7.93 (d, *J* = 5.2 Hz, 1H), 7.88 – 7.83 (m, 13H), 7.79 – 7.72 (m, 2H), 7.70 – 7.65 (m, 3H), 7.58 (d, *J* = 7.5 Hz, 2H), 7.37 – 7.27 (m, 1H), 7.25 – 7.04 (m, 22H), 7.04 – 6.93 (m, 2H), 5.44 – 5.40 (m, 1H), 5.26 – 5.19 (m, 1H), 5.16 – 5.10 (m, 1H), 4.96 – 4.92 (m, 1H), 4.86 – 4.79 (m, 1H), 4.79 – 4.71 (m, 1H), 4.62 – 4.52 (m, 2H), 4.47 – 4.41 (m, 1H), 4.34 – 4.29 (m, 6H), 4.26 – 4.22 (m, 2H), 4.15 – 4.08 (m, 1H), 3.99 – 3.95 (m, 1H), 3.92 – 3.86 (m, 2H), 3.84 (d, *J* = 5.8 Hz, 1H), 3.81 – 3.73 (m, 1H), 3.62 – 3.58 (m, 3H), 1.69 – 1.65 (m, 3H), 1.57 – 1.39 (m, 3H), 1.36 (t, *J* = 7.2 Hz, 3H), 1.23 (d, *J* = 7.0 Hz, 2H), 1.05 – 0.99 (m, 5H), 0.96 (t, *J* = 6.2 Hz, 3H); ^{13}C NMR (126 MHz, DMSO) δ 176.4, 171.9, 171.7, 171.6, 171.4, 171.2, 171.0, 171.0, 170.9, 170.9, 170.8, 170.1, 169.9, 169.7, 169.4, 168.6, 168.5, 137.8, 137.7, 137.6, 137.5, 136.2, 136.1, 129.2, 129.2, 129.1, 128.9, 128.9, 128.1, 128.1, 127.3, 127.1, 126.3, 123.7, 72.5, 69.8, 67.0, 66.9, 66.5, 65.8, 63.1, 61.8, 61.5, 57.8, 55.2, 55.1, 54.8, 54.0, 53.7, 53.5, 52.7, 52.4, 51.1, 49.6, 48.2, 43.2, 41.8, 38.7, 37.5, 37.2, 37.0, 36.6, 26.7, 26.6, 26.5, 26.5, 22.5, 22.2, 20.5, 19.4, 19.0, 17.3; IR (KBr, cm^{-1}) 3421(br), 2925(m), 1654(s), 1508(m), 1117(m), 1032(s), 1008(s).

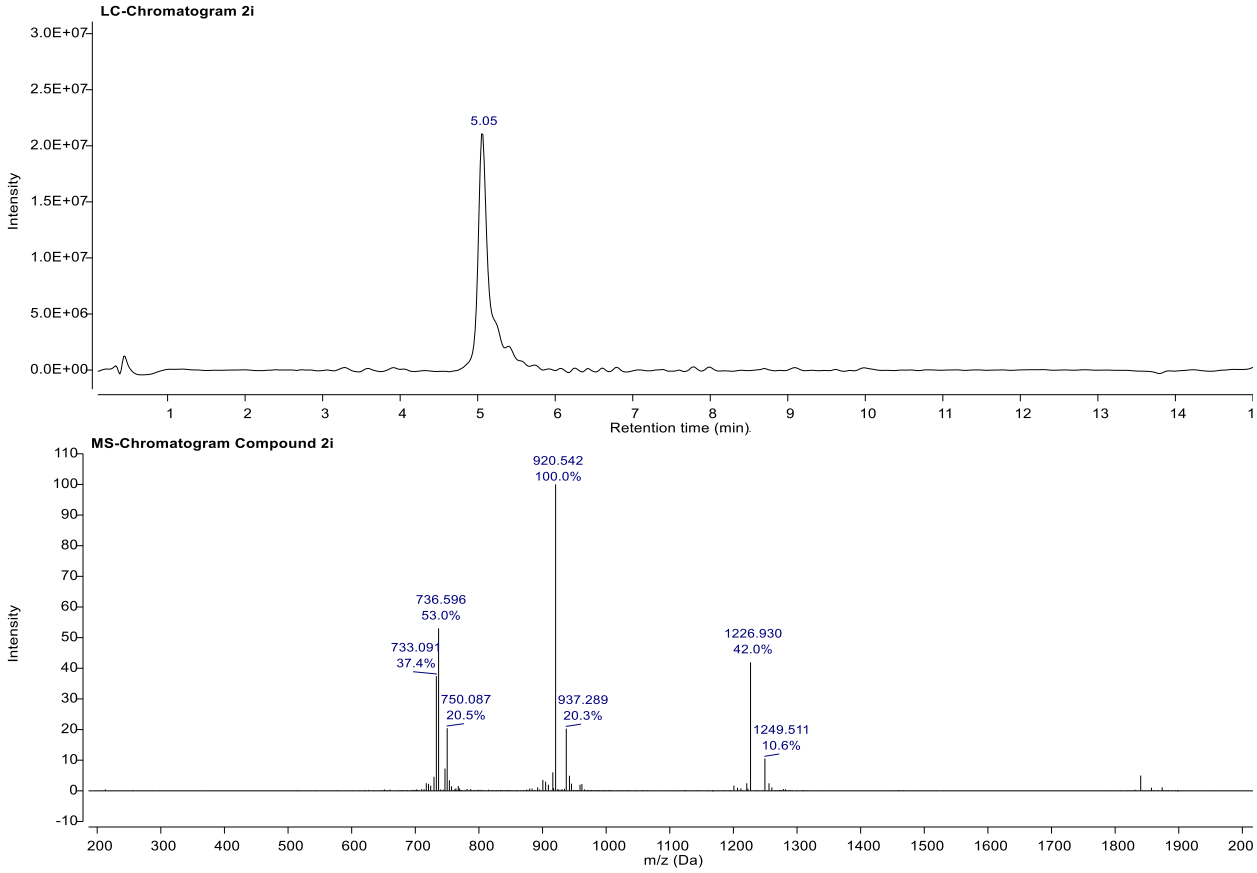
Gradient 5-60% MeCN, 15 min, 2mL/min

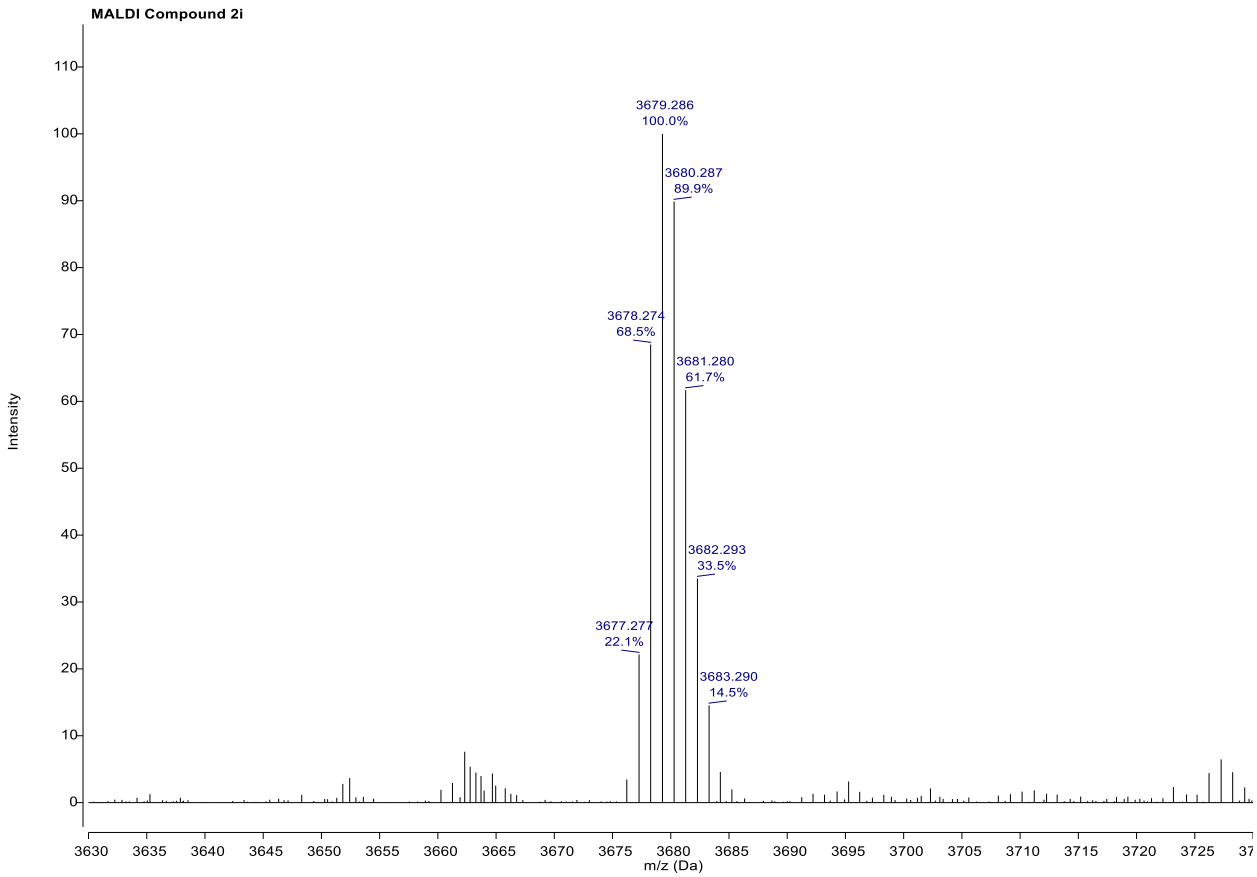




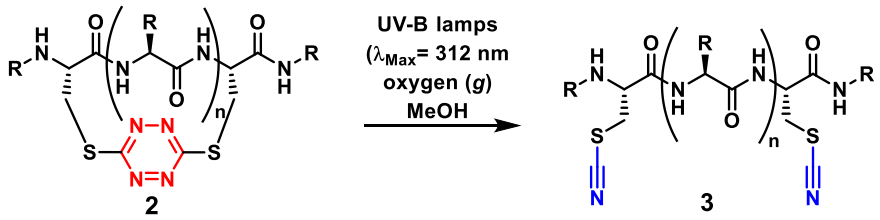
2i

Peptide 2i. Peptide **1i** (7.2 mg, 2 µmol) was subjected to the general phase-transfer protocol with 6M guanidine hydrochloride additive to construct **2i**, the salts were removed by dialysis and the crude reaction mixture was purified by reverse-phase HPLC (10 - 60% organic over 20 min) to give (1.5 mg, 21%) of an orange powder after lyophilization. MALDI-TOF Found *m/z* 3677.277 [$(M+H)^+$; calcd for $C_{158}H_{230}N_{49}O_{48}S_3$: 3677.622].




MALDI Compound 2i
Intensity
m/z (Da)
3679.286
100.0%
3680.287
89.9%
3678.274
68.5%
3681.280
61.7%
3682.293
33.5%
3677.277
22.1%
3683.290
14.5%

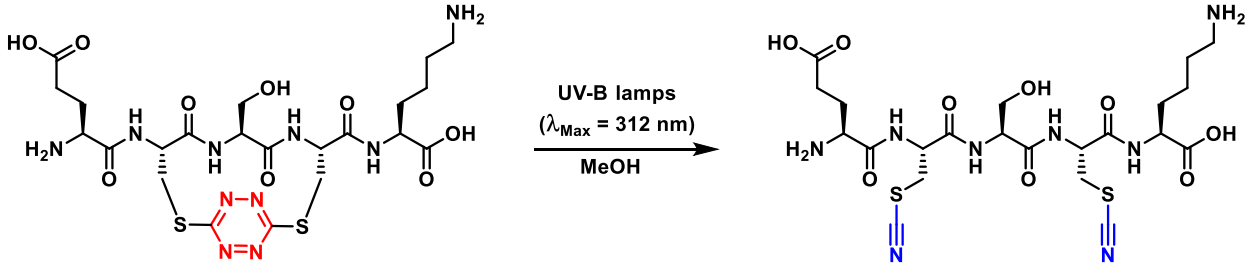
General Procedure for Unstapling S,S-Tetrazine Peptides Photochemically

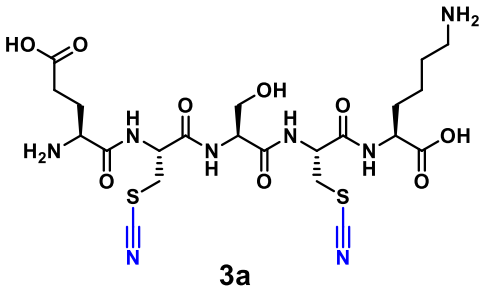


2 3

A 10 mL glass vial was charged with a solution of **2** in MeOH (1-2 mM). The contents were capped with a septum and sparged with oxygen gas for 15 minutes. The solution was then irradiated in a Rayonet® photoreactor equipped with six (7 watt) UV-B lamps (λ_{Max} = 312 nm) until the solution turned colorless. The MeOH was evaporated *in vacuo*, then redissolved in water and lyophilized to yield a white amorphous powder.

Colormetric Change that Occurs After Irradiation in a Rayonet® Photoreactor

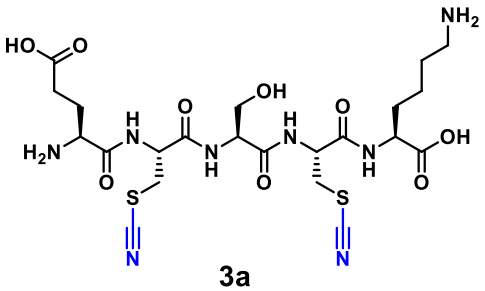




3a

Peptide 3a. Peptide **2a** (10.0 mg, 15 µmol) was subjected to the general photochemical unstapling protocol to yield 9.4 mg (98%). HRMS (ES) Found *m/z* 619.1961 [(M+H)$^+$; calcd for $C_{22}H_{35}N_8O_9S_2$: 619.1963]; ^{1}H NMR (500 MHz, Deuterium Oxide) δ 4.96 (dd, *J* = 7.4, 5.4 Hz, 1H), 4.89 (dd, *J* = 7.9, 5.0 Hz, 1H), 4.61 (t, *J* = 5.6 Hz, 1H), 4.36 (dd, *J* = 8.6, 5.1 Hz, 1H), 4.22 (t, *J* = 6.4 Hz, 1H), 3.93 (d, *J* = 5.7 Hz, 2H), 3.62 (ddd, *J* = 14.2, 5.2, 2.2 Hz, 2H), 3.45 (ddd, *J* = 14.2, 8.8, 7.7 Hz, 2H), 3.03 (t, *J* = 7.6 Hz, 2H), 2.59 (t, *J* = 7.4 Hz, 2H), 2.26 (q, *J* = 7.1 Hz, 2H), 1.93 (ddd, *J* = 13.5, 8.3, 5.1 Hz, 1H), 1.80 (td, *J* = 14.8, 14.2, 8.1 Hz, 1H), 1.72 (pd, *J* = 7.2, 2.2 Hz, 2H), 1.47 (p, *J* = 7.4, 6.8 Hz, 2H); ^{13}C NMR (126 MHz, D_2O) δ 177.7, 177.1, 172.8, 171.3, 171.1, 170.9, 115.4, 115.3, 62.5, 57.0, 54.6, 54.6, 54.5, 53.7, 40.6, 35.7, 35.6, 31.6, 30.5, 27.7, 27.3, 23.5; IR (KBr, cm^{-1}) 3425(br), 3286(br), 3077(br), 2950(br), 2159(w), 1682(s), 1628(s), 1535(m), 1429(m), 1207(s), 1132(s).

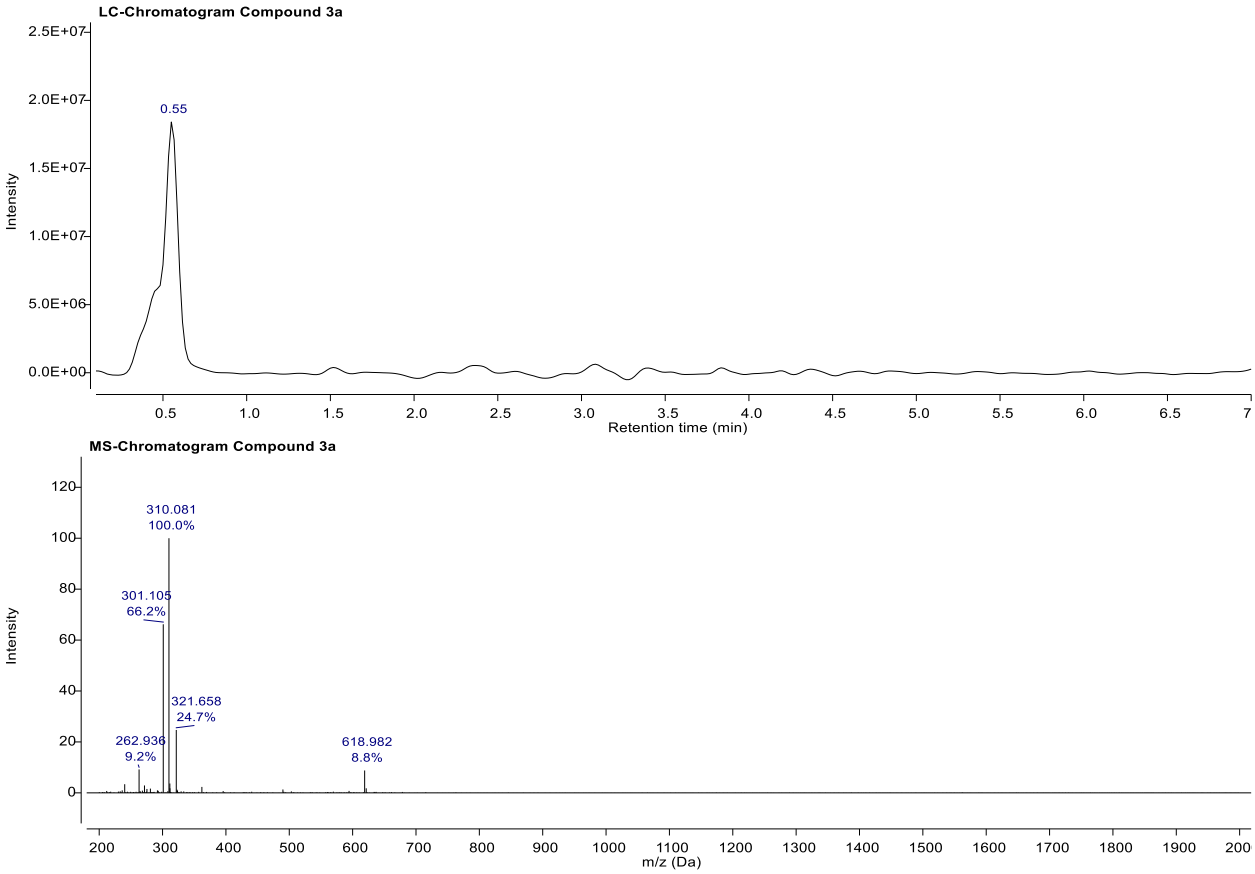
Photochemical Unstapling with UV-A Lamps (λ_{Max} = 365 nm)

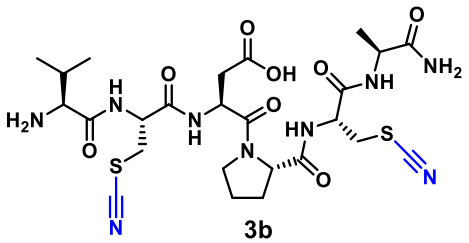


3a

Peptide 3a. Peptide **2a** (15.0 mg, 23 µmol) was dissolved in MeOH (25 mL) and transferred to a thin-walled pyrex tube. The contents were then irradiated in a Rayonet photoreactor with twelve (7 watt) UV-A lamps (λ_{Max}= 365 nm) for 24 hours, during which time the solution turned from red to colorless. The solvent was evaporated then redissolved in water and lyophilized to yield 13.8 mg (96%) of a white amorphous powder. Spectral data was identical to **3a** photolyzed with UV-B lamps.

Gradient 5-60% MeCN, 7 min, 2mL/min

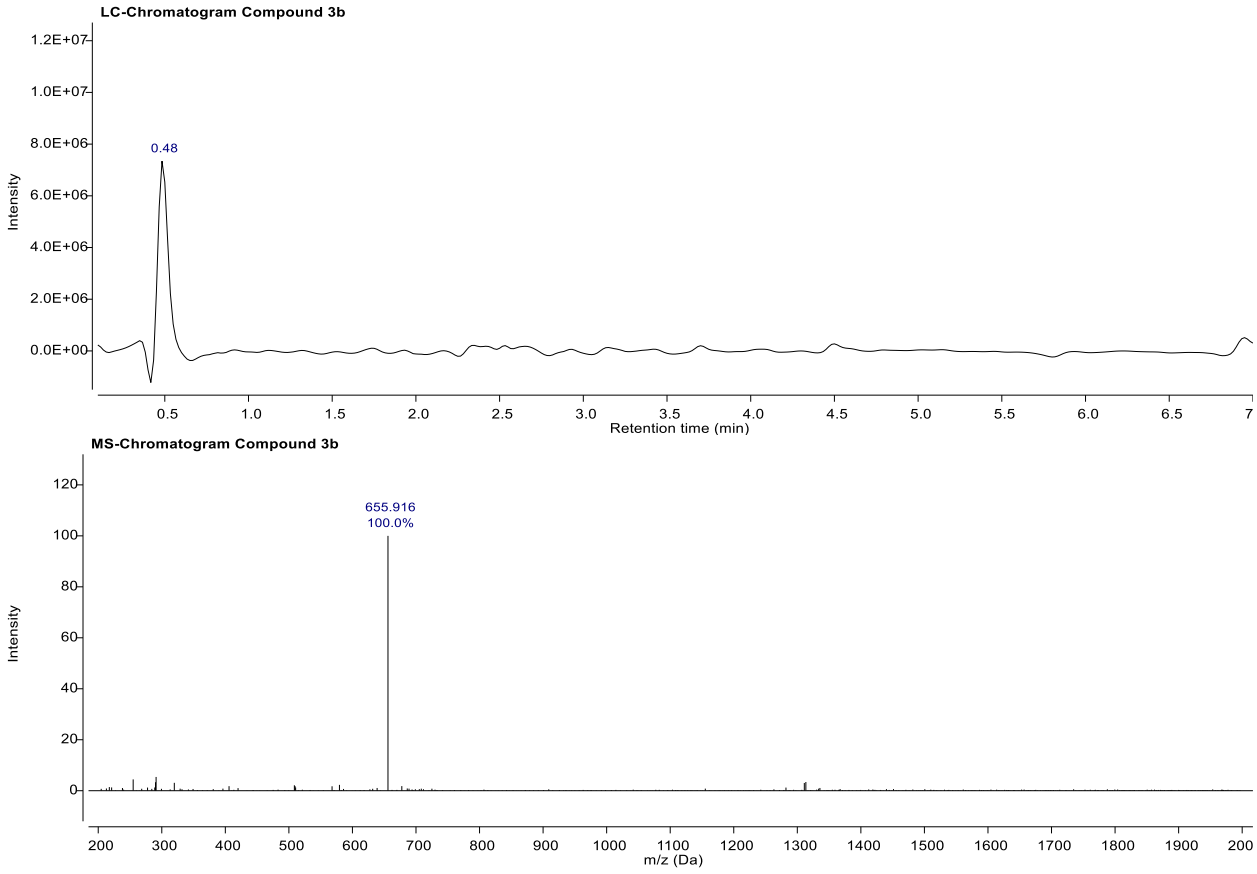


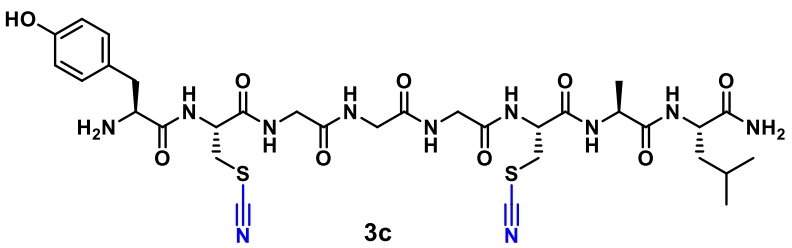


3b

Peptide 3b. Peptide **2b** (1.5 mg, 2.2 µmol) was subjected to the general photochemical unstapling protocol to yield 1.4 mg (96%): HRMS (ES) Found *m/z* 656.2283 [(M+H)$^+$; calcd for $C_{25}H_{38}N_9O_8S_2$: 656.2285]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.88 (d, *J* = 7.7 Hz, 1H), 8.84 (d, *J* = 7.9 Hz, 1H), 8.08 (d, *J* = 5.6 Hz, 3H), 7.98 (d, *J* = 8.0 Hz, 1H), 7.76 (d, *J* = 7.5 Hz, 1H), 7.14 (s, 1H), 7.02 (s, 1H), 4.85 (q, *J* = 7.1 Hz, 1H), 4.68 (q, *J* = 7.7 Hz, 1H), 4.51 (td, *J* = 8.6, 4.4 Hz, 1H), 4.29 (dd, *J* = 8.6, 3.6 Hz, 1H), 4.14 (p, *J* = 7.3 Hz, 1H), 3.67 (dt, *J* = 10.8, 5.8 Hz, 4H), 3.55 (dd, *J* = 13.5, 4.6 Hz, 2H), 3.45 (dd, *J* = 13.5, 5.4 Hz, 1H), 3.27 (dd, *J* = 13.3, 9.2 Hz, 1H), 3.20 (dd, *J* = 13.4, 8.1 Hz, 1H), 2.81 (dd, *J* = 16.9, 6.6 Hz, 1H), 2.09 (dt, *J* = 9.2, 4.5 Hz, 2H), 1.95 (tq, *J* = 10.2, 5.9, 5.3 Hz, 2H), 1.89 (dd, *J* = 11.3, 5.0 Hz, 1H), 1.24 (d, *J* = 7.0 Hz, 3H), 0.93 (dd, *J* = 12.7, 6.9 Hz, 6H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.6, 172.0, 171.7, 169.2, 168.0, 167.8, 167.6, 113.0, 112.4, 60.2, 57.2, 52.8, 52.1, 48.4, 47.7, 46.9, 35.7, 34.9, 34.9, 29.8, 28.8, 24.2, 18.2, 17.8, 17.3; IR (KBr, cm^{-1}) 3323(br), 3067(m), 2974(m), 2160(w), 1669(s), 1525(m), 1202(m).

Gradient 5-60% MeCN, 7 min, 2mL/min

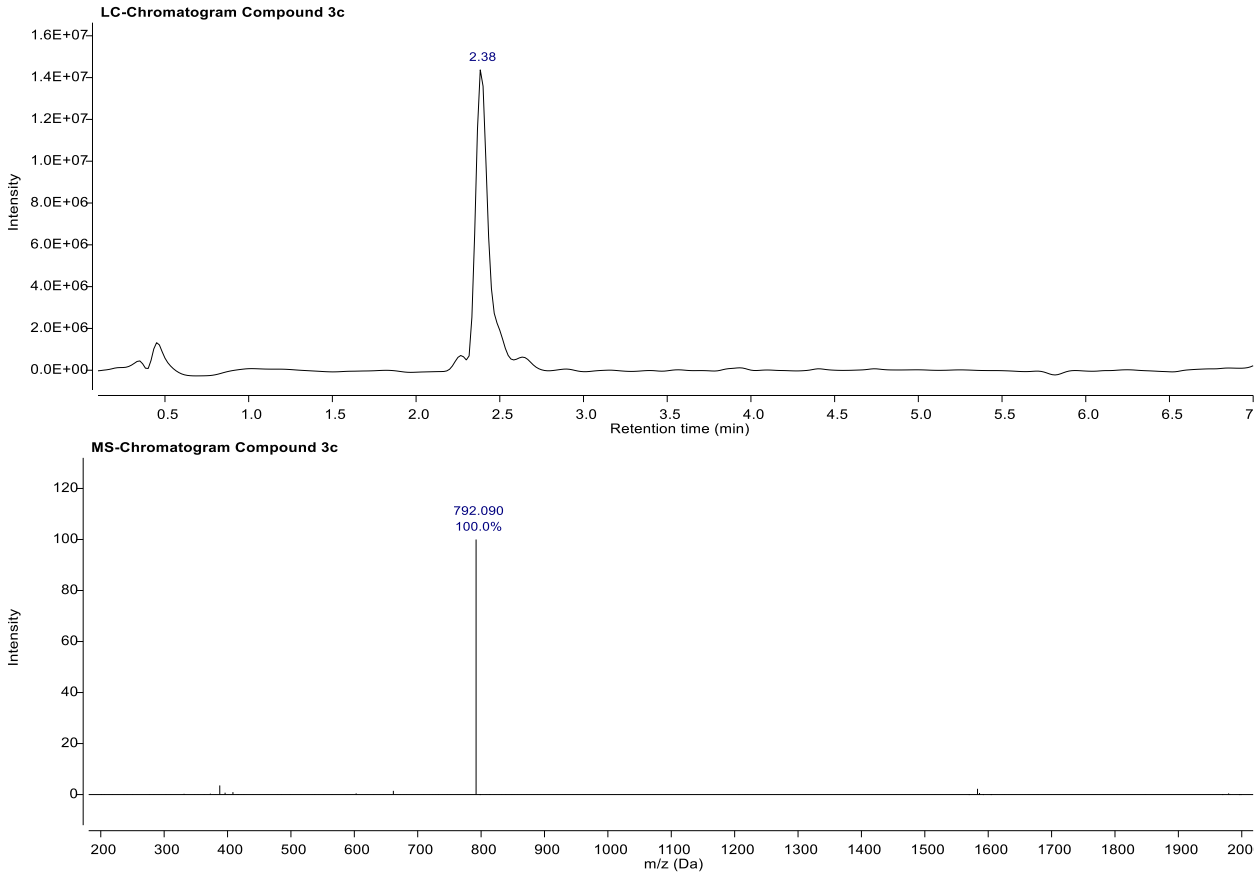


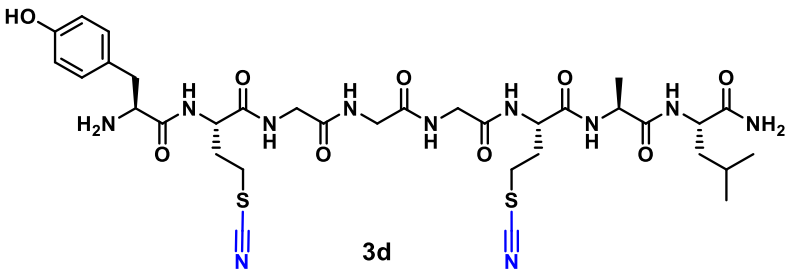


3c

Peptide 3c. Peptide **2c** (2.8 mg, 3.4 µmol) was subjected to the general photochemical unstapling protocol to yield 2.6 mg (97%): HRMS Found (ES) *m/z* 792.2917 [(M+H)$^+$; calcd for $C_{32}H_{46}N_{11}O_9S_2$: 792.2921]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.34 (s, 1H), 9.06 (d, *J* = 8.0 Hz, 1H), 8.52 (t, *J* = 5.7 Hz, 1H), 8.39 (d, *J* = 8.2 Hz, 1H), 8.33 (d, *J* = 7.2 Hz, 1H), 8.19 (dt, *J* = 11.2, 5.8 Hz, 2H), 8.08 (s, 3H), 7.77 (d, *J* = 8.4 Hz, 1H), 7.23 (s, 1H), 7.05 (d, *J* = 8.4 Hz, 2H), 6.96 (s, 1H), 6.71 (d, *J* = 8.4 Hz, 2H), 4.82 – 4.70 (m, 1H), 4.66 (td, *J* = 8.3, 4.7 Hz, 1H), 4.26 – 4.16 (m, 2H), 3.99 (d, *J* = 8.0 Hz, 1H), 3.89 – 3.71 (m, 6H), 3.67 – 3.58 (m, 1H), 3.49 (td, *J* = 13.5, 4.9 Hz, 2H), 3.27 (dd, *J* = 13.5, 7.8 Hz, 1H), 3.22 – 3.17 (m, 1H), 3.04 (dd, *J* = 14.3, 5.0 Hz, 1H), 2.82 (dd, *J* = 14.3, 8.2 Hz, 1H), 1.58 (dt, *J* = 13.5, 6.6 Hz, 1H), 1.45 (t, *J* = 7.2 Hz, 2H), 1.23 (d, *J* = 7.2 Hz, 3H), 0.86 (dd, *J* = 20.4, 6.6 Hz, 6H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.4, 171.9, 169.6, 169.5, 168.6, 168.5, 156.8, 130.8, 124.8, 115.6, 113.1, 54.0, 52.5, 52.4, 51.1, 49.1, 41.7, 41.5, 41.4, 41.1, 36.3, 35.6, 35.5, 24.5, 23.3, 21.8, 17.9; IR (KBr, cm^{-1}) 3294(br), 3067(br), 2962(m), 2159(w), 1676(s), 1518(s), 1431(w), 1205(m), 1136(m).

