Electronic Supplementary Material (ESI) for Chemical Science

Supporting Information

Chemoselective Sulfenylation and Peptide Ligation at Tryptophan

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General synthetic experimental

^{1}H NMR spectra were recorded at 300 K using a Bruker Avance DPX 400 spectrometer. Chemical shifts are reported in parts per million (ppm) and are referenced to solvent residual signals: D_2O (δ 4.79 [^{1}H]), MeOD (δ 3.31 [^{1}H]). ^{1}H NMR data is reported as chemical shift (δ_H), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, ddd = doublet of doublet of doublets), relative integral, coupling constant (*J* Hz) and assignment where possible.

Low-resolution mass spectra were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. High resolution MALDI-FTICR mass spectra were measured on a Bruker–Daltonics Apex Ultra 7.0T Fourier transform mass spectrometer (FTICR) using a matrix of 0.7 mg/mL α-cyano-4-hydroxycinnamic acid in water/acetonitrile (3:7 v/v) containing 0.1 vol.% TFA. UV absorption spectra were recorded on a Varian Cary 4000 UV-Vis Spectrophotometer using Scan software.

Analytical reverse-phase HPLC was performed on a Waters System 2695 separations module with an Alliance series column heater at 30 °C and 2996 photodiode array detector. Peptides were analyzed using a Waters Sunfire 5 μm, 2.1 x 150 mm column (C-18) at a flow rate of 0.2 mL min^{-1} using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B). Results were analyzed with Waters Empower software.

Preparative reverse-phase HPLC was performed using a Waters 600 Multisolvent Delivery System and Waters 500 pump with 2996 photodiode array detector or Waters 490E Programmable wavelength detector operating at 230 and 254 nm. Model Trp peptides **2**-**4**, **10**-**11** and CXCR1 peptides **18**-**20** were purified on a Waters Sunfire 5 μm (C-18) preparative column operating at a flow rate of 7 mL min^{-1} using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) and a linear gradient as specified. Model ligation and desulfurization products and compound **22** [2-thiol Trp CXCR1 (1-28)] were purified on a Waters Sunfire 5 μm (C-18) 10 x 250 mm semi-preparative column operating a flow rate of 4 mL min^{-1} using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) and a linear gradient of 0-50% B over 40 min. CXCR1 (1-28) **17** was purified on an Xbridge BEH300 5 μm (C-18) 10 x 250 mm semi-preparative column operating a

flow rate of 4 mL min^{-1} using a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) and a linear gradient of 0-50% B over 40 min.

LC-MS was performed on a Shimadzu LC-MS 2020 instrument consisting of a LC-M20A pump and a SPD-20A UV/Vis detector coupled to a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. Separations were performed on a Waters Sunfire 5 μm, 2.1 x 150 mm column (C18), operating at a flow rate of 0.2 mL min^{-1}. Separations were performed using a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) and a linear gradient of 0-50% B over 30 min.

Reaction yields are calculated based on the amount of isolated peptide product relative to the theoretical reaction yield. Yields are adjusted to account for the removal of aliquots for reaction monitoring (e.g. LC-MS, testing of pH).

Materials

Commercial materials were used as received unless otherwise noted. Amino acids, coupling reagents and resins were obtained from Novabiochem or GL Biochem. Reagents that were not commercially available were synthesized following literature procedures. Dichloromethane (DCM) was distilled from calcium hydride and *N*,*N*-dimethylformamide (DMF) was obtained as peptide synthesis grade from Merck or Labscan.

Solid-phase peptide synthesis

Preloading Rink amide resin

Rink amide resin was initially washed with DCM (5 x 3 mL) and DMF (5 x 3 mL), followed by removal of the Fmoc group by treatment with 10% piperidine/DMF (3 mL, 2 x 5 min). The resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL). PyBOP (4 eq.) and *N*-methylmorpholine (NMM) (8 eq.) were added to a solution of Fmoc-AA-OH (4 eq.) in DMF (final concentration 0.1 M). After 2 min of pre-activation, the mixture was added to the resin. After 2 h the resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL), capped with acetic anhydride/pyridine (1:9 v/v) (3 mL, 2 x 3 min) and washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL). The resin was subsequently submitted to iterative peptide assembly (Fmoc-SPPS).

Preloading 2-chloro-trityl chloride resin

2-chloro-trityl chloride resin (1.22 mmol/g loading) was swollen in dry DCM for 30 min then washed with DCM (5 x 3 mL) and DMF (5 x 3 mL). A solution of Fmoc-AA-OH (4.0 eq.) and *N*,*N*-diisopropylethylamine (DIPEA) (8.0 eq.) in DMF (final concentration 0.1 M) was added and the resin shaken at rt for 16 h. The resin was washed with DMF (5 x 3 mL) and DCM (5 x 3 mL) and treated with a solution of DCM/CH_3OH/DIPEA (17:2:1 v/v/v, 3 mL) for 0.5 h. The resin washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL) and subsequently submitted to iterative peptide assembly (Fmoc-SPPS).

General iterative peptide assembly (Fmoc-SPPS)

Deprotection: The resin was treated with 10% piperidine/DMF (3 mL, 2 x 3 min) and washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

General amino acid coupling: A preactivated solution of protected amino acid (4 eq.), PyBOP (4 eq.) and NMM (8 eq.) in DMF (final concentration 0.1 M) was added to the resin. After 1 h, the resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Capping: Acetic anhydride/pyridine (1:9 v/v) was added to the resin (3 mL). After 3 min the resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Cleavage: A mixture of TFA, triisopropylsilane (TIS) and water (90:5:5 v/v/v) was added to the resin. After 2 h, the resin was washed with TFA (3 x 2 mL). For peptide thioesters and peptides containing Met residues, a mixture of TFA, triisopropylsilane (TIS), thioanisole and water (85:5:5:5 v/v/v/v) was used instead.

Work-up: The combined solutions were concentrated under a stream of nitrogen. The residue was dissolved in water containing 0.1% TFA, filtered and purified by reverse-phase HPLC and analyzed by LC-MS and ESI mass spectrometry.

Coupling conditions for glycosylamino acids (Fmoc-Asn(GlcNAc)-OH)

A solution of glycosylamino acid **21** (1.2 eq.), HATU (1.2 eq.) and DIPEA (2.4 eq.) in DMF (final concentration 0.1 M) was added to the resin (1.0 eq.) and shaken. After 18 h, the resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL). A capping step was

performed as described above, and synthesis of the desired glycopeptide was completed using iterative Fmoc-SPPS.

On resin *O*-deacetylation

The resin (25 μmol) was washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL). A 5 vol.% solution of hydrazine hydrate in DMF was prepared and added to the resin (3 mL). The peptide was shaken at rt for 16 h and washed with DMF (10 x 3 mL), DCM (10 x 3 mL), and DMF (10 x 3 mL).

General Procedures

Peptide Sulfenylation with DNPS-Cl (solution phase)

The peptide (~20 mg) was dissolved in glacial acetic acid or an acetic acid/H_2O mixture (4:1 v/v) (10 mL) and treated with 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl) (10.0 eq.). The resultant solution was stirred at room temperature for 16 h, or until completion, as indicated by LC-MS analysis. Acetic acid was then removed under a stream of nitrogen gas and the peptide precipitated with cold diethyl ether. The crude pellet was dissolved in a mixture of 0.1% TFA in water and 0.1% TFA in acetonitrile and purified immediately *via* reverse-phase HPLC employing a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) using a linear gradient as specified. Sulfenylation products were isolated as yellow solids following lyophilization.

Peptide Sulfenylation with DNPS-Cl (on-resin)

A Rink amide-bound peptide bearing an N-terminal Trp residue (side-chain unprotected) was washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL). A solution of DNPS-Cl (20 eq.), glacial acetic acid (20 eq.) and DMF (0.1 mL/μmol resin) was added to the resin. The peptide was shaken for 2 h at rt and washed with DMF (10 x 3 mL) and DCM (10 x 3 mL). The peptide was cleaved from the resin using TFA, triisopropylsilane (TIS) and water (90:5:5 v/v/v) according to the standard conditions above. The crude residue was concentrated under a stream of nitrogen and purified immediately *via* reverse-phase HPLC employing a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) using a linear gradient as

specified. 2-DNPS Trp sulfenylation products were isolated as yellow solids following lyophilization.

Thiolysis of 2-DNPS Tryptophan containing peptides

The 2-DNPS substituted tryptophan peptide (~10 mg) was dissolved in degassed buffer (6 M guanidine hydrochloride, 100 mM Na_2HPO_4, adjusted to pH 8.0, 1.5 mM concentration) and treated with thiophenol (0.5 vol.%). The reaction was stirred at rt for 3 h and subsequently purified by reverse-phase HPLC employing a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) with a linear gradient of 0-50% B over 40 min. Peptides were isolated primarily as the peptide disulfides and as pale yellow solids following lyophilization.

Peptide Alkyl Thioesters (Ac-LYRANX-S$(CH_2)_2CO_2$Et, Ac-LYRC(Acm)NG-S$(CH_2)_2CO_2$Et, CXCR1 (1-9)-S$(CH_2)_2CO_2$Et)

Model peptide thioesters were prepared on 2-chloro-trityl chloride resin using conditions described by Kajihara and coworkers.[1]

Peptide Thiophenyl Thioesters (Ac-LYRANX-SPh, Ac-LYRC(Acm)NG-SPh, CXCR1 (1-9)-SPh)

Peptide thiophenyl thioesters **5-9**, **14**, and **19** were prepared from the corresponding alkyl thioesters by thiol exchange.[2] The alkyl thioester (~5 mg) was dissolved in degassed buffer (6 M guanidine hydrochloride, 100 mM Na_2HPO_4, pH 6.8, 1.2 mL) and treated with thiophenol (2 vol.%). The reaction was stirred at rt for 18 h (X = Gly, Ala, Met, Phe) or rt for 18 h followed by 37 ^oC for 48 h (X = Pro). The reaction mixtures were purified immediately *via* preparative reverse-phase HPLC employing a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) with a linear gradient of 0-50% B over 40 min, yielding the thiophenyl thioester and recovered alkyl thioesters as white solids following lyophilization.

Table S1. Isolated yields for the synthesis of model peptide thiophenyl thioesters (Ac-LYRANX-SPh) from the corresponding alkyl thioesters (Ac-LYRANX-S$(CH_2)_2CO_2Et$)

Entry	Thioester (X =)	Yield	Recovered alkyl thioester
1	Glycine	56%	22%
2	Glycine (LYRC(Acm)NG)	55%	30%
3	Alanine	43%	51%
4	Methionine	52%	-
5	Phenylalanine	73%	25%
6	Proline	49%	-

Ligation of 2-thiol Trp peptides with peptide thiophenyl thioesters

General Protocol: Peptide thiophenyl thioesters (~1.5 eq.) were dissolved in degassed buffer (6 M guanidine hydrochloride, 100 mM Na_2HPO_4, 100 mM TCEP, adjusted to pH 6.8, 4 mM concentration based on the 2-thiol Trp containing peptide fragment). The solution was added to the 2-thiol Trp containing peptide (~2 mg, 1.0 eq.). The final pH of the solution was measured and adjusted to 6.5-6.8, using 2 M NaOH or 1 M HCl solution, if necessary. The solution was flushed with argon and incubated at 37 ^oC. The progress of the reaction was monitored by LC-MS. If necessary, an additional aliquot of peptide thiophenyl thioester in ligation buffer was added (see model peptide reactions and analytical data section for further details). Upon completion, the reaction was quenched by the addition of 0.1% TFA in water (0.5 mL) and purified by semi-preparative reverse-phase HPLC employing a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) using a linear gradient of 0-50% B over 40 min. Ligation products were isolated primarily as the peptide disulfides and as white to pale yellow solids following lyophilization.

Desulfurization of peptide ligation products

The peptide ligation product (2-3 mg) was dissolved in degassed buffer (6 M guanidine hydrochloride, 100 mM Na_2HPO_4, adjusted to pH 5.8, concentration = 2 mg/mL). The solution was cooled to 0 ^oC, treated with 5% Pd/Al_2O_3 (10 times the weight of the peptide, e.g. 2 mg peptide = 20 mg Pd/Al_2O_3) and further degassed with argon. The reaction flask was evacuated

and flushed with H_2 (g). The reaction was stirred at 0 ^oC for 4 h (or as indicated) under an atmosphere of hydrogen, at which point LC-MS analysis indicated complete consumption of starting materials. The crude mixture was centrifuged (10,000 rpm for 2 min) to remove the Pd catalyst, and the supernatant collected and submitted to purification *via* semi-preparative reverse-phase HPLC employing a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) with a linear gradient of 0-50% B over 40 min. The desulfurized products were isolated as white solids following lyophilization.

UV spectroscopy and calculation of extinction coefficients for peptides 2-4

Peptides **2-4** were dissolved in water and diluted to the following final concentrations:

Peptide **2**: 0.65 mg/10 mL
Peptide **3**: 0.46 mg/10 mL
Peptide **4**: 0.71 mg /10 mL

A baseline of pure water was obtained, and each peptide solution was scanned from 800 – 200 nm and absorbance at λ_{max} recorded for each sample. Peptide **4** was let stand for 1 h at rt to facilitate formation of the 2-thiol Trp disulfide dimer. The sample was run again using the same conditions.

Extinction coefficients (ε) were calculated using Beer's Law: $A = \varepsilon cl$, where A = absorbance at λ_{max}, c = concentration (M) and l = pathlength (1 cm).

Table S2. Wavelength of maximum absorbance and extinction coefficients for peptides **2-4**.

Peptide	λ_{max} (nm)	A	c (M)	ε ($M^{-1}cm^{-1}$)[a]
2	279	0.484	9.36×10^{-5}	5170
3	278	0.642	5.18×10^{-5}	12400
4	313	0.529	9.77×10^{-5}	5410
4 (disulfide)	346	0.354	4.89×10^{-5}	7240

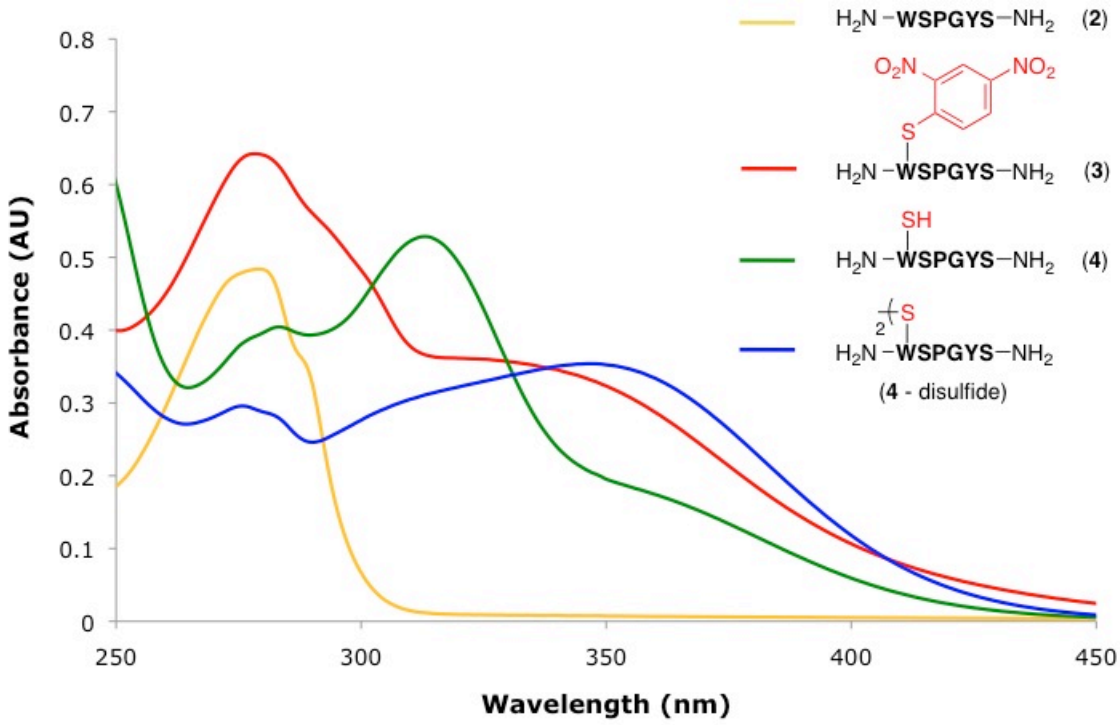
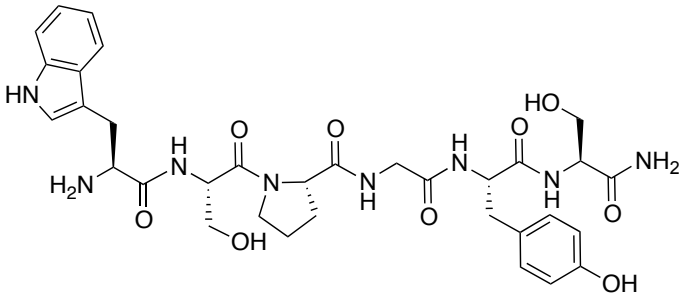


Figure S1. UV absorption spectra of peptides **2**-**4** in water.

