Supporting Information

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

Stephen P. Brown, Amos B. Smith, III*

Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104
Table of Contents

General Methods.......................................................................................................................... S-3
General Procedures for the Resin Loading .................................................................................... S-4
General Procedures for Manual Solid-Phase Peptide Synthesis ......................................................... S-5
General Procedures for Peptide Cleavage and Global Deprotection .................................................. S-5
General Phase-Transfer Protocol for Tetrazine Insertion ................................................................. S-24
  Colormetric Change that Occurs After Mixing the Biphasic Mixture for 1 Minute. ........ S-24
  UV-Vis Spectrum of Peptide 2a [73 μM] in pH 7.8 buffer ......................................................... S-28
  Calculating the Extinction Coefficient for Peptide 2a at 280, 410 and 532 nm .................. S-29
  Table of Stability Data for the S,S-Tetrazine Peptide 2a ............................................................. S-30
General Procedure for Unstapling S,S-Tetrazine Peptides Photochemically............................ S-47
  Colormetric Change that Occurs After Irradiation in a Rayonet® Photoreactor ............... S-47
General Nitrile Removal Protocol: Regeneration of the Native Peptide ........................................ S-60
Synthesis of Fluorescein Tethered Bicyclononyne ....................................................................... S-70
Inverse-Electron Demand Diels-Alder of S,S-Tetrazine Somatostatin ....................................... S-72
Tetrazine Stapling of the Thioredoxin Protein ............................................................................. S-75
Photochemical Unstapling and Regeneration of the Thioredoxin Protein .................................. S-77
  General Methods for FPLC ........................................................................................................... S-80
  C4-Liquid Chromatography-Mass Spectrometry Separation of Proteins ......................... S-84
  Measurement of Regenerated Thioredoxin Bioactivity ............................................................... S-87
Inverse-Electron Demand Diels-Alder Reaction of Bicyclononyne with Tetrazine Thioredoxin .. S-88
NMR-Spectra .................................................................................................................................. S-89
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 (Boc₂O), diisopropylethylamine (DIPEA), piperidine, Thioredoxin (Trx), were obtained from Aldrich Chemical Co. and used as received. Fmoc-protected amino acids, O-(Benzotriazol-1-yl)-N,N',N'-tetramethyluronium (HBTU), were purchased from Chem-Impex International and used as received. Dichloromethane (CH₂Cl₂), 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. ¹H NMR, ¹³C 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 CH₂Cl₂ (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 CH₂Cl₂ (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 CH₂Cl₂ (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 CH₂Cl₂ 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 Et₂O (15 mL) was added to precipitate the peptide. The white precipitate was collected by vacuum filtration and the solids wash with additional Et₂O. 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_{6}O_{9}S_{2}: 569.2063]; \(^1\)H NMR (500 MHz, D_{2}O) 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_{2}O) 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)+; calcld for C_{23}H_{40}N_{7}O_{8}S_{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 C₃₀H₄₈N₉O₉S₂: 742.3016]; 'H NMR (500MHz ,DMSO-d₆) 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); ¹³C NMR (126MHz ,DMSO-d₆) 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 C₄₀H₄₇N₇O₇S₂: 770.3336]; ¹H NMR (500 MHz, DMSO-d₆) 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); ¹³C 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]; ^1H 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); ^13C 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, 114.9, 57.9, 65.3, 55.0, 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 C₄₅H₆₁N₁₀O₈S₂: 933.4115]; ¹H NMR (500 MHz, DMSO-d₆) 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); ¹³C 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, 129.2, 128.5, 127.9, 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-chlorotiryl 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\textsubscript{38}H\textsubscript{69}N\textsubscript{12}O\textsubscript{13}S\textsubscript{3}: 997.4269]; ¹H NMR (500 MHz, DMSO-\textit{d\textsubscript{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), 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); ¹³C NMR (126 MHz, DMSO-\textit{d\textsubscript{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\textsuperscript{-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 – 7.01 (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), 3.10 – 3.05 (m, 9H), 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, 129.3, 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
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_{158}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
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 CHCl₃ (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.
**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$_{9}$S$_{2}$: 647.2030]; $^1$H NMR (500 MHz, D$_2$O) δ ppm 1.30 (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) 2.30 (ddd, $J$=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 $\lambda_{Max}$ 278 nm, 419 nm, 507 nm.
Crude LC Trace for 2a: Gradient 5-60% MeCN, 7 min, 2mL/min