Gradient 5-60% MeCN, 7 min, 2mL/min

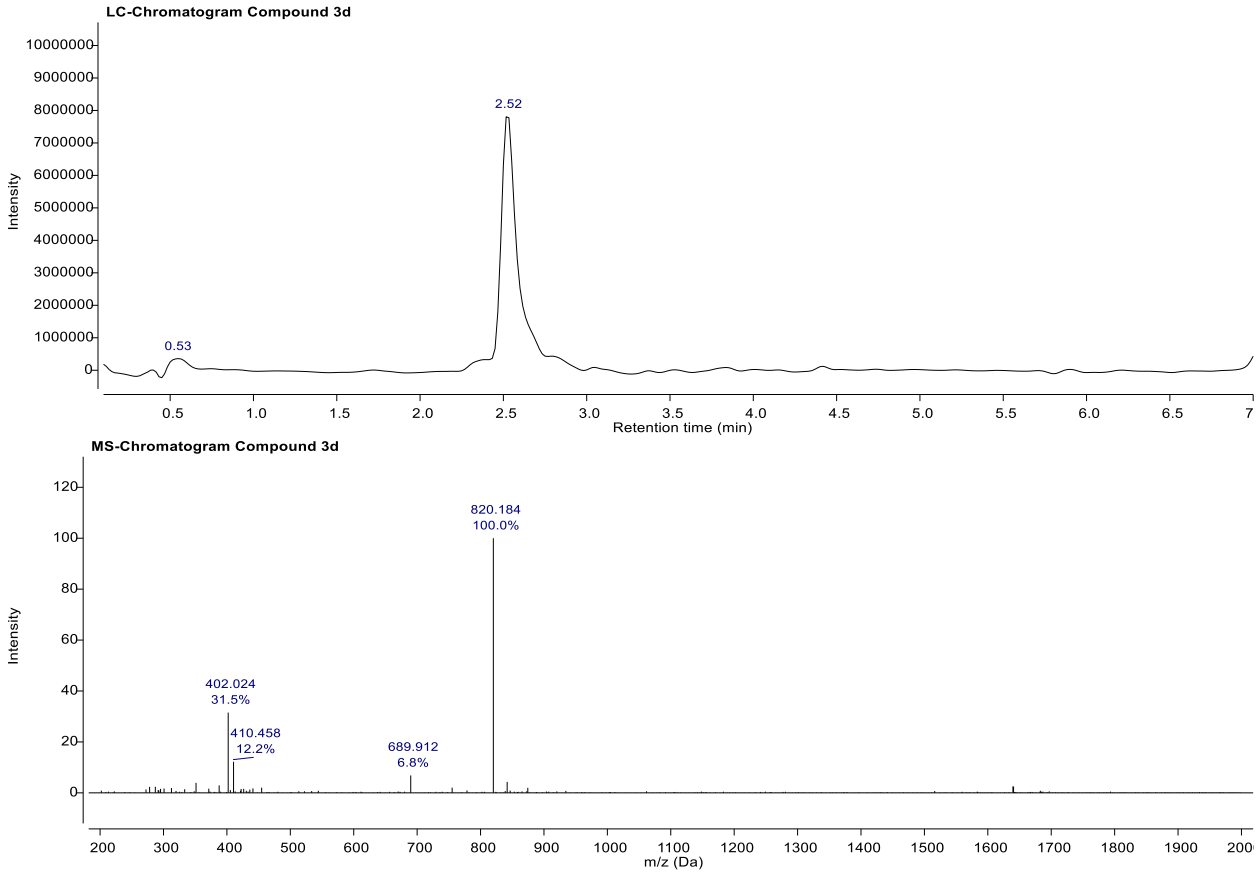


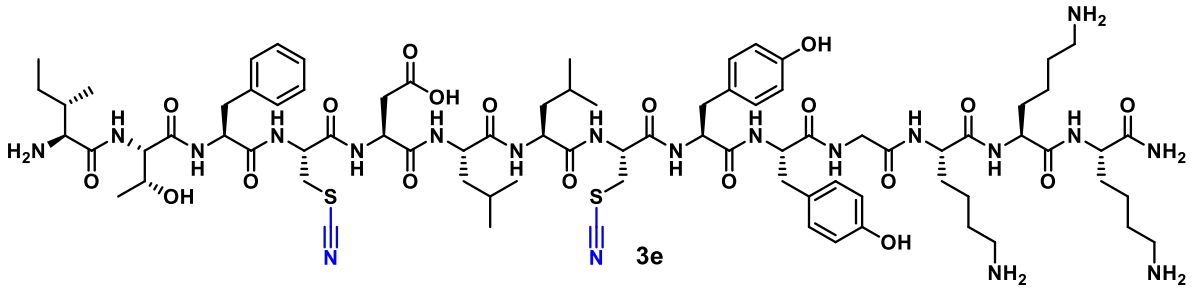


3d

Peptide 3d. Peptide **2d** (3.2 mg, 3.8 µmol) was subjected to the general photochemical unstapling protocol to yield 3.0 mg (96%). HRMS Found (ES) *m/z* 820.3257 [$(M+H)^+$; calcd for $C_{34}H_{50}N_{11}O_9S_2$: 820.3234]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.33 (s, 1H), 8.32 (t, *J* = 5.6 Hz, 1H), 8.25 (d, *J* = 7.2 Hz, 1H), 8.22 (d, *J* = 5.7 Hz, 1H), 8.19 – 8.13 (m, 1H), 7.80 (d, *J* = 8.3 Hz, 1H), 7.73 – 7.69 (m, 2H), 7.68 (d, *J* = 5.1 Hz, 1H), 7.29 (s, 1H), 7.02 (d, *J* = 8.1 Hz, 2H), 6.98 (s, 1H), 6.69 (d, *J* = 8.1 Hz, 2H), 6.56 (s, 1H), 4.55 – 4.47 (m, 1H), 4.44 (dd, *J* = 13.5, 8.1 Hz, 1H), 4.33 – 4.16 (m, 2H), 4.13 (t, *J* = 5.4 Hz, 1H), 3.83 (dd, *J* = 16.6, 5.7 Hz, 1H), 3.79 – 3.68 (m, 4H), 3.13 – 2.99 (m, 3H), 2.93 (dd, *J* = 14.5, 4.7 Hz, 1H), 2.82 – 2.65 (m, 1H), 2.21 – 2.10 (m, 1H), 2.10 – 2.04 (m, 1H), 2.04 – 1.92 (m, 2H), 1.69 – 1.53 (m, 2H), 1.44 (dt, *J* = 9.2, 4.5 Hz, 2H), 1.39 – 1.31 (m, 1H), 1.21 (d, *J* = 7.1 Hz, 3H), 0.87 (d, *J* = 6.3 Hz, 3H), 0.83 (d, *J* = 6.6 Hz, 3H); ^{13}C NMR (126 MHz, DMSO) δ 173.9, 171.6, 170.0, 169.1, 168.8, 168.8, 166.9, 156.3, 131.7, 131.5, 130.3, 128.6, 115.2, 112.9, 112.8, 67.4, 50.9, 50.8, 48.4, 42.0, 41.9, 41.0, 38.1, 29.8, 29.8, 28.3, 24.1, 23.2, 23.0, 22.3, 21.5, 17.6, 13.8, 10.8; IR (KBr, cm^{-1}) 3409(br), 2928(w), 2159(w), 1671(s), 1541(m), 1205(m), 1180(w), 1134(w).

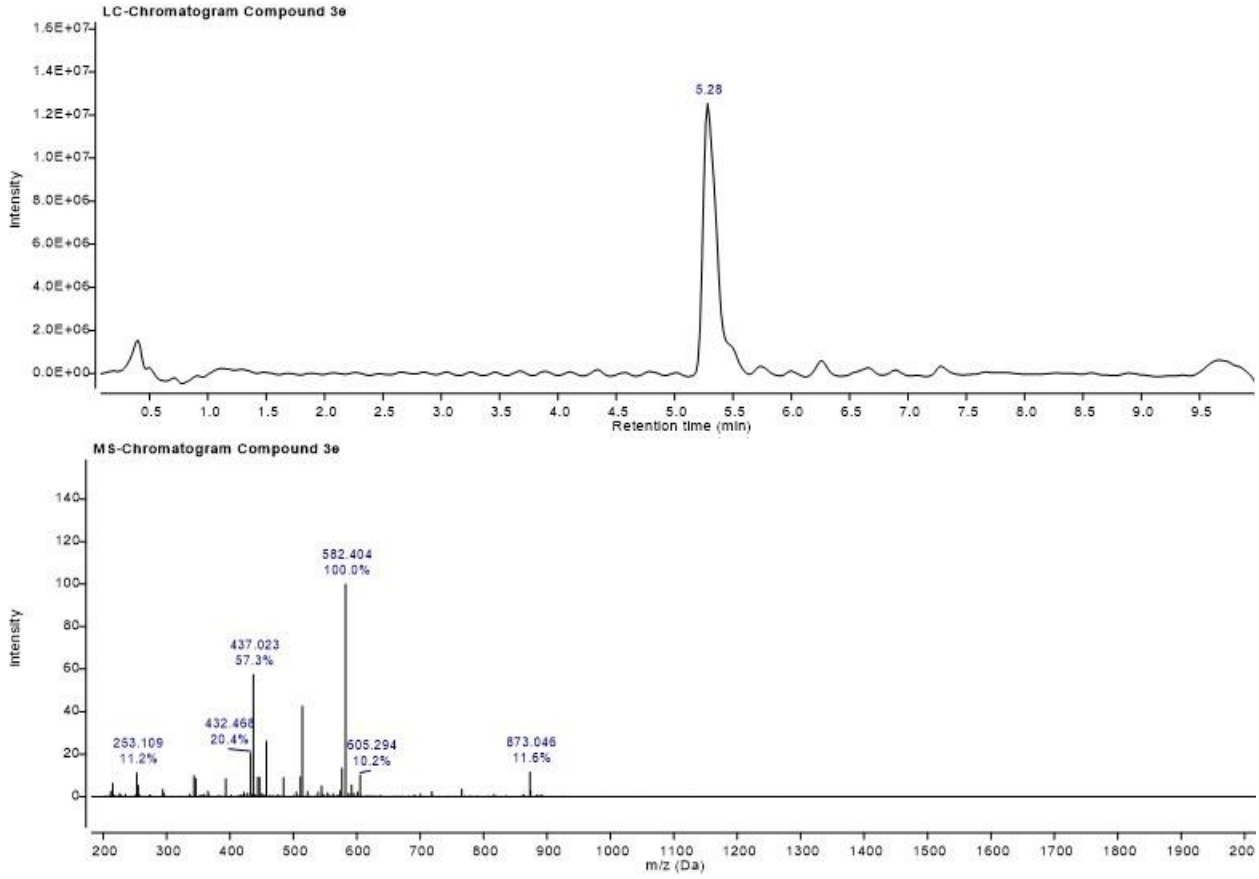
Gradient 5-60% MeCN, 7 min, 2mL/min

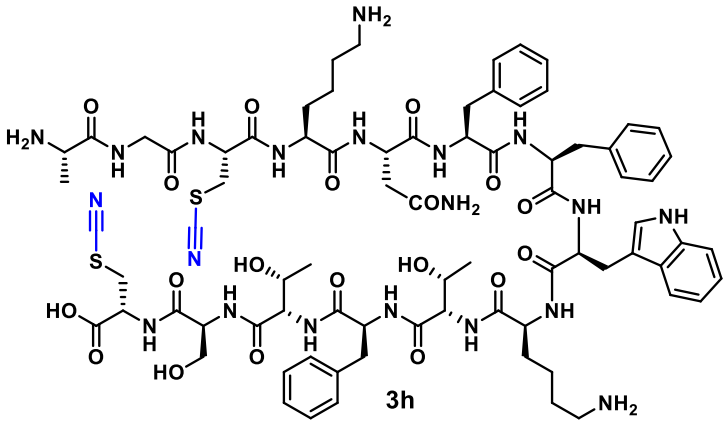




Peptide 3e. Peptide **2e** (4.0 mg, 2.3 µmol) was subjected to the general photochemical unstapling protocol to yield 3.9 mg (99%): MALDI-TOF Found *m/z* 1743.230 [$(M+H)^+$; calcd for $C_{81}H_{123}N_{20}O_{19}S_2$: 1743.871]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.21 (d, *J* = 12.0 Hz, 1H), 8.57 (d, *J* = 7.4 Hz, 1H), 8.32 (d, *J* = 8.3 Hz, 1H), 8.23 – 8.17 (m, 1H), 8.15 (d, *J* = 7.8 Hz, 1H), 8.13 – 8.00 (m, 4H), 7.97 (d, *J* = 7.6 Hz, 1H), 7.92 (d, *J* = 8.1 Hz, 1H), 7.86 (d, *J* = 7.7 Hz, 1H), 7.80 (s, 7H), 7.40 (s, 1H), 7.21 (d, *J* = 6.7 Hz, 3H), 7.16 (d, *J* = 6.3 Hz, 1H), 7.07 (s, 1H), 7.02 (d, *J* = 8.4 Hz, 2H), 6.92 (d, *J* = 8.2 Hz, 2H), 6.64 (d, *J* = 8.1 Hz, 2H), 6.60 (d, *J* = 8.3 Hz, 2H), 5.42 (s, 1H), 5.22 – 5.14 (m, 1H), 5.01 (d, *J* = 4.0 Hz, 1H), 4.66 – 4.57 (m, 3H), 4.55 (t, *J* = 7.0 Hz, 1H), 4.49 (d, *J* = 3.6 Hz, 1H), 4.44 (d, *J* = 6.6 Hz, 1H), 4.43 – 4.38 (m, 1H), 4.37 – 4.24 (m, 3H), 4.23 – 4.17 (m, 1H), 4.17 – 4.09 (m, 1H), 4.03 (q, *J* = 6.8 Hz, 1H), 3.98 – 3.90 (m, 1H), 3.81 – 3.68 (m, 2H), 3.15 (dd, *J* = 13.3, 8.7 Hz, 1H), 3.07 (dd, *J* = 14.5, 3.3 Hz, 1H), 2.91 (d, *J* = 11.3 Hz, 1H), 2.83 (dd, *J* = 13.9, 9.1 Hz, 1H), 2.75 (s, 6H), 2.65 – 2.62 (m, 1H), 1.78 – 1.39 (m, 17H), 1.31 (s, 5H), 1.23 (d, *J* = 6.8 Hz, 3H), 1.05 (d, *J* = 6.3 Hz, 3H), 0.86 (t, *J* = 6.8 Hz, 5H), 0.84 – 0.73 (m, 11H); ^{13}C NMR (126 MHz, DMSO) δ 176.4, 173.5, 171.8, 171.7, 171.6, 171.4, 171.3, 171.2, 170.6, 169.7, 169.2, 168.7, 168.4, 167.9, 167.9, 155.9, 155.8, 137.4, 130.1, 130.1, 129.2, 128.0, 127.7, 127.4, 126.3, 114.9, 114.9, 72.5, 67.0, 65.8, 63.1, 57.9, 56.3, 54.6, 53.6, 52.6, 52.6, 52.3, 52.3, 52.3, 52.2, 52.2, 52.1, 51.2, 51.2, 51.2, 51.1, 49.8, 38.7, 36.3, 31.5, 26.7, 26.6, 24.1, 23.9, 23.2, 23.1, 22.3, 22.2, 21.7, 21.6, 20.5, 19.4, 14.5, 11.1; IR (KBr, cm^{-1}) 3298(br), 3071(br), 2962(m), 2933(m), 2159(w), 1671(s), 1517(m), 1438(m), 1203(s), 1137(m).

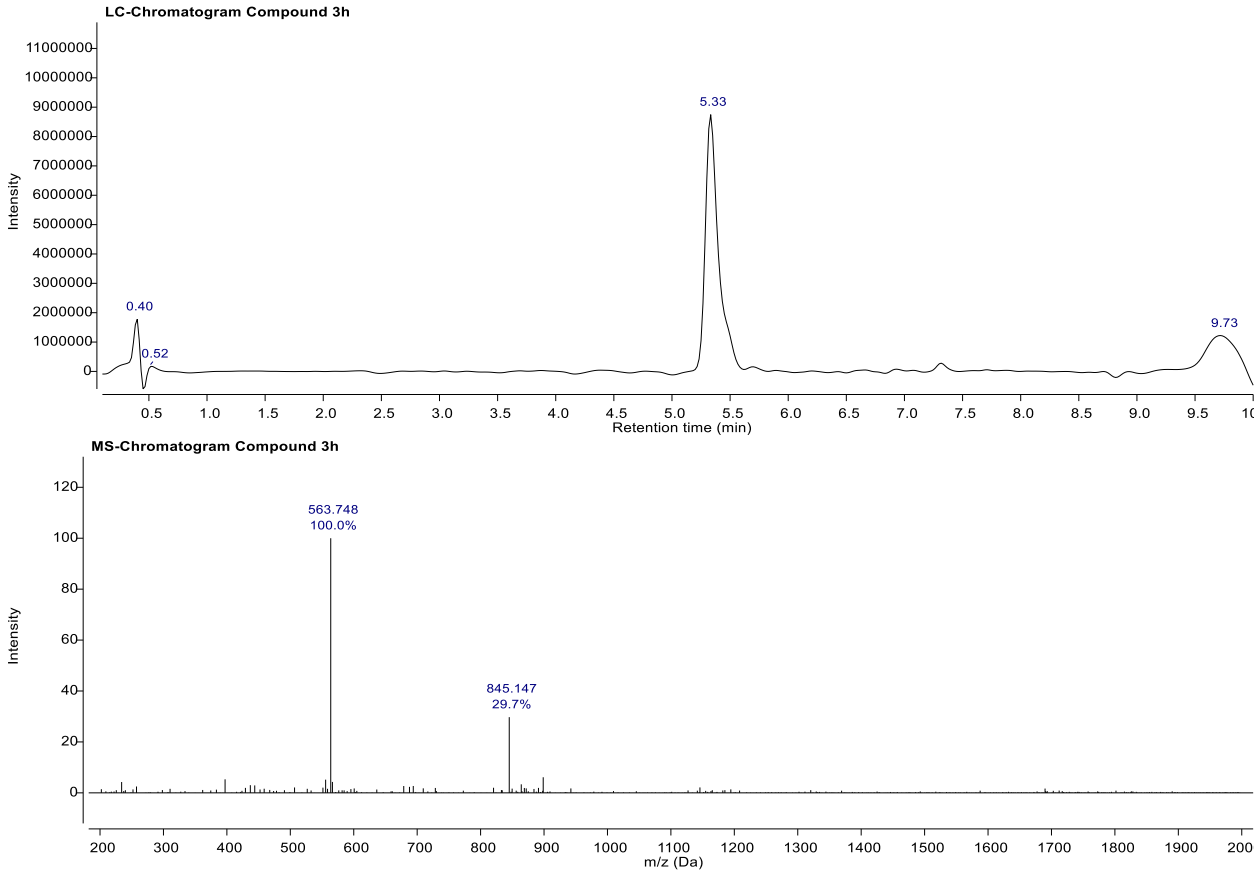
Gradient 5-60% MeCN, 10 min, 2mL/min



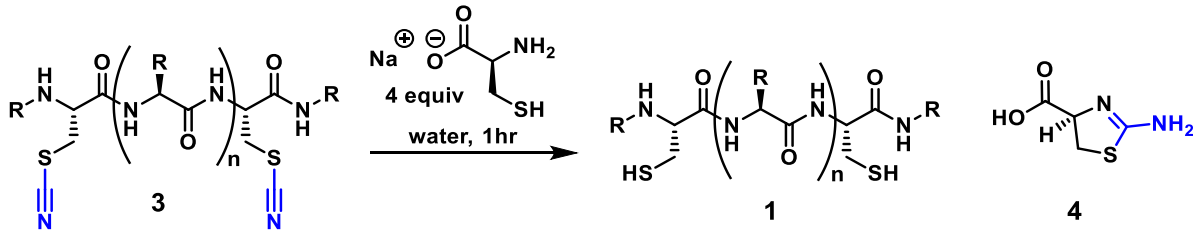


Peptide 3h. Peptide **2h** (8.3 mg, 5.1 µmol) was subjected to the general photochemical unstapling protocol, without sparging with oxygen gas (atmospheric oxygen was not excluded). The crude reaction mixture was purified by reverse-phase HPLC (gradient 10-60% organic over 15 min) to yield 2.1 mg (38%) of a white powder after lyophilization. MALDI-TOF *m/z* 1689.333 [$(M+H)^+$; calcd for $C_{78}H_{105}N_{20}O_{19}S_2$: 1689.730]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.77 (s, 1H), 8.67 (t, *J* = 5.7 Hz, 1H), 8.48 (d, *J* = 8.0 Hz, 1H), 8.34 (d, *J* = 8.0 Hz, 1H), 8.21 – 8.01 (m, 6H), 8.01 – 7.86 (m, 5H), 7.86 – 7.65 (m, 6H), 7.61 (d, *J* = 7.9 Hz, 1H), 7.50 – 7.41 (m, 2H), 7.32 (d, *J* = 7.9 Hz, 1H), 7.27 – 7.08 (m, 15H), 7.05 (t, *J* = 7.5 Hz, 1H), 7.00 (d, *J* = 9.7 Hz, 1H), 6.97 (d, *J* = 7.4 Hz, 1H), 4.88 (d, *J* = 4.5 Hz, 1H), 4.72 – 4.60 (m, 1H), 4.57 (d, *J* = 5.8 Hz, 1H), 4.54 – 4.42 (m, 2H), 4.42 – 4.26 (m, 4H), 4.21 (dd, *J* = 8.6, 3.9 Hz, 2H), 4.10 – 3.93 (m, 1H), 3.94 – 3.82 (m, 3H), 3.74 – 3.57 (m, 1H), 3.53 – 3.43 (m, 1H), 3.19 (dd, *J* = 13.7, 8.8 Hz, 2H), 3.14 – 3.06 (m, 1H), 3.03 – 2.95 (m, 2H), 2.94 – 2.88 (m, 1H), 2.88 – 2.76 (m, 2H), 2.71 (t, *J* = 7.3 Hz, 4H), 2.41 – 2.32 (m, 1H), 1.71 – 1.60 (m, 1H), 1.58 – 1.41 (m, 7H), 1.36 (d, *J* = 6.9 Hz, 3H), 1.32 – 1.18 (m, 5H), 1.14 (d, *J* = 6.9 Hz, 1H), 1.04 (d, *J* = 6.2 Hz, 3H), 0.97 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (126 MHz, DMSO) δ 171.8, 171.3, 171.2, 170.9, 170.8, 170.8, 170.6, 170.5, 170.3, 170.1, 169.8, 169.7, 169.7, 168.6, 168.4, 137.7, 137.6, 137.5, 136.0, 129.2, 129.2, 129.0, 128.0, 127.9, 127.3, 126.1, 126.1, 123.6, 120.8, 118.5, 118.4, 118.2, 116.1, 112.9, 111.2, 109.8, 70.5, 70.4, 66.6, 61.4, 57.9, 57.8, 54.1, 53.9, 53.7, 53.3, 53.3, 52.5, 52.3, 52.3, 49.5, 48.1, 41.8, 38.7, 37.3, 37.3, 37.2, 37.2, 37.2, 37.0, 35.6, 31.1, 26.6, 26.6, 22.1, 22.1, 19.3, 19.3, 17.1; IR (KBr, cm^{-1}) 3424(br), 2933(w), 2159(w), 1671(s), 1632(s), 1526(m), 1204(w), 1136(w).

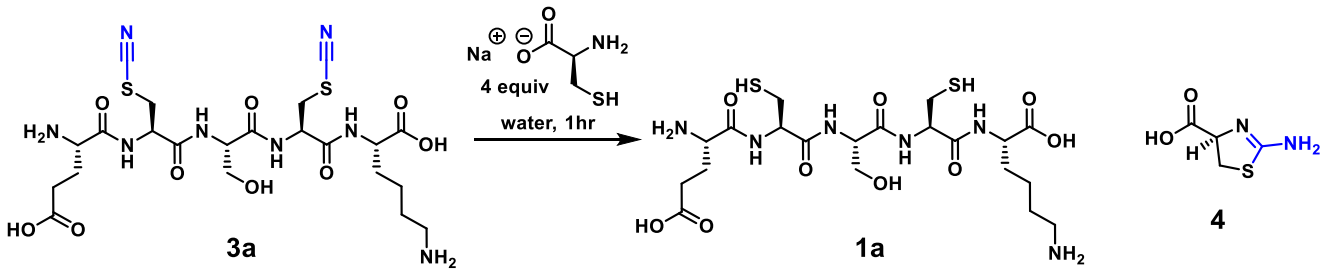
Gradient 5-60% MeCN, 10 min, 2mL/min



General Nitrile Removal Protocol: Regeneration of the Native Peptide

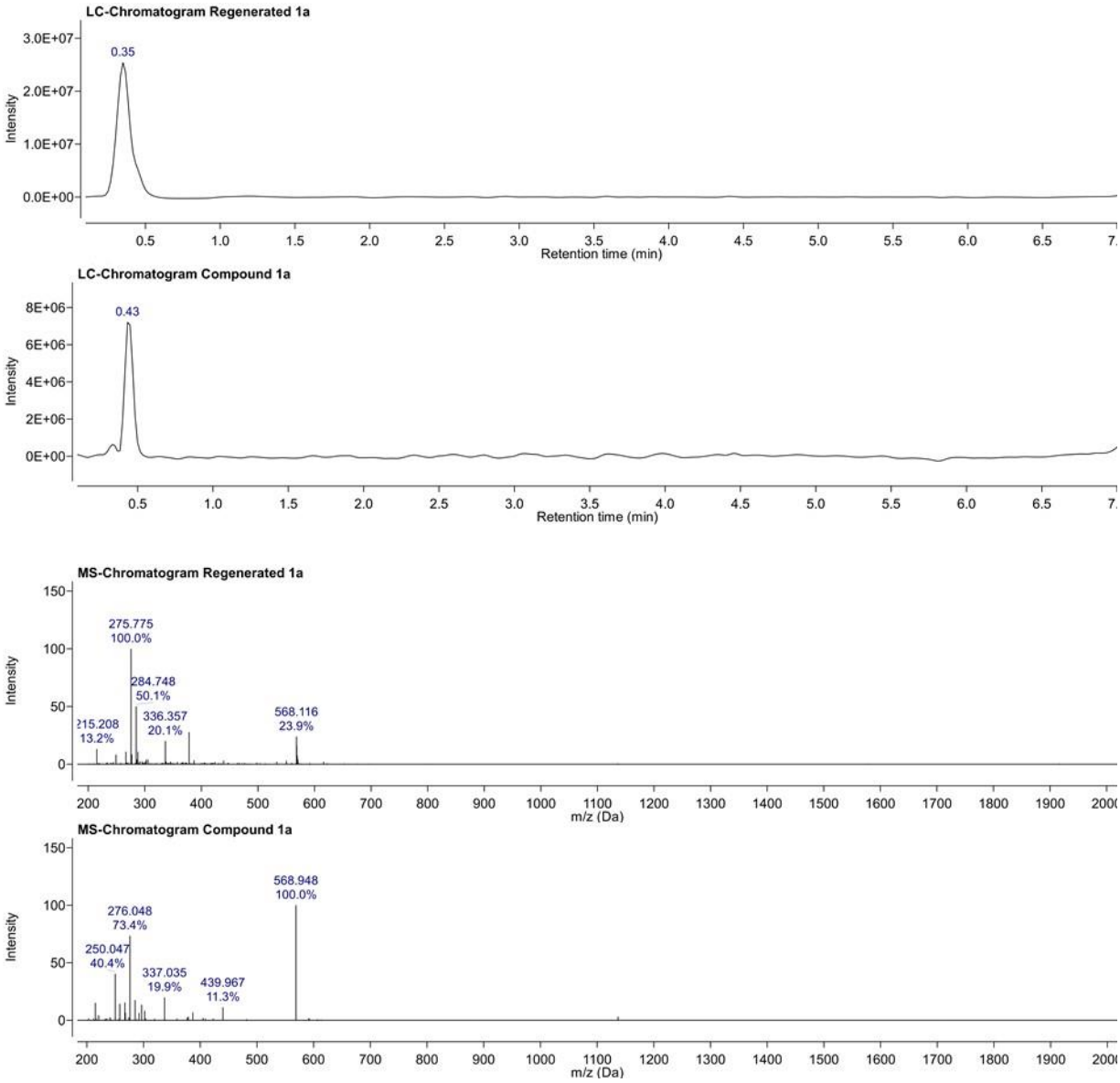


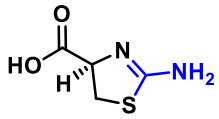
A 13 mm test tube was charged with peptide **3** (1-5 mg) and dissolved in water (1.0 mL). To this solution was added 4 equivalents of a pre-mixed 250 mM solution of sodium cysteine [prepared by dissolving cysteine (121 mg, 1.0 mmol) in 0.25M NaOH (4 mL, 1 equiv)]. The contents were stirred for 1 hour, then formic acid (4-8 equiv) was added and the reaction solution was purified by reverse-phase high-pressure liquid chromatography (HPLC) to yield peptide **1** as a white lyophilized powder and **4** was also separated from the reaction.



Regeneration of 1a. Peptide **3a** (6.2 mg, 10.0 μmol) was subjected to the general nitrile removal protocol then purified by reverse-phase HPLC (5 - 15% organic over 5 min) to yield 4.9 mg (87%) of peptide **1a** as a white lyophilized powder and 1.3 mg (45%) **4** is also separated from the reaction.

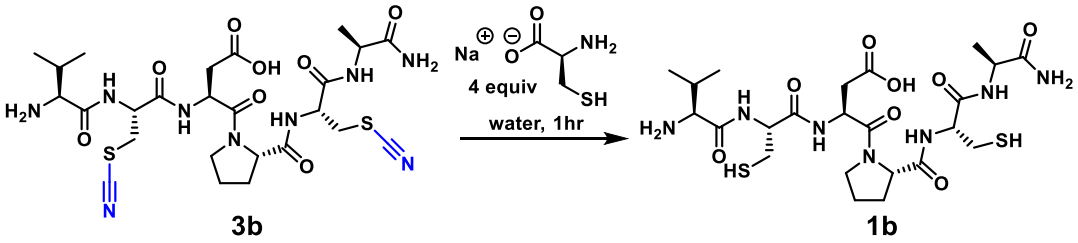
Comparison of the Regenerated (Top) and Native (Bottom) Peptides: Gradient 5-60% MeCN, 7 min, 2mL/min





4

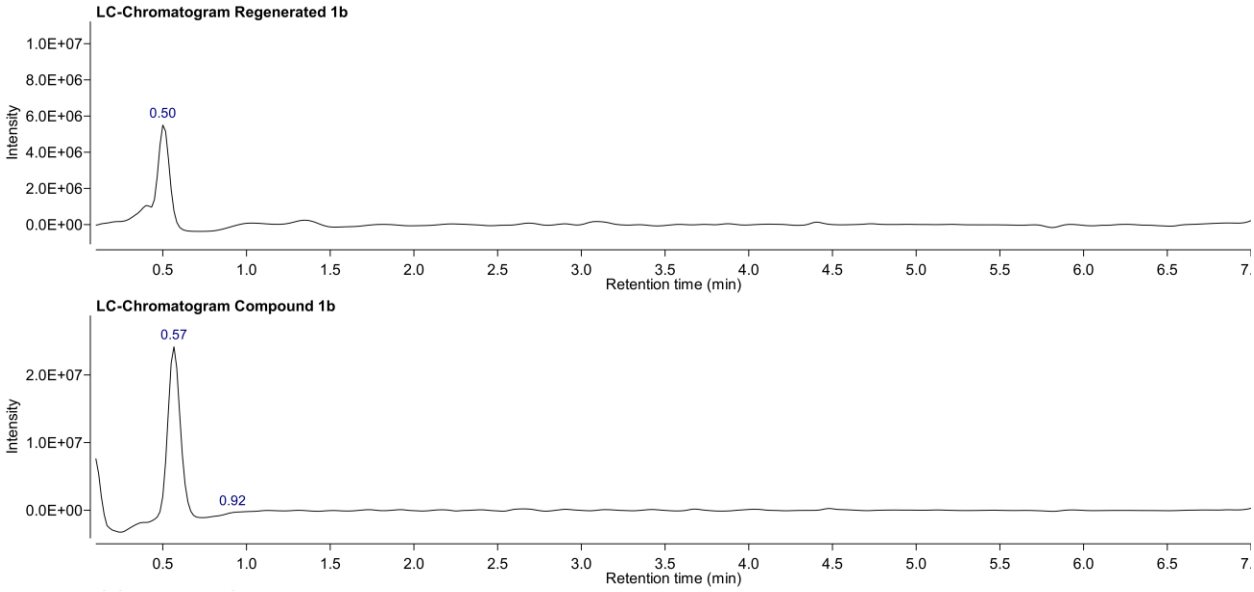
(R)-2-amino-4,5-dihydrothiazole-4-carboxylic acid (4): HRMS Found (ES) *m/z* 147.0228 [$(M+H)^+$; calcd for $C_4H_6N_2O_2S$: 147.0228]; ^{1}H NMR (500 MHz, Deuterium Oxide) δ 4.75 (dd, *J* = 8.8, 5.0 Hz, 1H), 3.92 (dd, *J* = 11.3, 8.9 Hz, 1H), 3.69 (dd, *J* = 11.4, 5.0 Hz, 1H); ^{13}C NMR (126 MHz, Deuterium Oxide) δ 175.5, 173.9, 63.8, 34.7; IR (KBr, cm^{-1}) 3153(br), 2980(br), 22840(br), 2347(m), 2281(m), 2222(m), 1638(s), 1588(s), 1442(m), 1392(s), 1290(m).

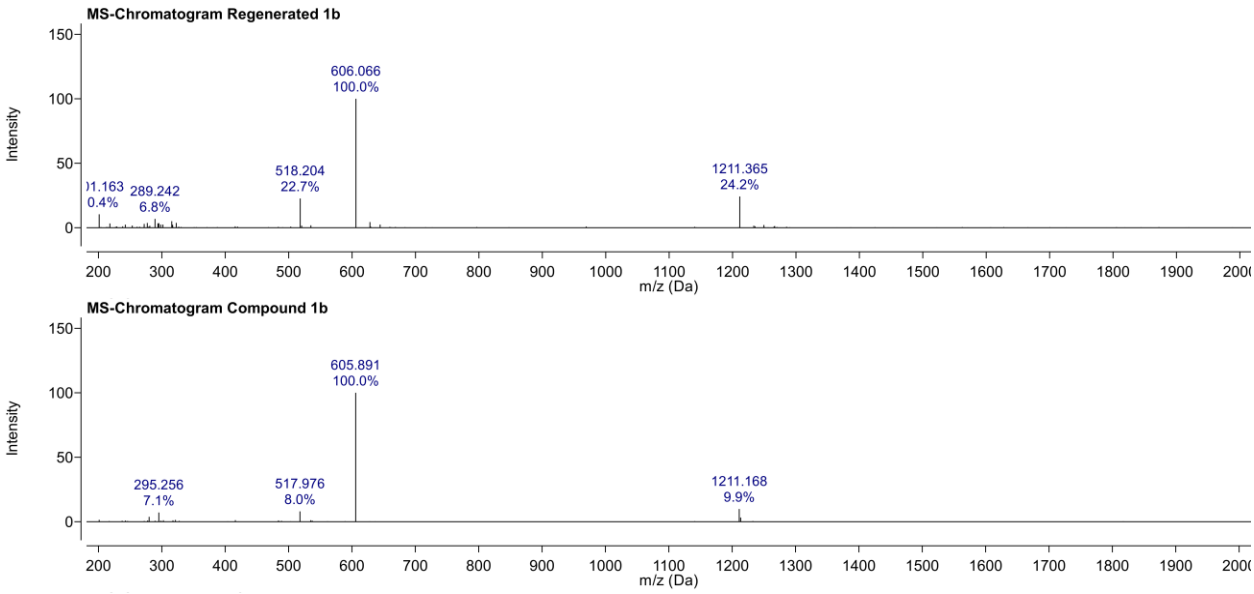


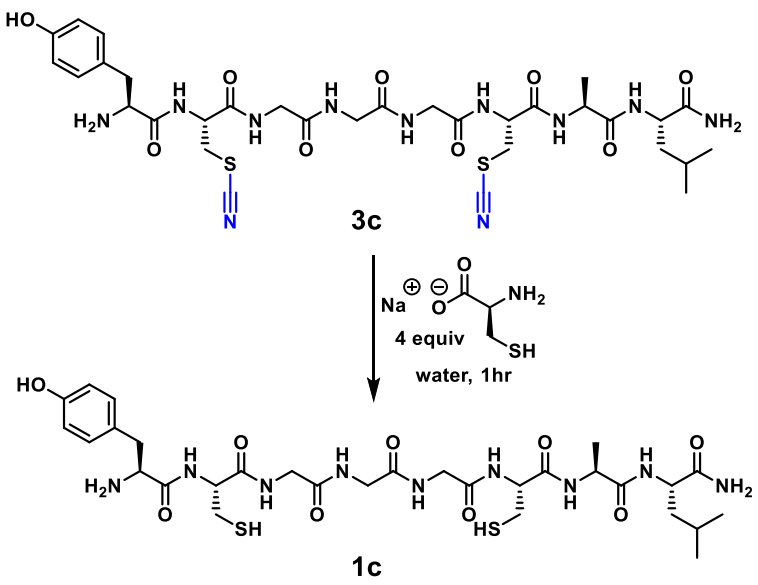
3b **1b**

Regeneration of 1b. Peptide **3b** (1.1 mg, 1.7 μmol) was subjected to the general nitrile removal protocol then purified by reverse-phase HPLC (5 - 35% organic over 12 min) to yield 0.7 mg (68%) of peptide **1b** as a white lyophilized powder.