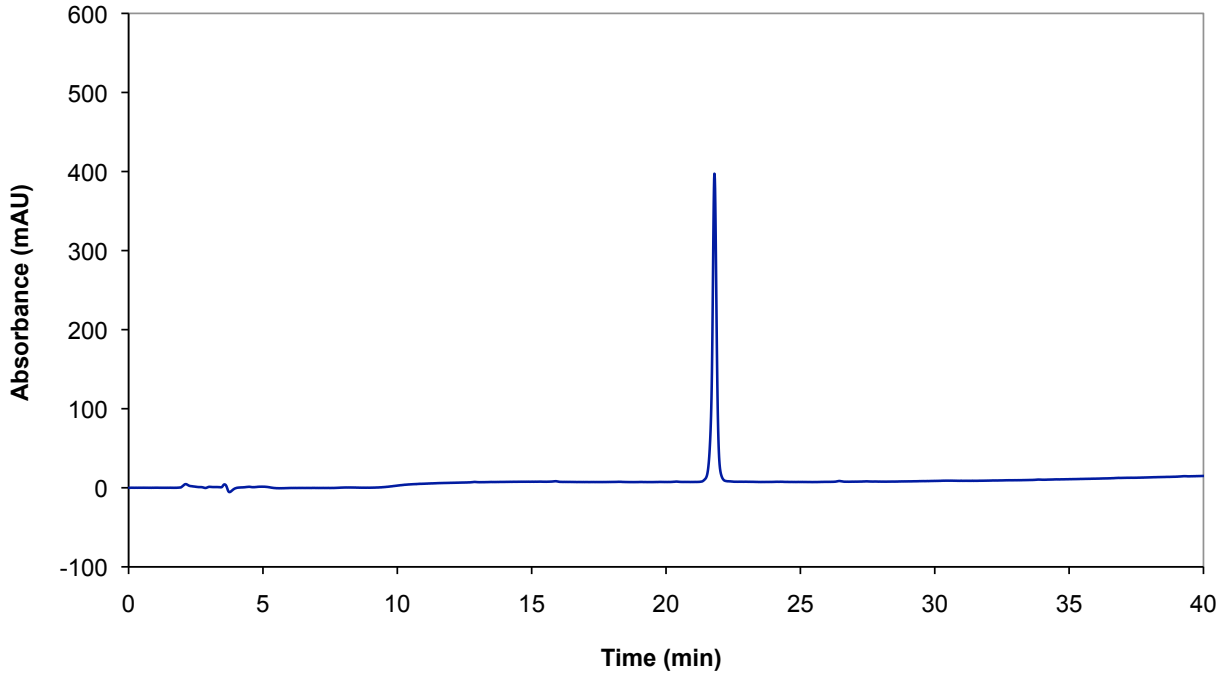
Model peptide reactions and analytical data

H-WSPGYS-NH_2 (2)

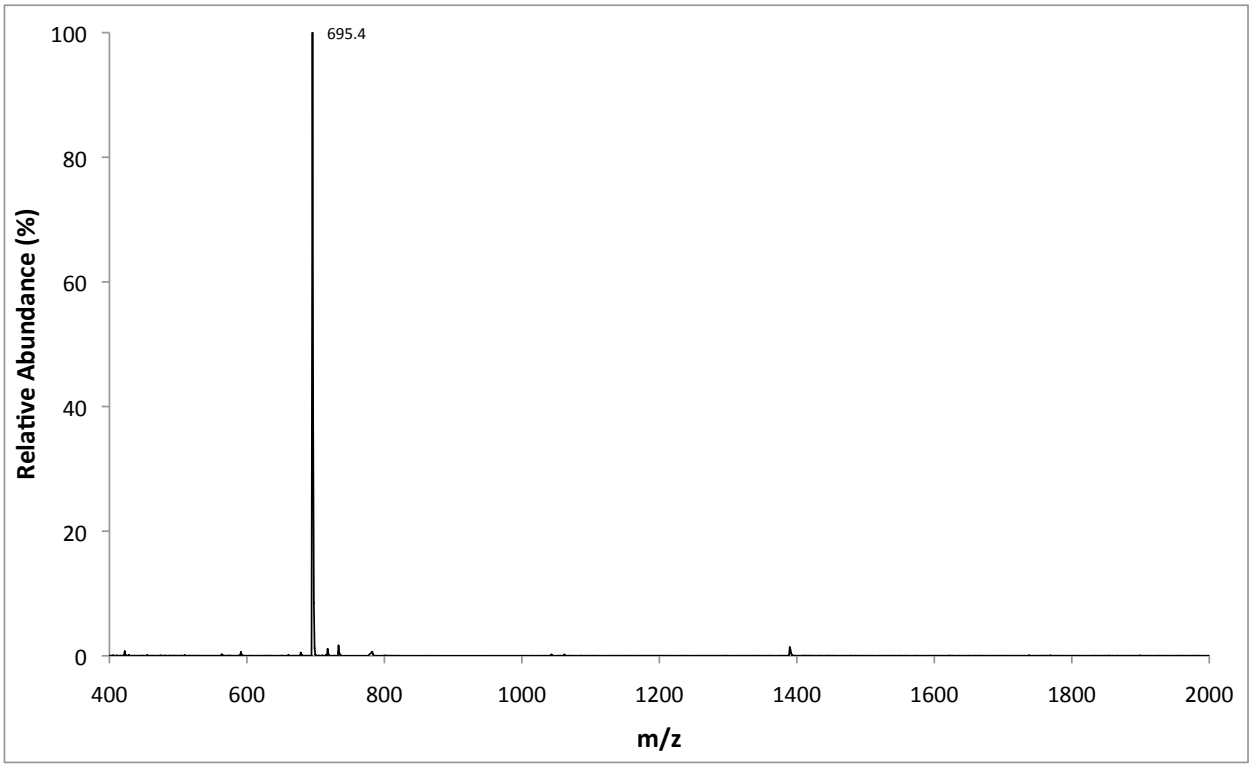


Peptide H-WSPGYS-NH_2 **2** was prepared on a 100 μmol scale on Rink amide resin according to Fmoc-strategy SPPS outlined in the general procedures and purified by preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) to afford the target compound as a white solid following lyophilization (61.0 mg, 88% yield).

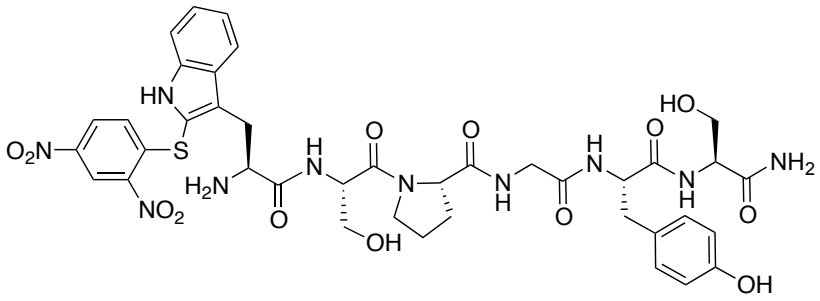
^{1}H NMR (400 MHz, D_2O, select aromatic signals) δ 7.51 (apparent t, 2H, *J* = 7.4 Hz, Trp Ar-H), 7.31 (s, 1H, Trp C2-H), 7.26 (apparent t, 1H, *J* = 7.9 Hz, Trp Ar-H), 7.13 (apparent t, 1H, *J* = 7.8 Hz, Trp Ar-H), 7.08 (d, 2H, *J* = 8.5 Hz, Tyr Ar-H), 6.78 (d, 2H, *J* = 8.5 Hz, Tyr Ar-H) ppm



Analytical HPLC: R_t 21.8 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 695.31; Mass Found (ESI^+); 695.4 $[M+H^+]^+$



H-(2-DNPS)WSPGYS-NH_2 (3)



Peptide H-(2-DNPS)WSPGYS-NH_2 **3** was prepared from H-WSPGYS-NH_2 **2** (29.5 mg, 42 µmol) *via* sulfenylation with DNPS-Cl in solution as outlined in the general procedures. The crude peptide was purified by preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) to afford the target compound as a yellow solid following lyophilization (21.3 mg, 56% yield).

Alternatively, the peptide was prepared on the solid phase from Rink Amide bound H_2N-WSPGYS **1** (15 µmol) in accord with the general procedures. Following cleavage from the resin and purification *via* preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA), the product was obtained as a yellow solid following lyophilization (5.0 mg, 38% yield).

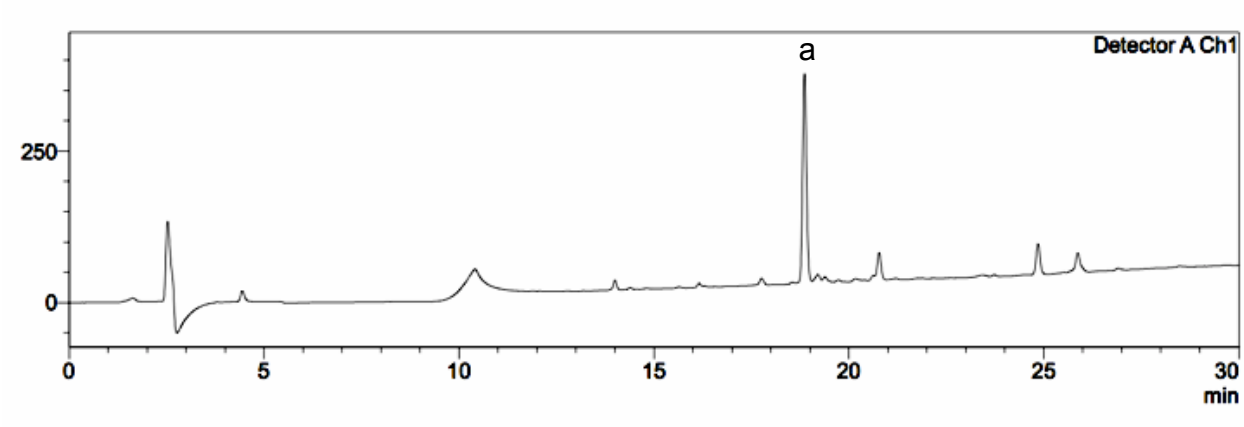


Figure S2. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of crude peptide cleavage following on-resin sulfenylation reaction; a = peptide **3**

^{1}H NMR (400 MHz, D_2O, select aromatic signals) δ 9.15 (d, 1H, *J* = 2.4 Hz, DNPS Ar-H), 8.16 (dd, 1H, *J* = 9.1, 2.4 Hz, DNPS Ar-H), 7.64 (d, 1H, *J* = 8.1 Hz, Trp Ar-H), 7.57 (d, 1H, *J* = 8.3 Hz, Trp Ar-H), 7.42 (apparent t, 1H, *J* = 7.8 Hz, Trp Ar-H), 7.26 (apparent t, 1H, *J* = 7.8 Hz, Trp

Ar-H), 7.04 (d, 2H, *J* = 8.4 Hz, Tyr Ar-H), 6.85 (d, 1H, *J* = 9.0 Hz, DNPS Ar-H), 6.78 (d, 2H, *J* = 8.5 Hz, Tyr Ar-H) ppm