89% 2a
1% y; 4 isomers
8% z; 2 isomers
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**

![UV-Vis Spectrum of Peptide 2a](image1)

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

![Zoom Region](image2)
Calculating the Extinction Coefficient for Peptide 2a at 280, 410 and 532 nm

Peptide 2a @ 280 nm

\[ y = 11369x - 0.0036 \]
\[ R^2 = 0.9999 \]

Peptide 2a @ 410

\[ y = 551.19x - 0.0054 \]
\[ R^2 = 1 \]

Peptide 2a @ 532

\[ y = 343.97x - 0.0059 \]
\[ R^2 = 0.9999 \]
# Table of Stability 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

| Condition                  | Stability                                                                 |
|----------------------------|---------------------------------------------------------------------------|
| ambient temperature        | stable for months as a lyophilized powder                                 |
| elevated temperature       | stable after reflux in water (100°C) 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 10°C |

## Organic solvents/Reagents

| Solvent/Reagent                  | 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**

![Proposed Mechanism](image)
**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_{8}S_{2}: 684.2346; {^1}H NMR (500MHz, DMSO-d6) 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), 4.62 - 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, 181.71, 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
Peptide 2c. Peptide 1c (15.3 mg, 21 µmol) 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 C₃₂H₄₆N₁₃O₉S₂: 820.2983; ¹H NMR (500MHz, DMSO-d₆) 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); ¹³C NMR (126MHz, DMSO-d₆) 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
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 C₃₄H₅₀N₁₃O₉S₂: 848.3296; ^1H NMR (500MHz, DMSO-d₆) 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); ^13C NMR (126MHz, DMSO-d₆) 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 [elu

cent 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 C₈₁H₁₂₂N₂₂O₁₉S₂: 1793.860]; ¹H NMR (500 MHz, DMSO-d₆) 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); ¹³C NMR (126 MHz, DMSO-d₆) 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
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_{8}S_{2}: 1011.4082]; 1H 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); 13C 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 [eluent 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_{9}S_{2}: 1075.4236] \^1H NMR (500 MHz, DMSO-\textit{d}_6) \delta 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-\textit{d}_6) \delta 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]. 1H NMR (500 MHz, DMSO-d6) 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); 13C NMR (126 MHz, DMSO) δ 176.4, 171.9, 171.7, 171.6, 171.4, 171.2, 171.0, 170.9, 170.8, 170.1, 169.9, 169.7, 169.4, 169.3, 168.6, 168.5, 137.8, 137.7, 137.6, 137.5, 136.2, 136.1, 129.2, 129.1, 128.9, 128.9, 128.1, 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
**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].
General Procedure for Unstapling S,S-Tetrazine Peptides Photochemically

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 (λMax = 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
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_{8}O_{9}S_{2}: 619.1963]; ¹H 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); ¹³C NMR (126 MHz, D₂O) 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 (λMax = 365 nm)

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 (λMax = 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
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_{9}O_{8}S_{2}: 656.2285]; \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 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-\(d_6\)) \(\delta\) 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
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 C₃₂H₄₆N₁₁O₉S₂: 792.2921]; ¹H NMR (500 MHz, DMSO-d₆) 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); ¹³C NMR (126 MHz, DMSO-d₆) 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
**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 C₃₄H₅₀N₁₁O₉S₂: 820.3234]; ¹H NMR (500 MHz, DMSO-d₆) 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); ¹³C 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]; 1H 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); 13C 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, 52.2, 52.2, 52.1, 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, 170.6, 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, 125.3, 123.6, 120.8, 118.5, 118.4, 118.2, 116.1, 112.9, 111.2, 109.8, 107.5, 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, 37.0, 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
(R)-2-amino-4,5-dihydrothiazole-4-carboxylic acid (4): HRMS Found (ES) m/z 147.0228 [(M+H)+; calcd for C₄H₆N₂O₂S: 147.0228]; ¹H 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); ¹³C 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), 1638(s), 1588(s), 1442(m), 1392(s), 1290(m).