Comparison of the Regenerated (Top) and Native (Bottom) Peptides: Gradient 5-60% MeCN, 7 min, 2mL/min

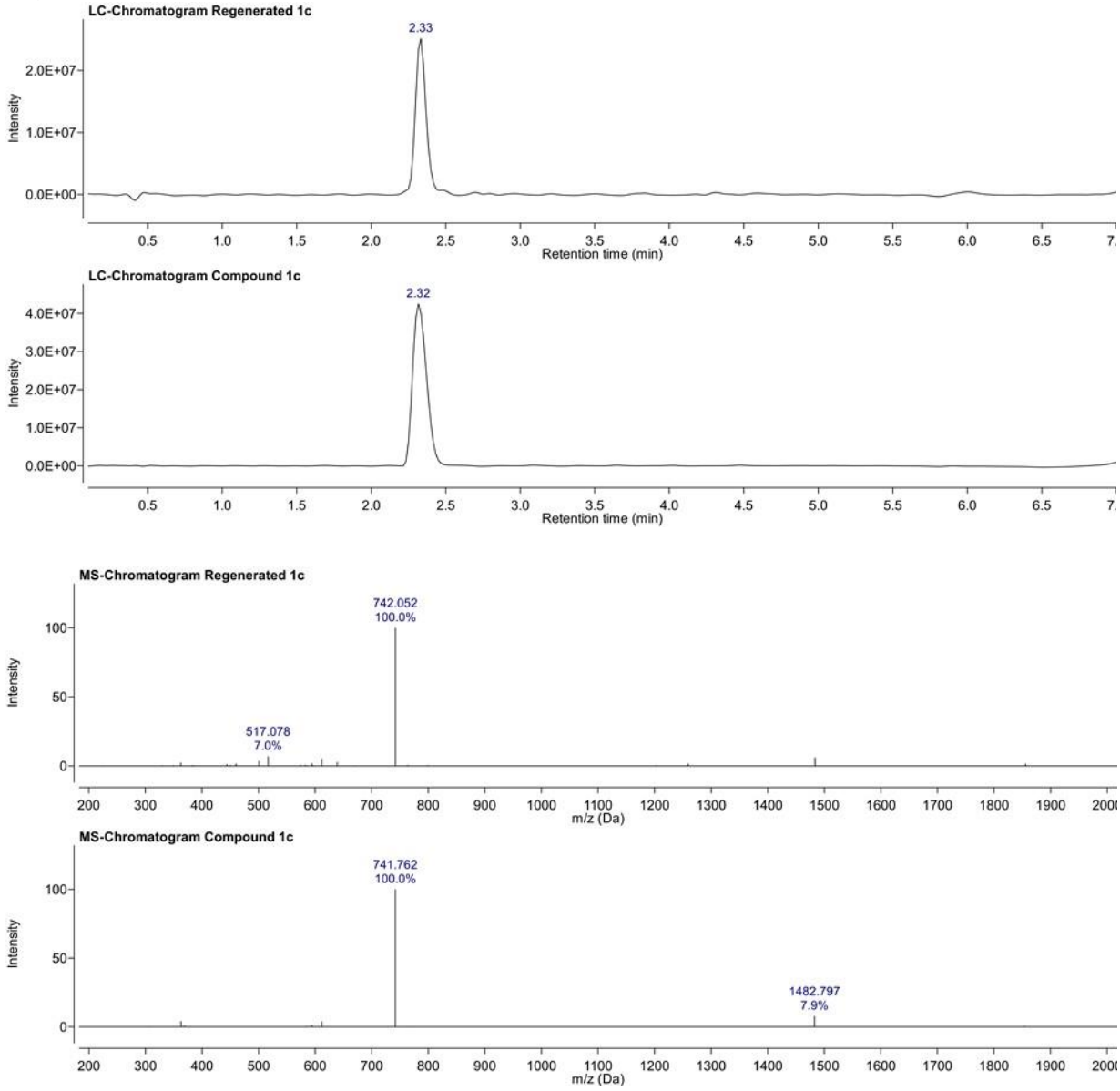


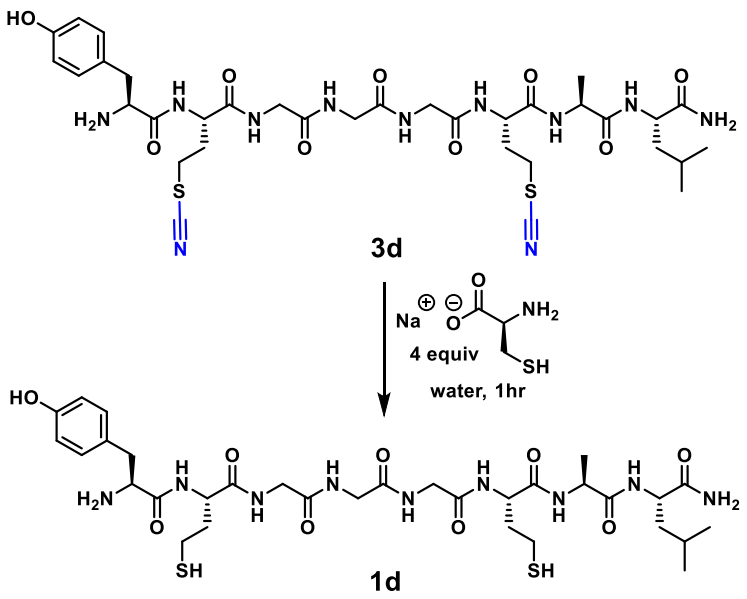




Regeneration of 1c. Peptide **3c** (1.5 mg, 1.9 μmol) was subjected to the general nitrile removal protocol then purified by reverse-phase HPLC (5 - 60% organic over 12 min) to yield 1.1 mg (78%) of peptide **1c** as a white lyophilized powder.

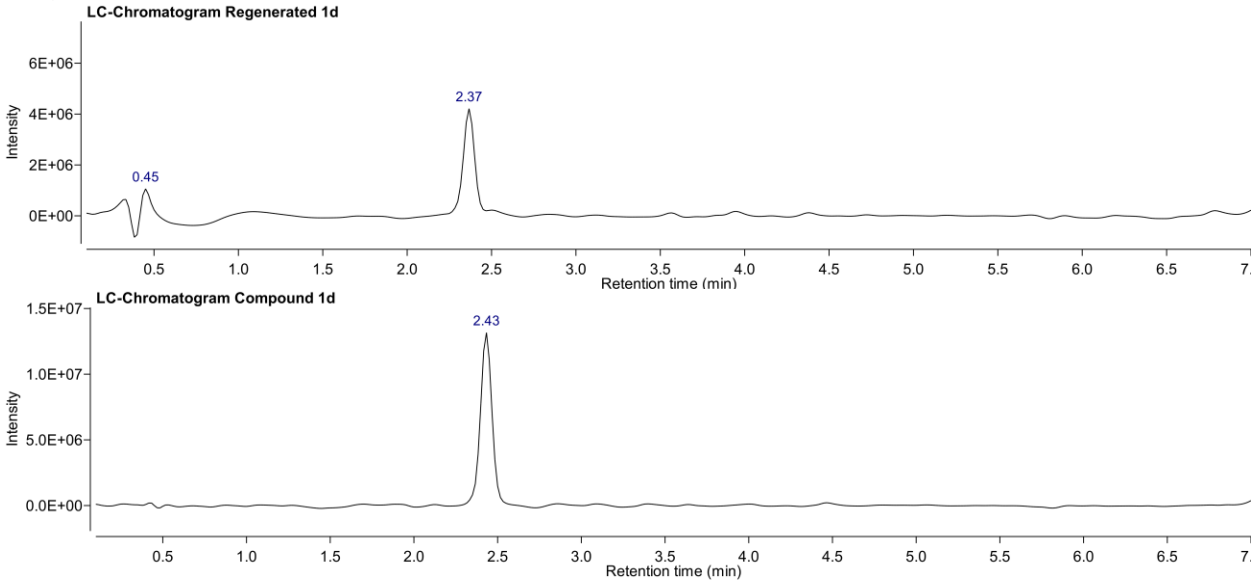
Comparison of the Regenerated (Top) and Native (Bottom) Peptides: Gradient 5-60% MeCN, 7 min, 2mL/min

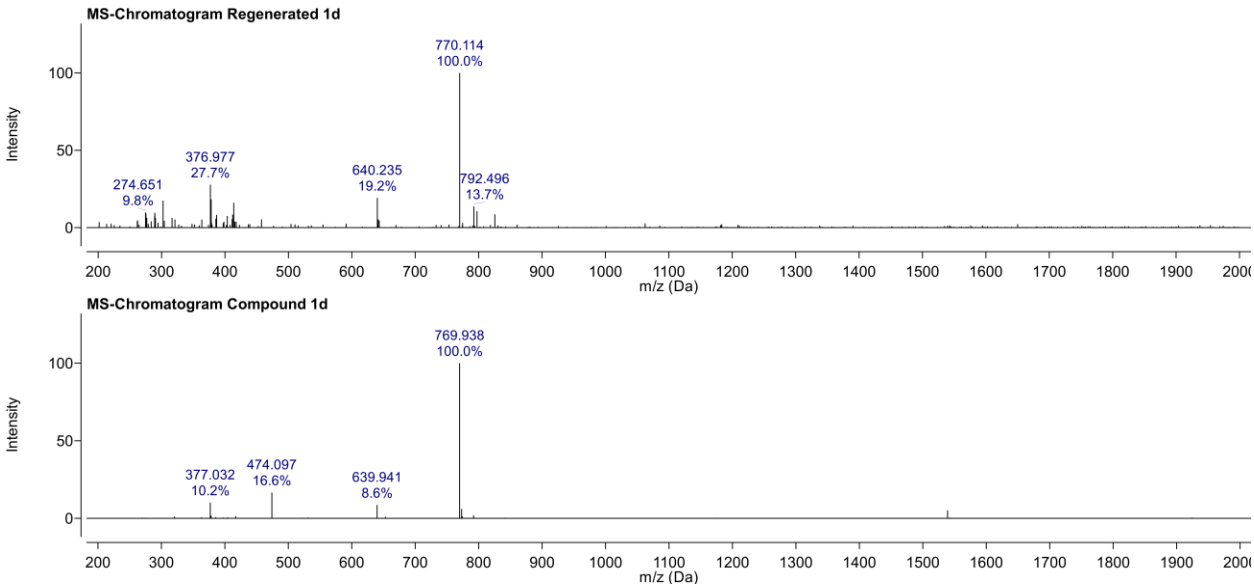


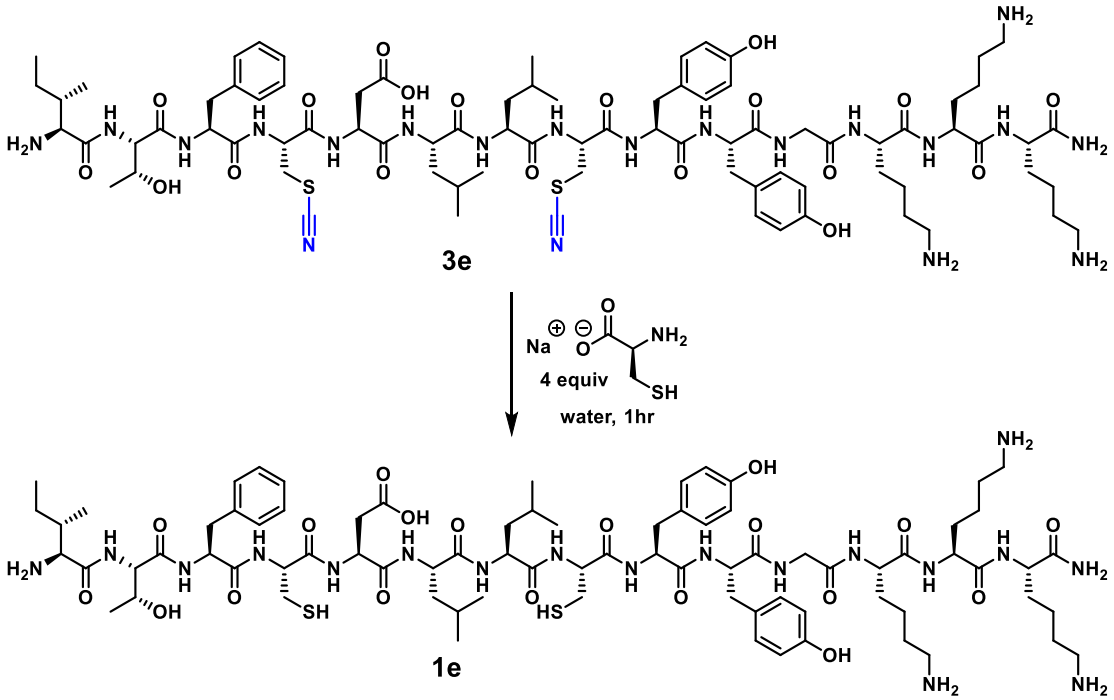


Regeneration of 1d. Peptide **3d** (5.0 mg, 6.1 μmol) was subjected to the general nitrile removal protocol then purified by reverse-phase HPLC (5 - 60% organic over 12 min) to yield 2.7 mg (58%) of peptide **1e** as a white lyophilized powder.

Comparison of the Regenerated (Top) and Native (Bottom) Peptides: Gradient 5-60% MeCN, 7 min, 2mL/min

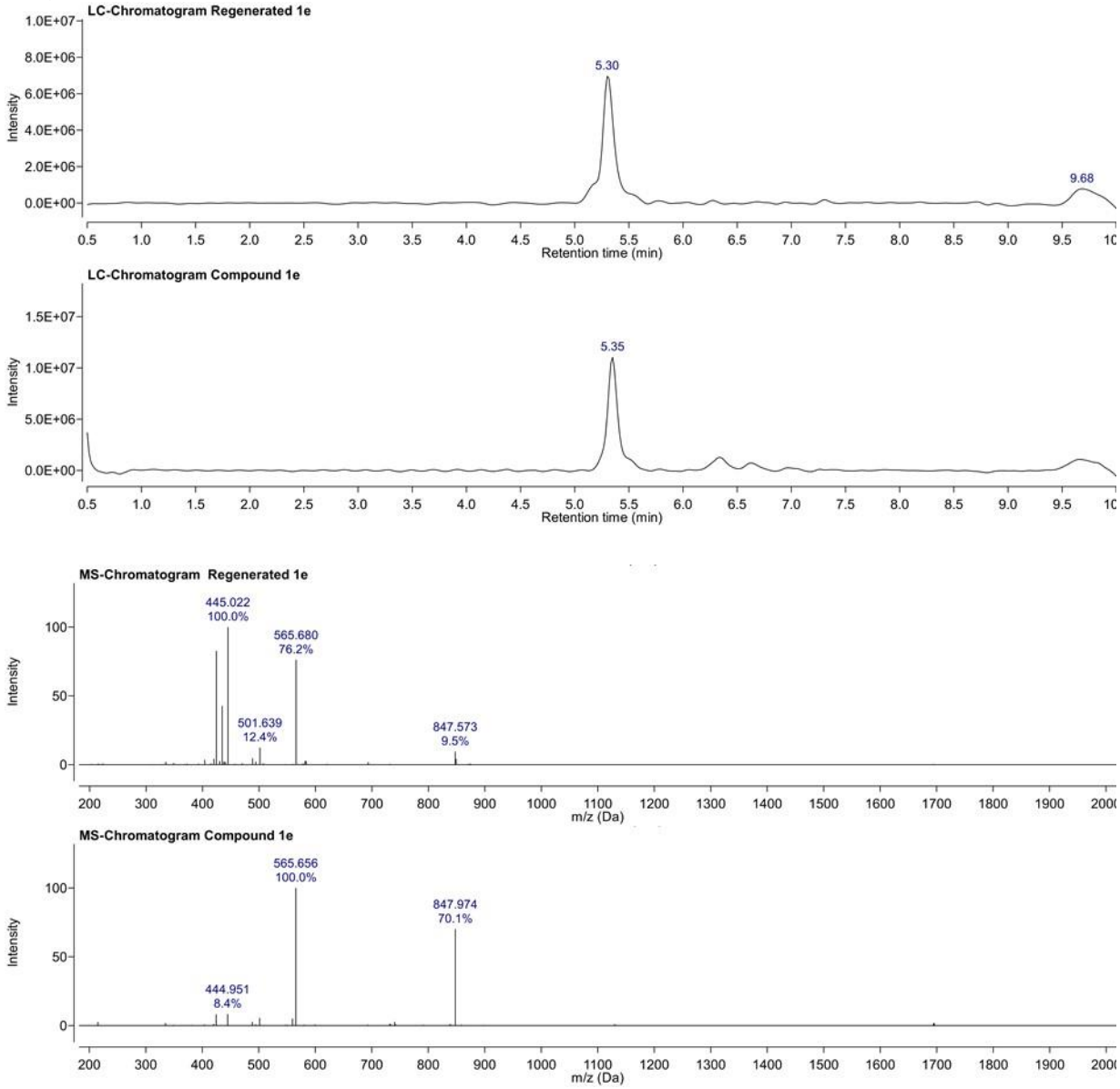


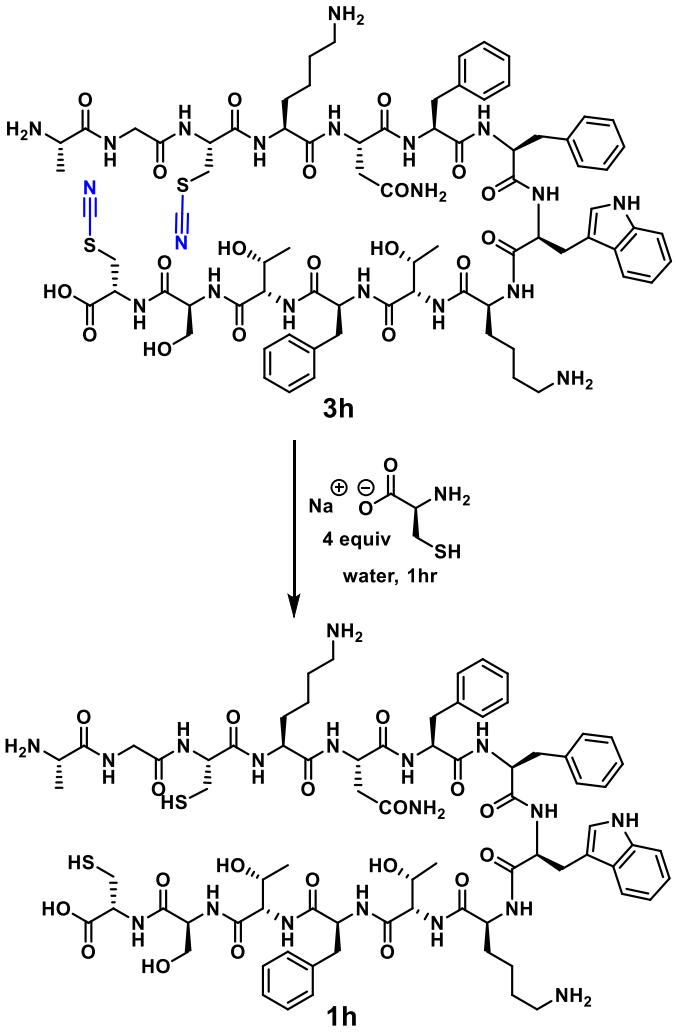




Regeneration of 1e. Peptide **3e** (1.7 mg, 1.0 μmol) was subjected to the general nitrile removal protocol then purified by reverse-phase HPLC [eluent water/MeCN/AcOH (85:10:5) and MeCN (organic) buffered with 0.1%TFA] (5 - 40% organic over 15 min) to yield 0.9 mg (55%) of peptide **1e** as a white lyophilized powder.

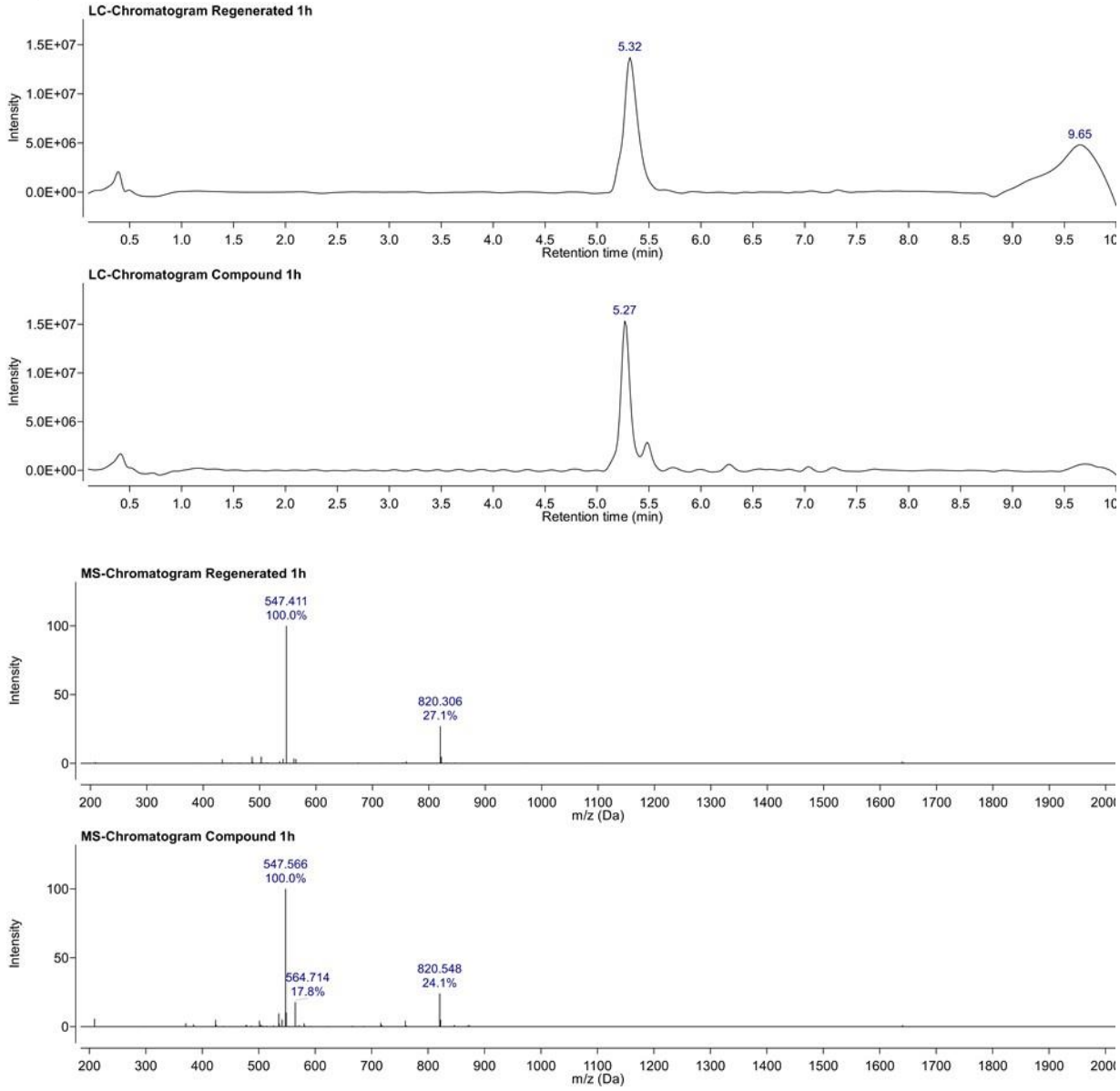
Comparison of the Regenerated (Top) and Native (Bottom) Peptides: Gradient 5-60% MeCN, 10 min, 2mL/min





Regeneration of 1h. Peptide **3h** (3.2 mg, 1.9 μmol) was subjected to the general nitrile removal protocol then purified by reverse-phase HPLC (10 - 60% organic over 15 min) to yield 2.1 mg (68%) of peptide **1h** as a white lyophilized powder.

Comparison of the Regenerated (Top) and Native (Bottom) Peptides: Gradient 5-60% MeCN, 10 min, 2mL/min

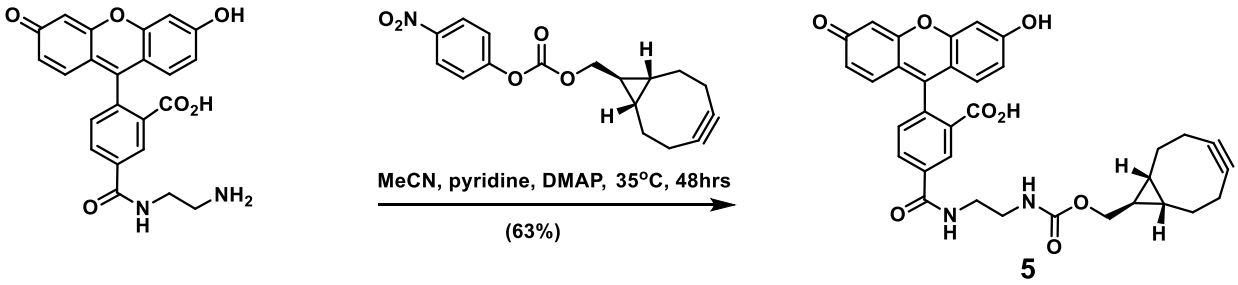


Synthesis of Fluorescein Tethered Bicyclononyne

The starting materials were prepared from previously reported procedures listed below.

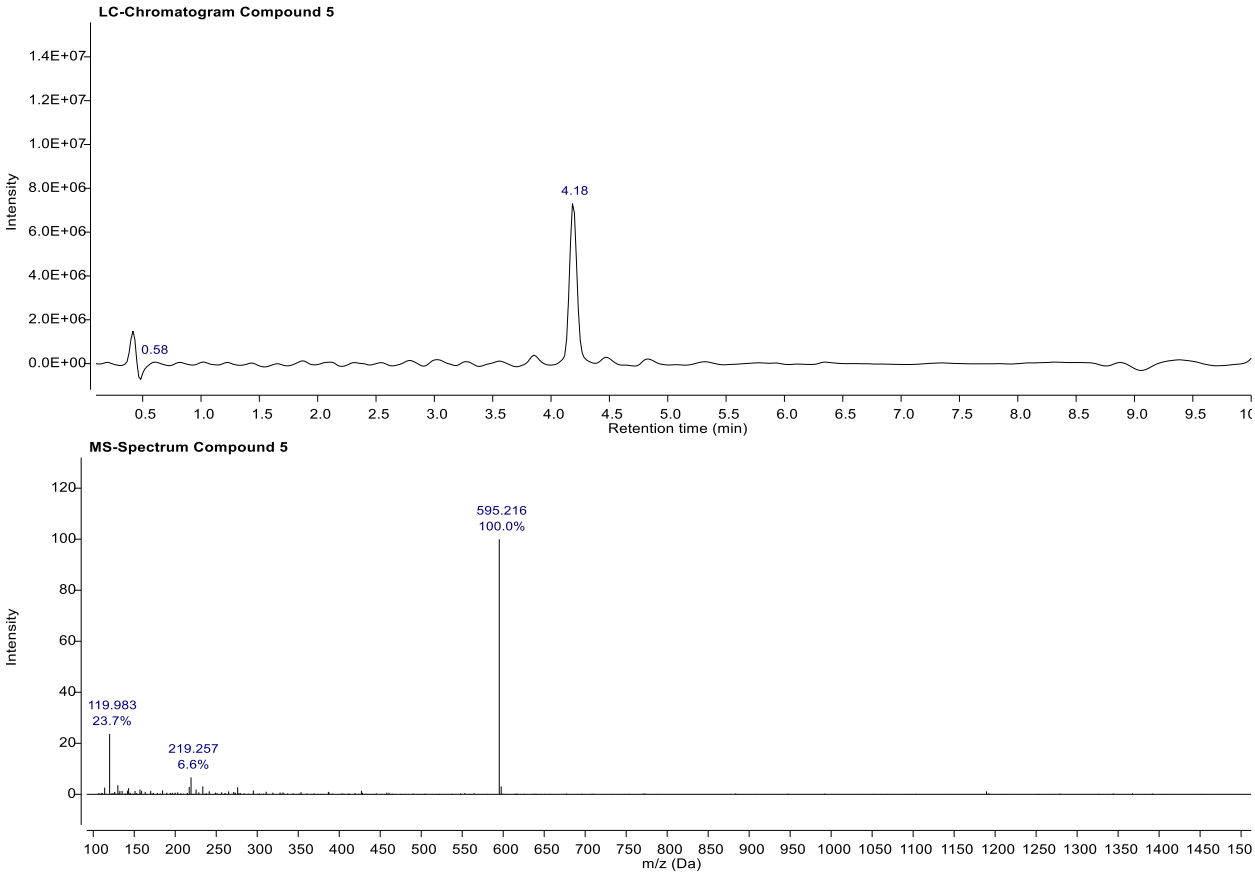
5-((2-aminoethyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid: Gasparini, G.; Bang, E. K.; Molinard, G.; Tulumello, D. V.; Ward, S.; Kelley, S. O.; Roux, A.; Sakai, N.; Matile, S. *J. Am. Chem. Soc.* **2014**, 136, 6069 – 6074.

(1R,8S,9r)-Bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate: Schieber, Christine; Bestetti, Alessandra; Lim, Jet Phey; Ryan, Anneke D.; Nguyen, Tich-Lam; Eldridge, Robert; White, Anthony R.; Gleeson, Paul A.; Donnelly, Paul S.; Williams, Spencer J.; Mulvaney, Paul *Angew. Chem. Int. Ed.* **2012** , 51, 10523 – 10527.

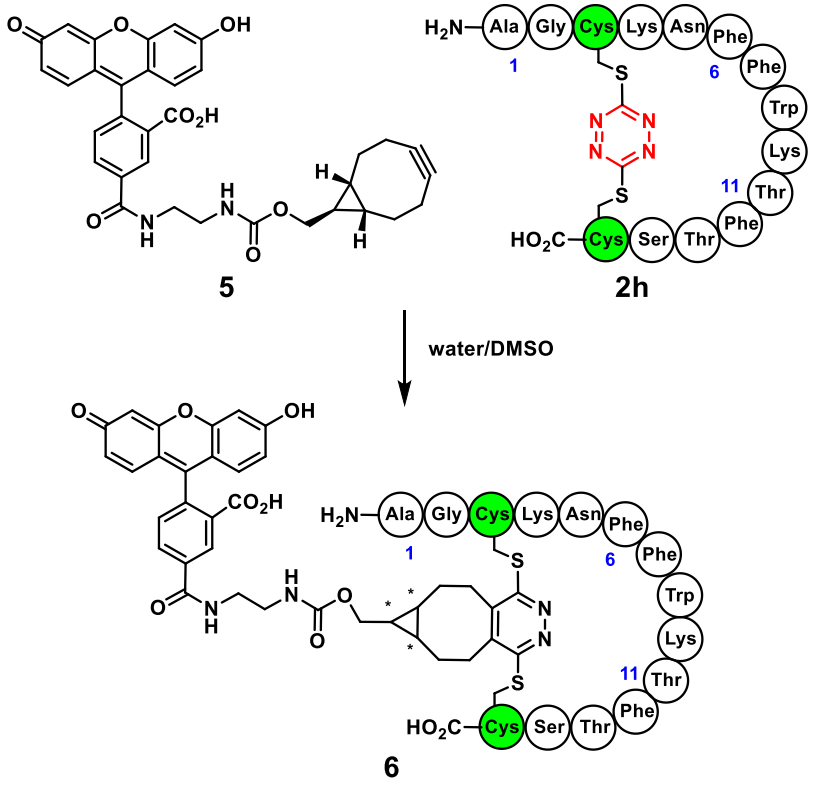


5-((2-(((((1R,8S,9r)-bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)ethyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (5). To a 5 mL round bottom flask containing 5-((2-aminoethyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (9.0 mg, 22 μmol) dissolved in MeCN (500 μL) was added bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate (8.3 mg, 26 μmol, 1.2 equiv) in MeCN (250 μL) followed by the addition of pyridine (16 μL, 200 μmol, 10 equiv) and DMAP (2.7 mg, 22 μmol, 1 equiv). The contents were then stirred at 35ºC for 48 hours. The reaction mixture was evaporated and the crude re-dissolved in water/MeCN (7:3, 1000 μL) and purified by reverse-phase HPLC (gradient 10-80% organic over 15 minutes) to give 8.2 mg (63%) after lyophilization. HRMS (ES) *m/z* 595.2069 [$(M+H)^+$; calcd for $C_{34}H_{31}N_2O_8$: 595.2080]. ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.49 (s, 1H), 8.21 (d, *J* = 8.0 Hz, 1H), 7.34 (d, *J* = 8.0 Hz, 1H), 6.79 (s, 2H), 6.73 (d, *J* = 8.3 Hz, 2H), 6.64 (d, *J* = 8.8 Hz, 2H), 4.15 (d, *J* = 8.2 Hz, 2H), 3.54 (t, *J* = 5.7 Hz, 2H), 3.39 (t, *J* = 6.0 Hz, 2H), 2.19 (d, *J* = 12.8 Hz, 4H), 2.09 (d, *J* = 15.3 Hz, 2H), 1.56 (dd, *J* = 21.8, 9.7 Hz, 2H), 1.36 (dt, *J* = 17.3, 8.6 Hz, 1H), 0.87 (t, *J* = 10.0 Hz, 2H).