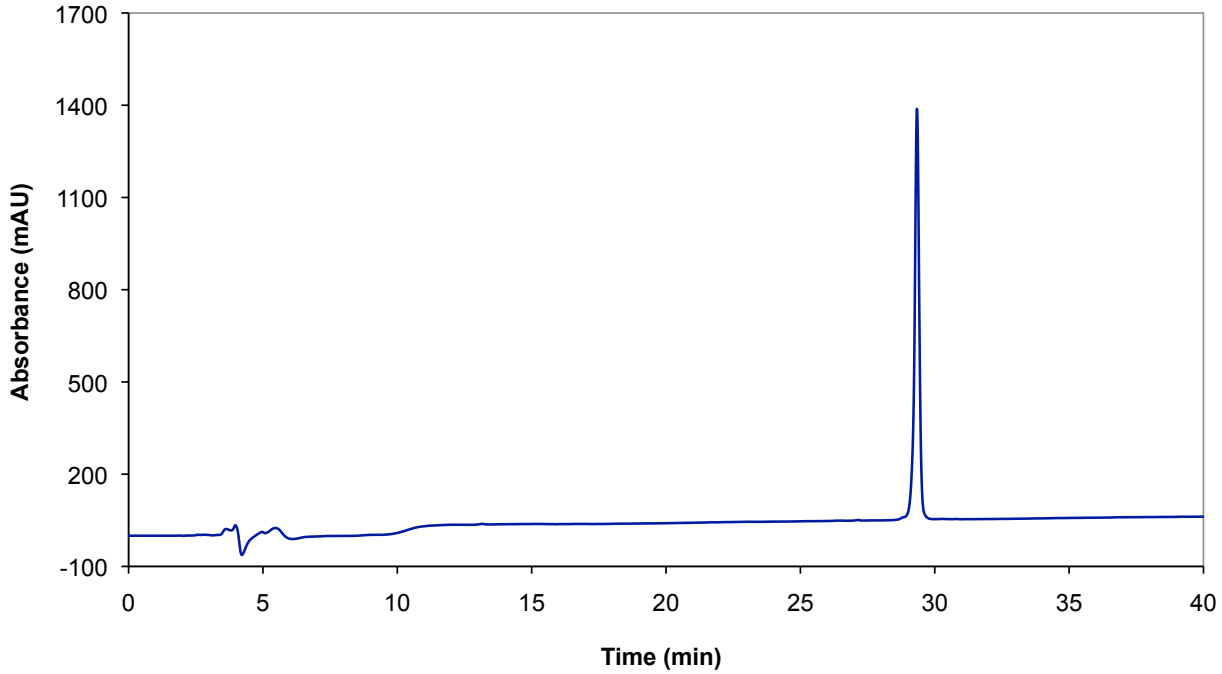
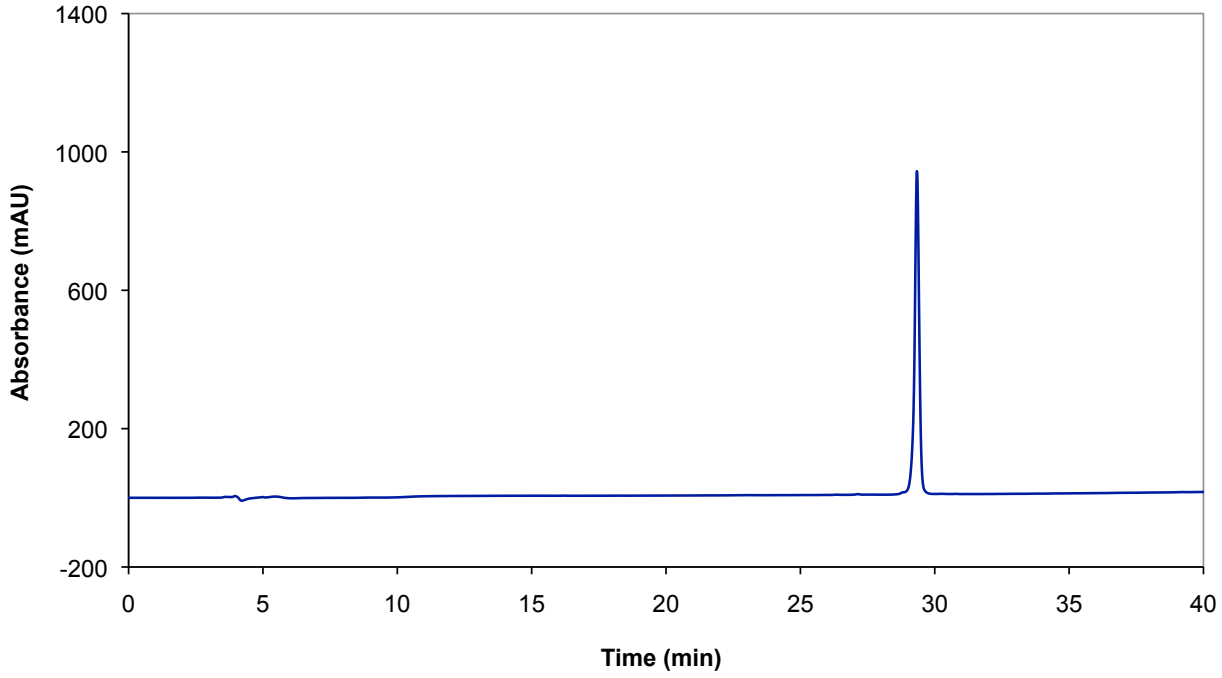
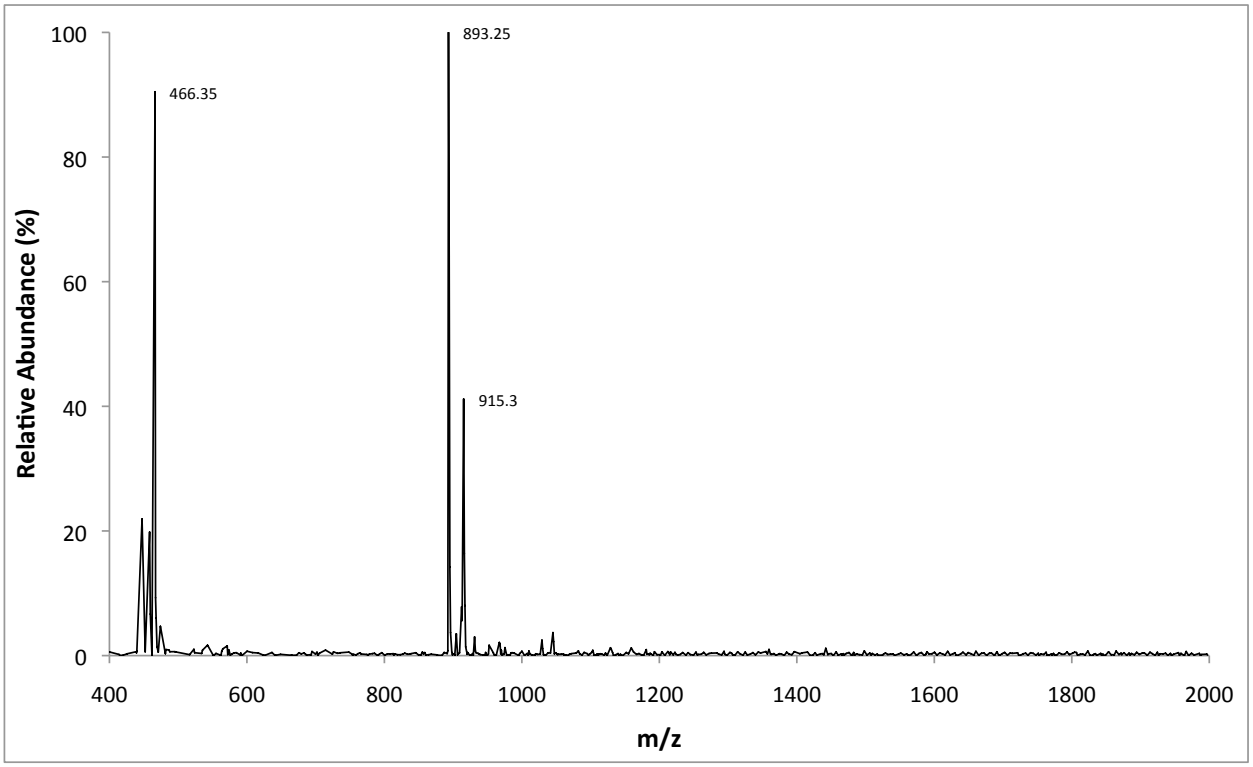


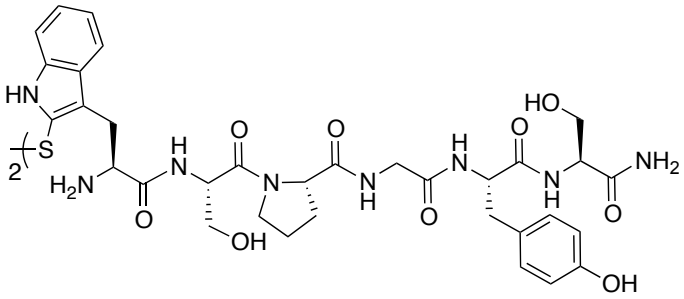
Figure S3. Analytical HPLC: R_t 29.3 min (0-50% B over 40 min, 0.1% TFA, λ = 210 nm).



Analytical HPLC: R_t 29.3 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 893.28, $[M+Na^+]^+$: 915.27, $[M+H^++K^+]^{2+}$: 466.13; Mass Found (ESI^+); 893.25 $[M+H^+]^+$, 915.3 $[M+Na^+]^+$, 466.35 $[M+H^++K^+]^{2+}$



H-(2-SH)WSPGYS-NH_2 (4 - disulfide)



Peptide **4** was prepared from the thiolysis of H-(2-DNPS)WSPGYS-NH_2 **3** (15.0 mg, 16.8 μmol) with thiophenol as outlined in the general procedures. The crude peptide was treated with TCEP to reduce disulfides (see Figure S4, below) and immediately purified by preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA). The purified product oxidized spontaneously in air to afford the target compound (disulfide) as a white solid following lyophilization (10.3 mg, 84% yield).

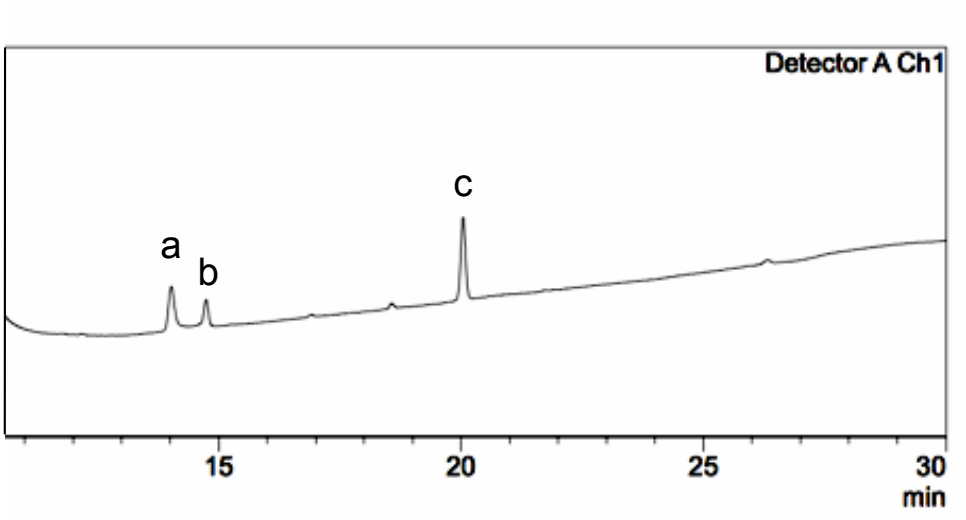
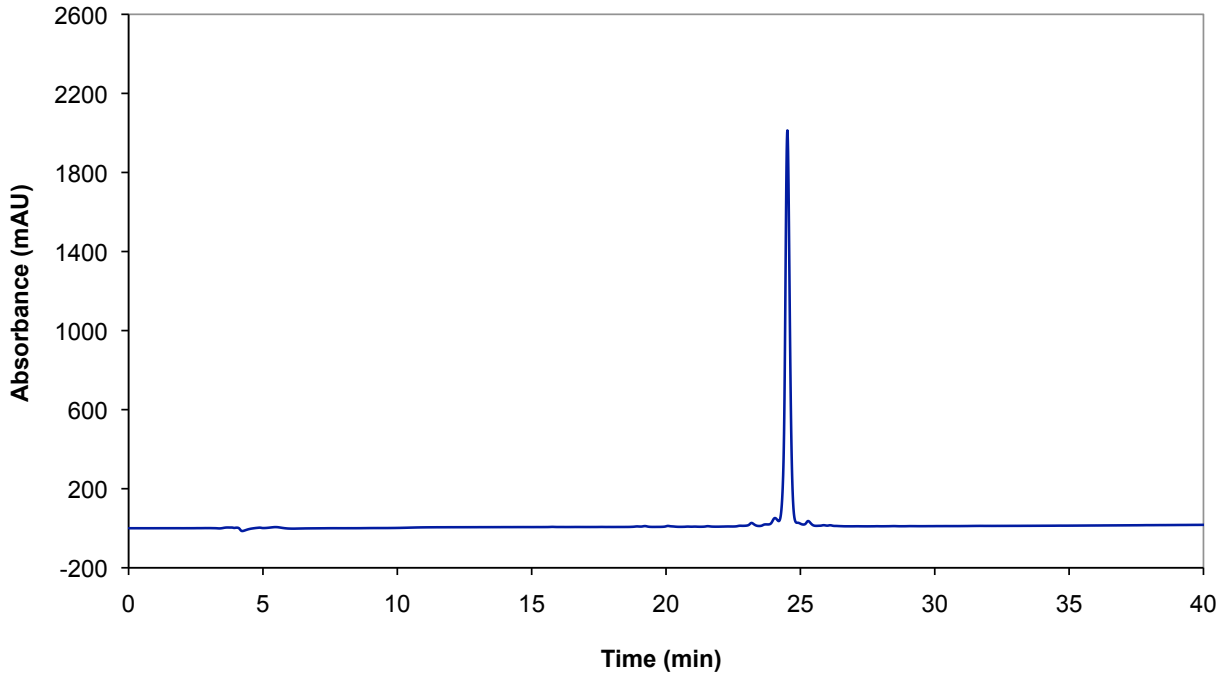
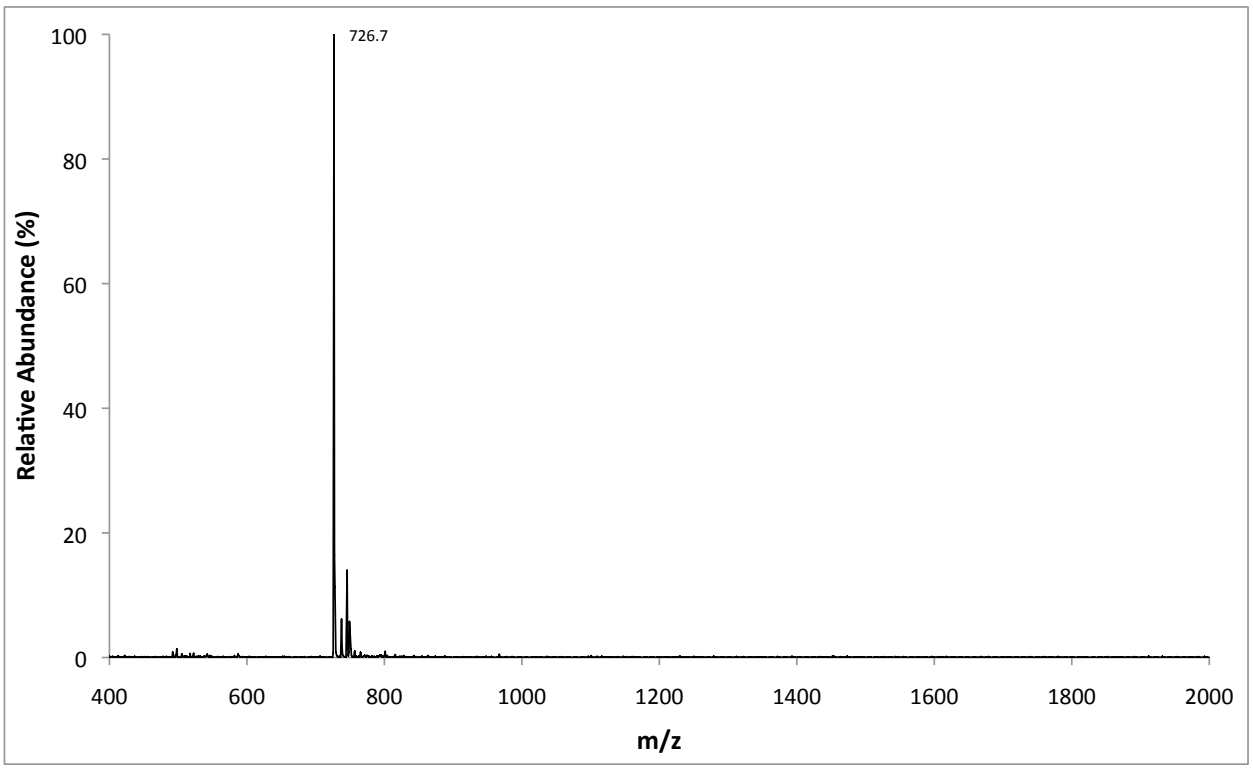


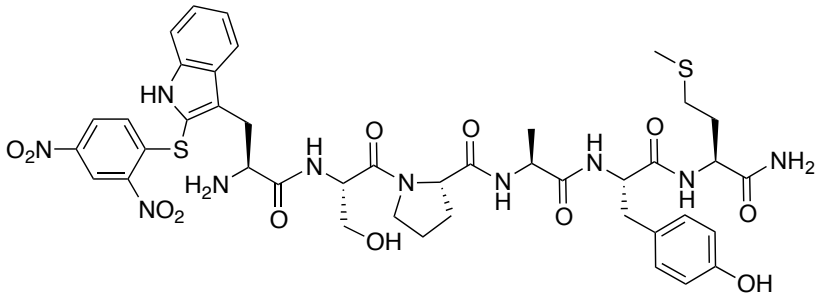
Figure S4. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of crude thiolysis reaction (t = 7 h); a = peptide **4** (free thiol); b = peptide **4** (symmetrical disulfide); c = peptide **4**-thiophenol asymmetric disulfide.