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-aminoethyl)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 C34H31N2O8: 595.2080]. 1H NMR (500 MHz, Methanol-d4) 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].
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 4°C. Bradford assay (88% yield). MALDI-TOF m/z 1175.782 [(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

1) UV-B lamps
   $\lambda_{\text{Max}} = 312$ nm
   50 mM Tris, pH 7.8
   150 mM NaCl
2) cysteine [1 mM]
   TCEP [1 mM]
3) Ellman's Reagent [1 mM]

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 comparison.

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, 35° fixed angle rotor @ 7000×G, 30 minutes, 4°C), 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, 35° fixed angle rotor @ 7000×G, 30 minutes, 4°C). 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].

S-77
MALDI Chromatogram of Tetrazine-Thioredoxin After Photolysis (mixture of products)

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

11690.168 ± 0.1%
11690.168 ± 0.1%
11690.168 ± 0.3%

Thioredoxin

Thioredoxin

Thioredoxin

S

S

HS

SH

SH

11678.5

11703.5

11728.5
MALDI Chromatogram of Regenerated Thioredoxin (calculated (M+H)^+ = 11676.44)

MALDI Comparison of Regenerated with Authentic Thioredoxin

Regenerated Thioredoxin

11676.188 ± 0.002%

Authentic (Native) Thioredoxin

11674.191 ± 0.02%
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 4°C 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.

![Molecular Weight Calibration for Superdex75 Analytical Column](image_url)
FPLC Chromatogram of Tetrazine Thioredoxin

FPLC Chromatogram of Regenerated Thioredoxin
Approximated extinction coefficient ($\varepsilon$) values were calculated for thioredoxin (Trx) and tetrazine-thioredoxin (tet-Trx). The Trx protein contains 2 Trp and 2 Tyr residues, the $\varepsilon$ values used in the calculations are listed below.

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

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

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

| Sample        | Approximated $\varepsilon$ |
|---------------|-----------------------------|
| 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 ($A_{\text{meas}}$) was normalized ($A_{\text{norm}}$) to account for the extinction coefficient contributed by the tetrazine to permit comparison with the regeneration thioredoxin using the equation below.

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

The regenerated thioredoxin yield was calculated using the equation below.

$$\frac{A_{\text{regen-Trx}}}{A_{\text{norm-Trx}}} \frac{8.92\text{mAU} \ast \text{mL}^{-1}}{10.53\text{mAU} \ast \text{mL}^{-1}} \times 100\% = 84.7\%$$
The FPLC chromatograms of the regenerated thioredoxin and normalized tetrazine thioredoxin have been overlaid for comparison. 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

LC-Chromatogram Commercial Thioredoxin

LC-Chromatogram Tetrazine Thioredoxin

LC-Chromatogram Regenerated 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 turbidity from the reduction of insulin was monitored at 650 nm at 30°C by a Tecan plate reader.

The kinetic curves were baseline 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, 35° fixed angle rotor @ 7000×G, 30 minutes, 4°C), 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) – N2 (28.01)].

S-88
Compound 1a

1a ($^1$H-NMR, D$_2$O, 500 MHz)
Compound 1a

1a (\(^{13}\text{C-NMR, D}_2\text{O, 126 MHz}\))
Compound 1b

\[ \text{H}_2\text{N} - \text{N} - \text{N} - \text{O} - \text{O} - \text{O} - \text{NH}_2 \]

\[ \text{O} - \text{NH} - \text{NH} - \text{SH} \]

1b (\text{\textsuperscript{1}H-NMR, DMSO, 126 MHz})
Compound 1b

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

\[ \text{H}_2\text{N} - \text{HO} - \text{H} - \text{N} - \text{H} - \text{O} - \text{N} - \text{H} - \text{O} - \text{N} - \text{H} - \text{O} - \text{N} - \text{H} - \text{O} - \text{N} - \text{H}_2 \]