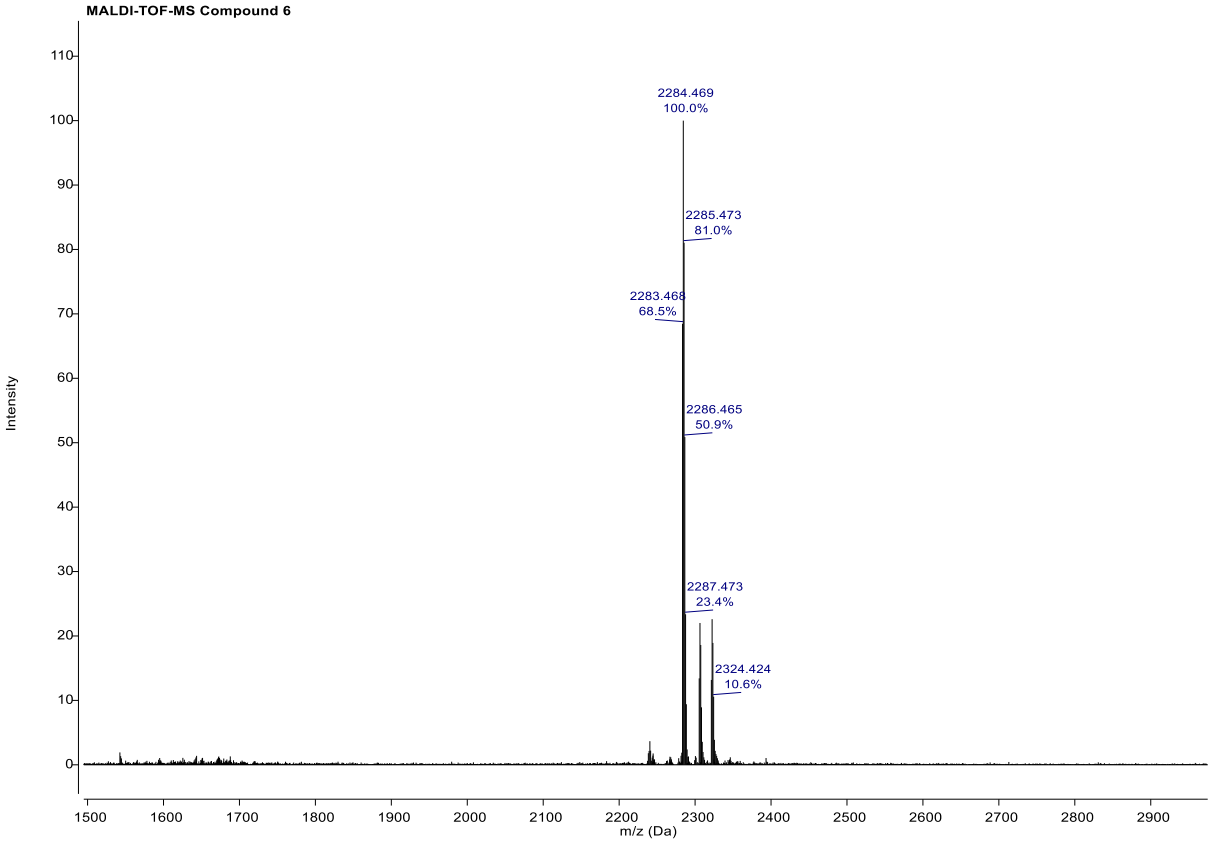
Gradient 10-90% MeCN, 10 min, 2mL/min

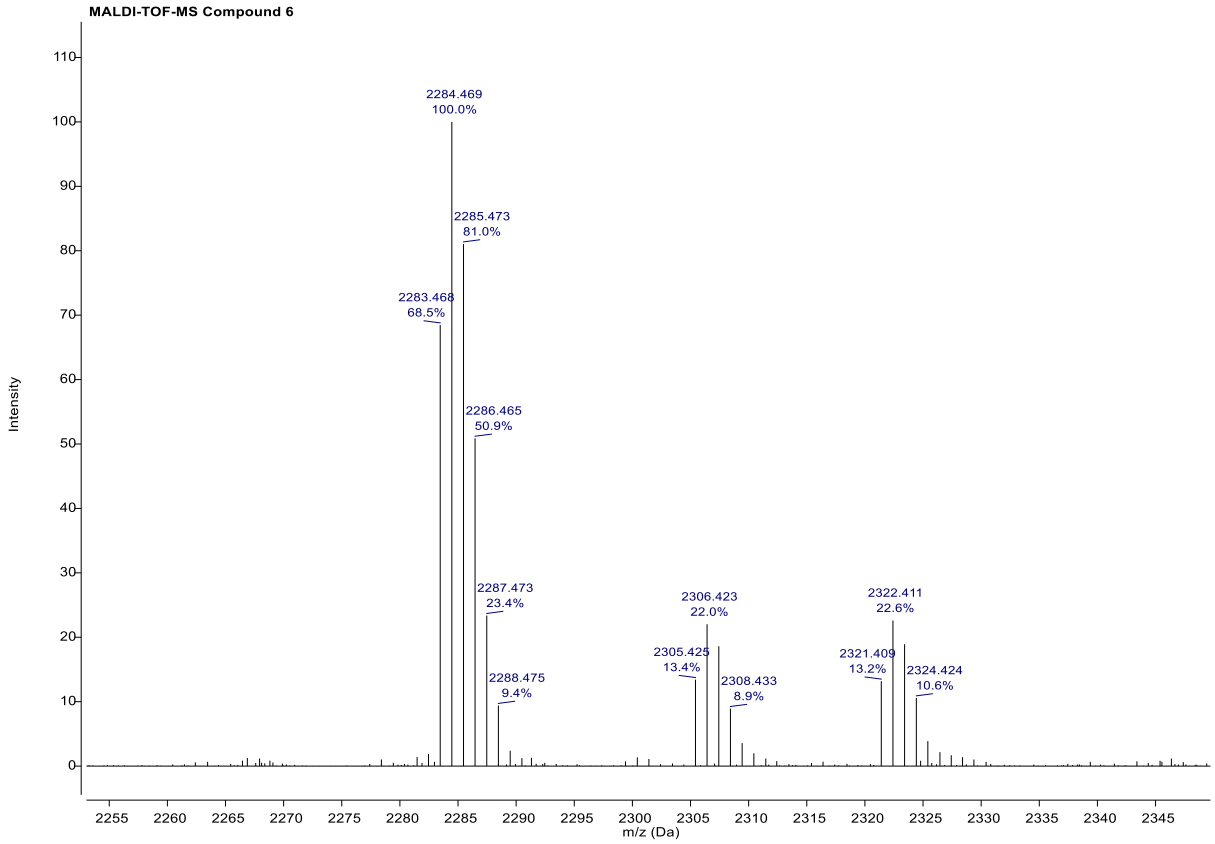


Inverse-Electron Demand Diels-Alder of S,S-Tetrazine Somatostatin

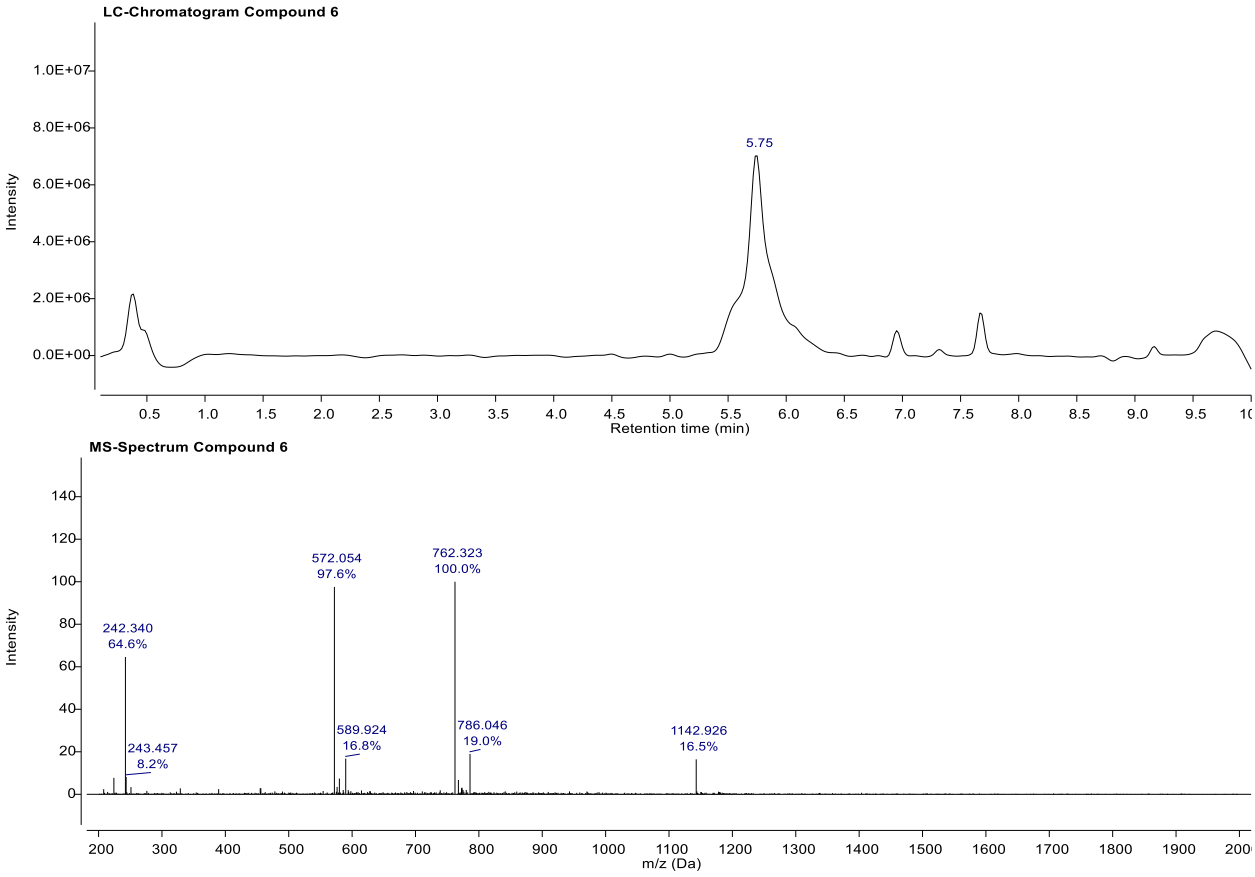


Peptide 6. To a 5 mL round bottom flask was added a solution (1.1 mM) of peptide **2h** dissolved in water (500 µL) followed by a solution (1.2 mM) of bicyclononyne **5** in DMSO (500 µL). The contents were stirred at room temperature for 4 days and the solvent removed *in vacuo*. The residue was purified by reverse-phase HPLC (gradient 10-60% organic over 15 min) to give (0.9 mg, 68%) of a yellow-orange powder after lyophilization. MALDI-TOF *m/z* 2283.468 [$(M+H)^+$; calcd for $C_{112}H_{135}N_{22}O_{27}S_2$: 2283.930].

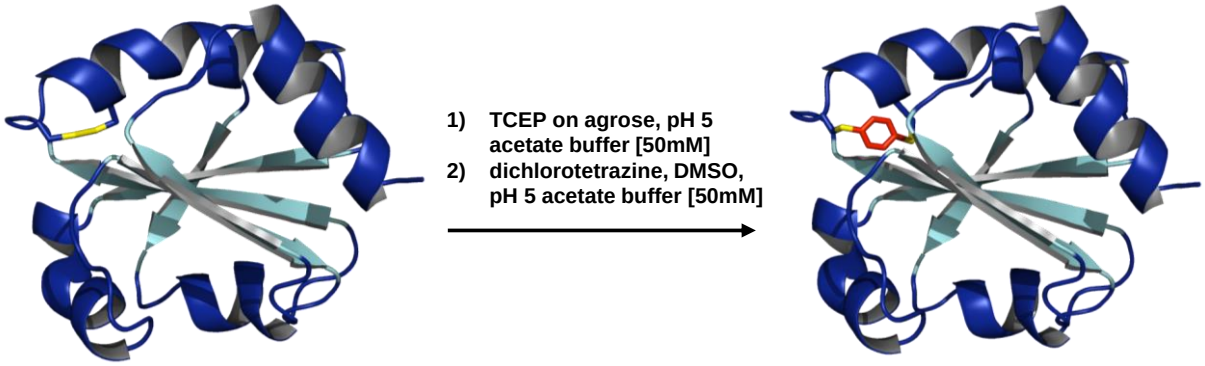

MALDI-TOF-MS Compound 6
Intensity
m/z (Da)
2284.469
100.0%
2285.473
81.0%
2283.468
68.5%
2286.465
50.9%
2287.473
23.4%
2324.424
10.6%


MALDI-TOF-MS Compound 6
Intensity
m/z (Da)
2284.469
100.0%
2285.473
81.0%
2283.468
68.5%
2286.465
50.9%
2287.473
23.4%
2288.475
9.4%
2306.423
22.0%
2305.425
13.4%
2308.433
8.9%
2322.411
22.6%
2321.409
13.2%
2324.424
10.6%

Gradient 5-60% MeCN, 10 min, 2mL/min

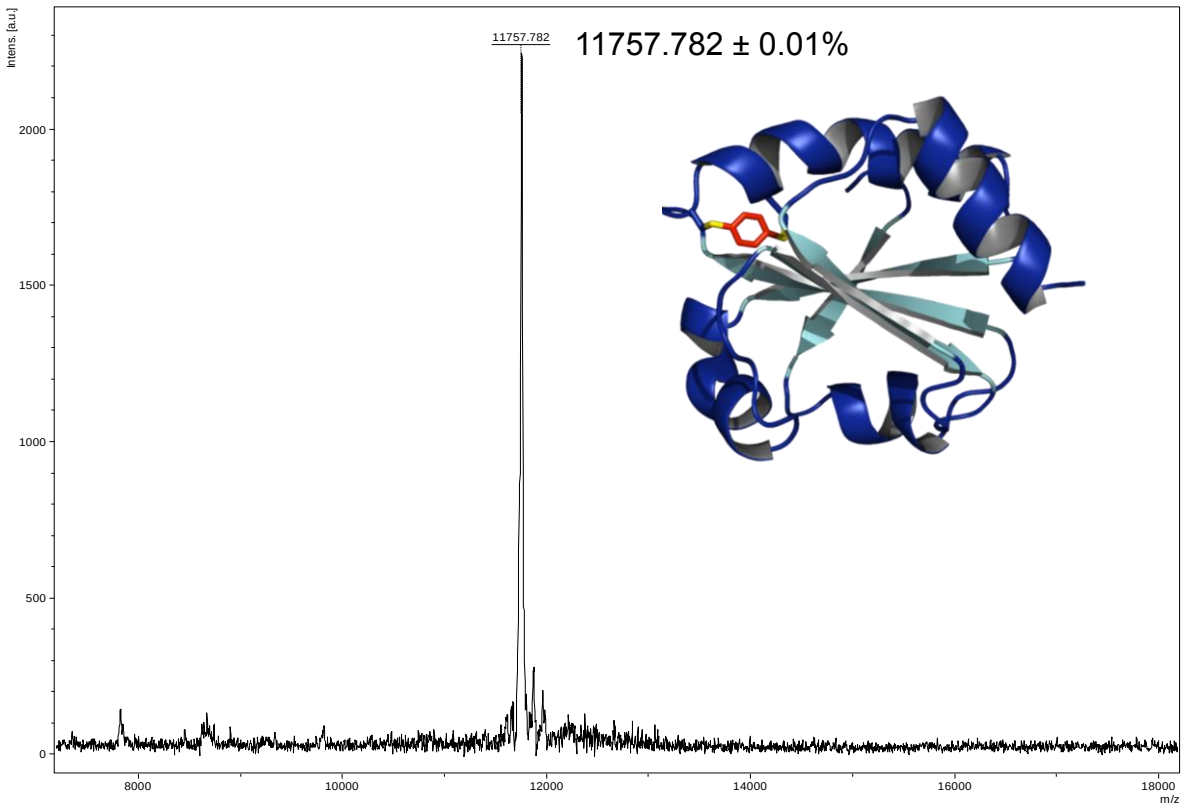


Tetrazine Stapling of the Thioredoxin Protein

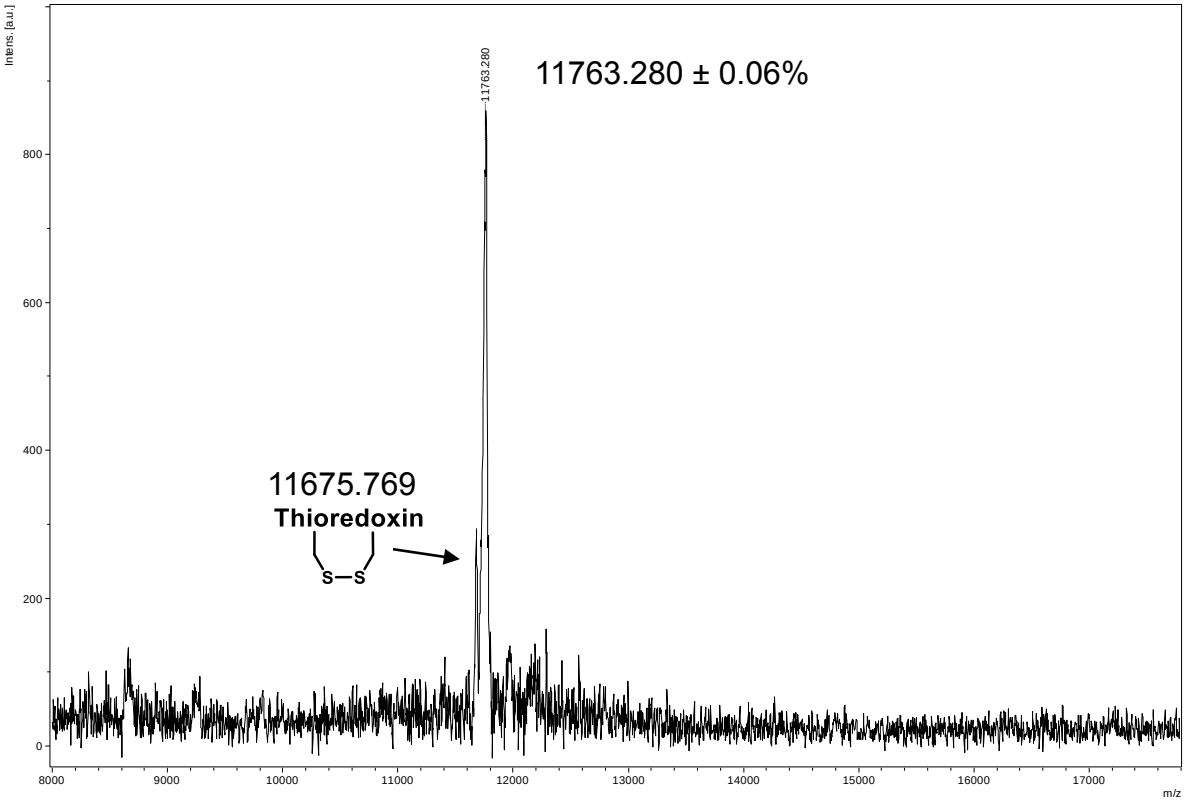


To a 1.7 mL mini-centrifuge tube containing thioredoxin (0.25 mg, 21 nmol) dissolved in acetate buffer pH 5 (200 mM, 100 µL), was added TCEP immobilized on agrose (300 µL, 8 µmol/mL, 2.4 µmol, 112 equiv); the final buffer concentration was 50 mM. The reaction was stirred at room temperature for 2.0 hours under an argon atmosphere. The contents were kept under a blanket of argon, then filtered through a plastic pipet tip with a cotton plug and rinsed with degassed 50 mM acetate buffer pH 5 (3 × 200 µL). To the pooled filtrates (1.0 mL) in a 1.7 mL mini-centrifuge tube was added a pre-mixed solution of dichlorotetrazine in DMSO (20 µL, 87 nmol, 4 equiv, [0.67 mg/mL]) and stirred for 1 minute. The solution was then transferred to a pre-equilibrated disposable PD-10 desalting column and eluted with 50 mM Tris, pH 7.8, 150 mM NaCl. The fractions containing protein were pooled and stored at 4ºC. Bradford assay (88% yield). MALDI-TOF *m/z* 11757.782 [$(M+H)^+$; 11756.45; calculated for Trx-1 (11675.43 Da) + tetrazine (80.01 Da) + H^+ (1.01)].

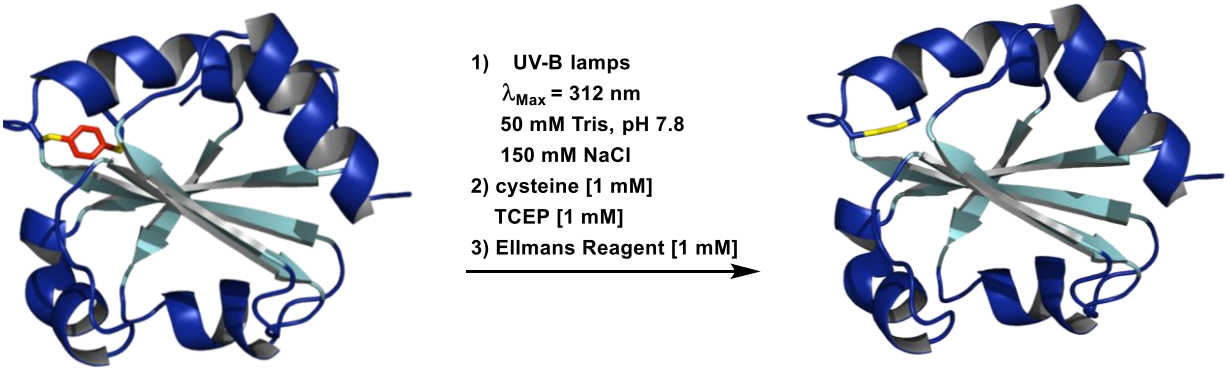
MALDI Spectrum of Tetrazine Thioredoxin (calculated $(M+H)^+$ = 11756.45)



MALDI Spectrum of Tetrazine Thioredoxin (same sample as above using lower ionization energy, shows some starting material)



Photochemical Unstapling and Regeneration of the Thioredoxin Protein



Sample Preparation for Comparison

A sample of tetrazine thioredoxin (0.1 mg, 8 nmol), from the desalting column in 50 mM Tris, pH 7.8, 150 mM NaCl (1000 µL) was divided between two 1.7 mL mini-centrifuge tubes. One sample underwent photolysis and the other used as a comparsion.

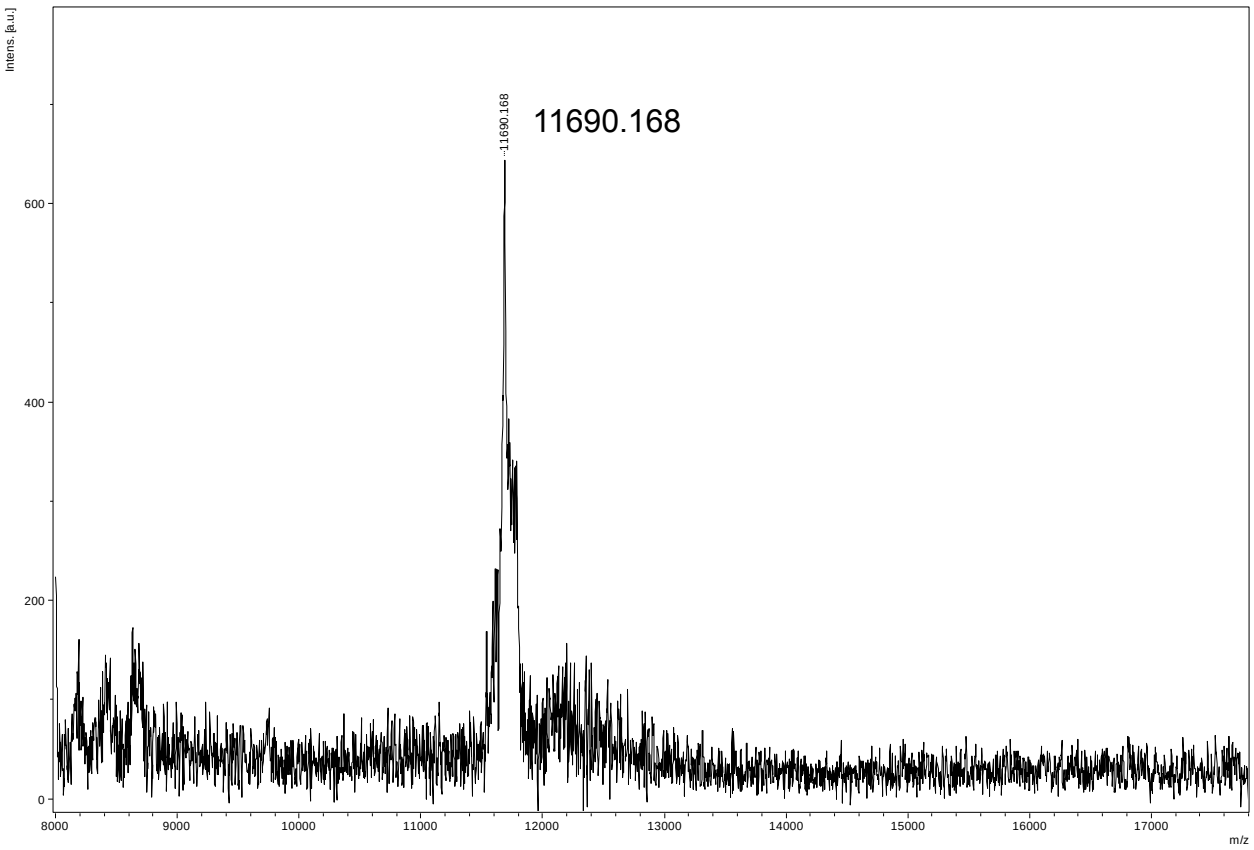
Tetrazine Thioredoxin Photolysis

A 1.7 mL mini-centrifuge tube containing tetrazine thioredoxin (0.05 mg, 4 nmol) dissolved in 50 mM Tris, pH 7.8, 150 mM NaCl (500 µL) was suspended in a Rayonet® photoreactor equipped with three UV-B lamps. The contents were irradiated for 1.0 hour, MALDI indicated consumption of the starting material with partial loss of the nitrile groups.

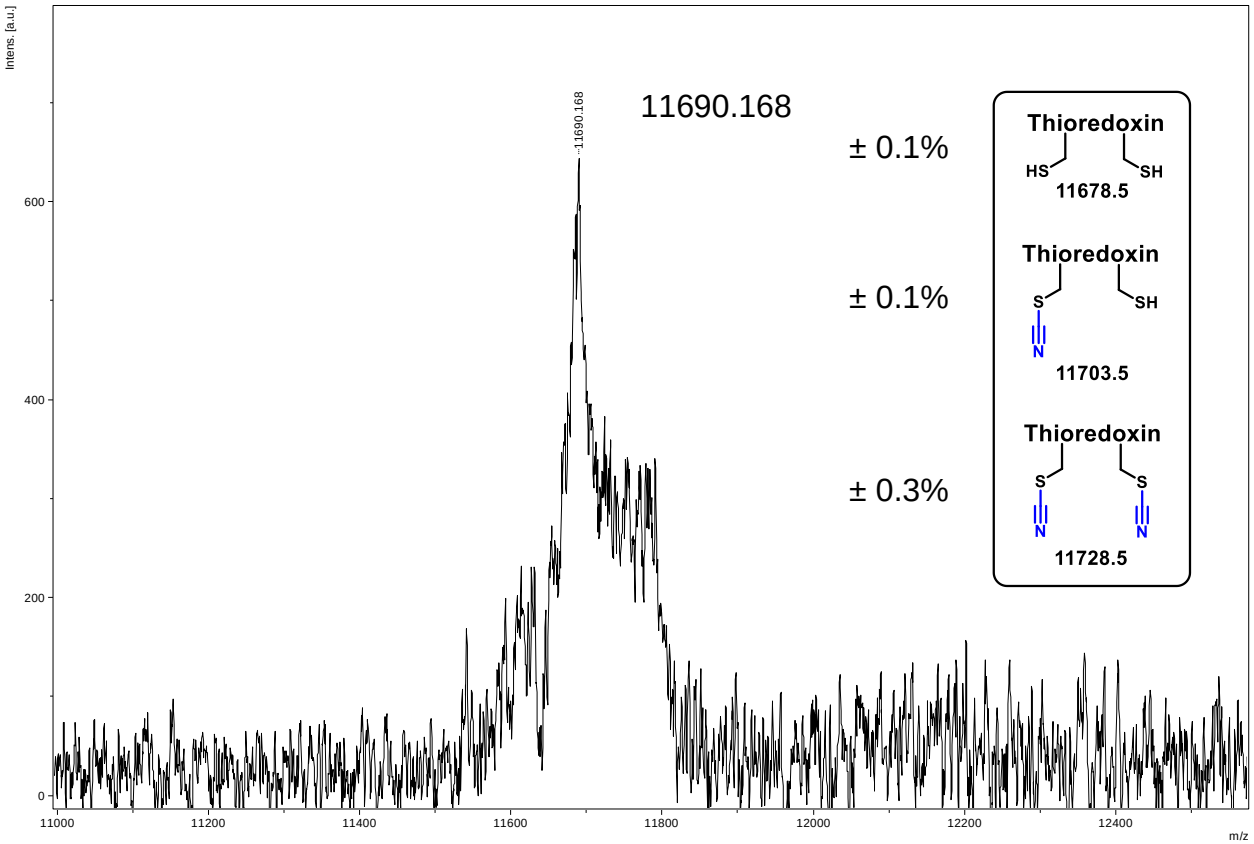
Regeneration of the Protein

To the photolyzed sample dissolved in 50 mM Tris, pH 7.8, 150 mM NaCl (500 µL), was added cysteine (25 µL) of a 20 mM solution in the Tris buffer system and TCEP (25 µL) of a 20 mM solution in the Tris buffer system. The contents were allowed to stand for 4.0 hours and then diluted to 3 mL with the Tris buffer system and transferred for centrifugation in an Amicon centrifugal filter (MWCO 3000, 35º fixed angle rotor @ 7000×G, 30 minutes, 4ºC), the concentrated sample (150 µL) was diluted to 3 mL and repeated. The retenate was collected and diluted to 500µL with the Tris buffer system, then Ellman's reagent [5,5'-dithio-bis-(2-nitrobenzoic acid)] (25 µL of a 20 mM solution in the Tris buffer system) was added and allowed to stand for 6.0 hours. The contents were then diluted to 3 mL with the Tris buffer system and transferred for centrifugation in an Amicon centrifugal filter (MWCO 3000, 35º fixed angle rotor @ 7000×G, 30 minutes, 4ºC). The retenate was collected and diluted to 500 µL with the Tris buffer solution and analyzed by MALDI-TOF and FPLC. Bradford assay (73% yield). MALDI-TOF *m/z* 11676.188 [$(M+H)^+$; 11676.44].

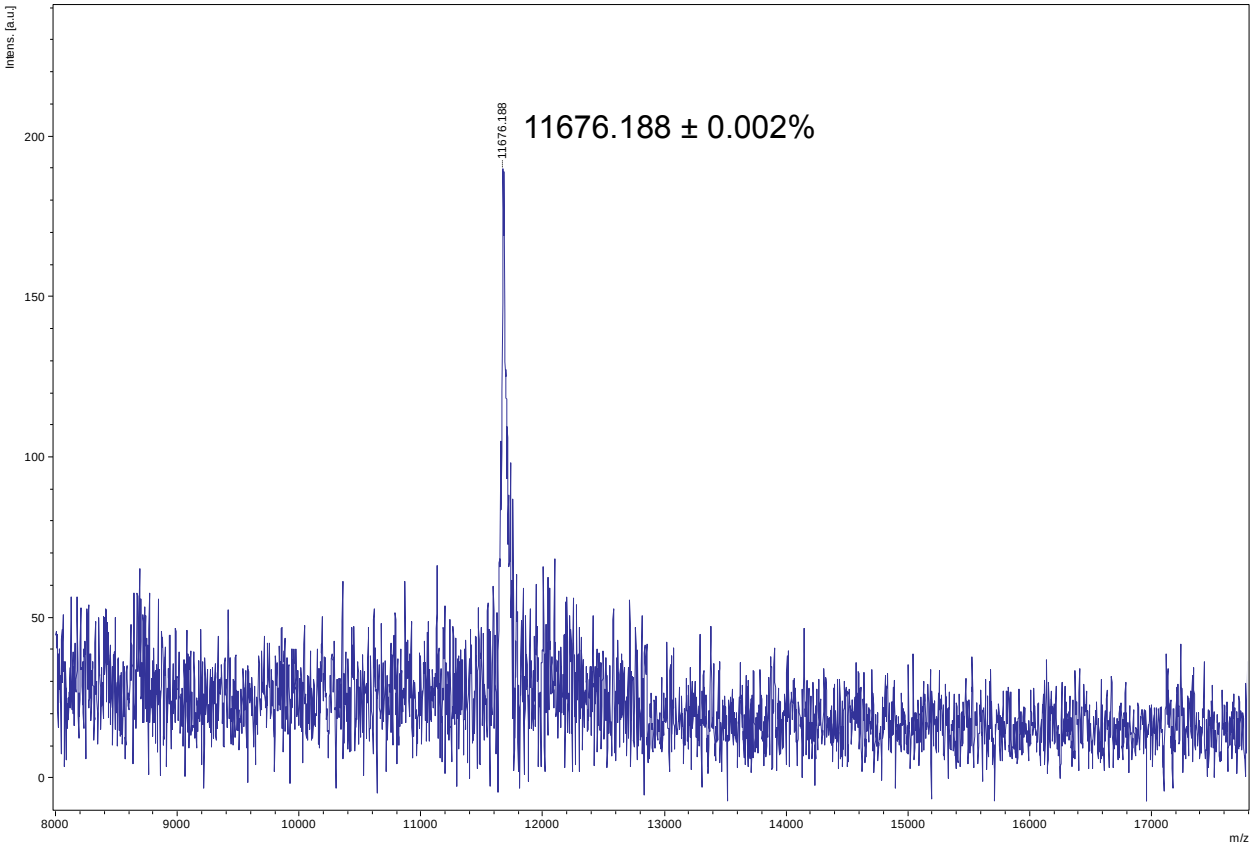
MALDI Chromatogram of Tetrazine-Thioredoxin After Photolysis (mixture of products)



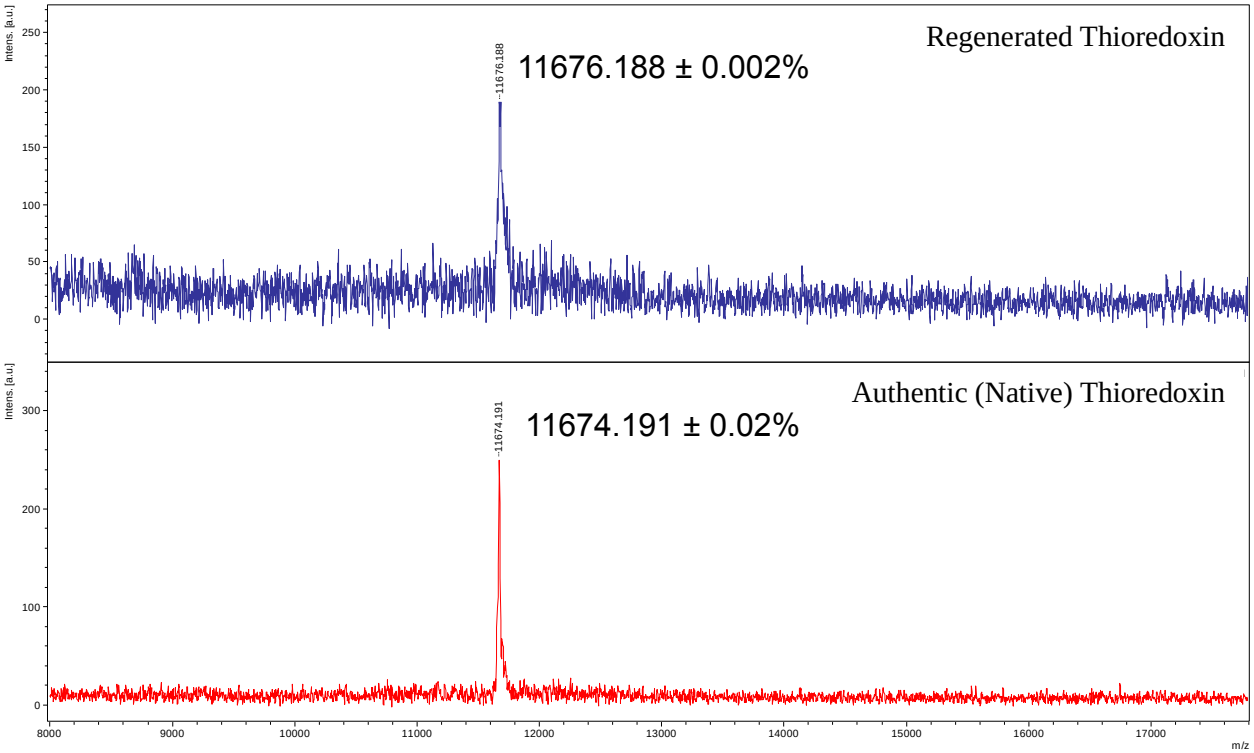
Zoom region 11000 – 12500: Tetrazine-Thioredoxin after Photolysis (mixture of products)



MALDI Chromatogram of Regenerated Thioredoxin (calculated $(M+H)^+$ = 11676.44)



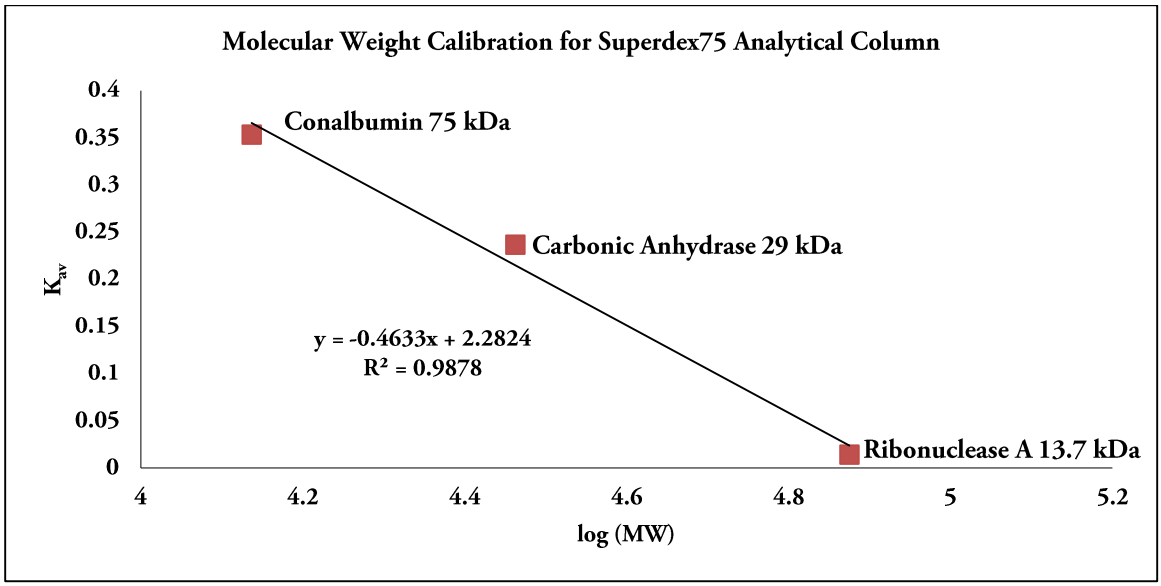
MALDI Comparison of Regenerated with Authentic Thioredoxin



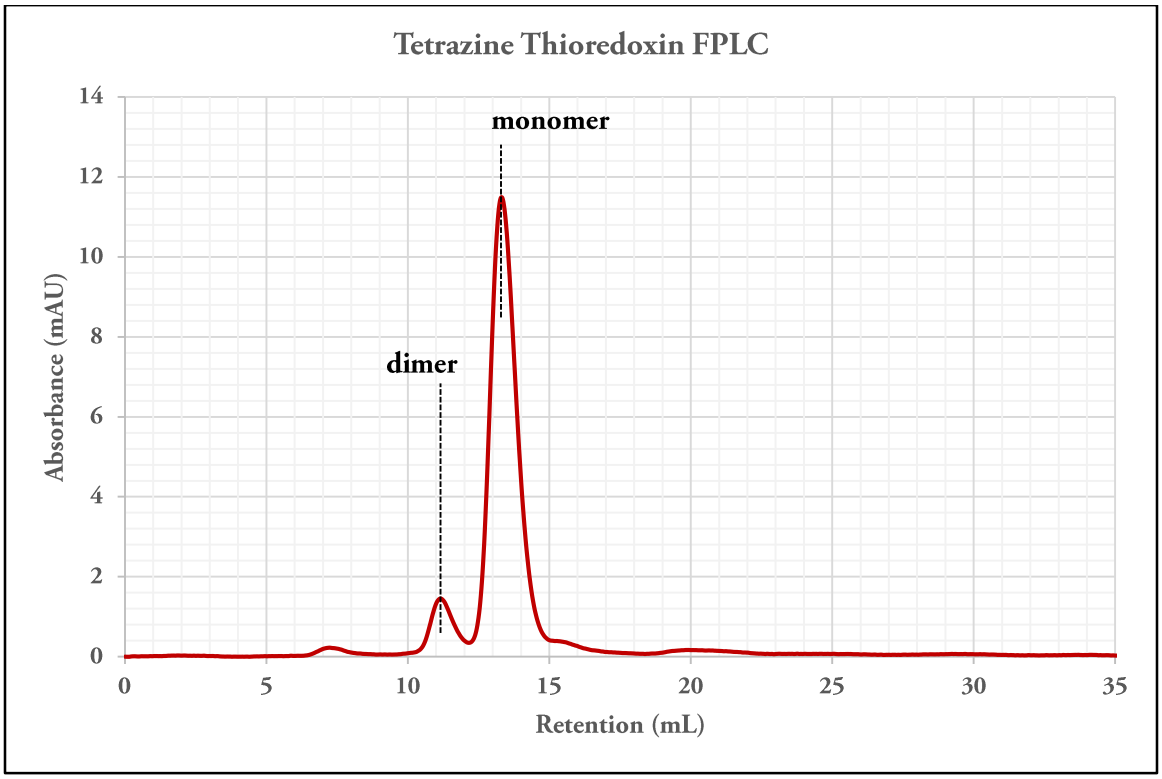
General Methods for FPLC

Fast protein liquid chromatography (FPLC) was conducted with an AKTA FPLC equipped with a P 920 pump and UPC-900 control box. Proteins were separated with a Superdex 75/10/300 column at 4ºC and eluted with 50 mM Tris, pH 7.8, 150 mM NaCl (isocratic) at 0.5 mL/min.