Analytical HPLC: R_t 24.5 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 1451.55, $[M+2H^+]^{2+}$: 726.28; Mass Found (ESI^+); 726.7 $[M+2H^+]^{2+}$



H-(2-DNPS)WSPAYM-NH$_2$ (11)



Peptide H-(2-DNPS)WSPAYM-NH$_2$ **11** was prepared from Rink Amide bound H$_2$N-WSPAYM (54 μmol) *via* solid-phase sulfenylation in accord with the general procedures. Following cleavage from the resin and purification *via* preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA), the product was obtained as a yellow solid following lyophilization (18 mg, 34% yield).

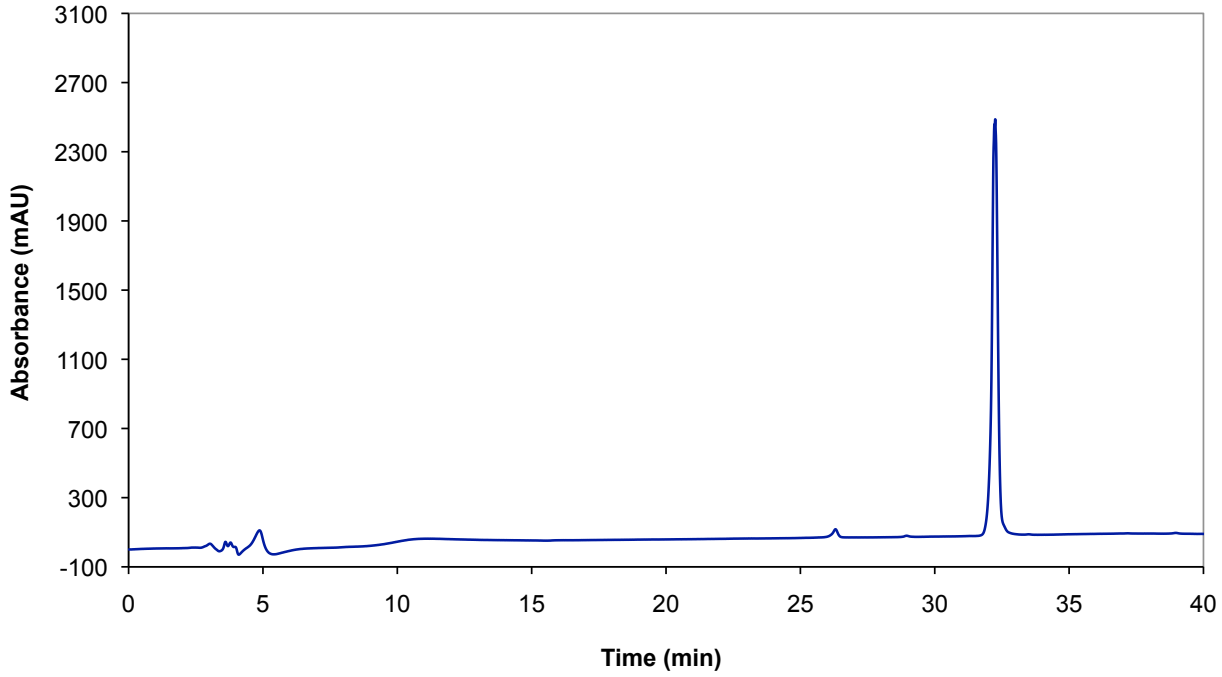
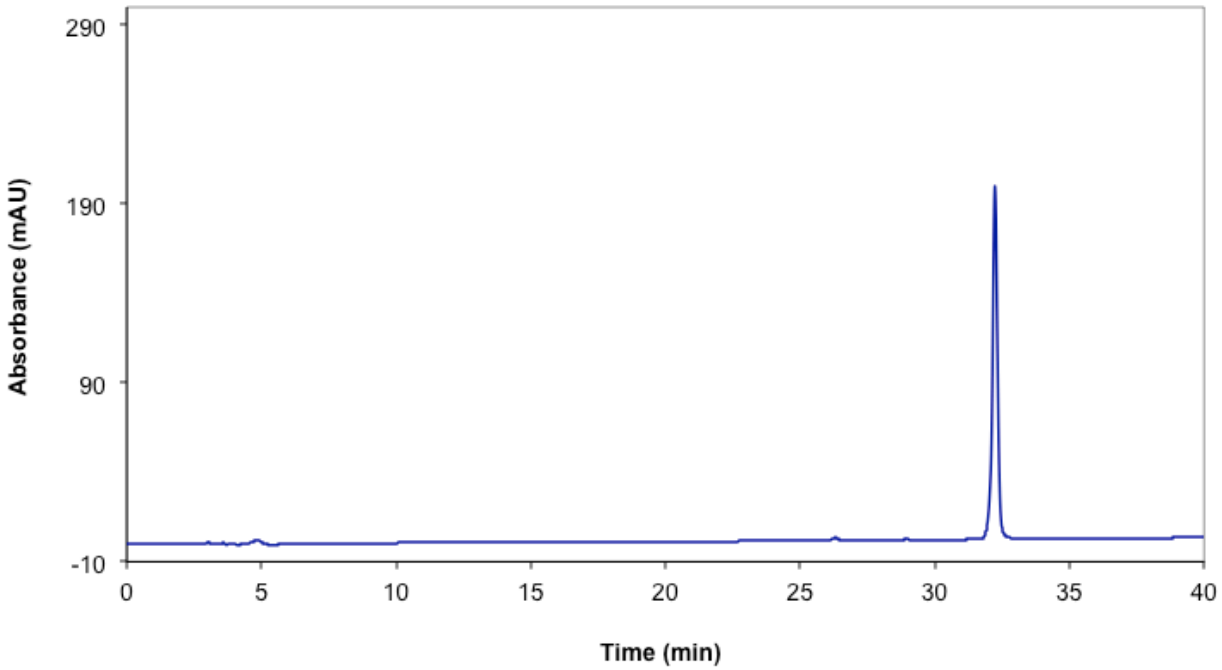
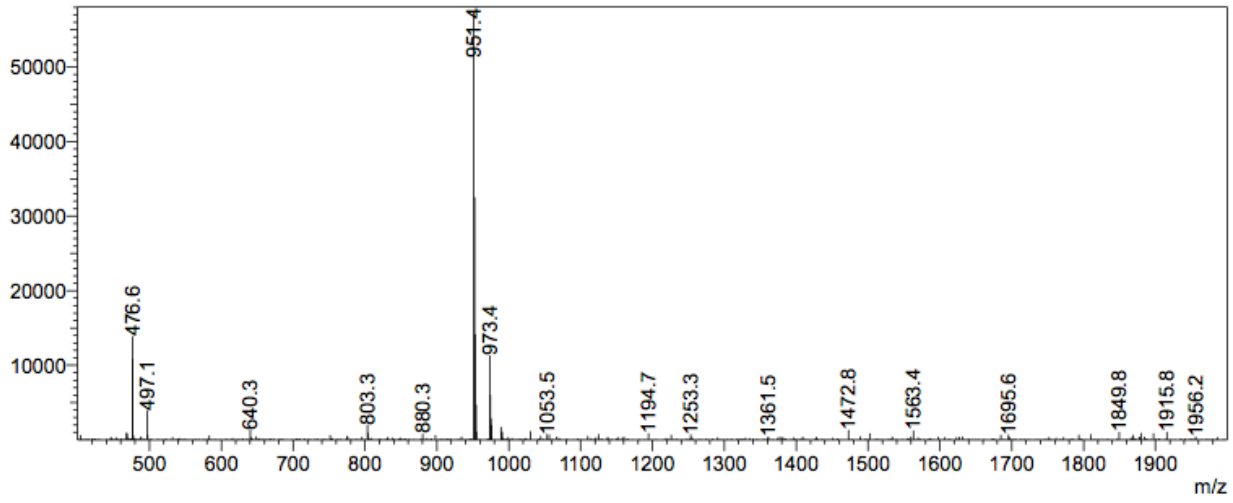


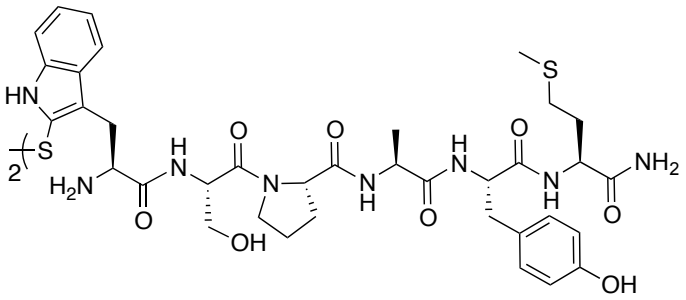
Figure S5. Analytical HPLC: R_t 32.2 min (0-50% B over 40 min, 0.1% TFA, λ = 210 nm)



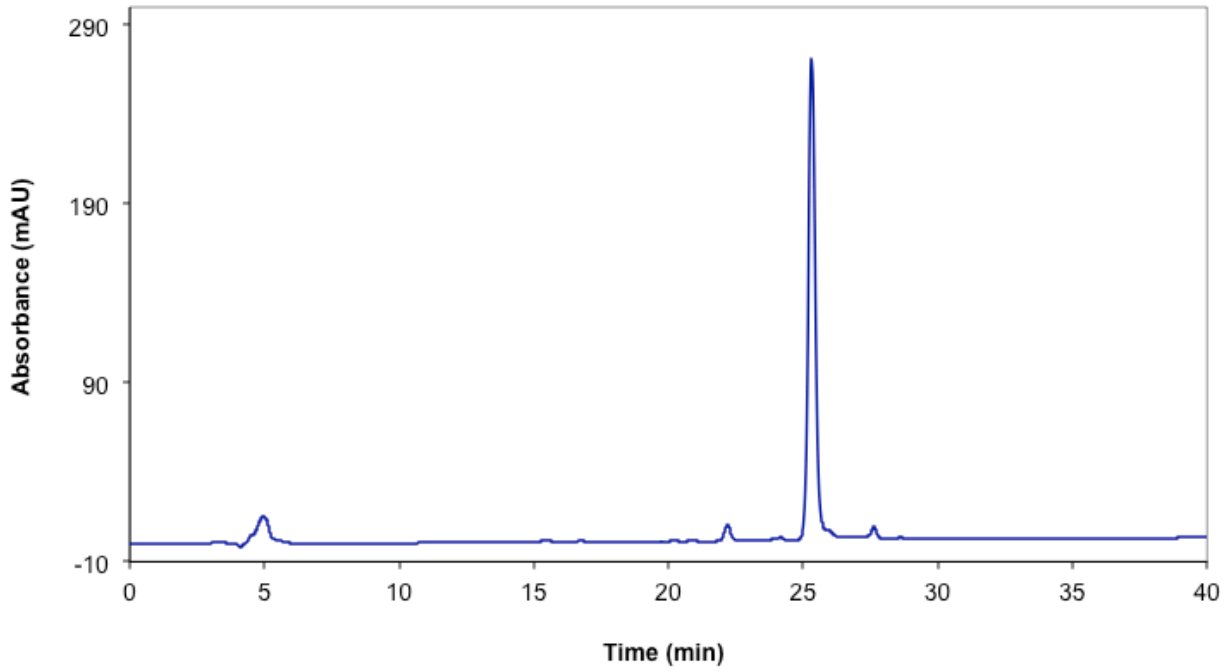
Analytical HPLC: R_t 32.2 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 951.31; Mass Found (ESI^+); 951.40 $[M+H^+]^+$



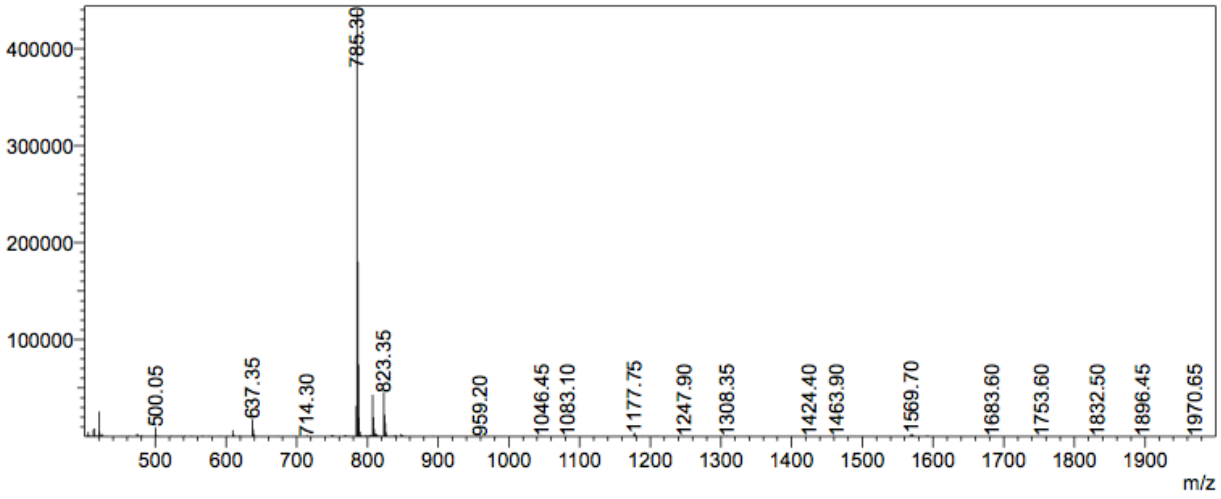
H-(2-SH)WSPAYM-NH_2 (10 - disulfide)



Peptide **10** was prepared from the thiolysis of H-(2-DNPS)WSPAYM-NH_2 **11** (8.7 mg, 9.1 μmol) with thiophenol as outlined in the general procedures. The crude peptide was treated with TCEP to reduce disulfides and immediately purified by preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA). The purified product oxidized spontaneously in air to afford the target compound (disulfide) as a white solid following lyophilization (5.1 mg, 71% yield).



Analytical HPLC: R_t 25.3 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass (reduced thiol) $[M+H^+]^+$: 785.31; Mass Found (ESI^+); 785.30 $[M+H^+]^+$



Screening of ligation conditions

Ac-LYRANG-S$(CH_2)_2CO_2$Et (0.8 mg, 0.9 μmol, 1.3 eq.) and peptide **4** (0.5 mg, 0.7 μmol, 1.0 eq.) were dissolved in degassed buffer (6 M guanidine hydrochloride, 100 mM Na_2HPO_4, 100 mM TCEP, adjusted to pH 7.3, 0.15 mL, [5 mM concentration based on 2-thiol Trp peptide **4**]). Thiophenol (3 μL, 2 vol.%) was added to the reaction and the solution was incubated at 37 °C.

The progress of the reaction was monitored by LC-MS. Significant formation of the 2-thiophenyl thioether derivative of peptide **4** (peak a, Figure S6) was detected at t = 26 h.