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

\[ \text{HO} - \text{C} - \text{NH} - \text{C} - \text{NH} - \text{C} - \text{NH} - \text{C} - \text{NH} - \text{C} - \text{NH} - \text{C} - \text{NH}_2 \]

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

1c (DEPT135, DMSO, 126 MHz)
Compound 1d

1d (1H-NMR, DMSO, 500 MHz)
Compound 1d

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

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

S-98
Compound 1e

\[
\text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO} - \text{HNCOO}
\]

1e (\text{\textsuperscript{13}C-NMR, DMSO, 126 MHz})

S-99
Compound 1f

If (1H-NMR, DMSO, 500 MHz)
Compound 1f

If (\textsuperscript{13}C-NMR, DMSO, 126 MHz)

S-101
Compound 1g

\[ S \]\n
$1g$ ($^1H$-NMR, DMSO, 500 MHz)
Compound 1g

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

1h (1H-NMR, DMSO, 500 MHz)
Compound 1h

$^{13}$C-NMR, DMSO, 126 MHz

1h ($^{13}$C-NMR, DMSO, 126 MHz)
Compound 2a

2a (\(^1\text{H-NMR, D}_2\text{O, 500 MHz}\))

S-106
Compound 2a

$^{13}$C-NMR, D$_2$O, 126 MHz
Compound 2a

2a (DEPT135, D$_2$O, 126 MHz)
Compound 2b

2b (1H-NMR, DMSO, 500 MHz)
Compound 2b

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

2c (1H-NMR, DMSO, 500 MHz)
Compound 2d

H-NMR, DMSO, 500 MHz
Compound 2d

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

2e (1H-NMR, DMSO, 500 MHz)
Compound 2f

2f (1H-NMR, DMSO, 500 MHz)
Compound 2f

2f (13C-NMR, DMSO, 126 MHz)
Compound 2g

2g (1H-NMR, DMSO, 500 MHz)
Compound 2g

$^{13}$C-NMR, DMSO, 126 MHz
Compound 2h

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

S-122
Compound 3a

3a (1H-NMR, D₂O, 500 MHz)
Compound 3b

3b (1H-NMR, DMSO, 500 MHz)
Compound 3b

$\text{H}_2\text{N}\text{C}-\text{S}\text{C}-\text{N}\text{H}_2$

$\text{O}\text{C}\text{H}\text{O}\text{N}\text{H}-\text{S}\text{C}\text{N}\text{H}_2$

$3\text{b} (^{13}\text{C-NMR, DMSO, 126 MHz})$
Compound 3c

3c (1H-NMR, DMSO, 500 MHz)
Compound 3c

$^{13}$C-NMR, DMSO, 126 MHz

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

S-128
Compound 3d

3d (1H-NMR, DMSO, 500 MHz)
Compound 3e
Compound 3e

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

S-132
Compound 3h

3h ($^1$H-NMR, DMSO, 500 MHz)
Compound 3h

\[ ^{13}C\text{-NMR, DMSO, 126 MHz} \]

3h
Compound 4

4 (\textsuperscript{1}H-NMR, D\textsubscript{2}O, 500 MHz)
Compound 4

\[ \text{HO} \quad \text{N} \quad \text{S} \quad \text{NH}_2 \]

\( ^{13}\text{C}-\text{NMR}, \text{D}_2\text{O}, 126 \text{ MHz} \)

\( 4 \)
Regenerated 1b

Compound 1b

Regenerated 1b ($^1$H-NMR, D$_2$O, 500 MHz)
Regenerated 1c

\[ \text{Compound 1c} \]

Regenerated 1c (\(^1\)H-NMR, DMSO, 500 MHz)
Regenerated 1e

Compound 1e

\[ \text{Regenerated 1e} \ (\text{\textsuperscript{1}H-NMR, DMSO, 500 MHz}) \]
Regenerated 1h

Compound 1h

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