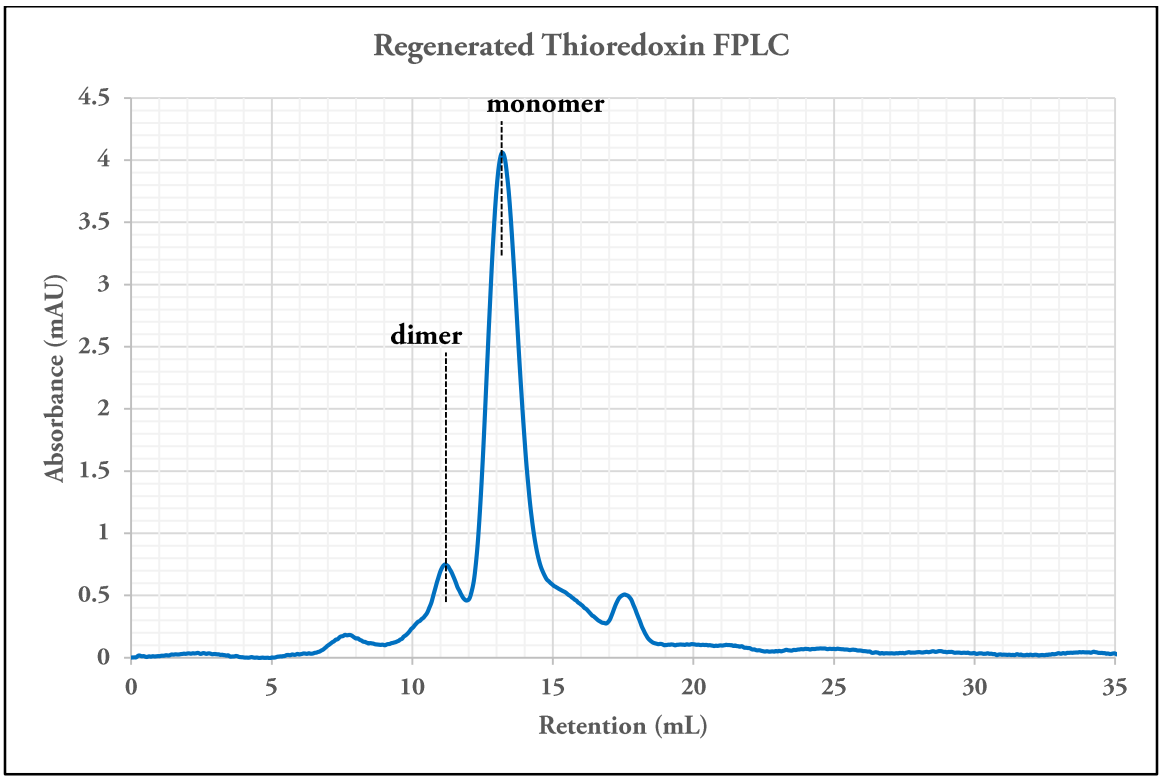
A calibration curve of Superdex 75/10/300 column was prepared for molecular weight estimation.



FPLC Chromatogram of Tetrazine Thioredoxin



FPLC Chromatogram of Regenerated Thioredoxin



Approximated extinction coefficient (ε) values were calculated for thioredoxin (Trx) and tetrazine-thioredoxin (tet-Trx). The Trx protein contains 2 Trp and 2 Tyr residues, the ε values used in the calculations are listed below.

Residue	Extinction Coefficient (ε) Used
Tyrosine	1280 $cm^{-1}M^{-1}$
Tryptophan	5690 $cm^{-1}M^{-1}$
S,S-tetrazine	11369 $cm^{-1}M^{-1}$

$$\boldsymbol{\varepsilon_{Trx} = (2 \times 5690 cm^{-1}M^{-1}) + (2 \times 1280 cm^{-1}M^{-1}) = 13940 cm^{-1}M^{-1}}$$

$$\boldsymbol{\varepsilon_{tet-Trx} = (2 \times 5690 cm^{-1}M^{-1}) + (2 \times 1280 cm^{-1}M^{-1}) + (11369 cm^{-1}M^{-1}) = 25309 cm^{-1}M^{-1}}$$

Sample	Approximated ε
Thioredoxin	13940 $cm^{-1}M^{-1}$
Tetrazine-Trx	25309 $cm^{-1}M^{-1}$

The measured areas from the FPLC chromatograms are:

Sample	Area of peaks at 13 min
Tetrazine-Trx	19.11 mAU/mL
Regenerated-Trx	8.92 mAU/mL

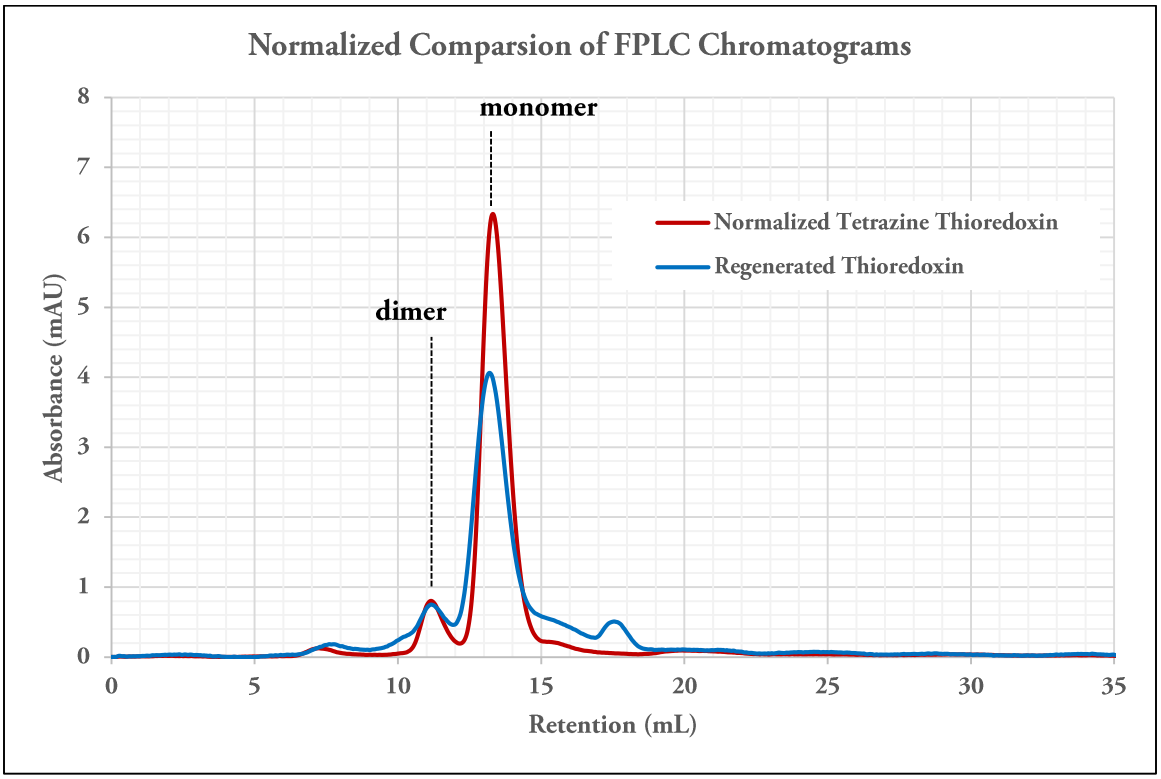
The measured tetrazine-thioredoxin absorbance (A_{meas}) was normalized (A_{norm}) to account for the extinction coefficient contributed by the tetrazine to permit comparison with the regeneration thioredoxin using the equation below.

$$A_{norm} = \frac{A_{meas}\varepsilon_{Trx}}{\varepsilon_{tet-Trx}} = \frac{19.11 mAU * mL^{-1} * 13940 cm^{-1}M^{-1}}{25309 cm^{-1}M^{-1}} = 10.53 mAU * mL^{-1}$$

The regenerated thioredoxin yield was calculated using the equation below.

$$\frac{A_{regen-Trx}}{A_{norm-Trx}} \frac{8.92 mAU * mL^{-1}}{10.53 mAU * mL^{-1}} \times 100\% = 84.7\%$$

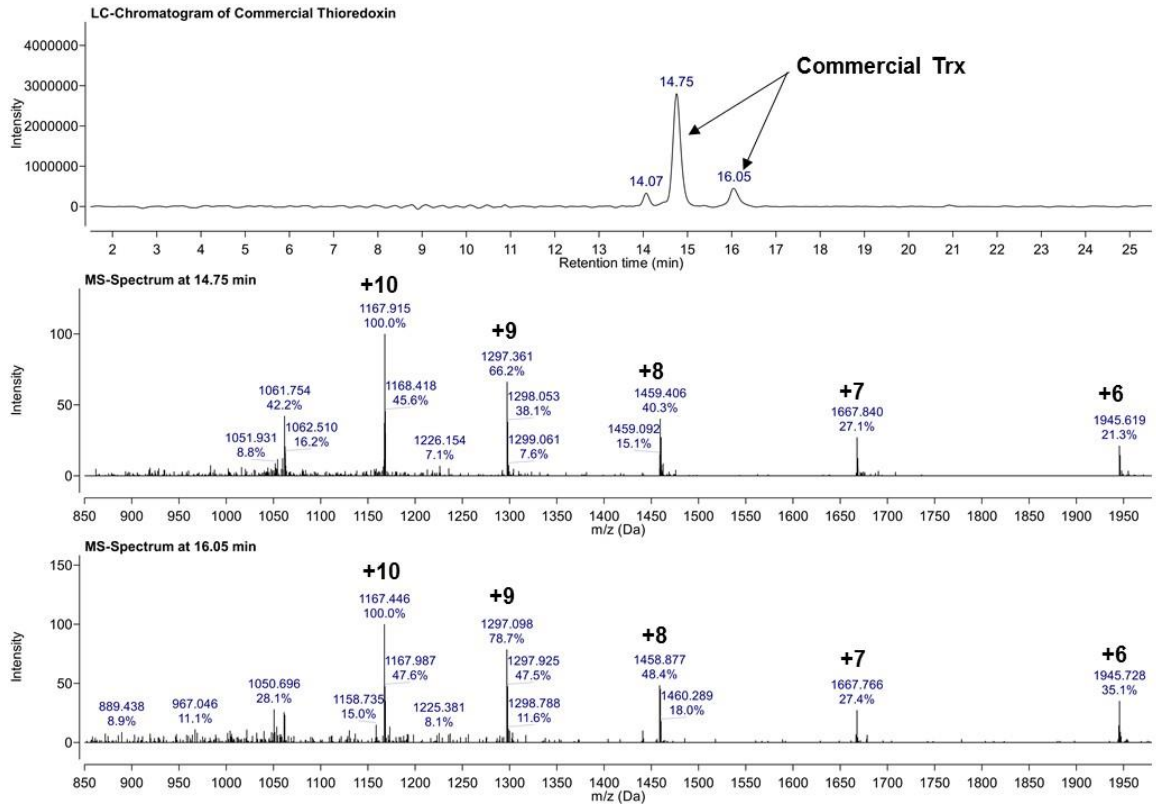
The FPLC chromatograms of the regenerated thioredoxin and normalized tetrazine thioredoxin have been overlaid for comparsion. The calibration curve was used to approximate molecular weight which indicated a monomer/dimer system as illustrated on the figure below. The yield of the regenerated thioredoxin was also found to be ~80%, relative to the normalized tetrazine thioredoxin after calculating the measured areas from the FPLC chromatograms.



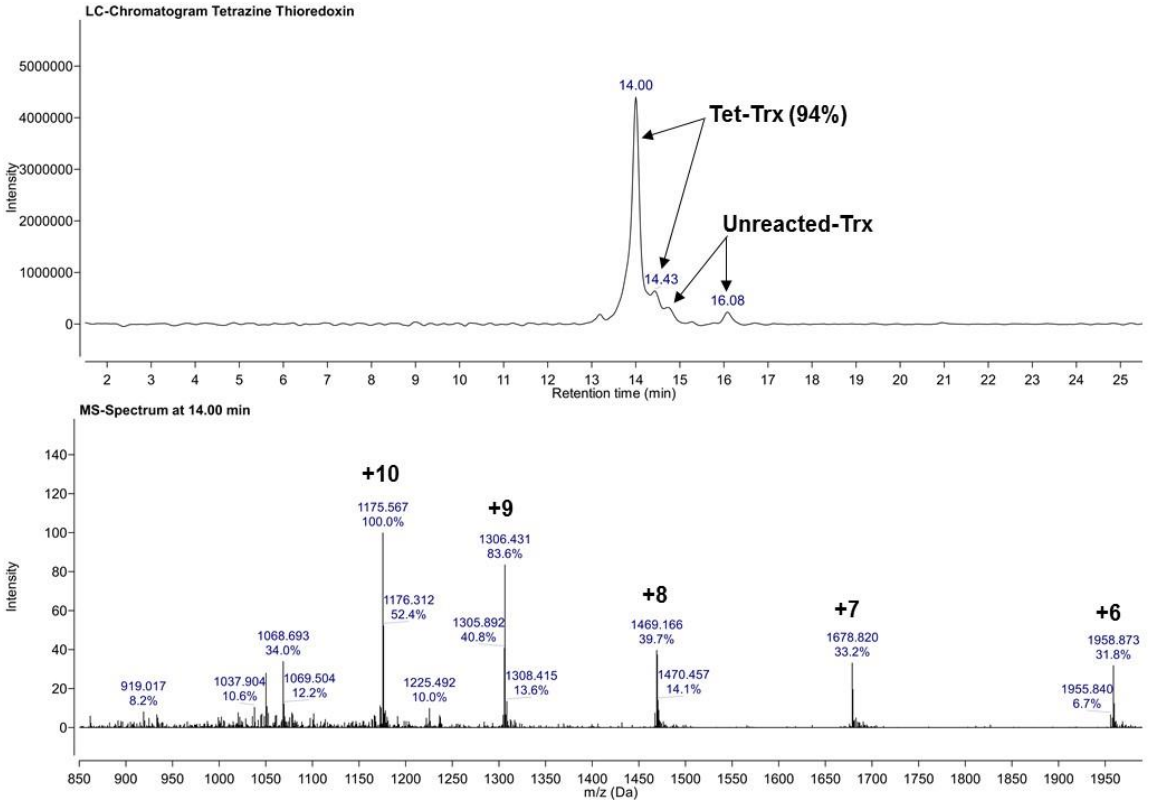
C4-Liquid Chromatography-Mass Spectrometry Separation of Proteins

The LC-MS chromatography was carried out on the same instrument set-up mentioned in the General Methods section; however equipped with Vydac 214MS C4 column (4.6 × 150 mm; 5 µm, part # 214MS5415) with linear gradient of 20%(B) – 70%(B) over 27 minutes at 1.5 mL/min; eluent was 0.1% TFA water(A) and 0.1% TFA in acetonitrile(B).

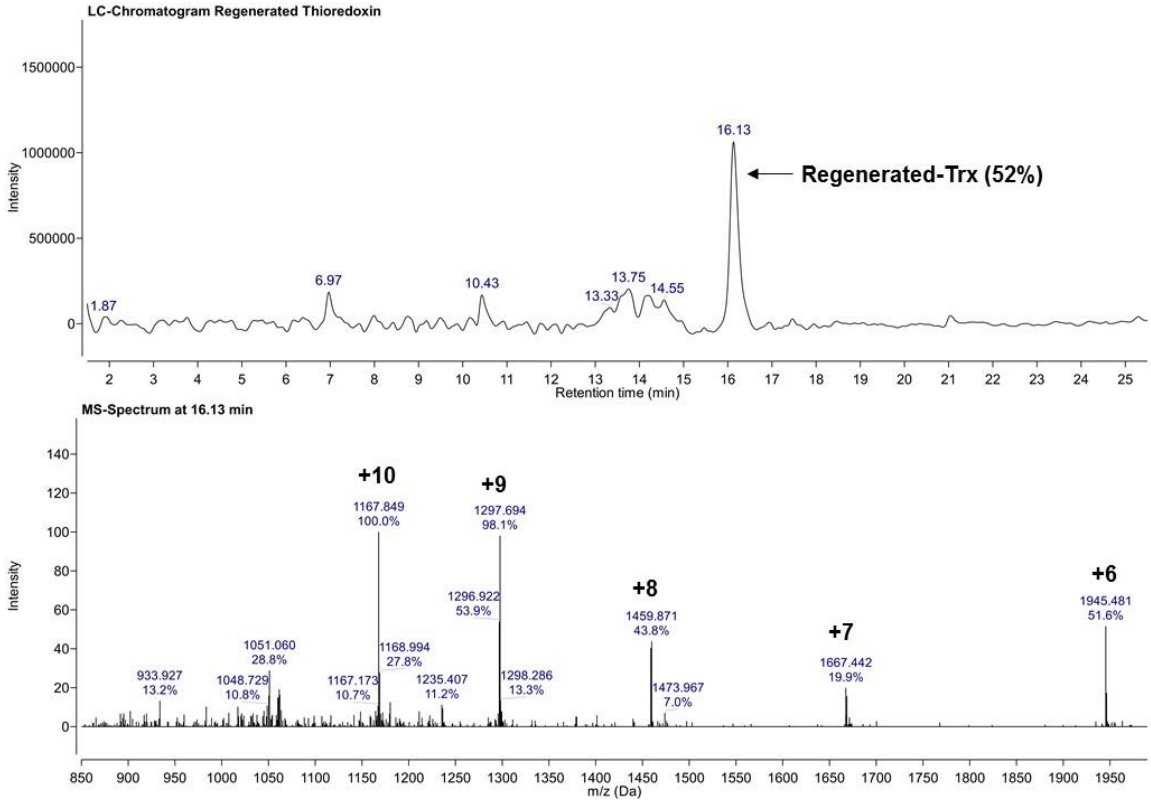
Commercial Thioredoxin: C4-column, Gradient 20-70% MeCN, 27 min, 1.5 mL/min



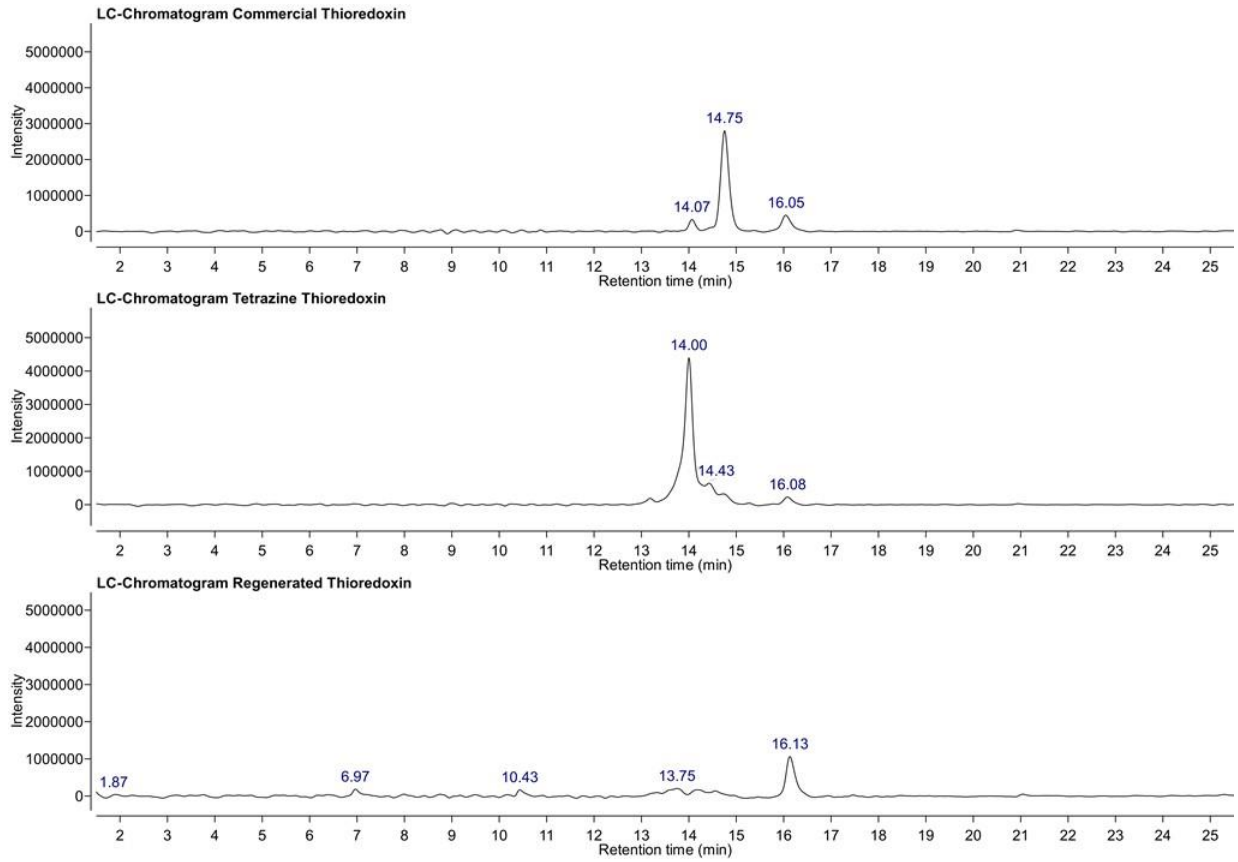
Tetrazine Thioredoxin: C4-column, Gradient 20-70% MeCN, 27 min, 1.5 mL/min



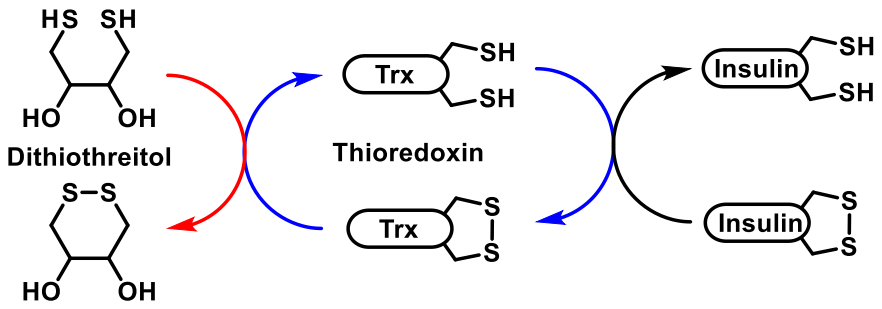
Regenerated Thioredoxin: C4-column, Gradient 20-70% MeCN, 27 min, 1.5 mL/min



Stacked Comparsion of the Stapling and Unstapling of Thioredoxin



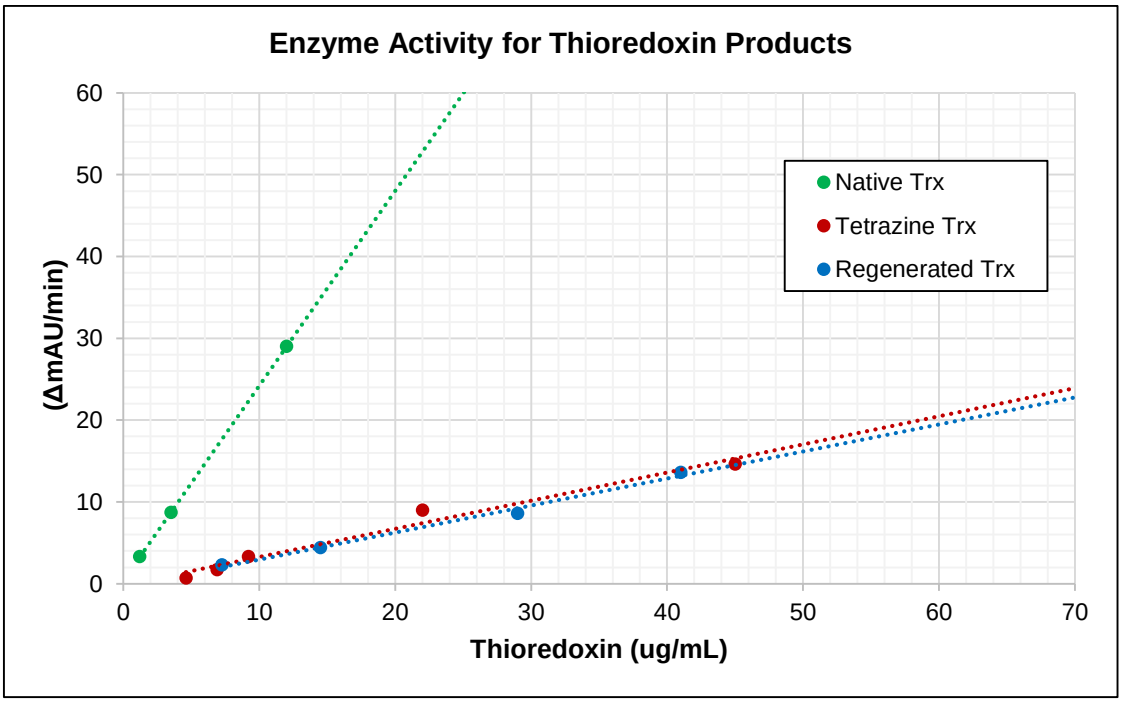
Measurement of Regenerated Thioredoxin Bioactivity



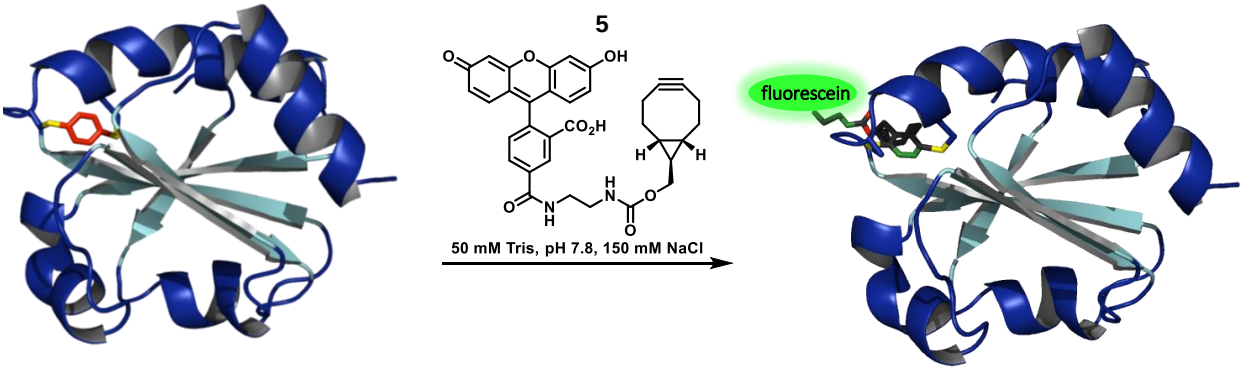
Thioredoxin activity was measured by following the reduction of insulin described by the method of Xianqin Yang and Kesen Ma, *Journal of Bacteriology*, **2010**, 192(5), 1370–1376.

The standard thioredoxin assay mixture, prepared in 200 µL overall volume, contained 50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.15 mM human insulin, 1 mM dithiothreitol. The amounts of native thioredoxin *E. coli*, tetrazine thioredoxin, and regenerated thioredoxin were varied, concentrations of protein were determined by Bradford assay. Sample were run in duplicate, the increase in turbity from the reduction of insulin was monitored at 650 nm at 30°C by a Tecan plate reader.

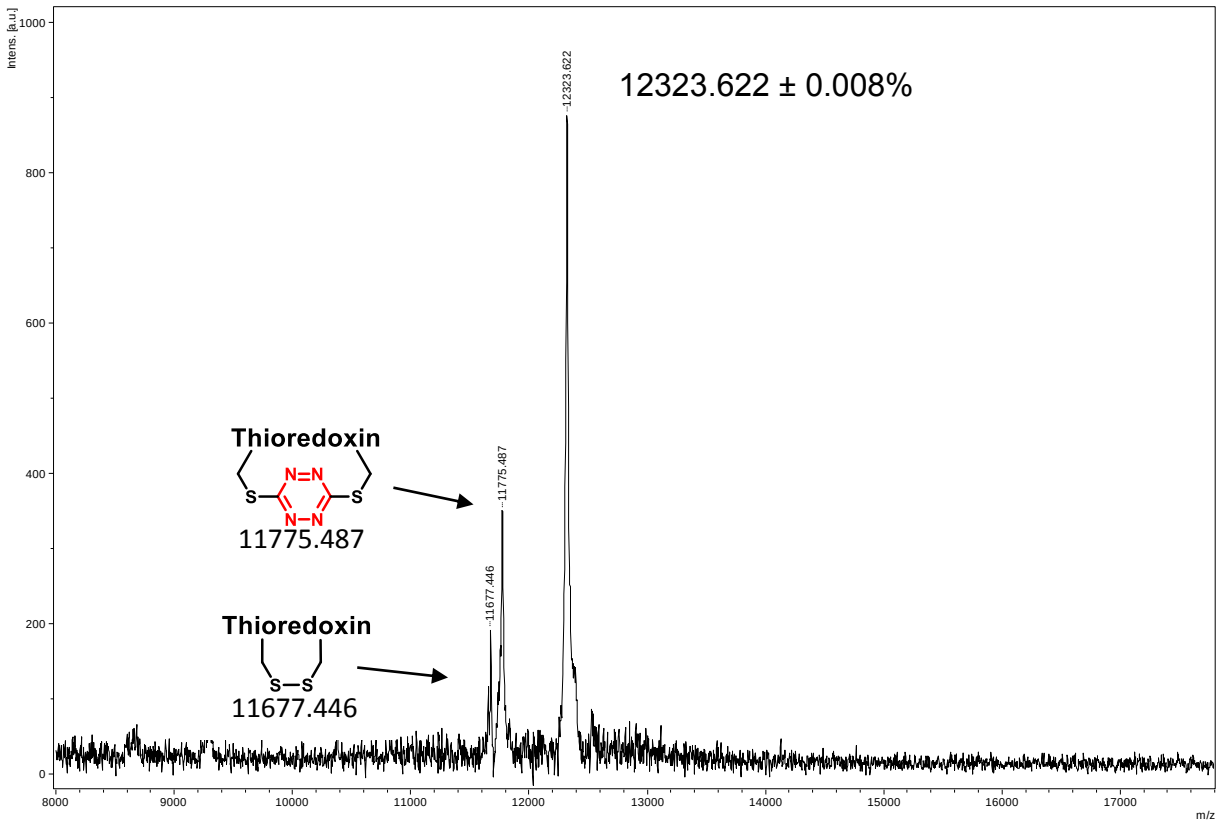
The kinetic curves were basline corrected by subtracting from insulin reduction by dithiothreitol alone. The corrected slopes from the kinetic data (ΔmAU/min), in the linear region, were plotted as a function of concentration of protein.



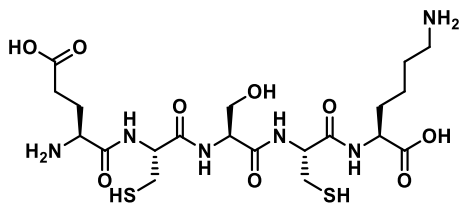
Inverse-Electron Demand Diels-Alder Reaction of Bicyclononyne with Tetrazine Thioredoxin



To a 1.7 mL mini-centrifuge tube containing tetrazine thioredoxin (0.05 mg, 4 nmol) dissolved in 50 mM Tris, pH 7.8, 150 mM NaCl (500 µL) was added a solution of **5** (100 µL, 0.024 mg, 40 nmol, 10 equiv) dissolved in the Tris buffer system. The contents were allowed to stand at ambient temperature for 10 days. The contents were next transferred for centrifugation in an Amicon centrifugal filter (MWCO 3000, 35º fixed angle rotor @ 7000×G, 30 minutes, 4ºC), the concentrated sample (150 µL) was diluted to 3 mL with the Tris buffer and repeated. The reaction was monitored by mass spectrometry, which illustrated the loss of nitrogen and an additional mass equal to **5** . MALDI-TOF *m/z* 12323.622 [$(M+H)^+$; 12322.64; calculated for tetrazine Trx (11755.44) + **5** (594.20 Da) + H^+ (1.01) – N_2 (28.01)].