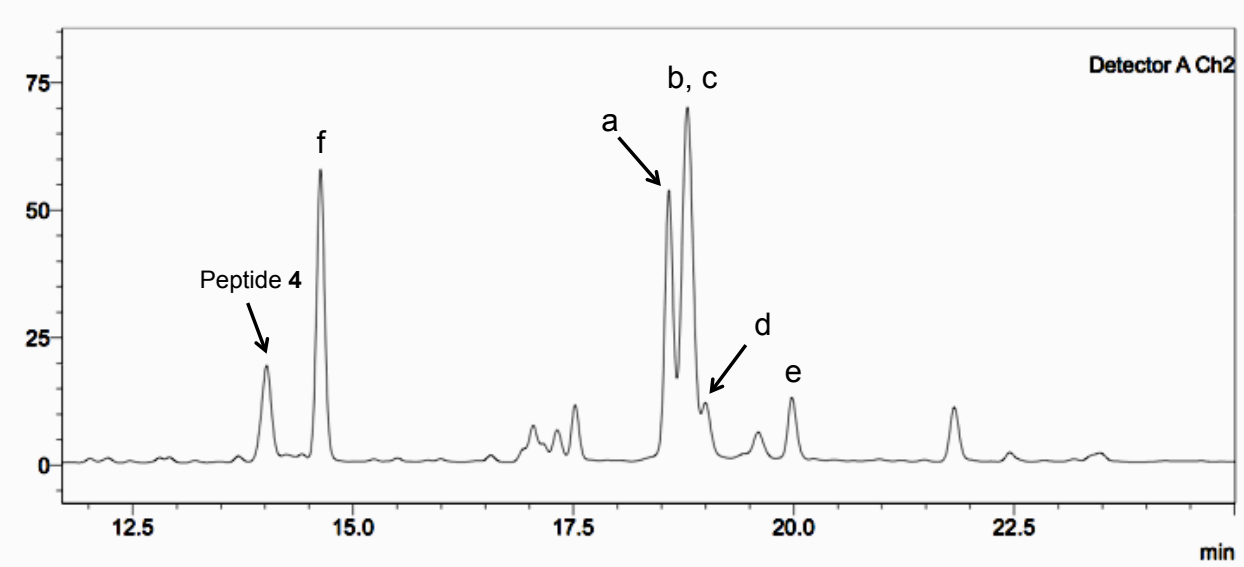
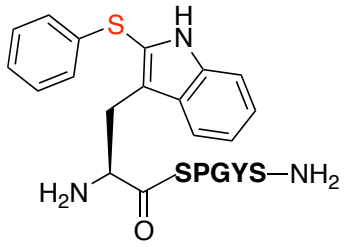
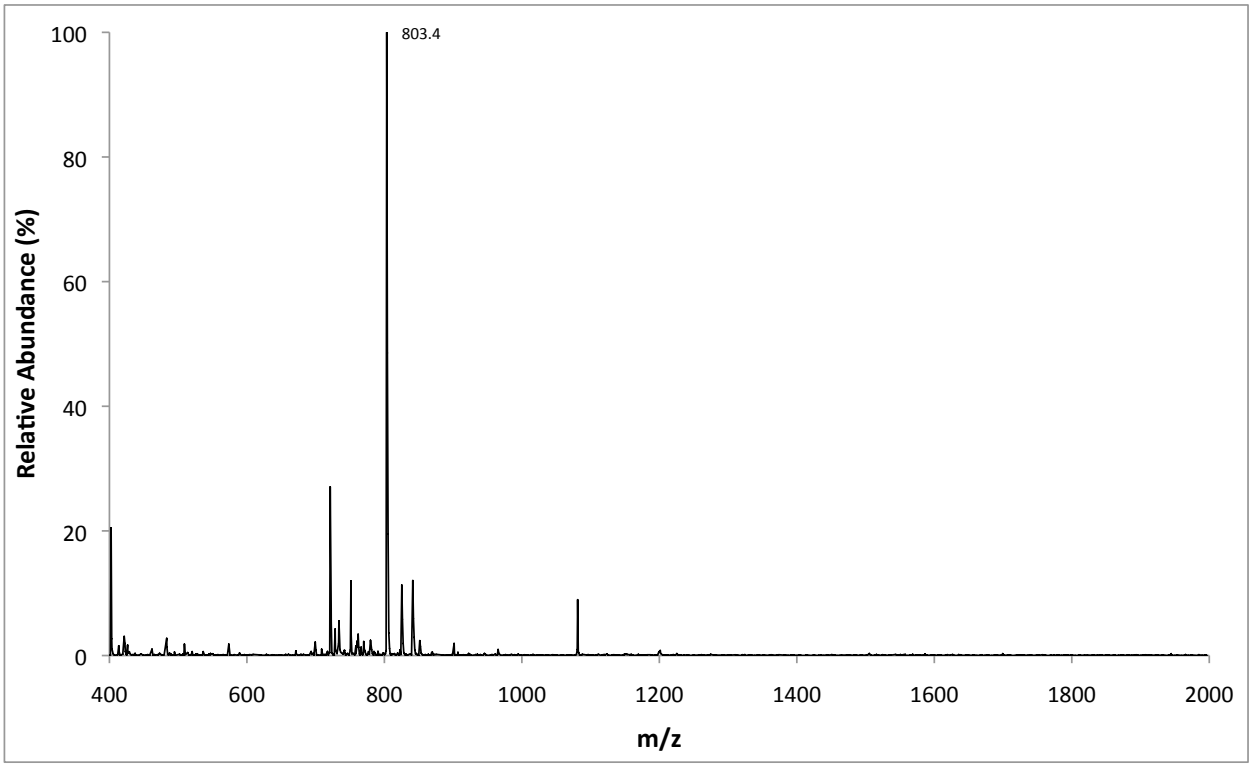


Figure S6. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation between Ac-LYRANG-S$(CH_2)_2CO_2Et$ and peptide **4** in the presence of thiophenol (t = 26 h). a = 2-thiophenyl thioether derivative of peptide **4,** $[M + H^+]^+$ = 803.4; b, c = ligation product (free thiol) + Ac-LYRANG-S$(CH_2)_2CO_2Et$; d = ligation product (disulfide); e = Ac-LYRANG-SPh; f = Ac-LYRANG-OH.

Peak a:



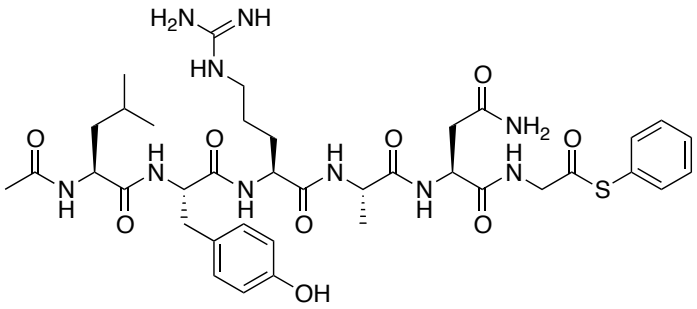
Calculated Mass $[M+H^+]^+$ = 803.32
Mass Found (ESI^+) = 803.4



Thiophenyl Thioesters

Thiophenyl thioesters were prepared as outlined in the general procedures.

Ac-LYRANG-SPh (5)



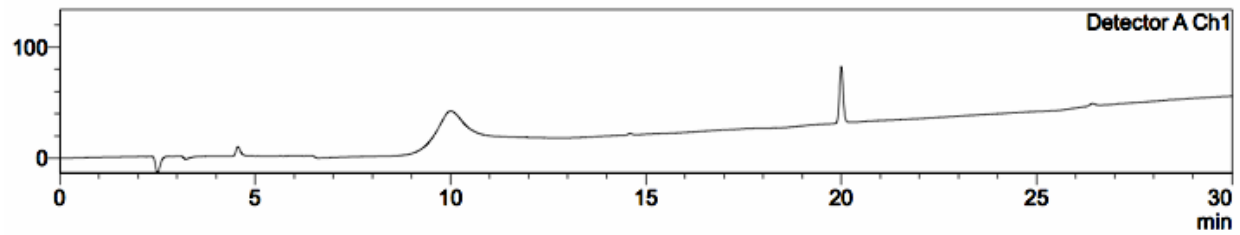
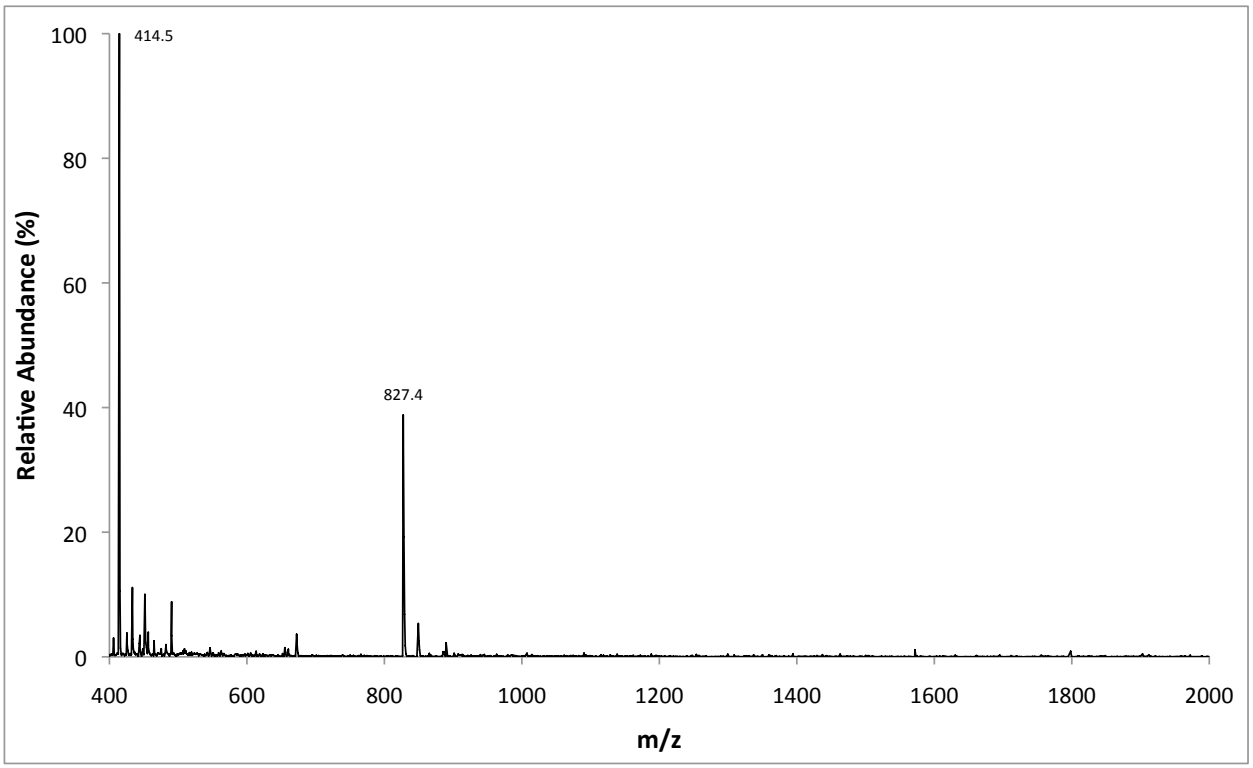


Figure S7. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of purified Ac-LYRANG-SPh (R_t 20.0 min)

Calculated Mass $[M+H^+]^+$: 827.39, $[M+2H^+]^{2+}$: 414.20; Mass Found (ESI^+); 827.4 $[M+H^+]^+$, 414.5 $[M+2H^+]^{2+}$



Ac-LYRANA-SPh (6)

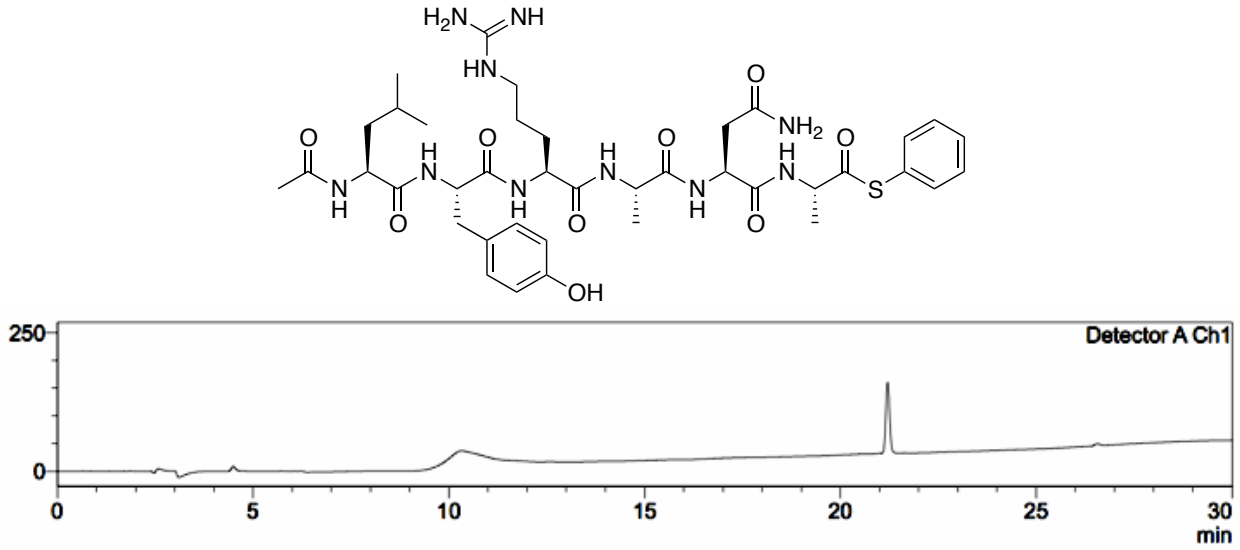
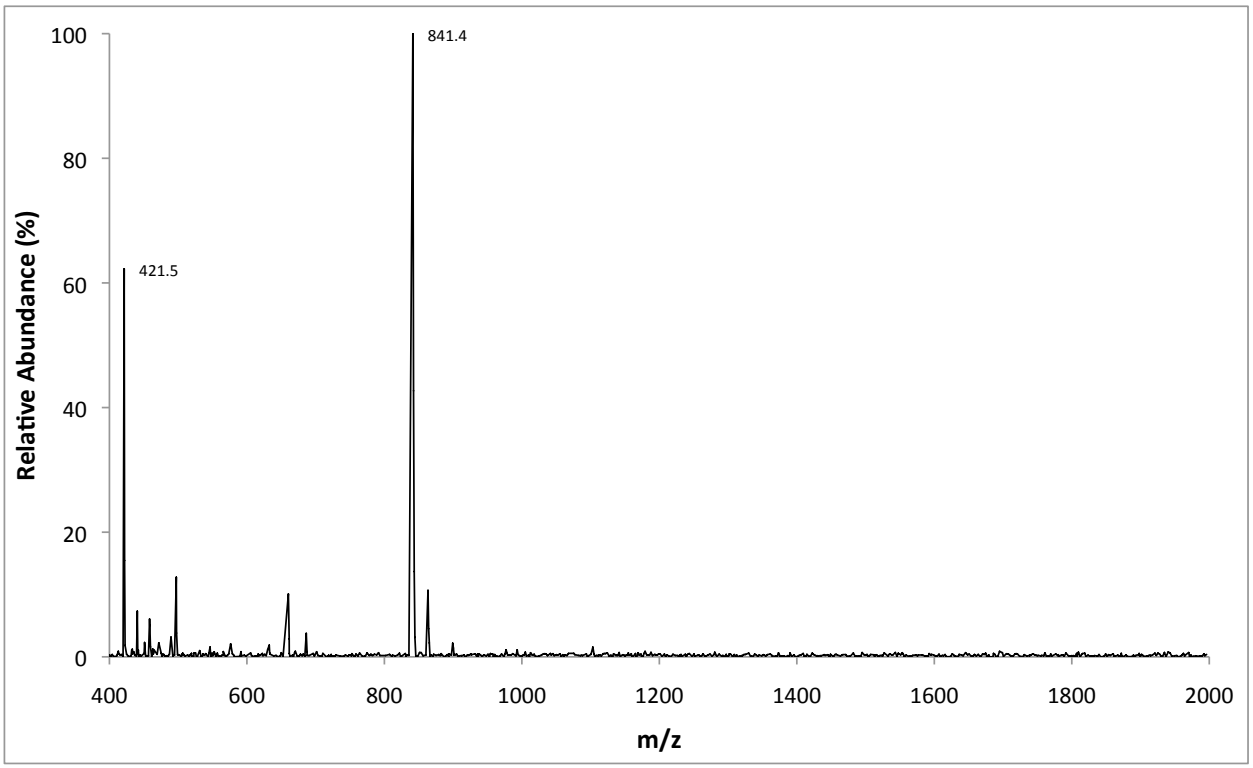
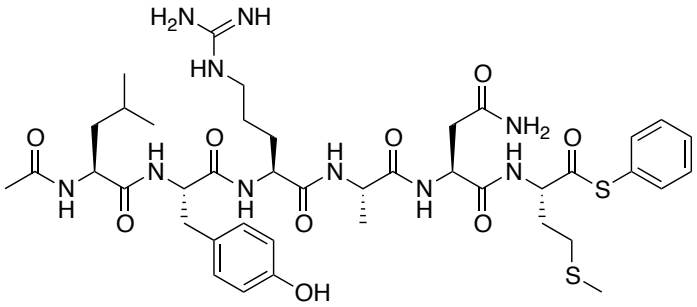