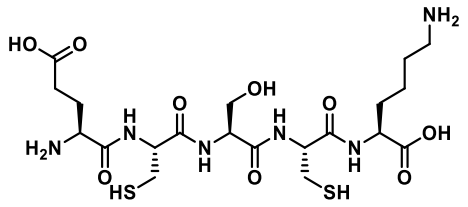


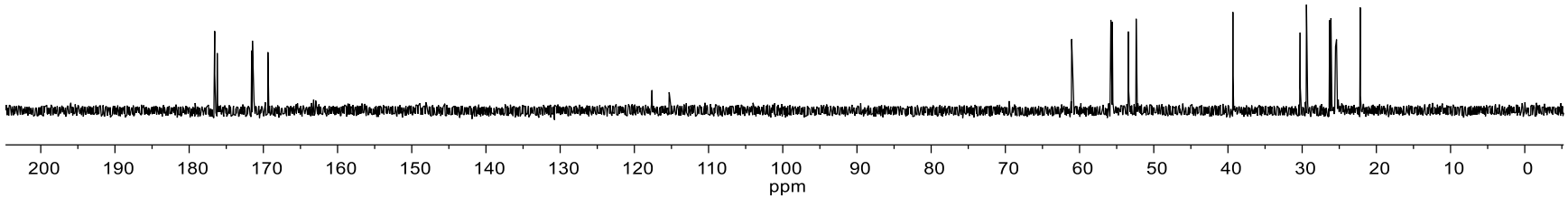
Compound 1a



1a (^{1}H-NMR, D_2O, 500 MHz)

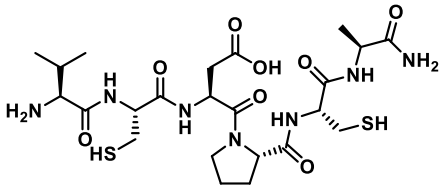
Compound 1a

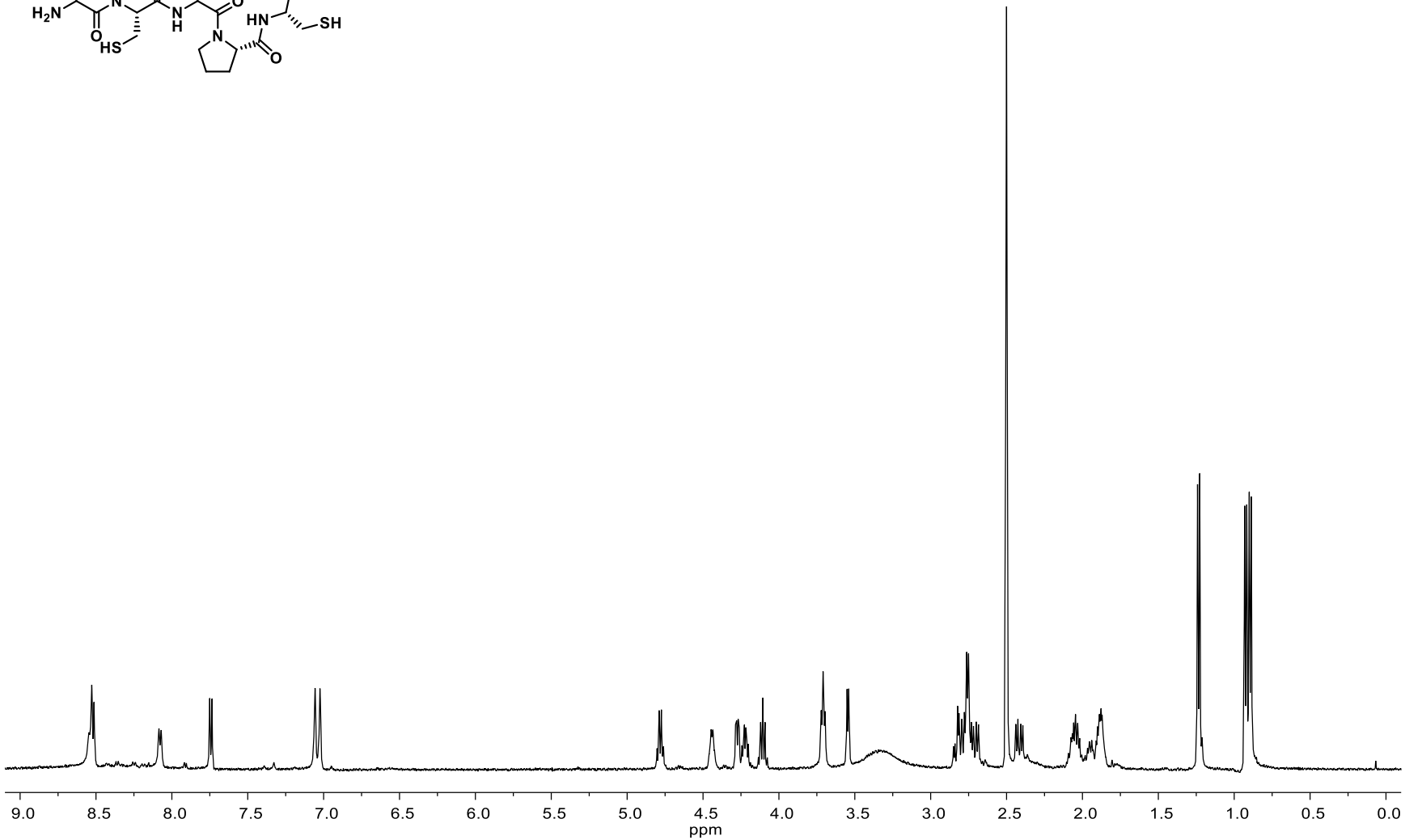




1a (^{13}C-NMR, D_2O, 126 MHz)

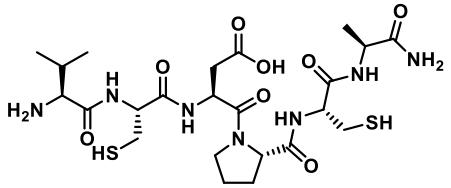
Compound 1b





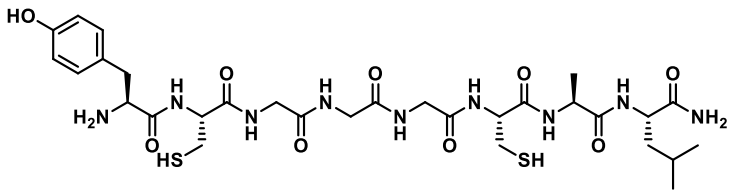
1b (^{1}H-NMR, DMSO, 126 MHz)

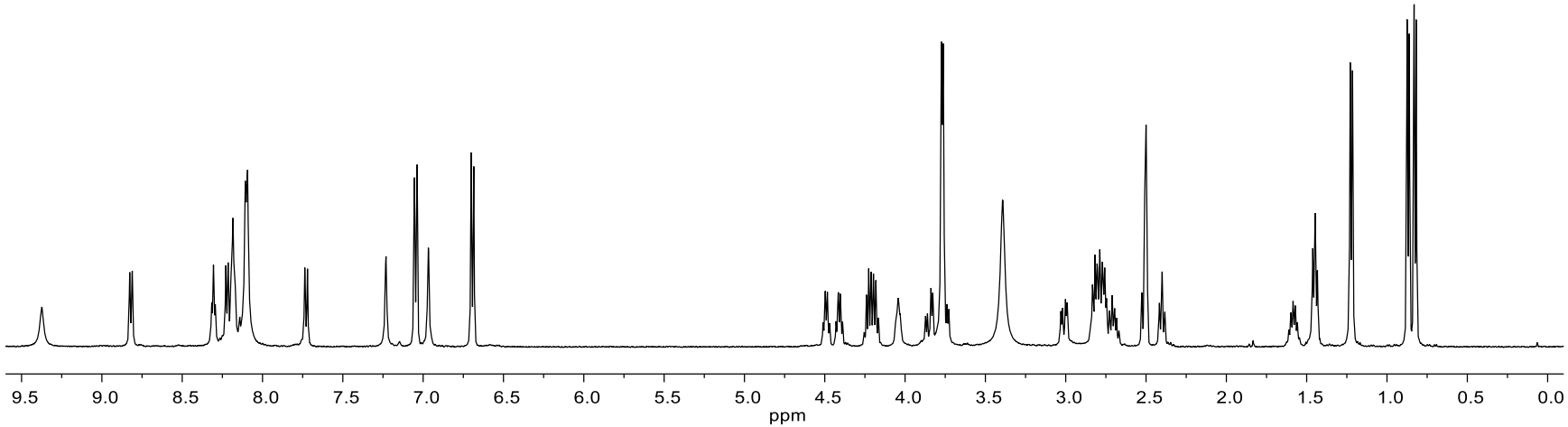
Compound 1b



1b (^{13}C-NMR, DMSO, 126 MHz)

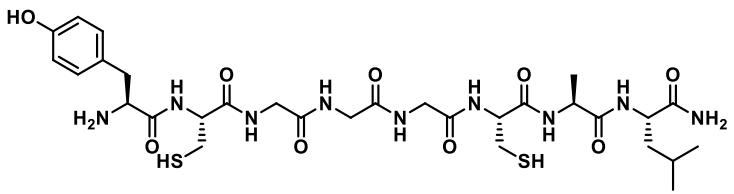
Compound 1c





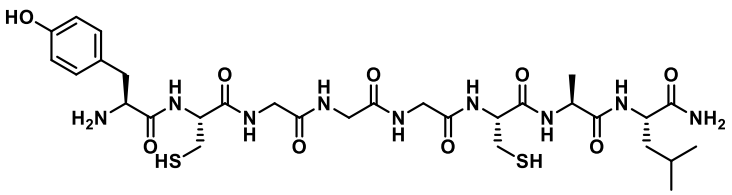
1c (^{1}H-NMR, DMSO, 500 MHz)

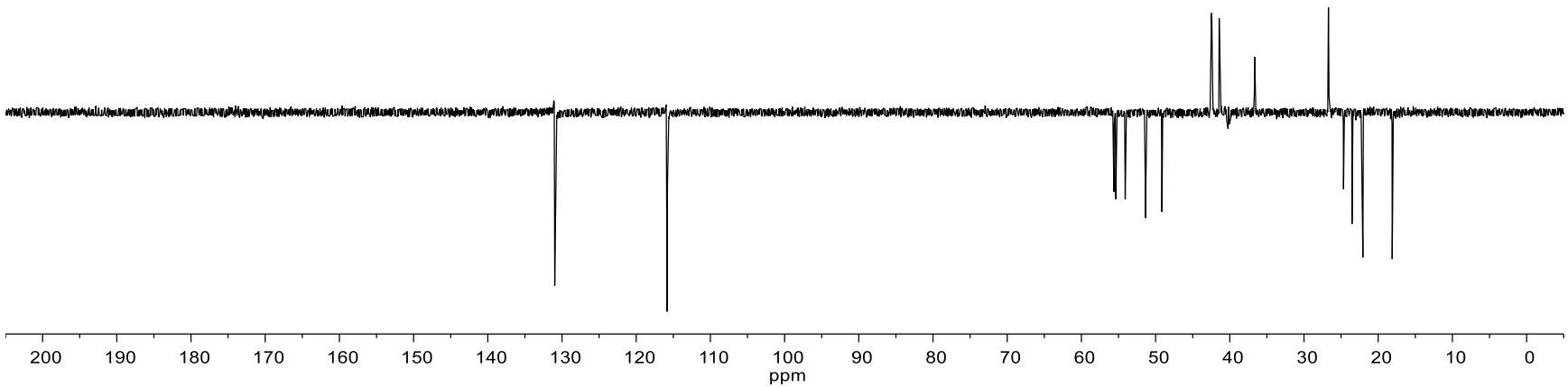
Compound 1c



1c (^{13}C-NMR, DMSO, 126 MHz)

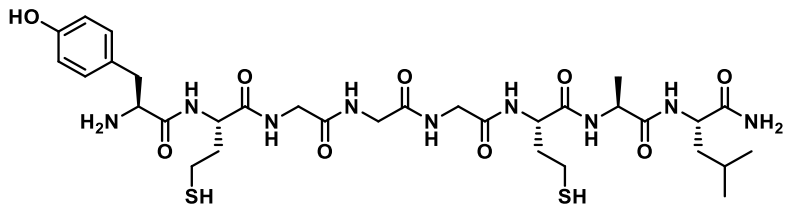
Compound 1c

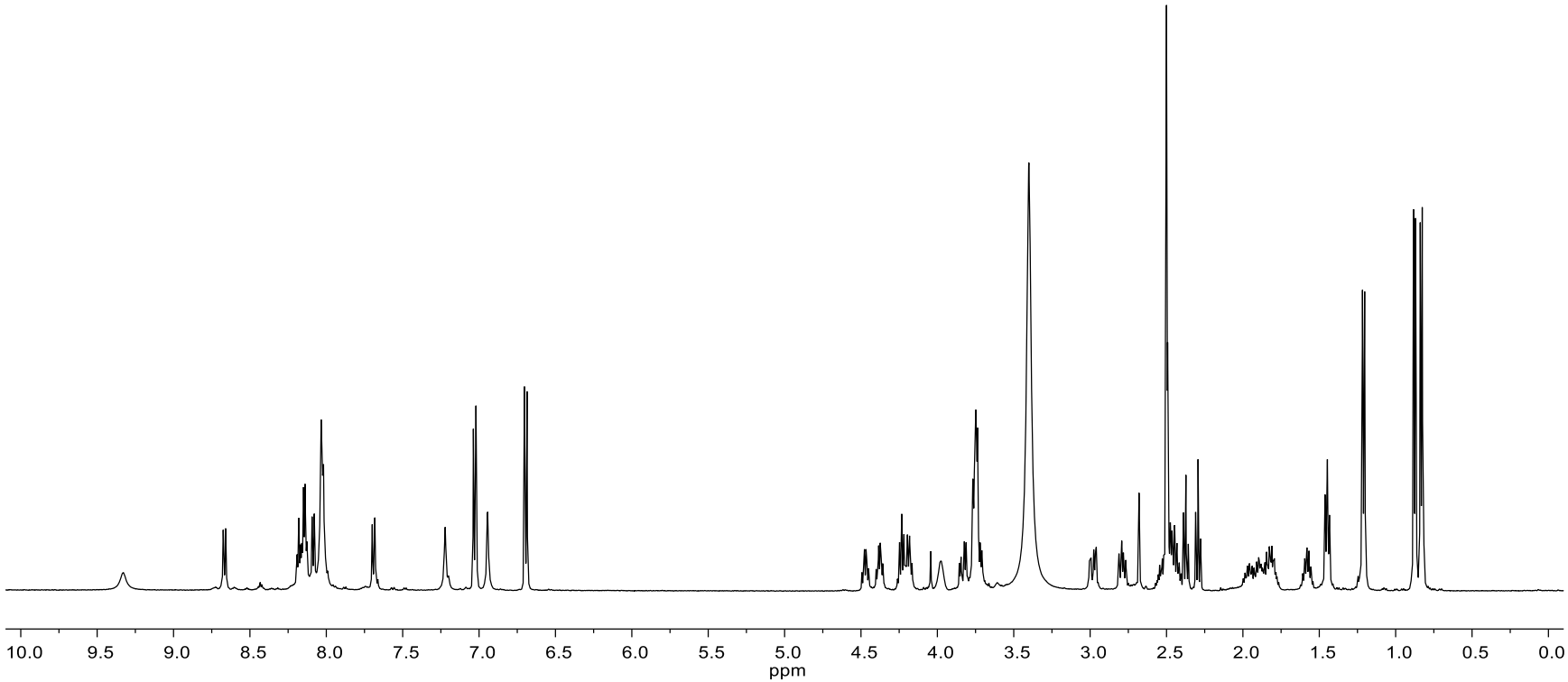




1c (DEPT135, DMSO, 126 MHz)

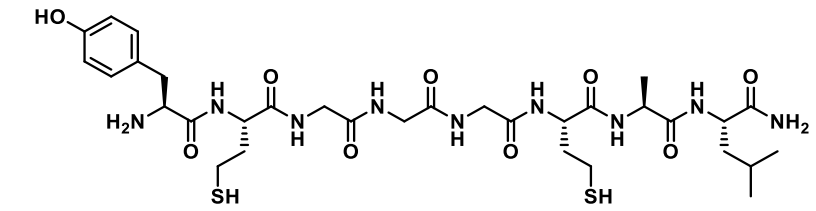
Compound 1d





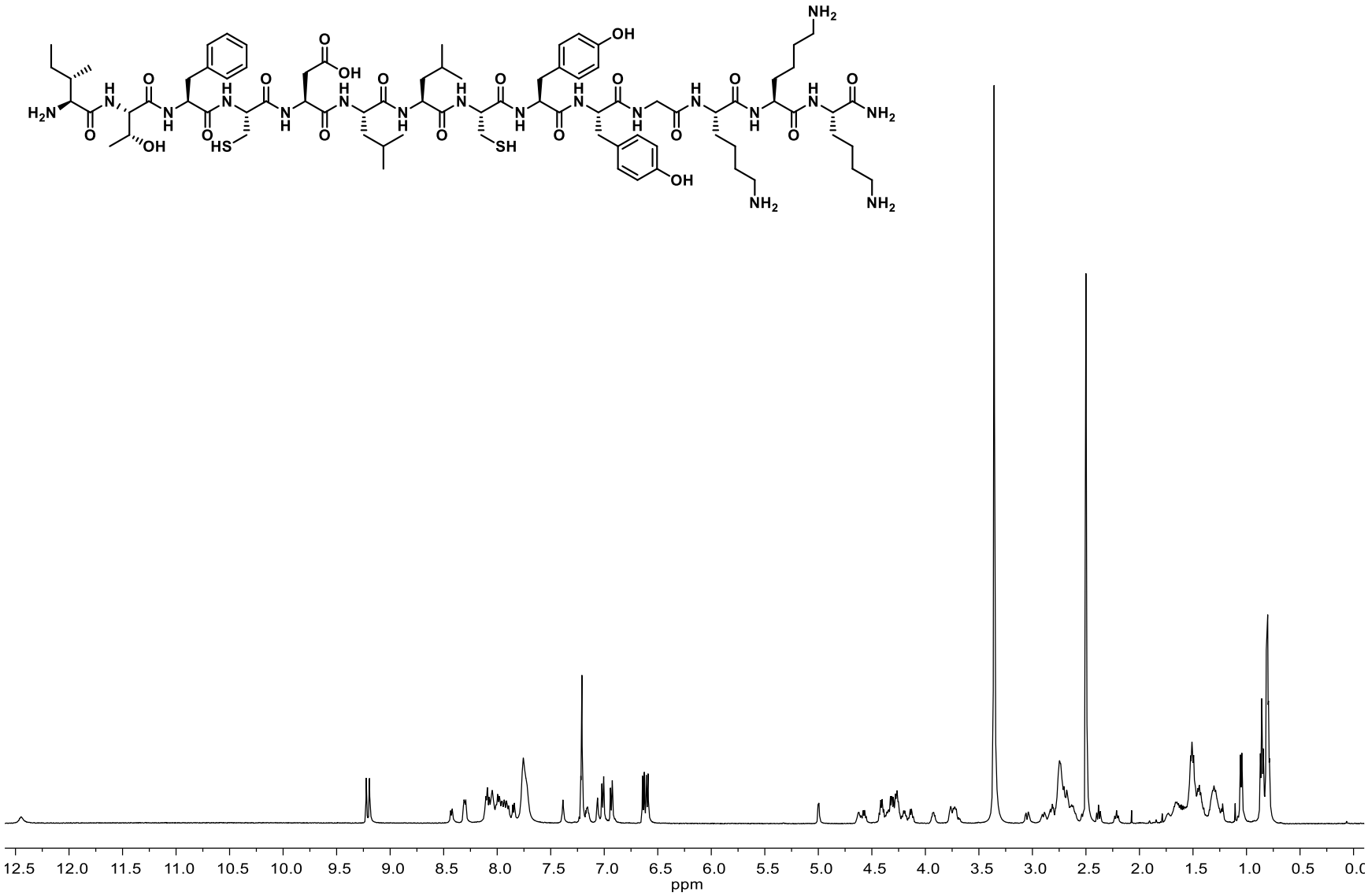
1d (^{1}H-NMR, DMSO, 500 MHz)

Compound 1d



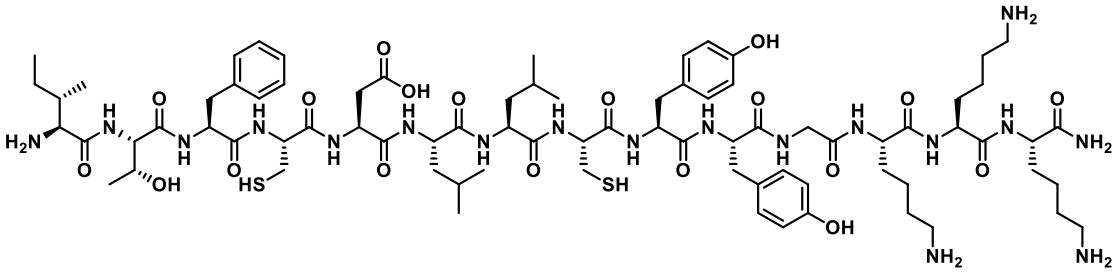
1d (^{13}C-NMR, DMSO, 126 MHz)

Compound 1e



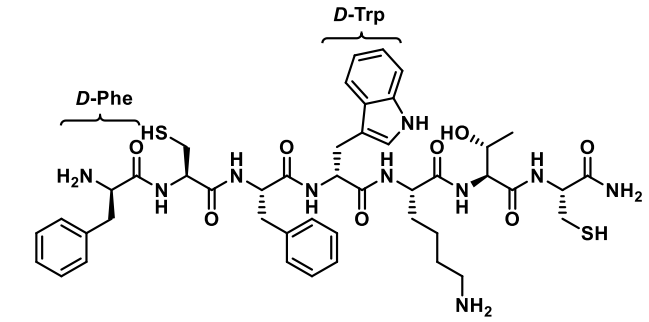
1e (^{1}H-NMR, DMSO, 500 MHz)

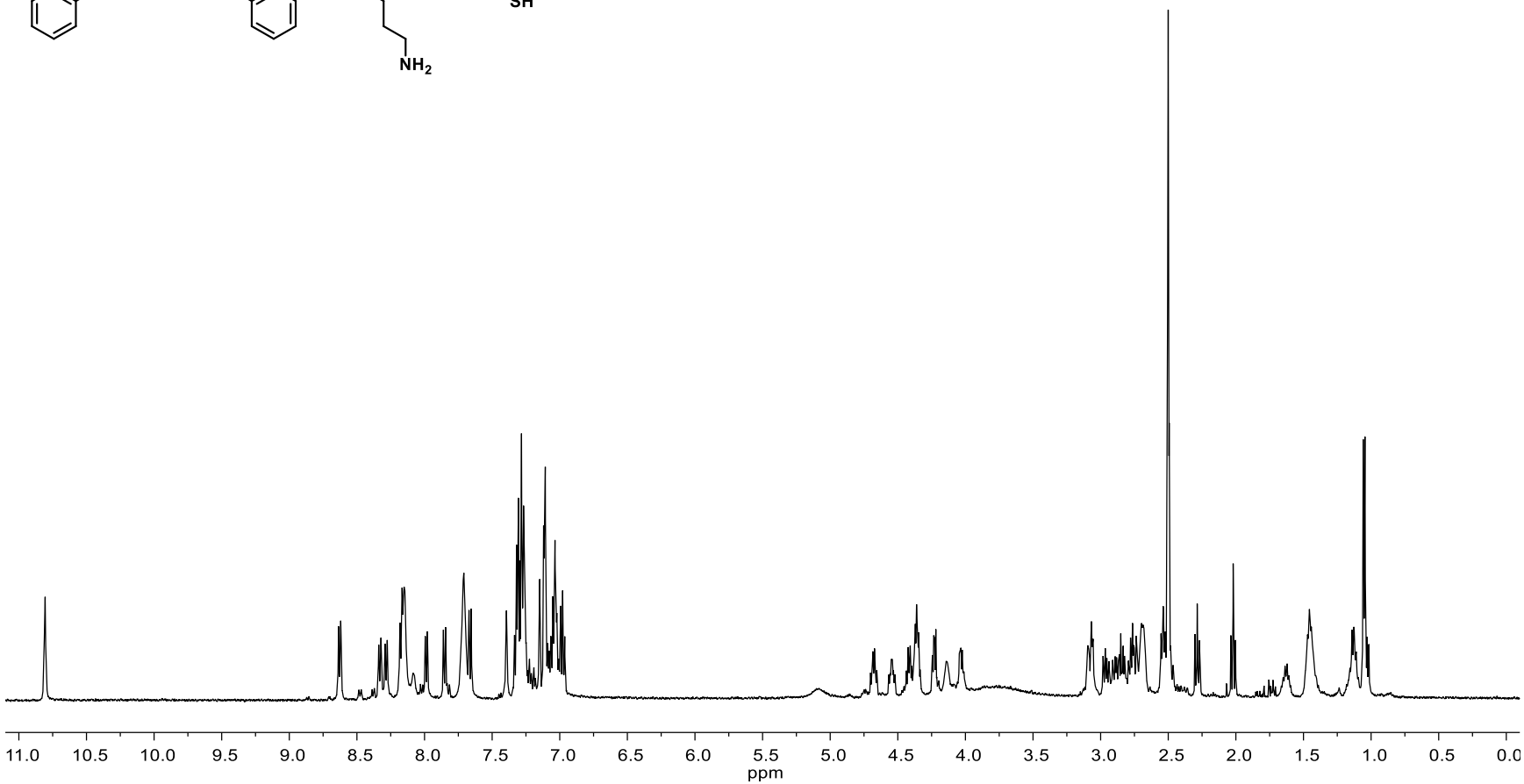
Compound 1e



1e (^{13}C-NMR, DMSO, 126 MHz)

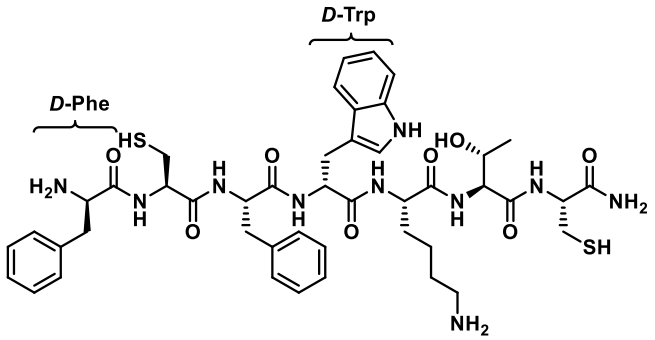
Compound 1f





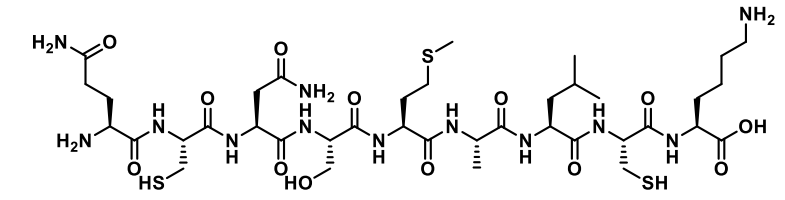
1f (^{1}H-NMR, DMSO, 500 MHz)

Compound 1f



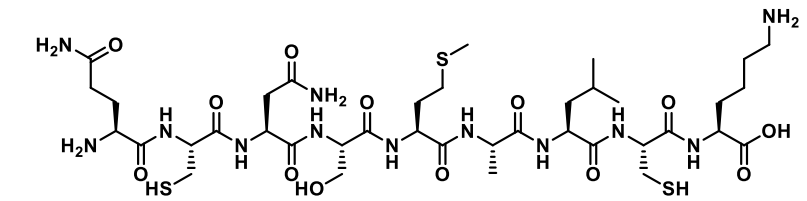
1f (^{13}C-NMR, DMSO, 126 MHz)

Compound 1g



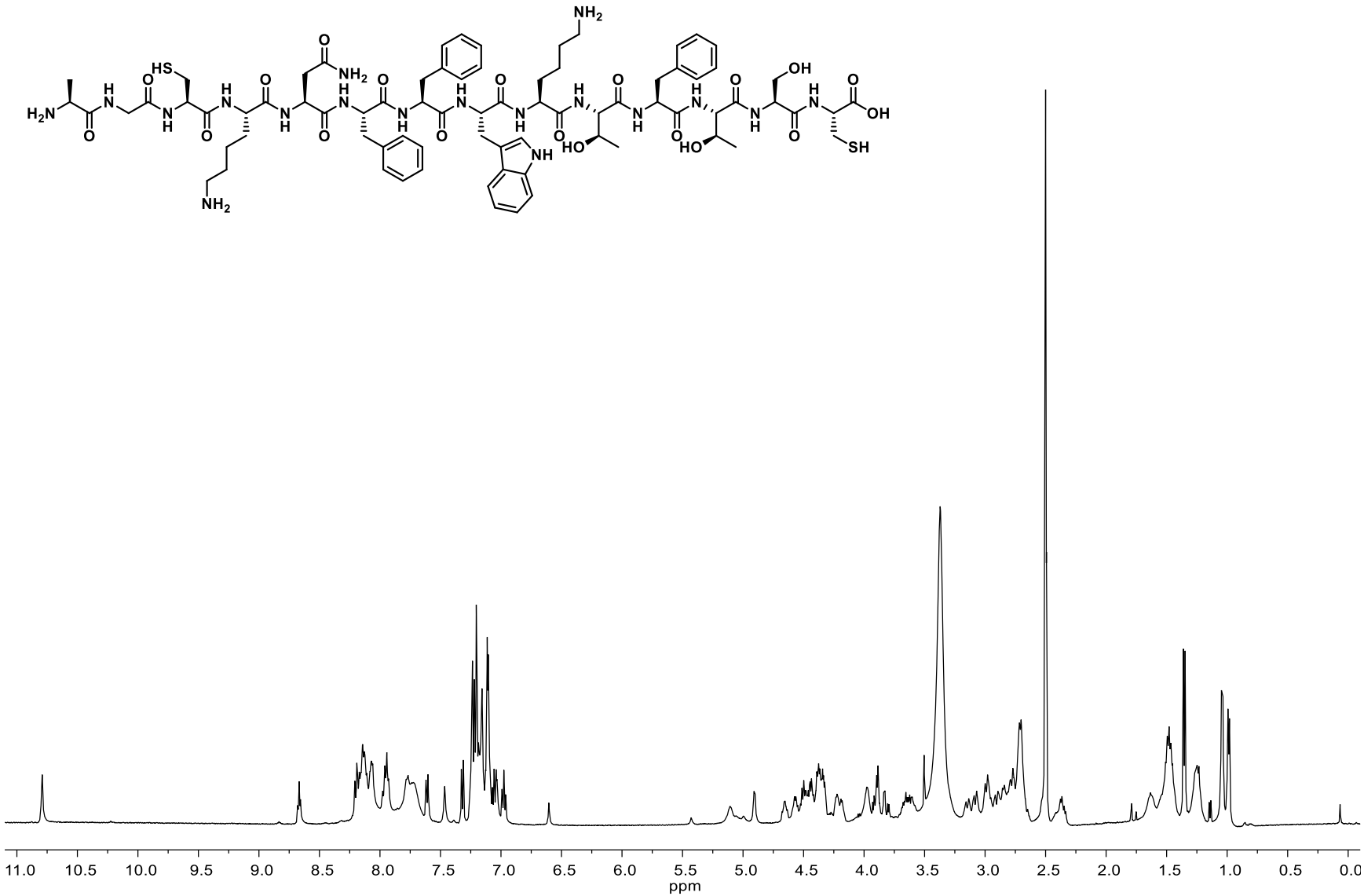
1g (^{1}H-NMR, DMSO, 500 MHz)

Compound 1g



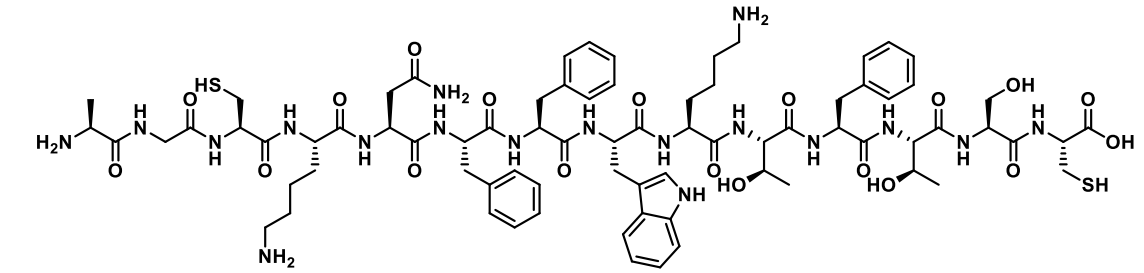
1g (^{13}C-NMR, DMSO, 126 MHz)

Compound 1h



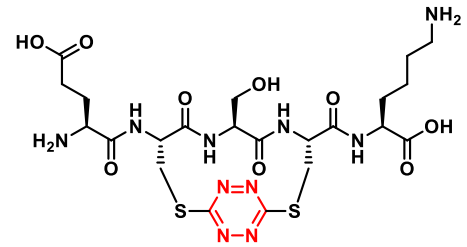
1h (^{1}H-NMR, DMSO, 500 MHz)

Compound 1h



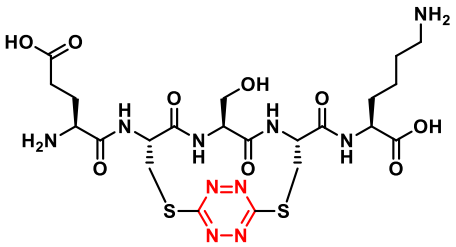
1h (^{13}C-NMR, DMSO, 126 MHz)

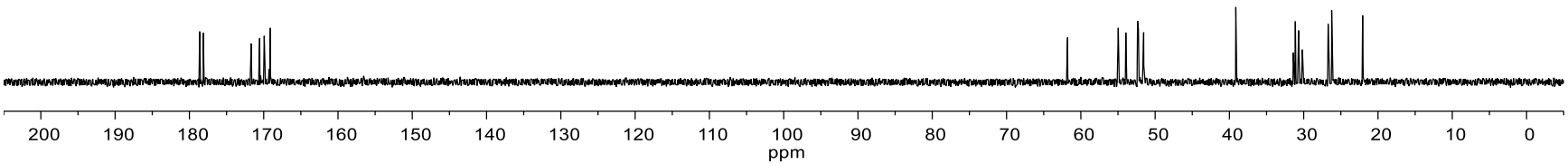
Compound 2a



2a (^{1}H-NMR, D_2O, 500 MHz)

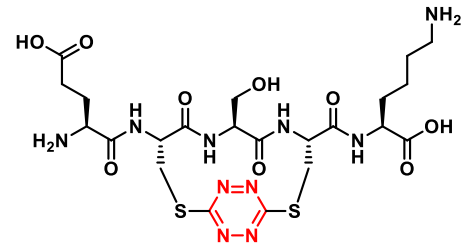
Compound 2a

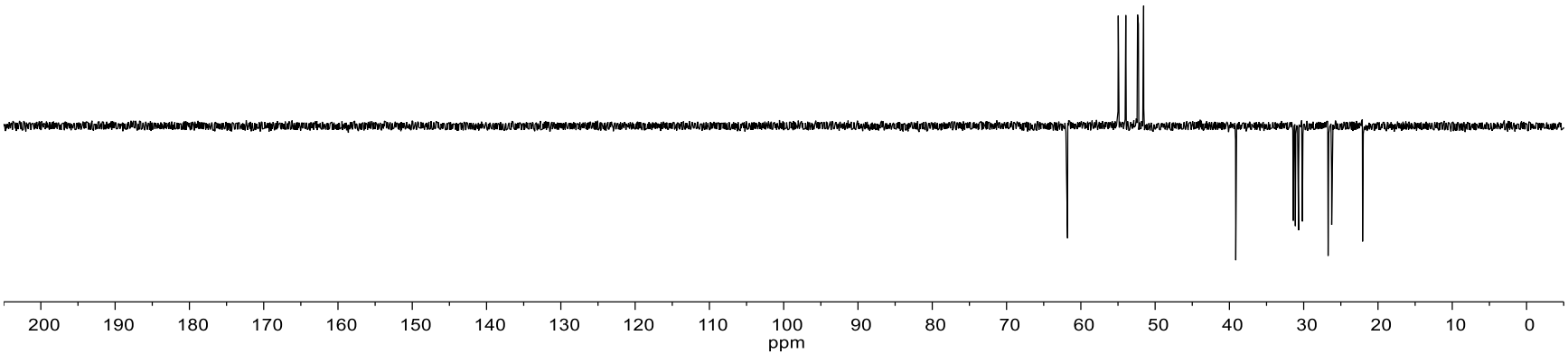




2a (^{13}C-NMR, D_2O, 126 MHz)

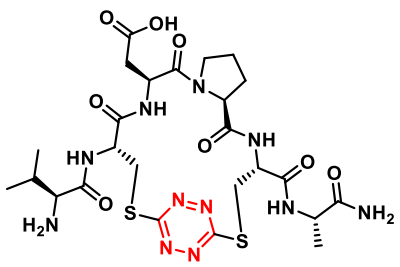
Compound 2a

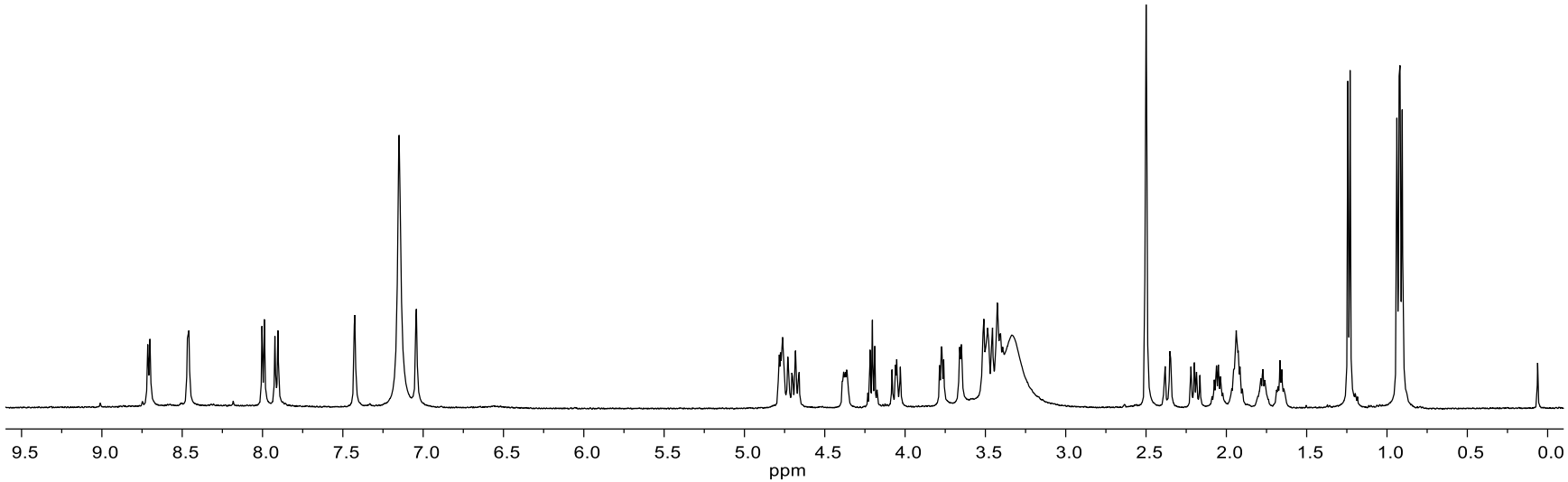




2a (DEPT135, D_2O, 126 MHz)

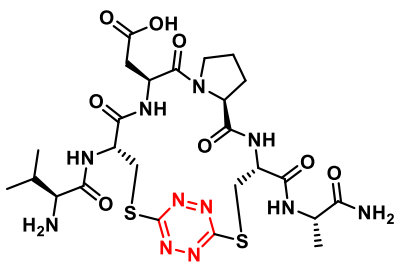
Compound 2b





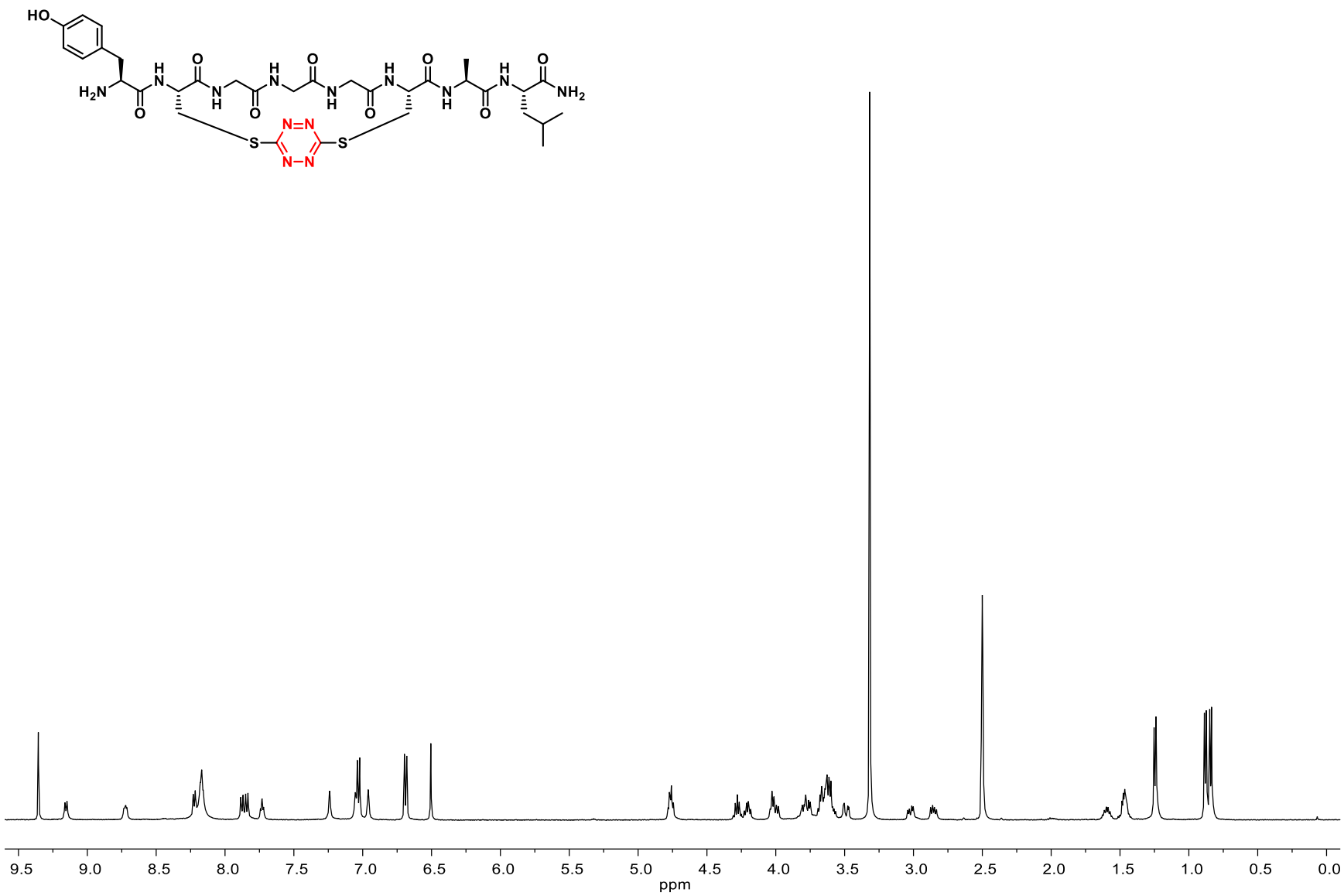
2b (^{1}H-NMR, DMSO, 500 MHz)

Compound 2b



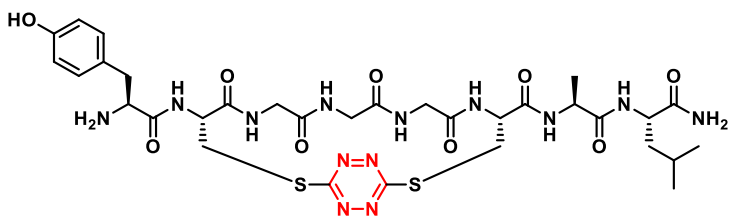
2b (^{13}C-NMR, DMSO, 126 MHz)

Compound 2c



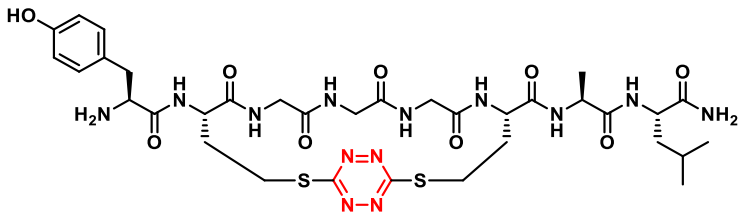
2c (^{1}H-NMR, DMSO, 500 MHz)

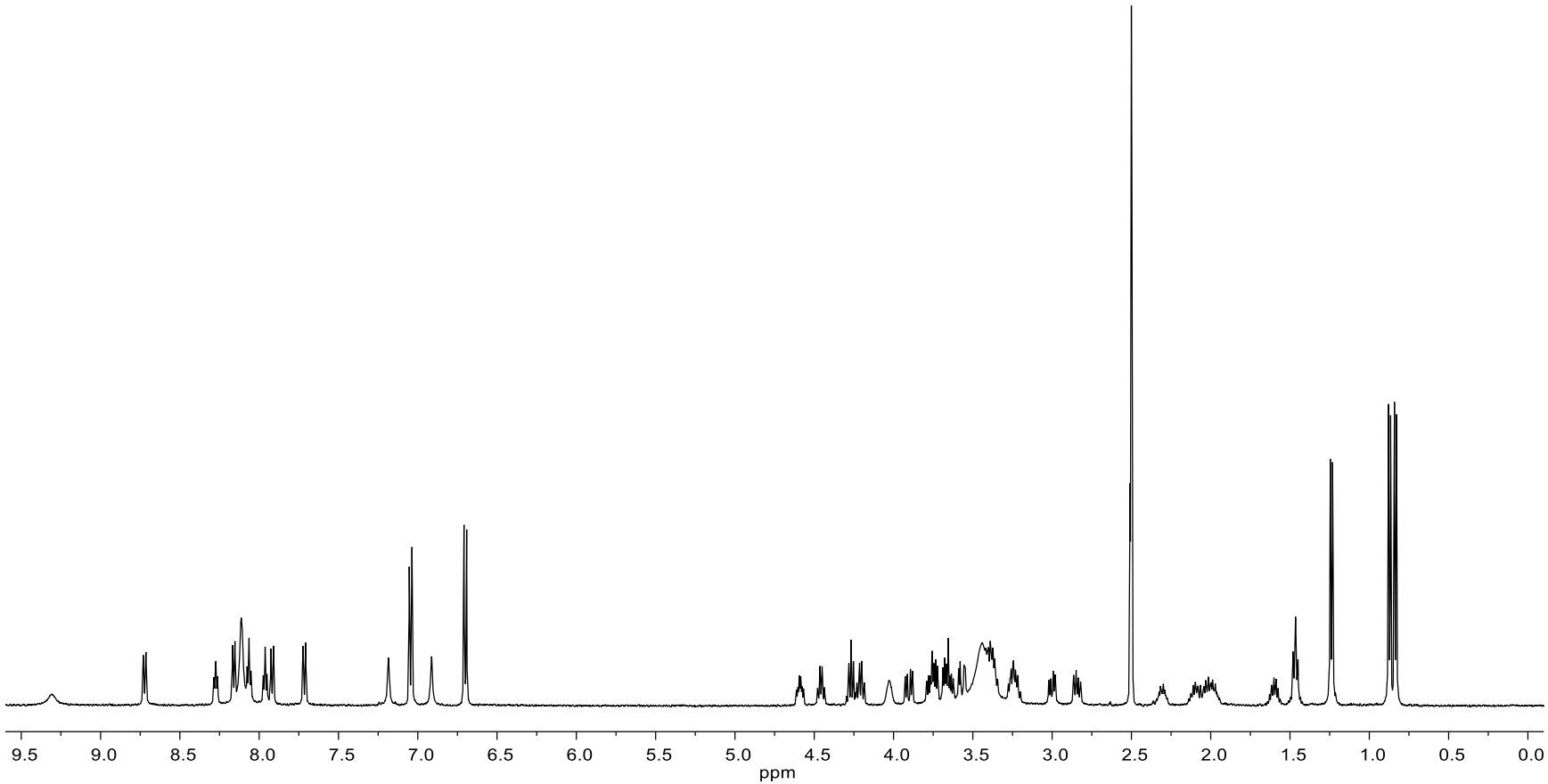
Compound 2c



2c (^{13}C-NMR, DMSO, 126 MHz)

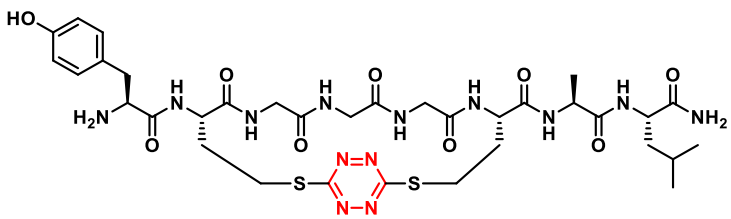
Compound 2d





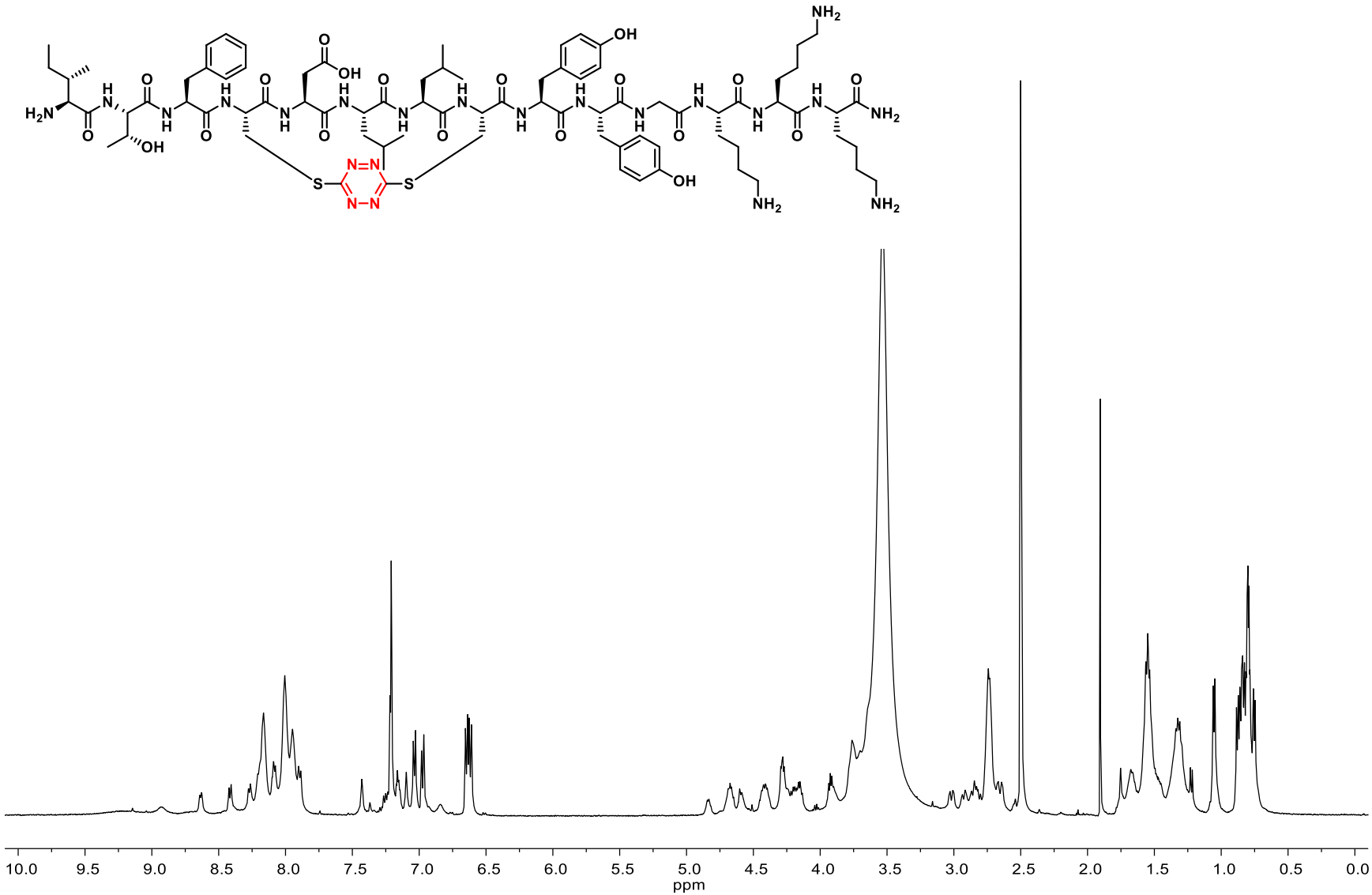
2d (^{1}H-NMR, DMSO, 500 MHz)

Compound 2d



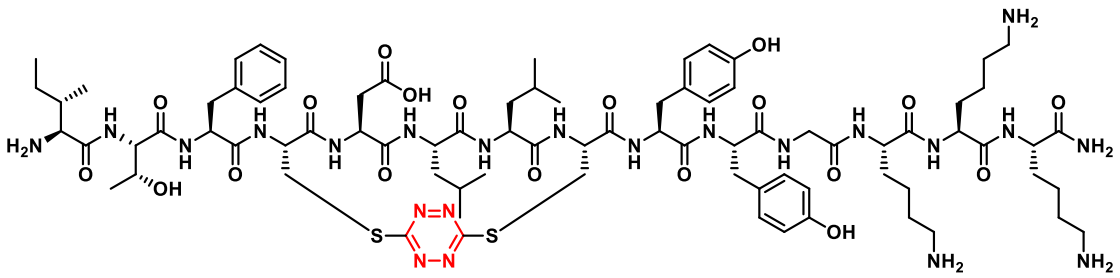
2d (^{13}C-NMR, DMSO, 126 MHz)

Compound 2e



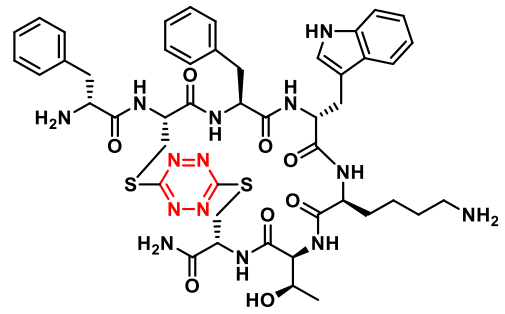
2e (^{1}H-NMR, DMSO, 500 MHz)

Compound 2e



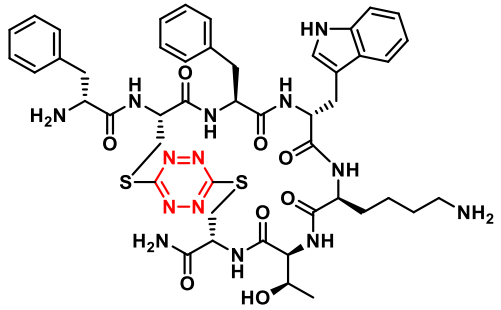
2e (^{13}C-NMR, DMSO, 126 MHz)

Compound 2f



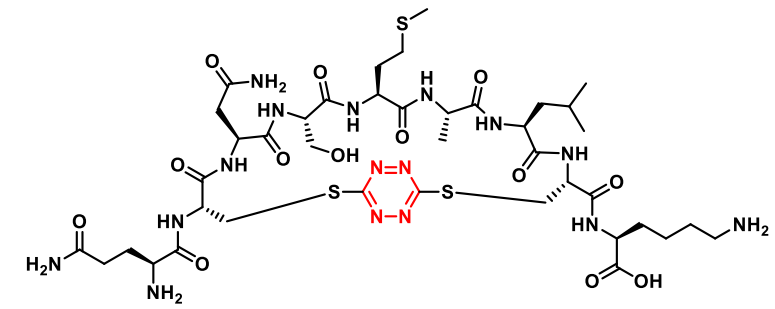
2f (^{1}H-NMR, DMSO, 500 MHz)

Compound 2f



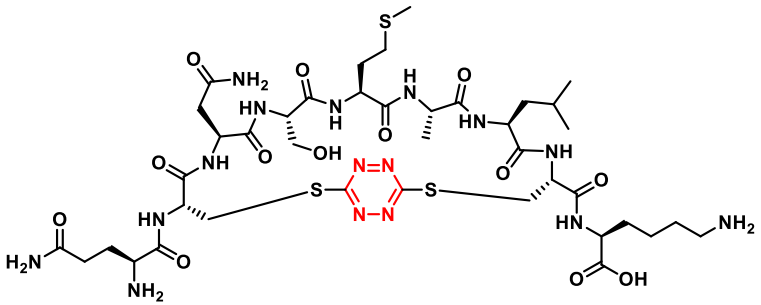
2f (^{13}C-NMR, DMSO, 126 MHz)

Compound 2g



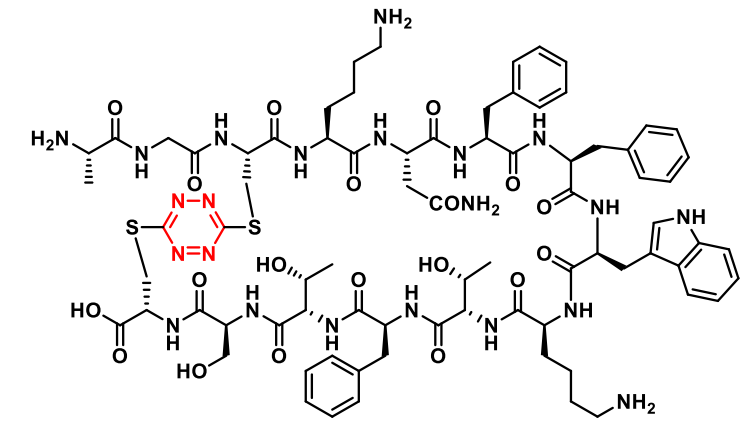
2g (^{1}H-NMR, DMSO, 500 MHz)

Compound 2g



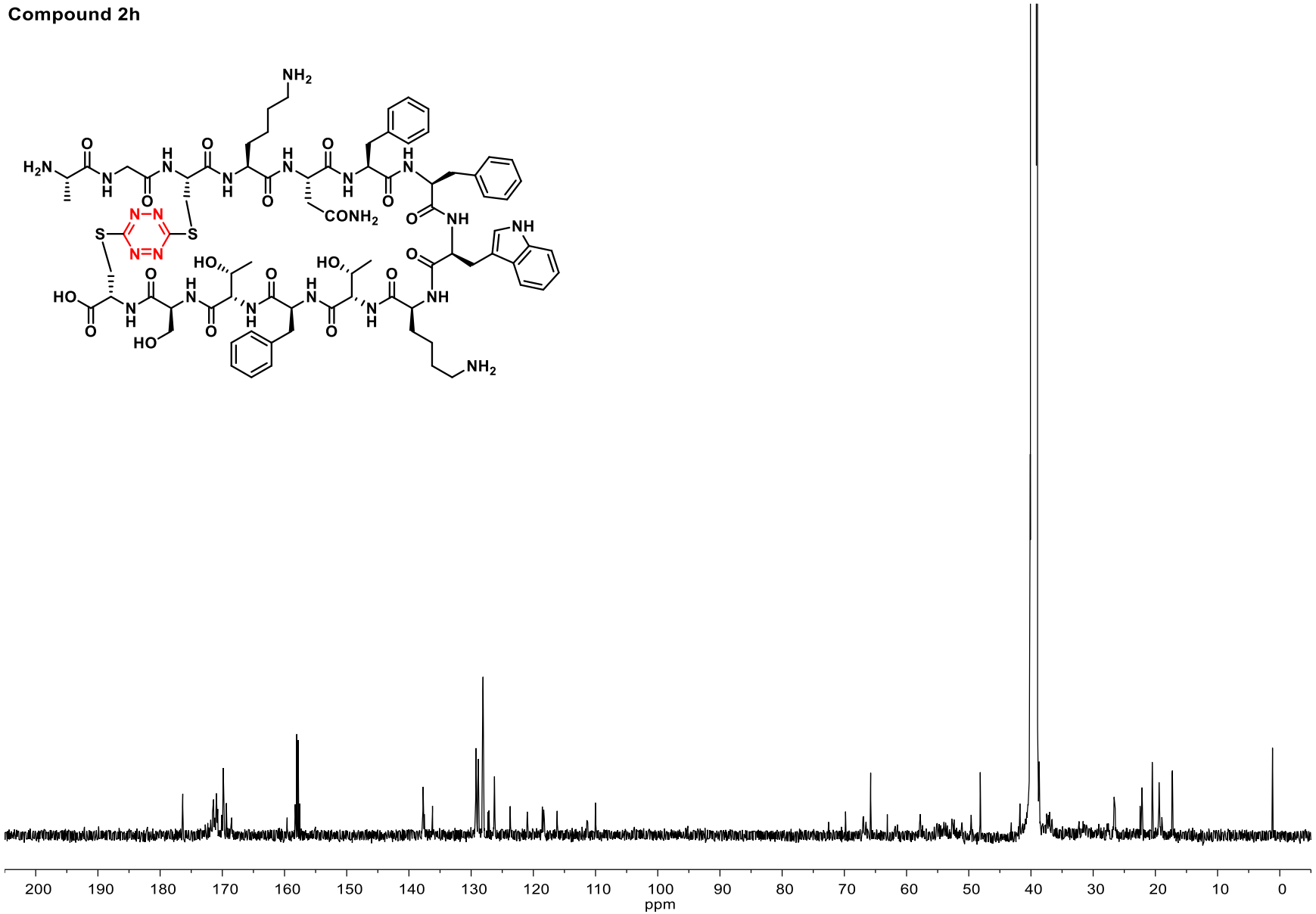
2g (^{13}C-NMR, DMSO, 126 MHz)

Compound 2h



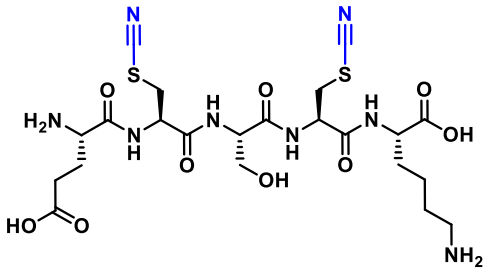
2h (^{1}H-NMR, DMSO, 500 MHz)

Compound 2h



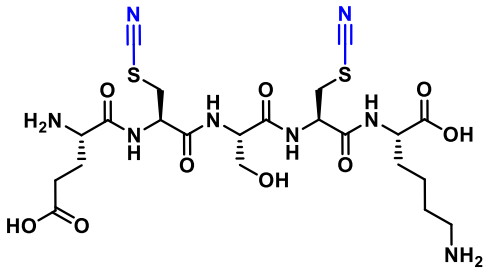
2h (^{13}C-NMR, DMSO, 126 MHz)

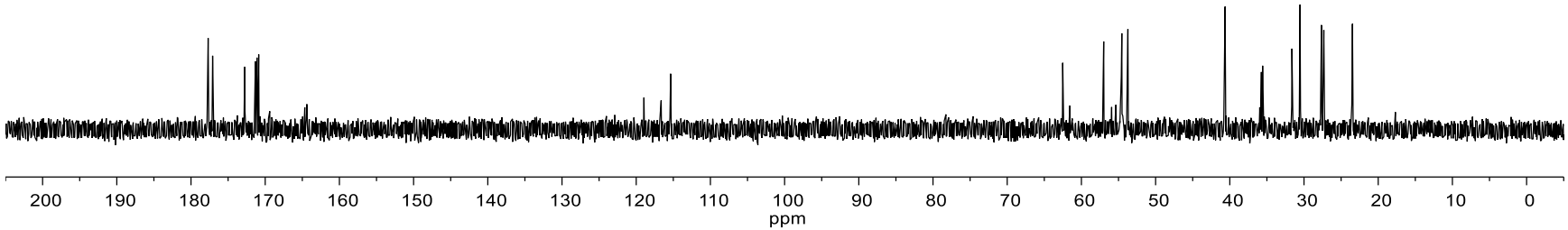
Compound 3a



3a (^{1}H-NMR, D_2O, 500 MHz)

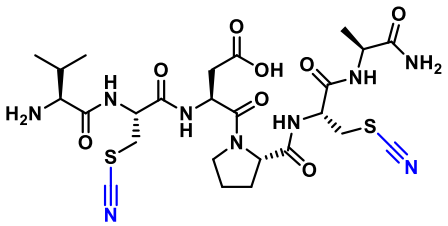
Compound 3a





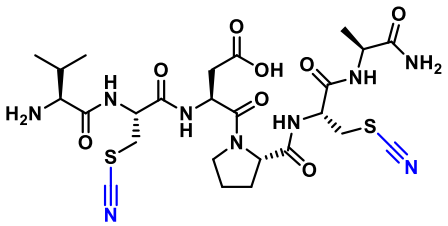
3a (^{13}C-NMR, D_2O, 126 MHz)

Compound 3b



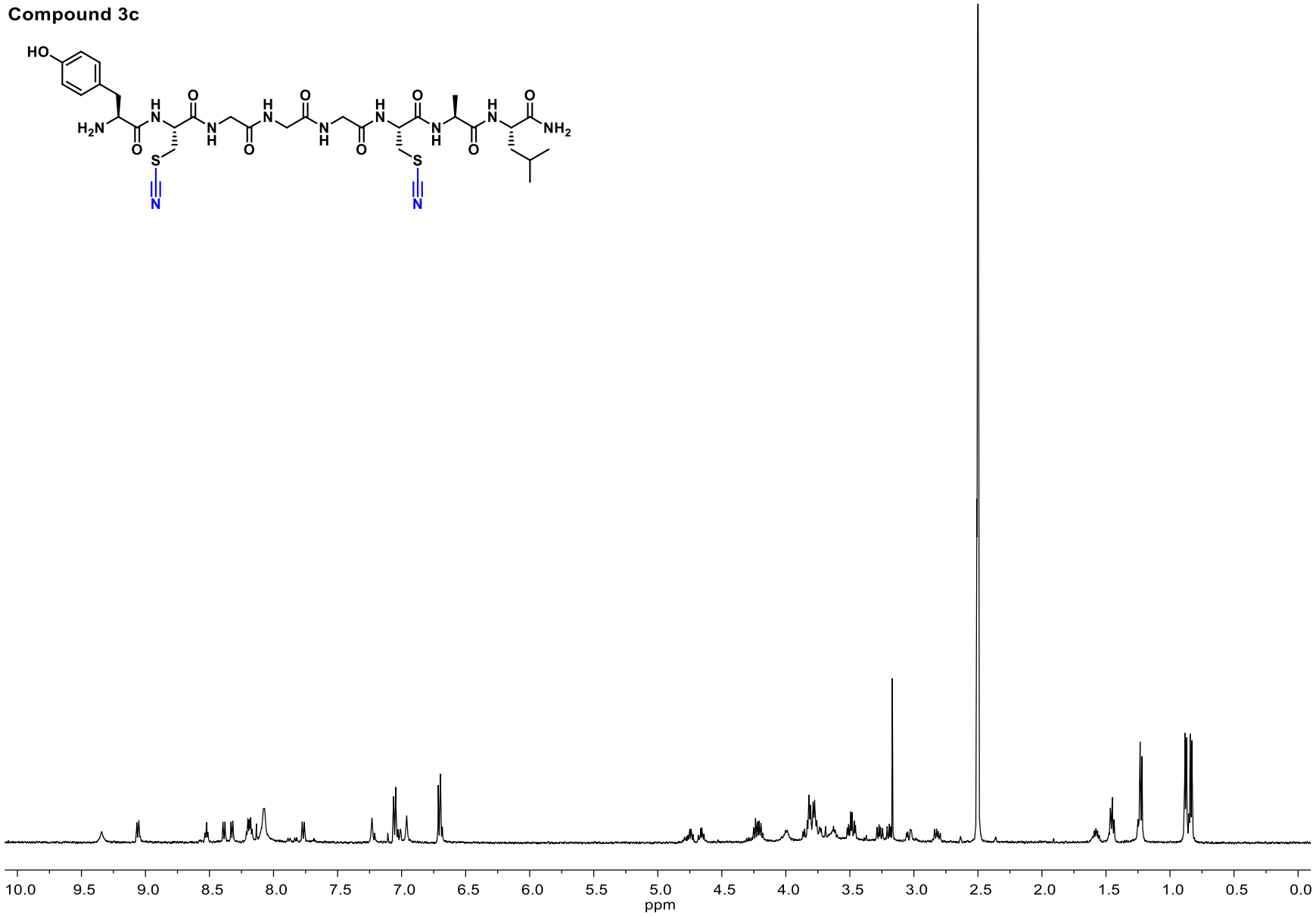
3b (^{1}H-NMR, DMSO, 500 MHz)

Compound 3b



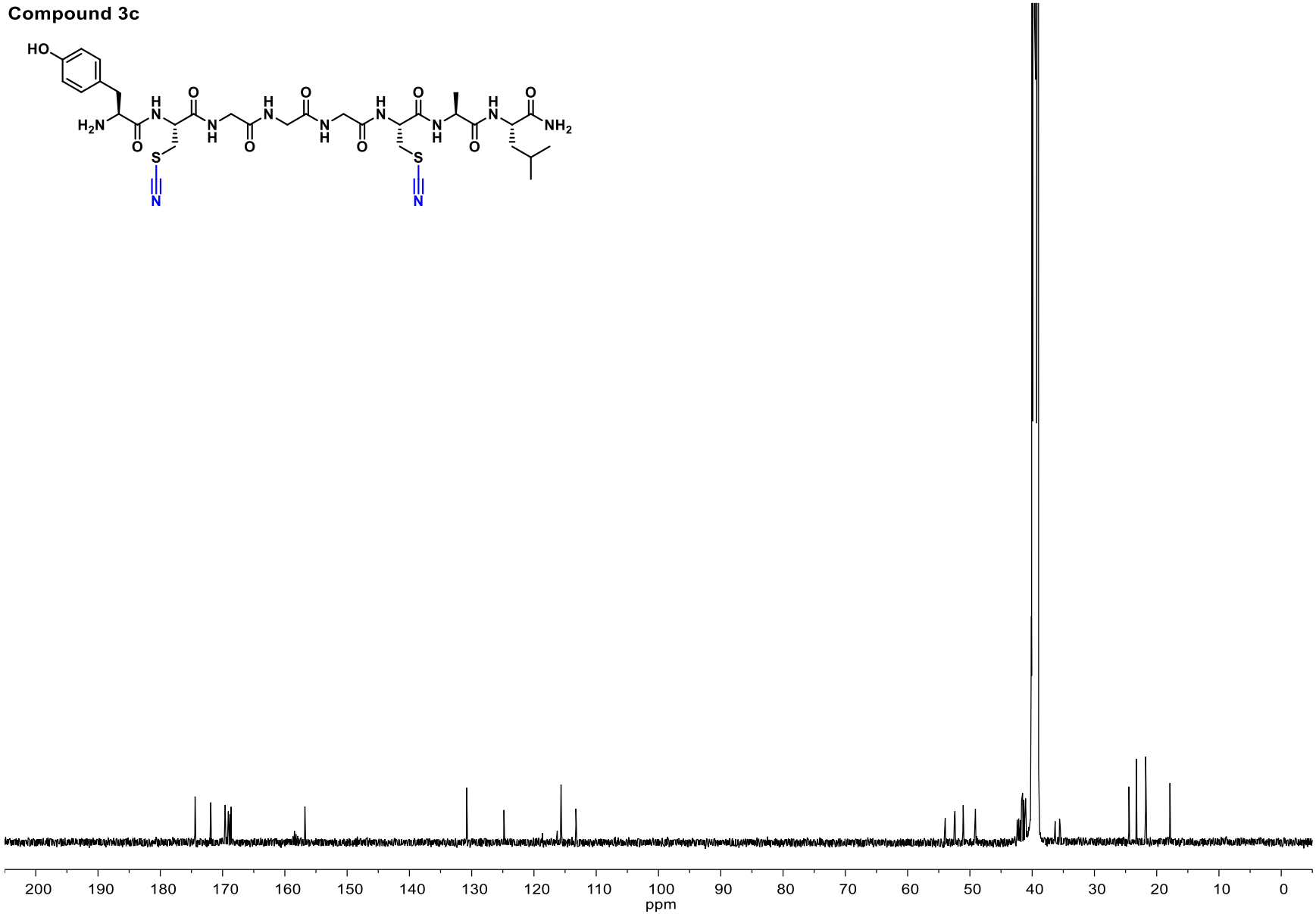
3b (^{13}C-NMR, DMSO, 126 MHz)