Figure S8. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of purified Ac-LYRANA-SPh (R_t 21.2 min)

Calculated Mass $[M+H^+]^+$: 841.40, $[M+2H^+]^{2+}$: 421.20; Mass Found (ESI^+); 841.4 $[M+H^+]^+$, 421.5 $[M+2H^+]^{2+}$



Ac-LYRANM-SPh (7)



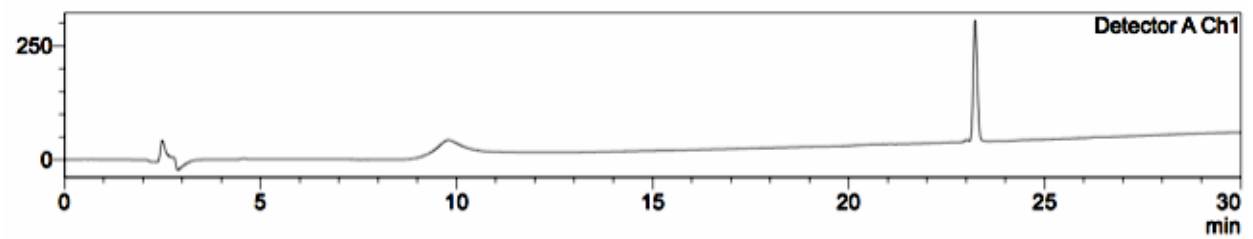
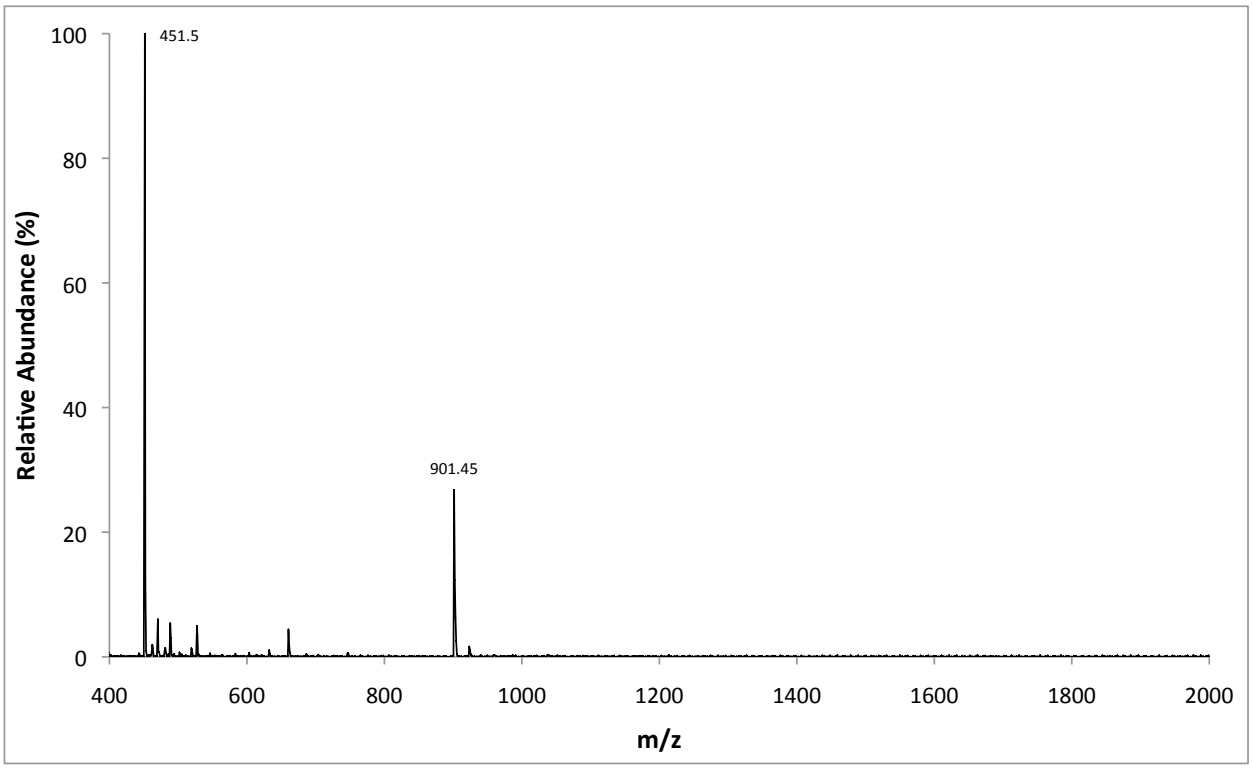
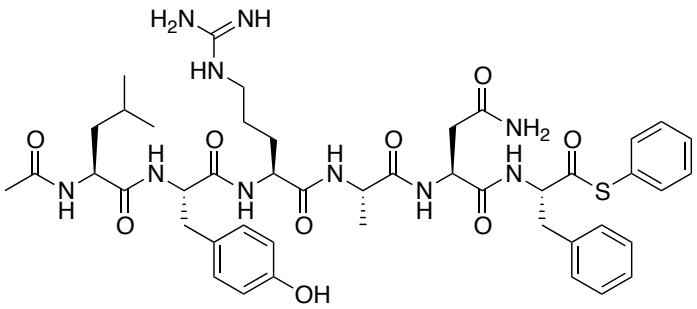


Figure S9. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of purified Ac-LYRANM-SPh (R_t 23.2 min)

Calculated Mass $[M+H^+]^+$: 901.41, $[M+2H^+]^{2+}$: 451.21; Mass Found (ESI^+); 901.45 $[M+H^+]^+$, 451.5 $[M+2H^+]^{2+}$



Ac-LYRANF-SPh (8)



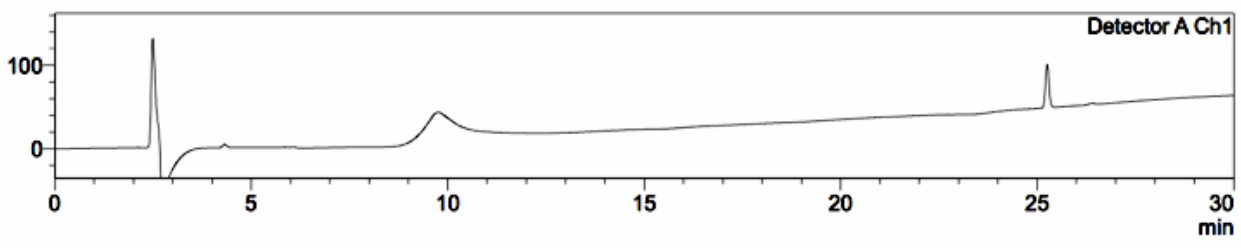
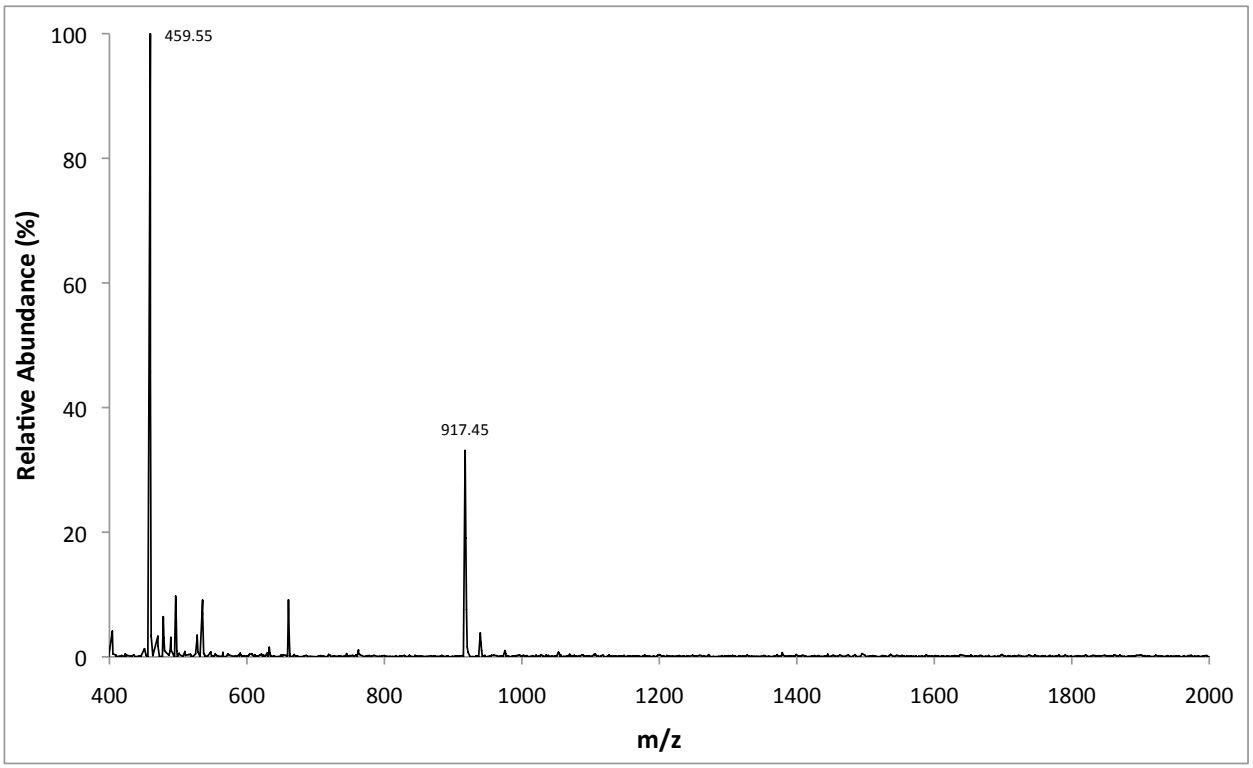
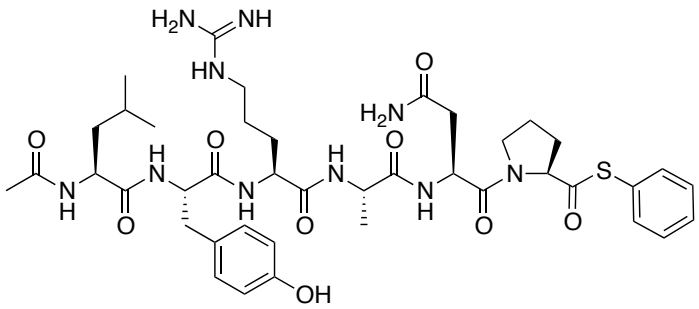


Figure S10. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of purified Ac-LYRANF-SPh (R_t 25.3 min)

Calculated Mass $[M+H^+]^+$: 917.43, $[M+2H^+]^{2+}$: 459.22; Mass Found (ESI^+); 917.45 $[M+H^+]^+$, 459.55 $[M+2H^+]^{2+}$



Ac-LYRANP-SPh (9)



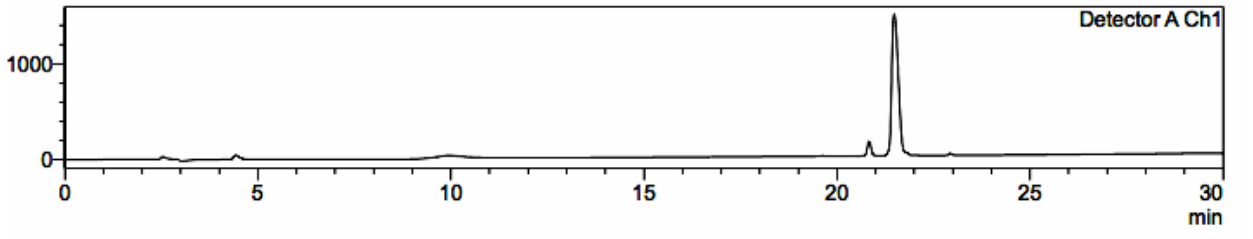
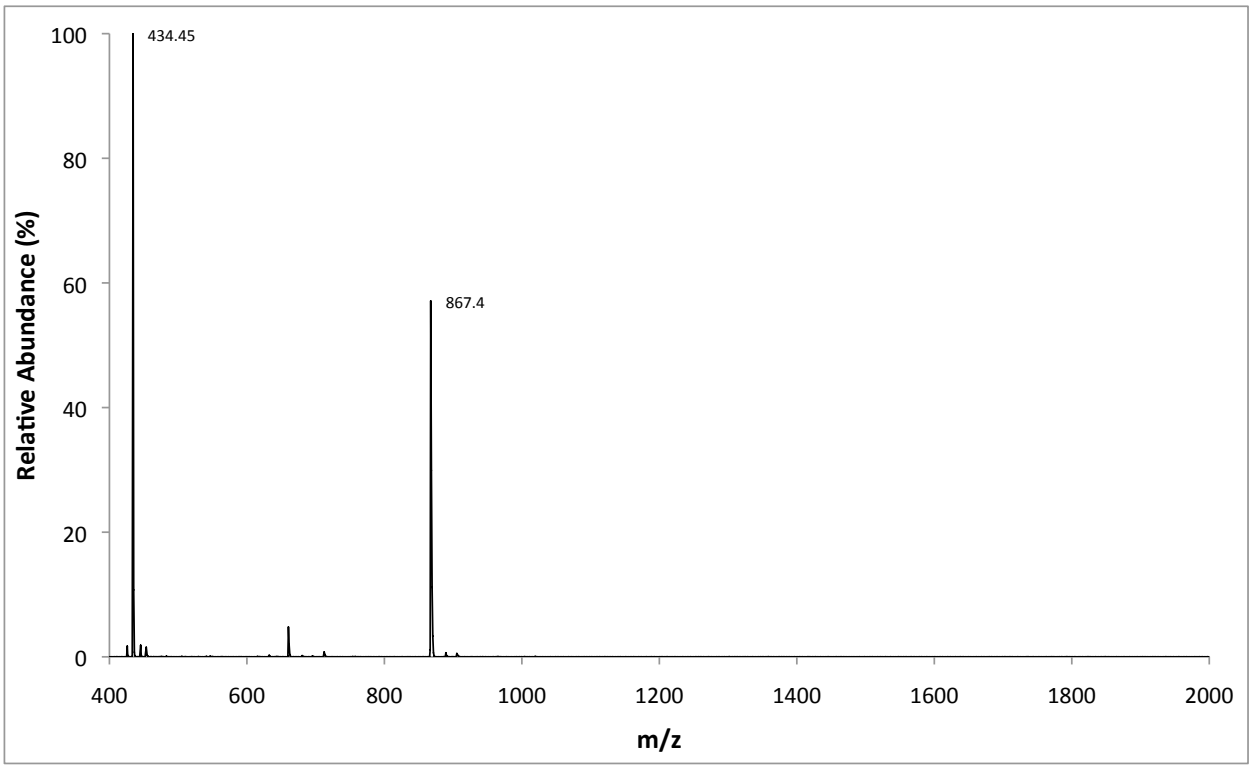
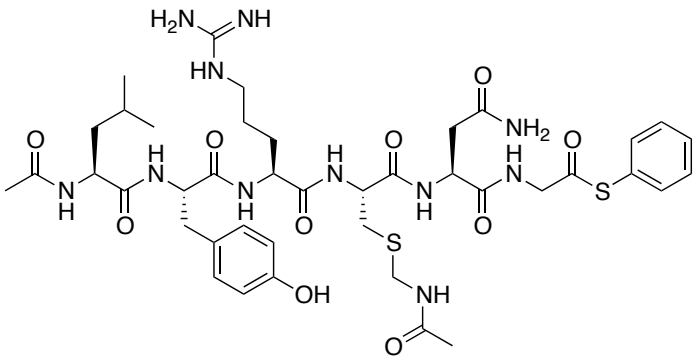


Figure S11. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of purified Ac-LYRANP-SPh (R_t 21.5 min)

Calculated Mass $[M+H^+]^+$: 867.42, $[M+2H^+]^{2+}$: 434.21; Mass Found (ESI^+); 867.4 $[M+H^+]^+$, 434.45 $[M+2H^+]^{2+}$



Ac-LYRC(Acm)NG-SPh (14)



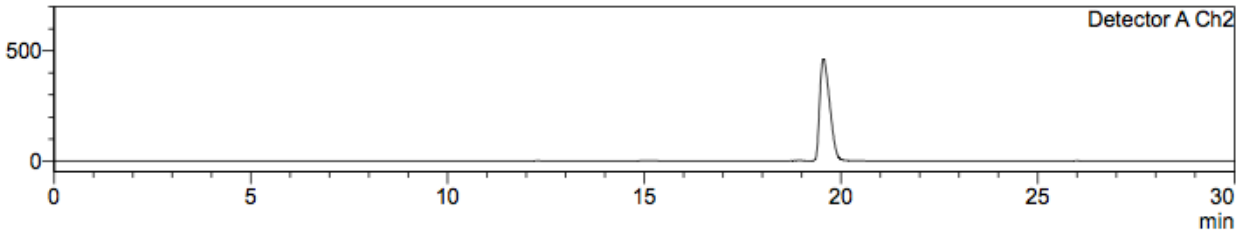
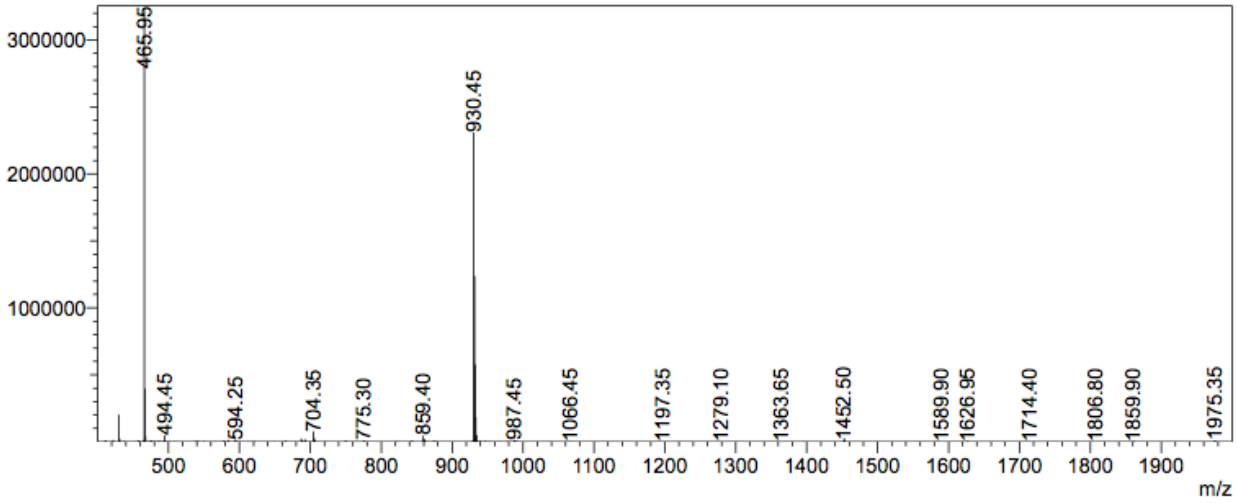


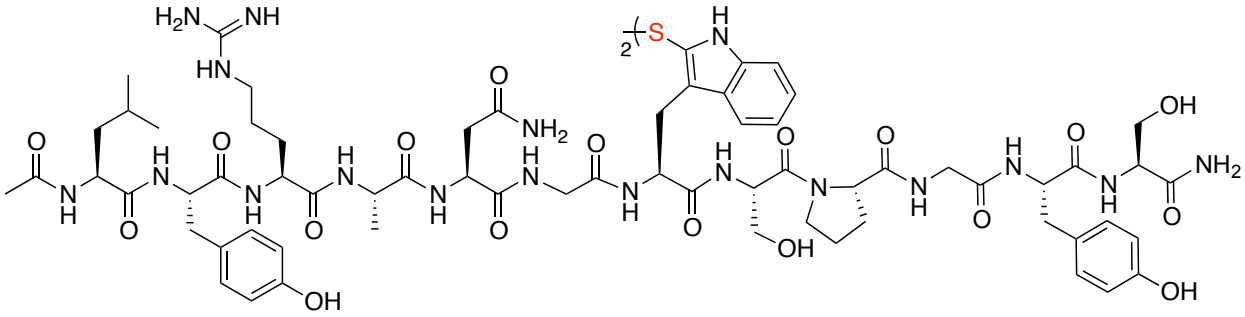
Figure S12. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of purified Ac-LYRC(Acm)NG-SPh (R_t 19.7 min)

Calculated Mass $[M+H^+]^+$: 930.40, $[M+2H^+]^{2+}$: 465.70; Mass Found (ESI^+); 930.45 $[M+H^+]^+$, 469.95 $[M+2H^+]^{2+}$



Ligations (optimized conditions)

Ac-LYRANG(2-SH)WSPGYS-NH_2 (Disulfide)



Ligation of H-(2-SH)WSPGYS-NH_2 **4** (2.1 mg, 2.9 µmol) and Ac-LYRANG-SPh **5** (4.4 mg, 5.3 µmol) was performed as outlined in the general procedures. At t = 8 h, LC-MS analysis showed incomplete consumption of **4** and considerable hydrolysis of the thiophenyl thioester. Additional Ac-LYRANG-SPh **5** (1.3 mg, 1.5 µmol) was dissolved in 50 µL ligation buffer and added to the ligation reaction. The resulting mixture was shaken for an additional 16 h before quenching and purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA). Lyophilization afforded the title compound as a white solid (2.9 mg, 71% yield), which quickly oxidized in air to yield the disulfide dimer.

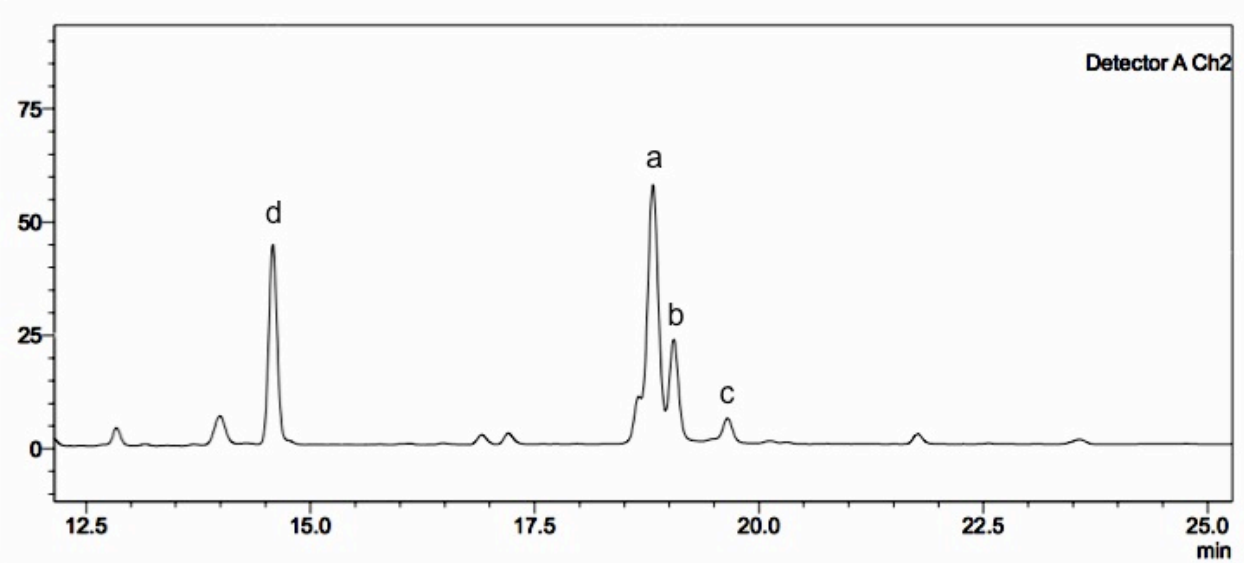


Figure S13. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 24 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANG-SPh **5**; d = Ac-LYRANG-OH.

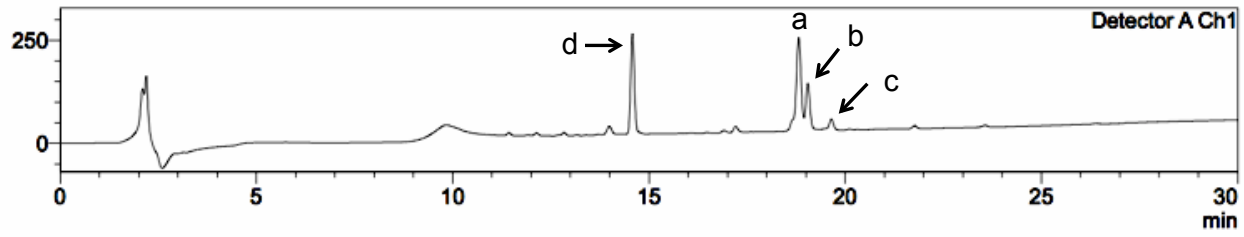
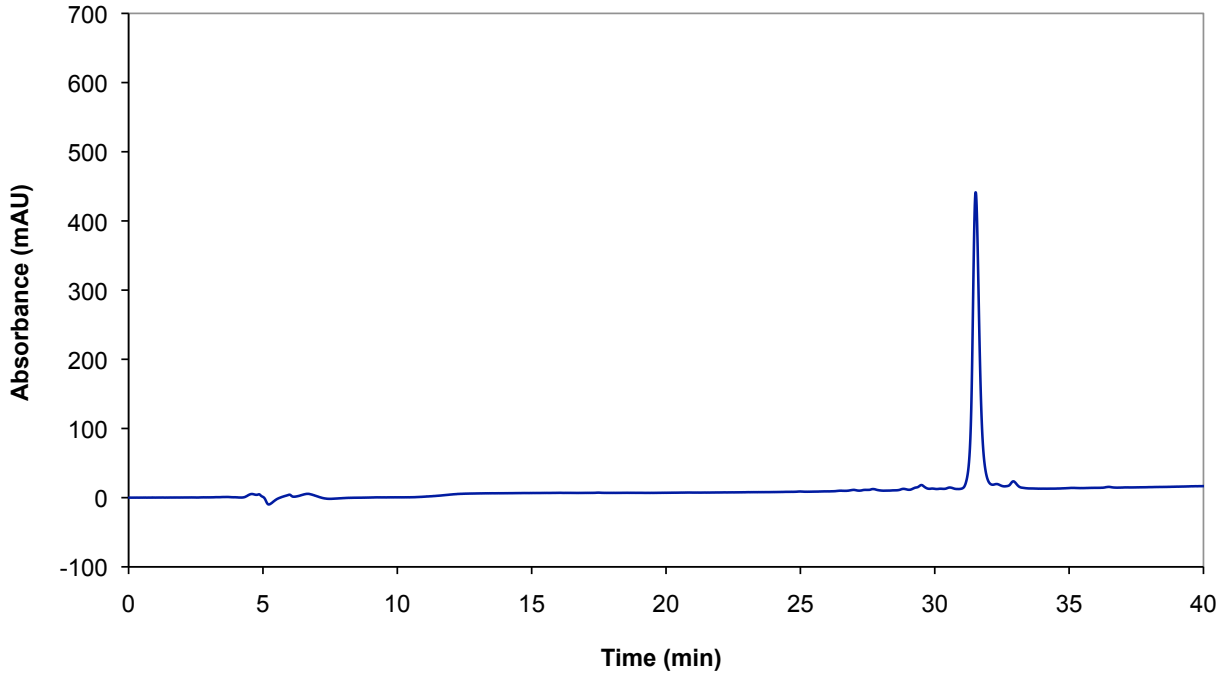
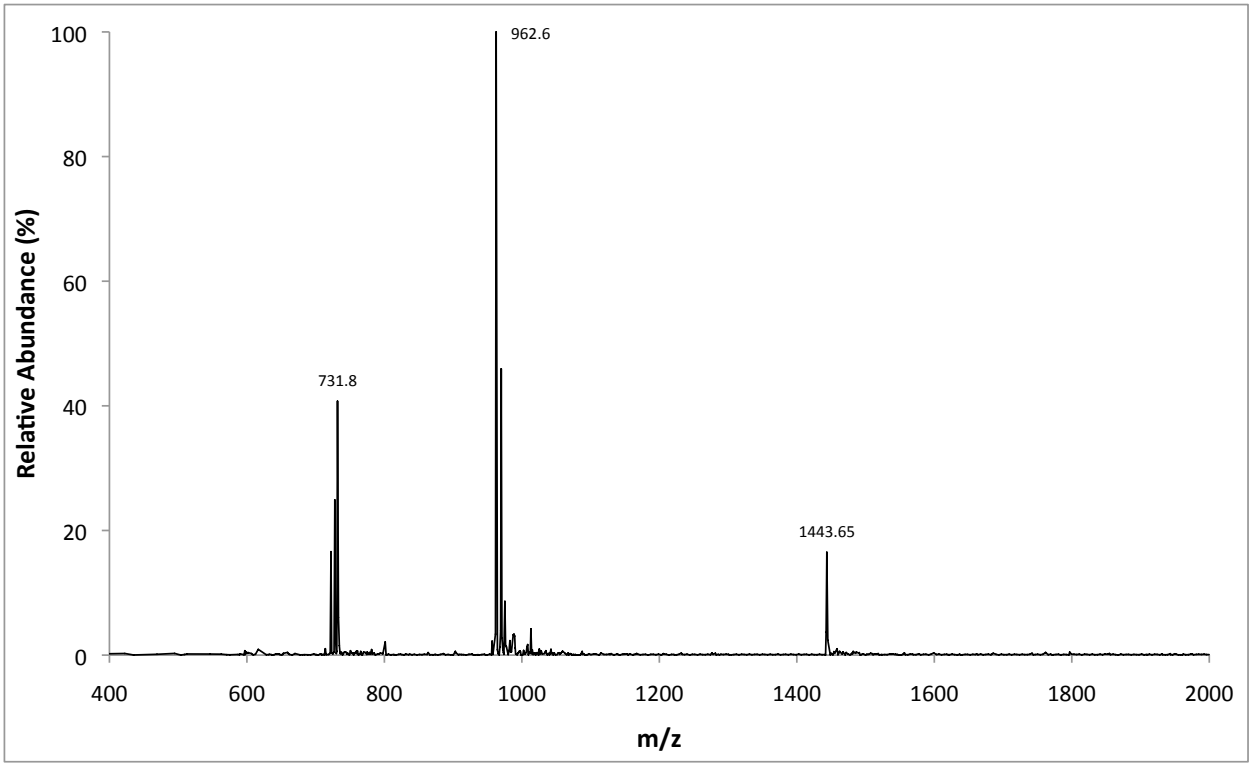


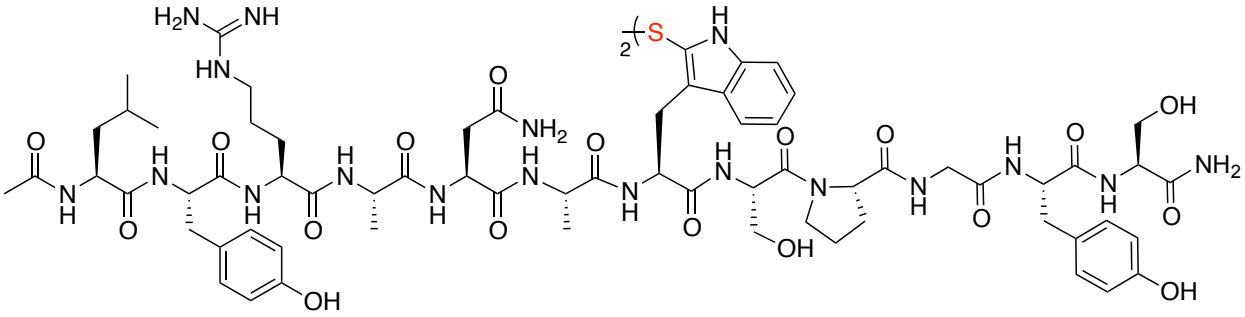
Figure S14. Full LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of crude ligation mixture (t = 24 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANG-SPh **5**; d = Ac-LYRANG-OH.