Compound 3c



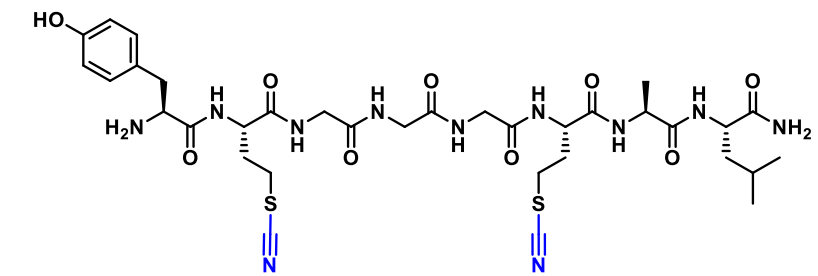
3c (^{1}H-NMR, DMSO, 500 MHz)

Compound 3c



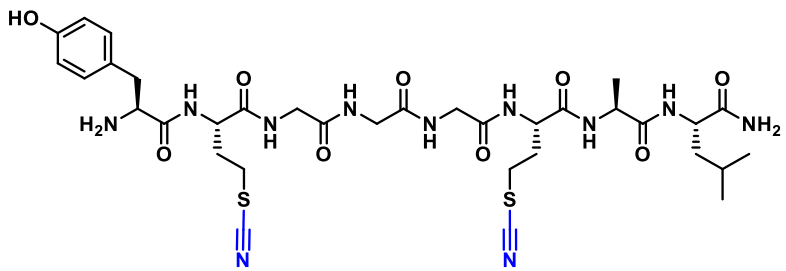
3c (^{13}C-NMR, DMSO, 126 MHz)

Compound 3d



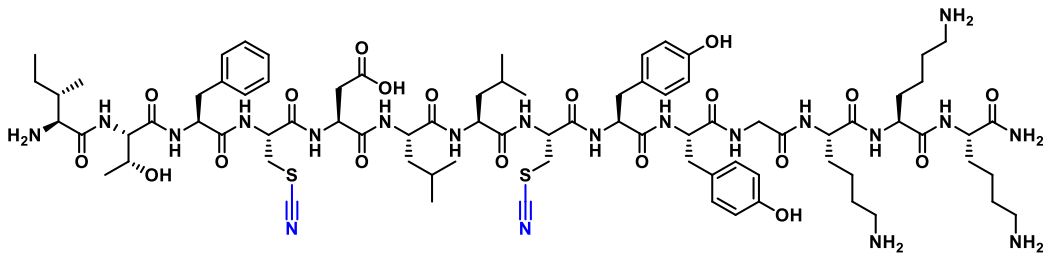
3d (^{1}H-NMR, DMSO, 500 MHz)

Compound 3d



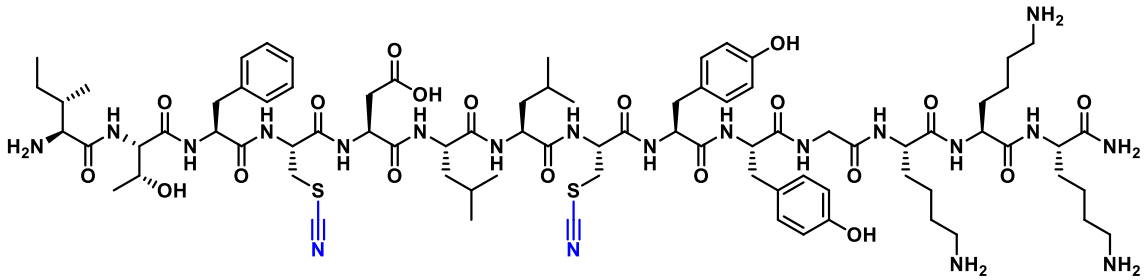
3d (^{13}C-NMR, DMSO, 126 MHz)

Compound 3e



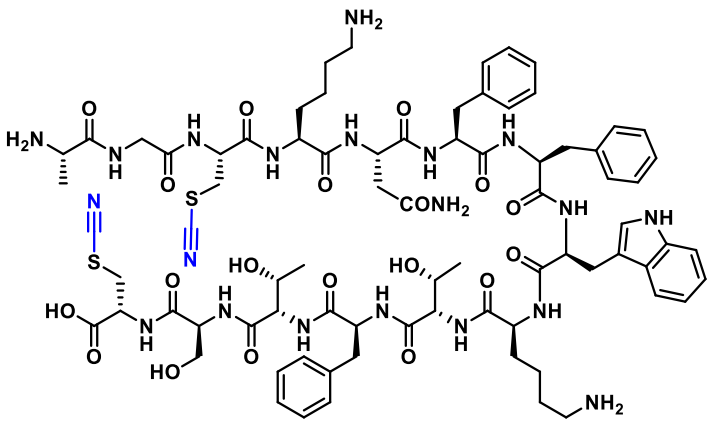
3e (^{1}H-NMR, DMSO, 500 MHz)

Compound 3e



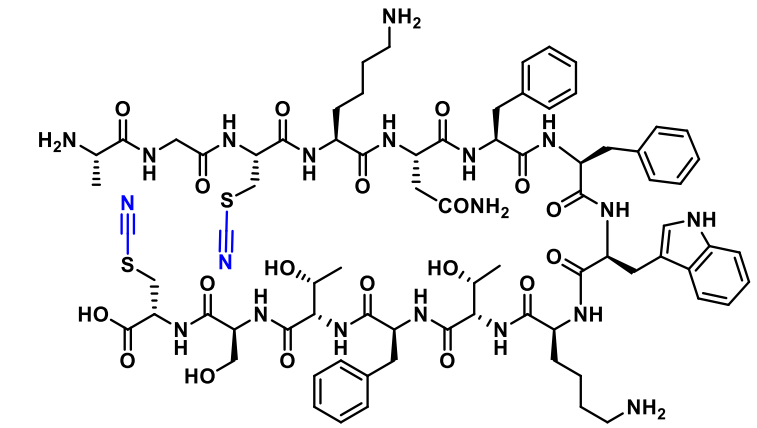
3e (^{13}C-NMR, DMSO, 126 MHz)

Compound 3h



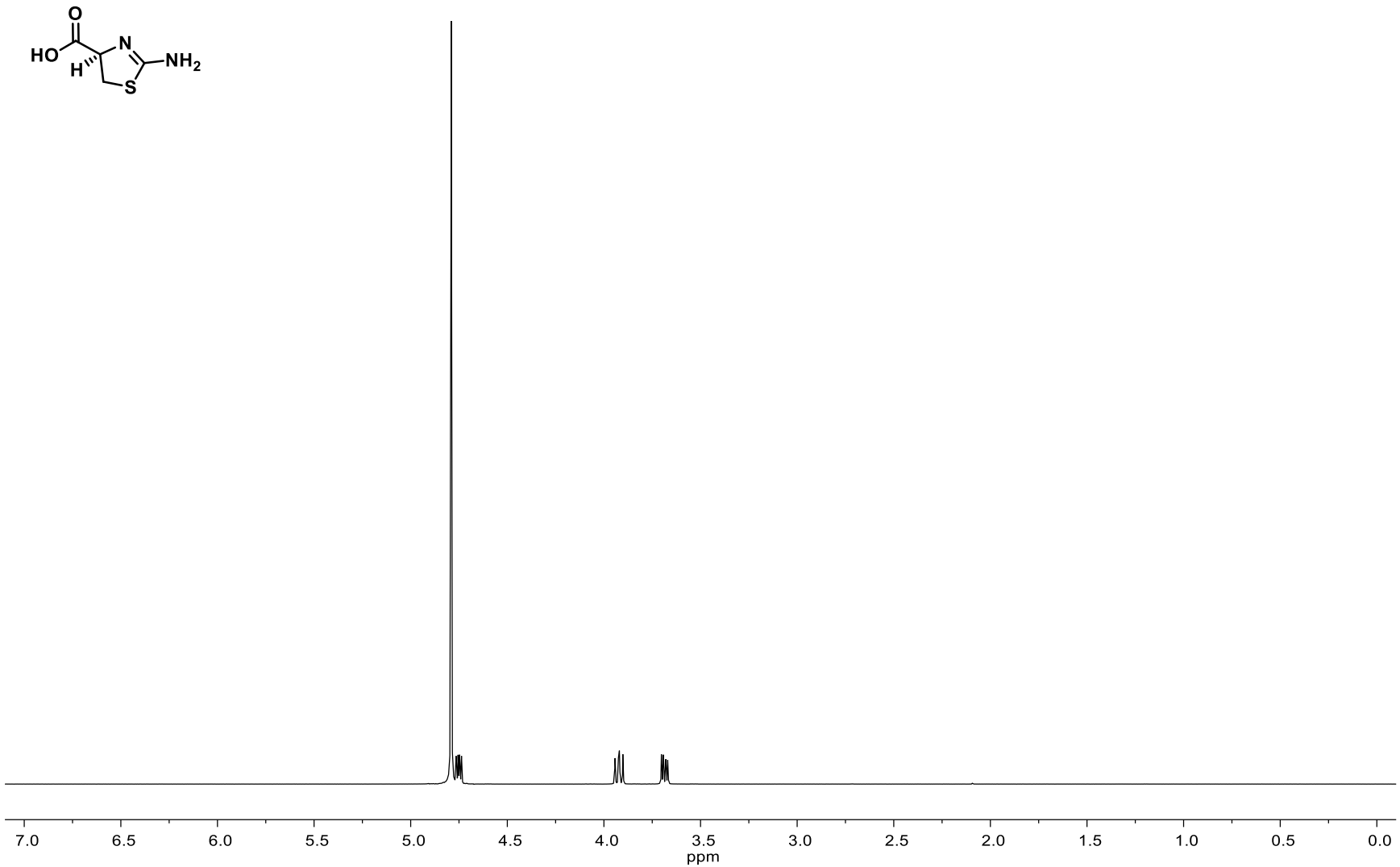
3h (^{1}H-NMR, DMSO, 500 MHz)

Compound 3h



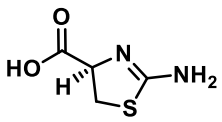
3h (^{13}C-NMR, DMSO, 126 MHz)

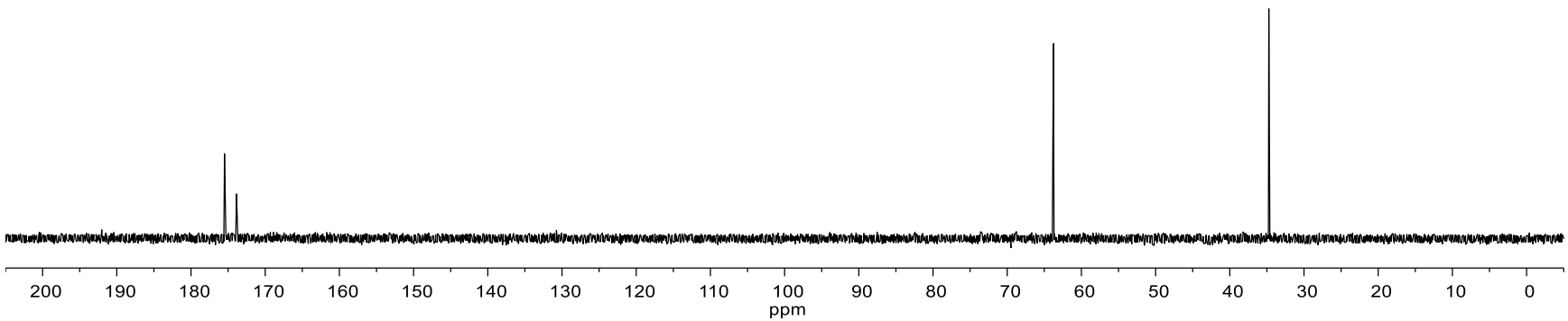
Compound 4



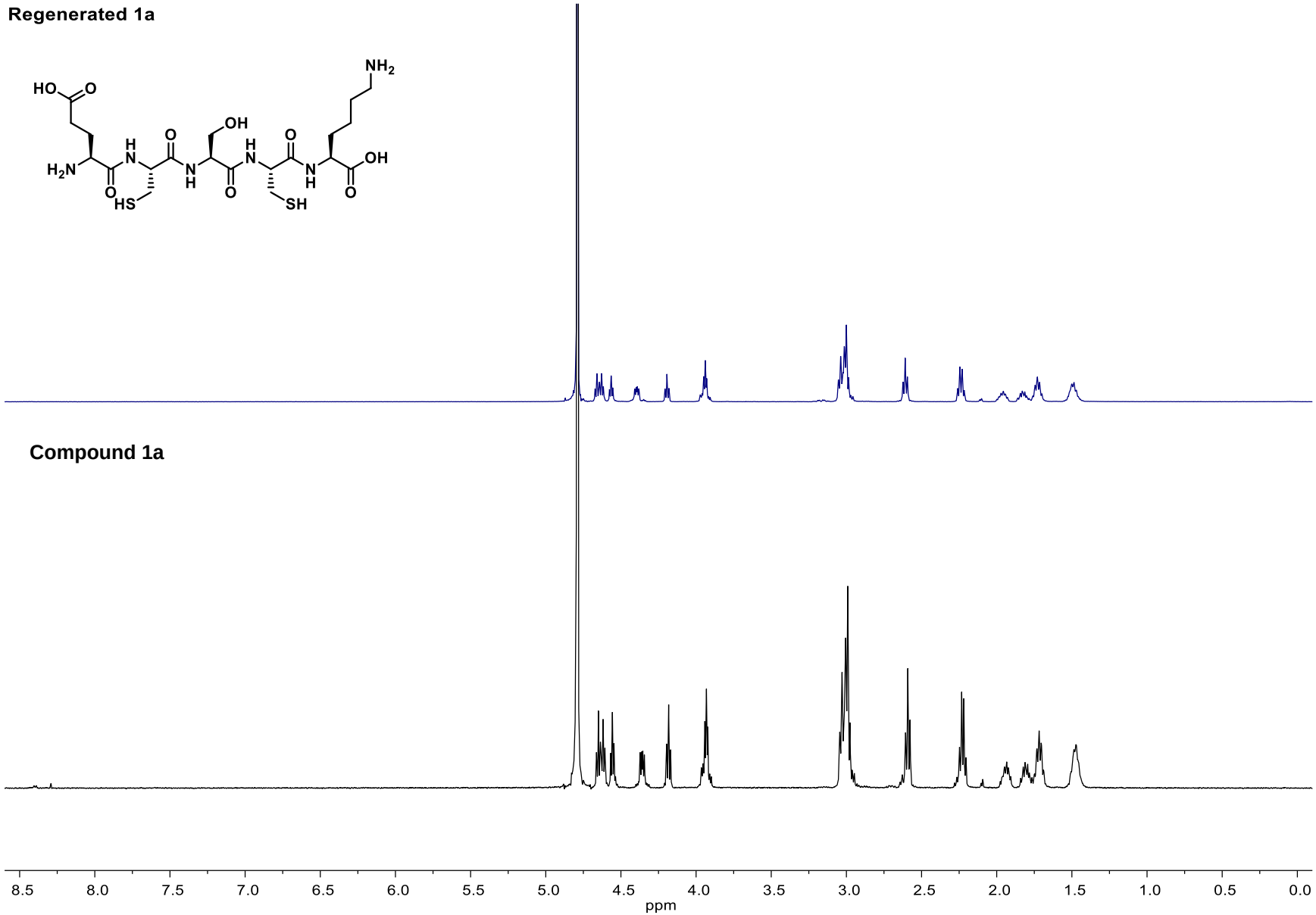
4 (^{1}H-NMR, D_2O, 500 MHz)

Compound 4



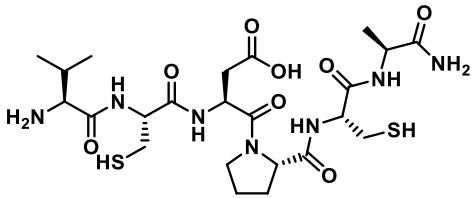


4 (^{13}C-NMR, D_2O, 126 MHz)

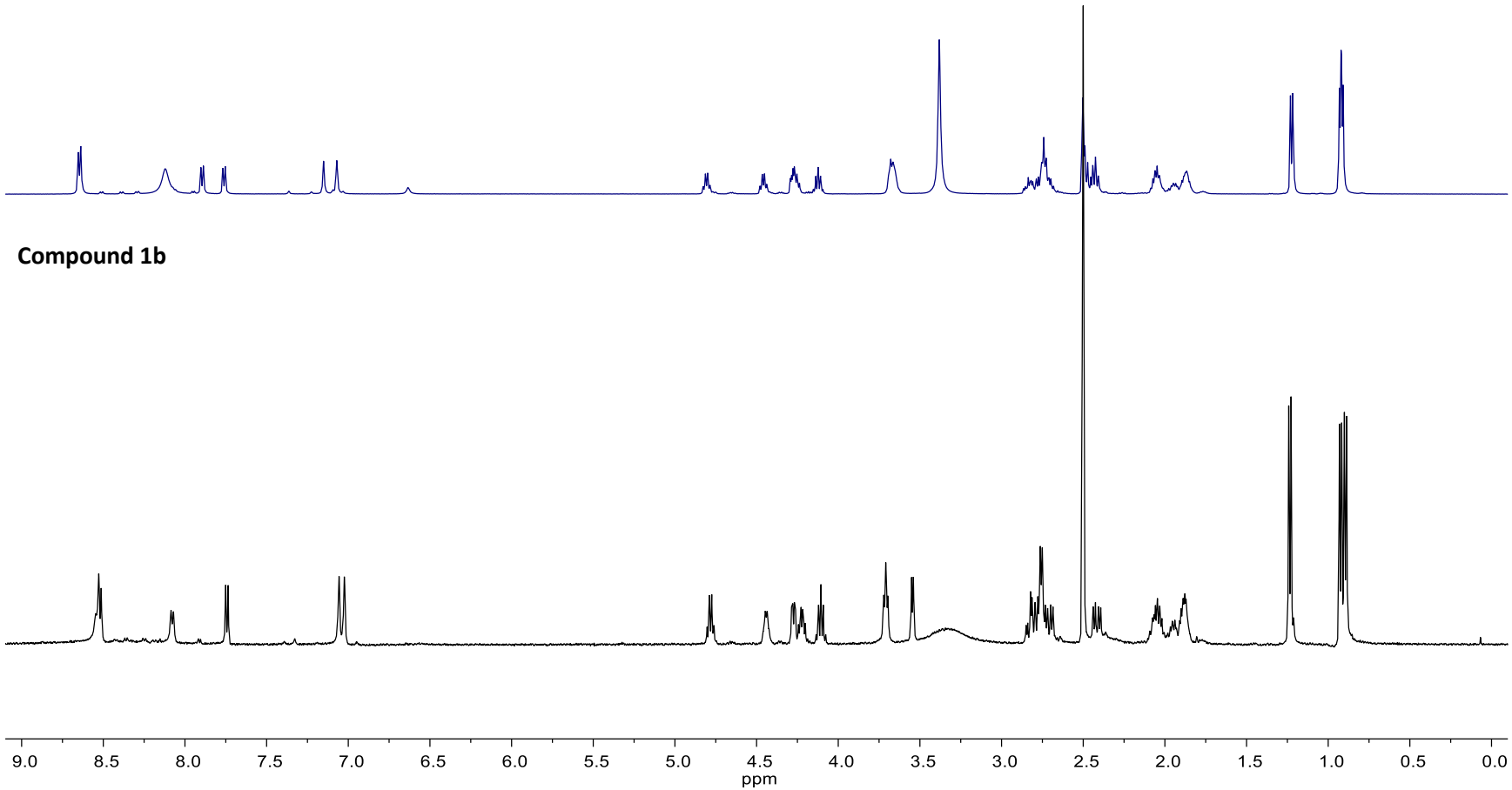


Regenerated 1a (^{1}H-NMR, D_2O, 500 MHz)

Regenerated 1b

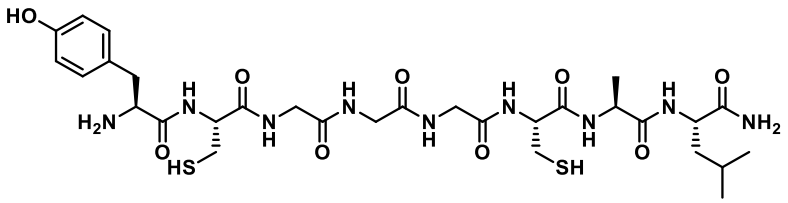


Compound 1b

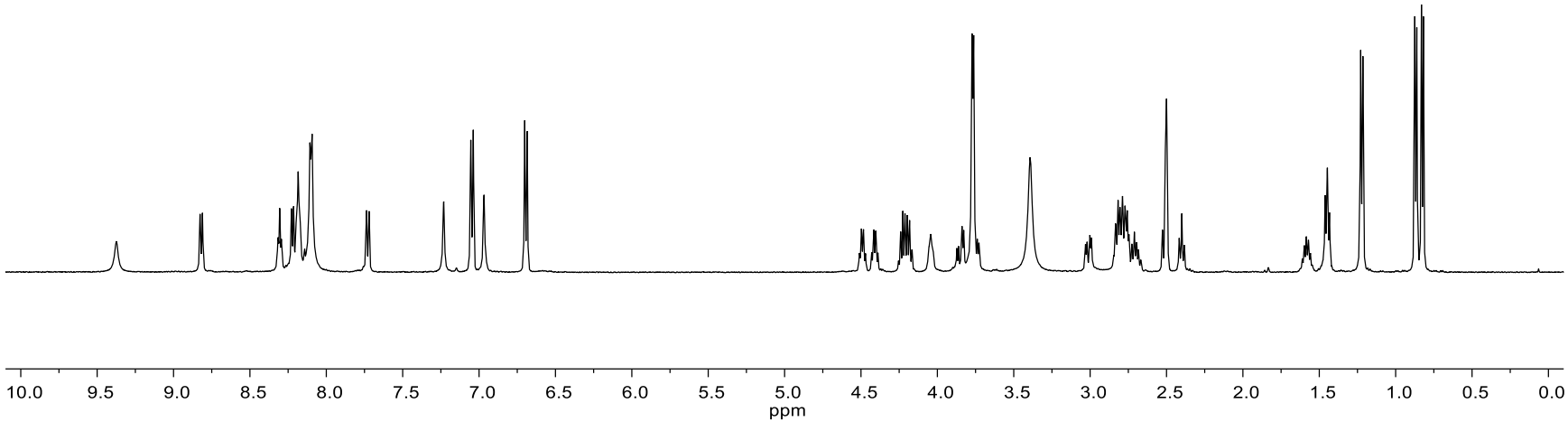


Regenerated 1b (^{1}H-NMR, D_2O, 500 MHz)

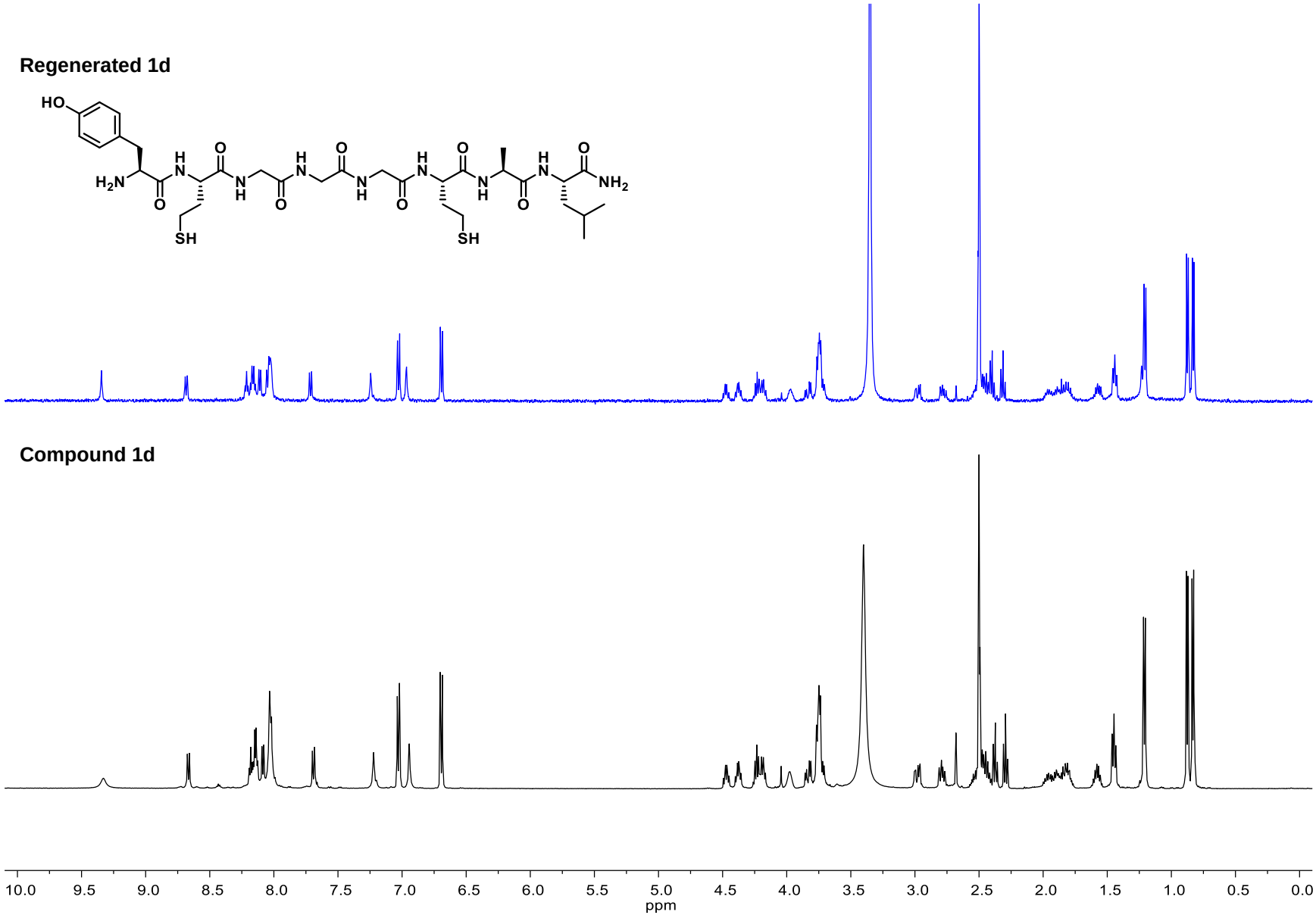
Regenerated 1c



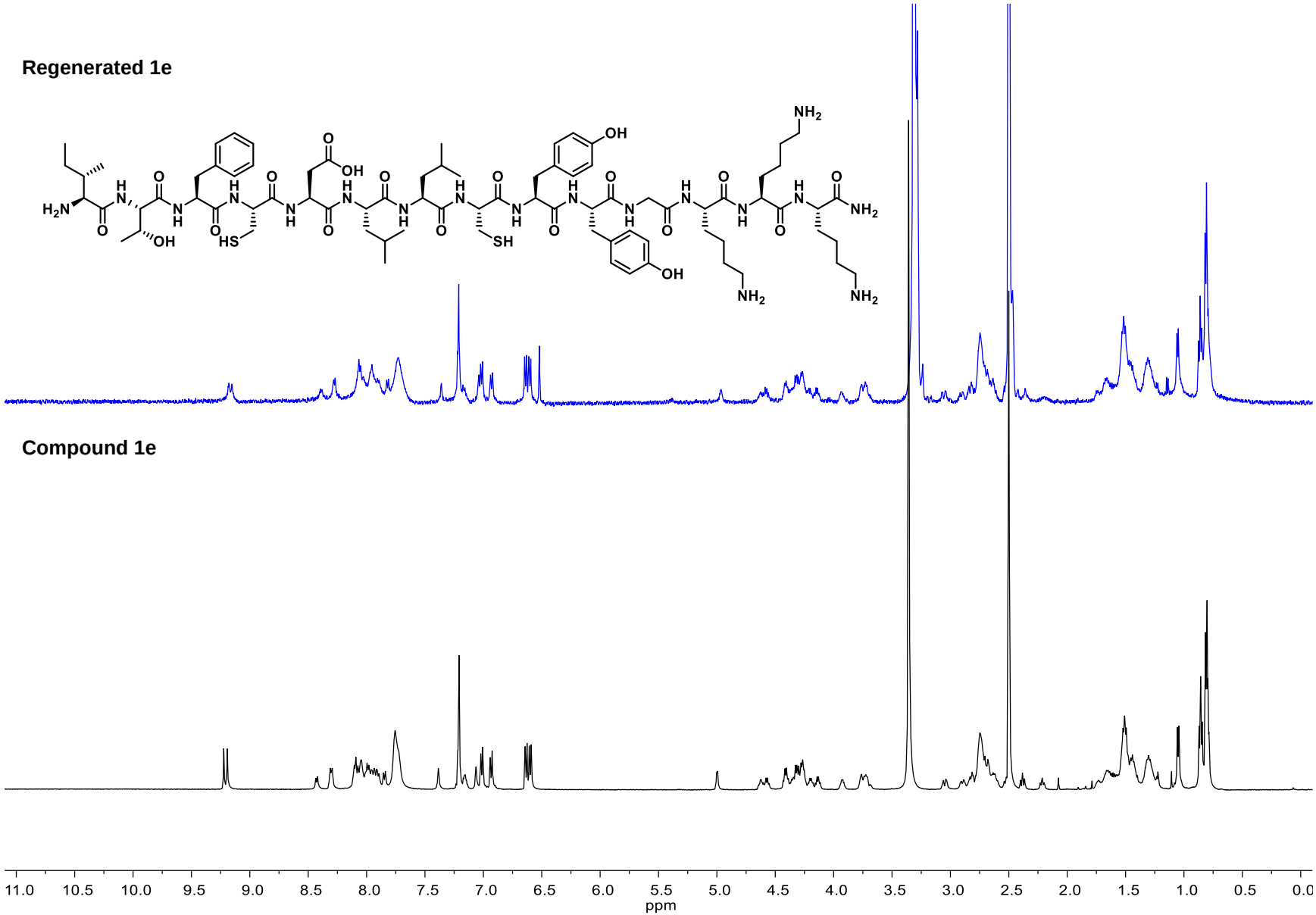
Compound 1c



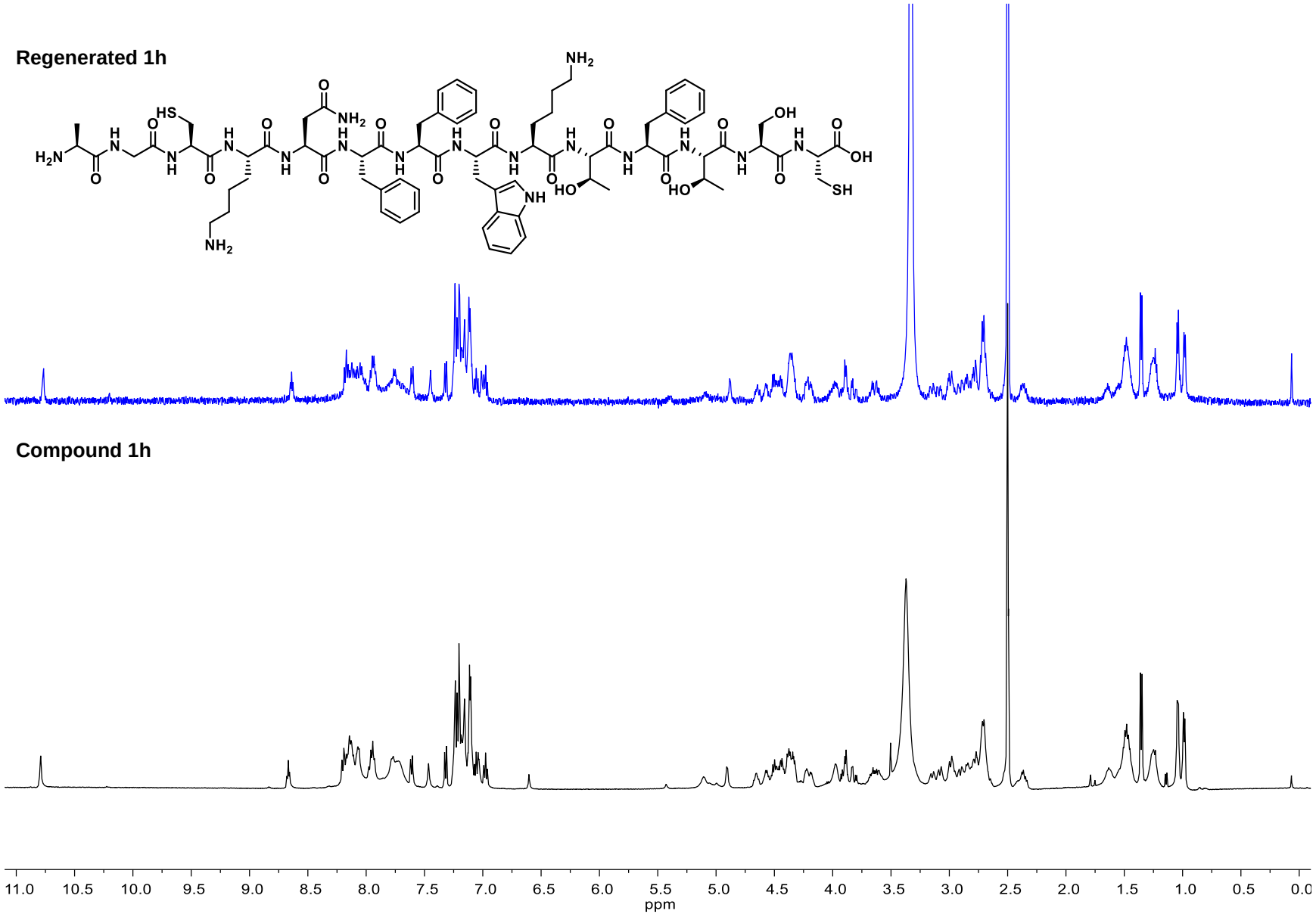
Regenerated 1c (^{1}H-NMR, DMSO, 500 MHz)



Regenerated 1d (^{1}H-NMR, DMSO, 500 MHz)

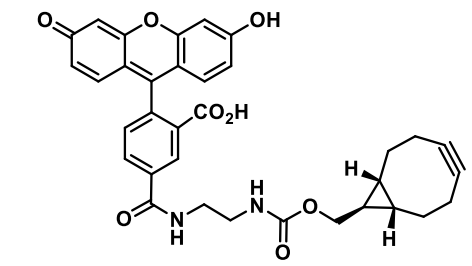


Regenerated 1e (^{1}H-NMR, DMSO, 500 MHz)



Regenerated 1h (^{1}H-NMR, DMSO, 500 MHz)

Compound 5



5 (^{1}H-NMR, CD_3OD, 500 MHz)