Analytical HPLC: R_t 31.5 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 2885.28, $[M+2H^+]^{2+}$: 1443.14, $[M+3H^+]^{3+}$: 962.43, $[M+3H^+ +K^+]^{4+}$: 731.56; Mass Found (ESI^+); 1443.65 $[M+2H^+]^{2+}$, 962.6 $[M+3H^+]^{3+}$, 731.8 $[M+3H^+ +K^+]^{4+}$



Ac-LYRANA(2-SH)WSPGYS-NH_2 (Disulfide)



Ligation of H-(2-SH)WSPGYS-NH_2 **4** (1.9 mg, 2.6 µmol) and Ac-LYRANA-SPh **6** (2.8 mg, 3.3 µmol) was performed as outlined in the general procedures. At t = 15 h, LC-MS analysis showed incomplete consumption of **4**. Additional Ac-LYRANA-SPh **6** (1.5 mg, 1.8 µmol) was dissolved in 70 µL ligation buffer (pH = 6.4) and added to the ligation reaction. The resulting mixture was shaken for an additional 8 h before quenching and purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA). Lyophilization afforded the title compound as a white solid (3.0 mg, 81% yield), which quickly oxidized in air to yield the disulfide dimer.

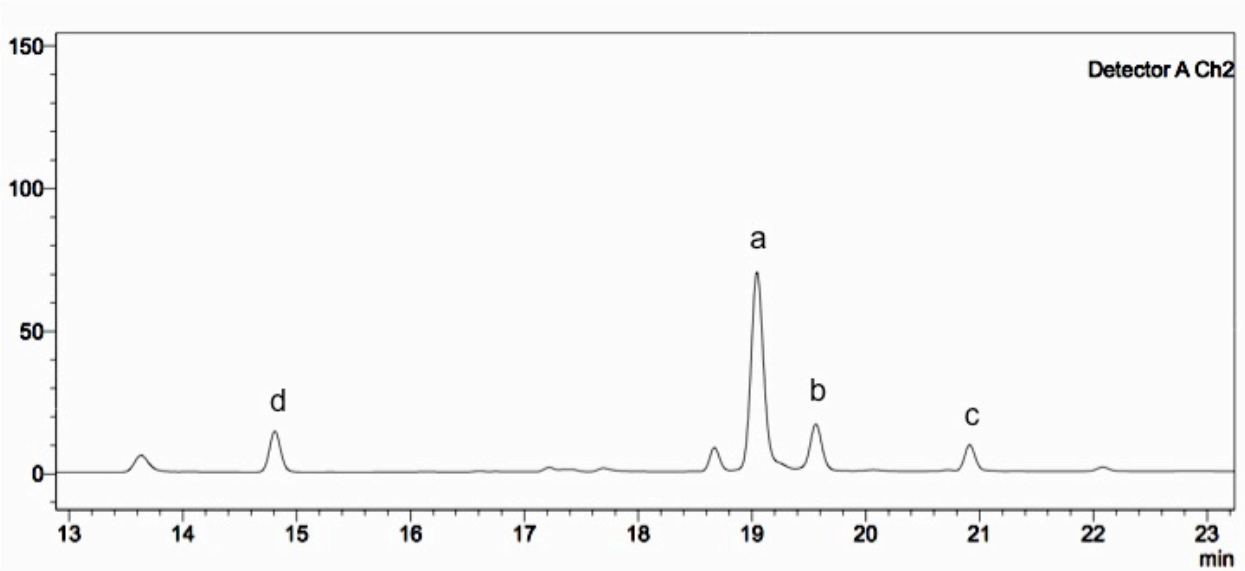
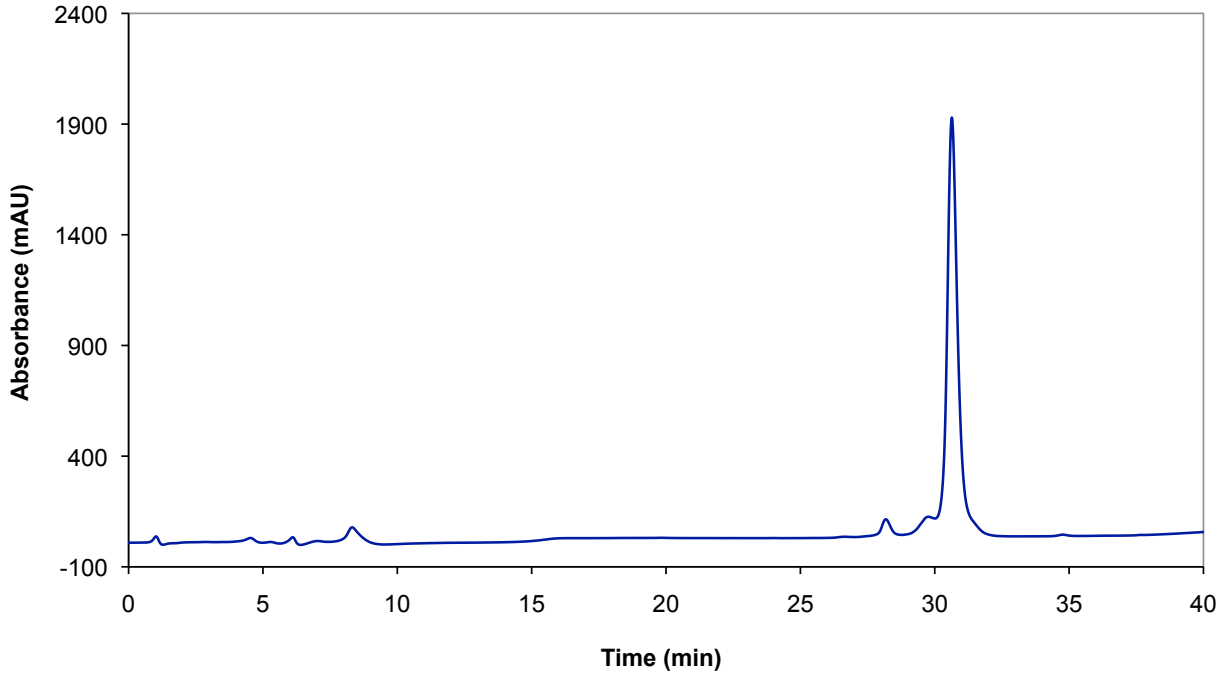
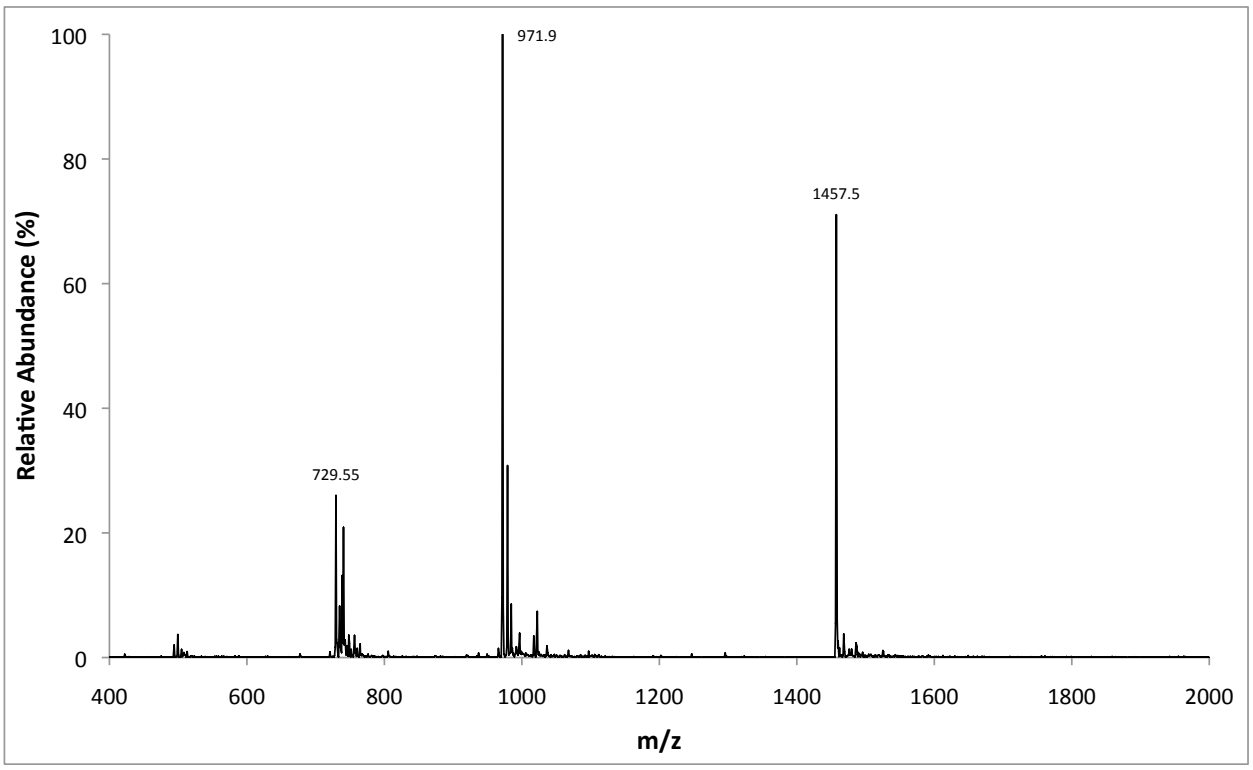


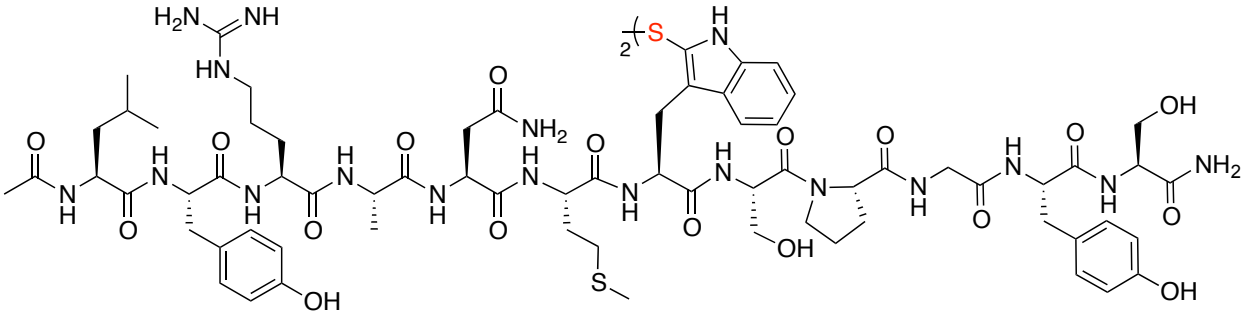
Figure S15. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 24 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANA-SPh **6**; d = Ac-LYRANA-OH.



Analytical HPLC: R_t 30.6 min (0-70% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 2913.31, $[M+2H^+]^{2+}$: 1457.16, $[M+3H^+]^{3+}$: 971.77, $[M+4H^+]^{4+}$: 729.08; Mass Found (ESI^+); 1457.5 $[M+2H^+]^{2+}$, 971.9 $[M+3H^+]^{3+}$, 729.55 $[M+4H^+]^{4+}$



Ac-LYRANM(2-SH)WSPGYS-NH_2 (Disulfide + free thiol)



Ligation of H-(2-SH)WSPGYS-NH_2 **4** (1.3 mg, 1.8 μmol) and Ac-LYRANM-SPh **7** (2.8 mg, 3.1 μmol) was performed as outlined in the general procedures. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (2.2 mg, 80% yield), which partially oxidized in air to yield a mixture of free thiol and disulfide dimer.

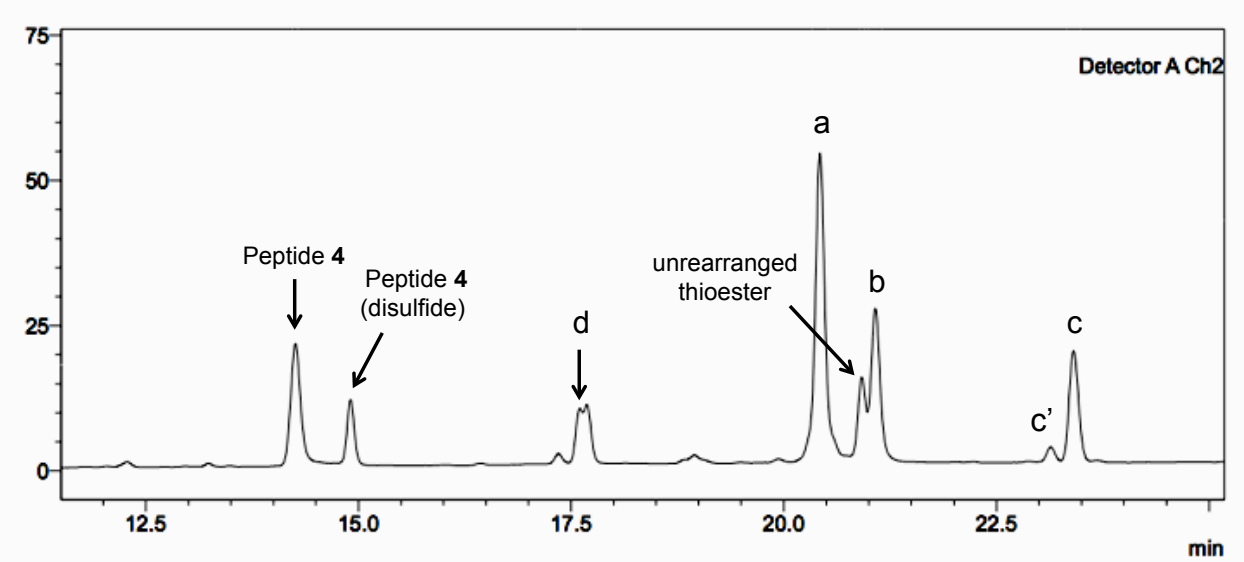


Figure S16. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 1.5 h) depicting unrearranged thioester intermediate; a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANM-SPh **7**; c' = Ac-LYRANM-SPh (Met epimer); d = Ac-LYRANM-OH.

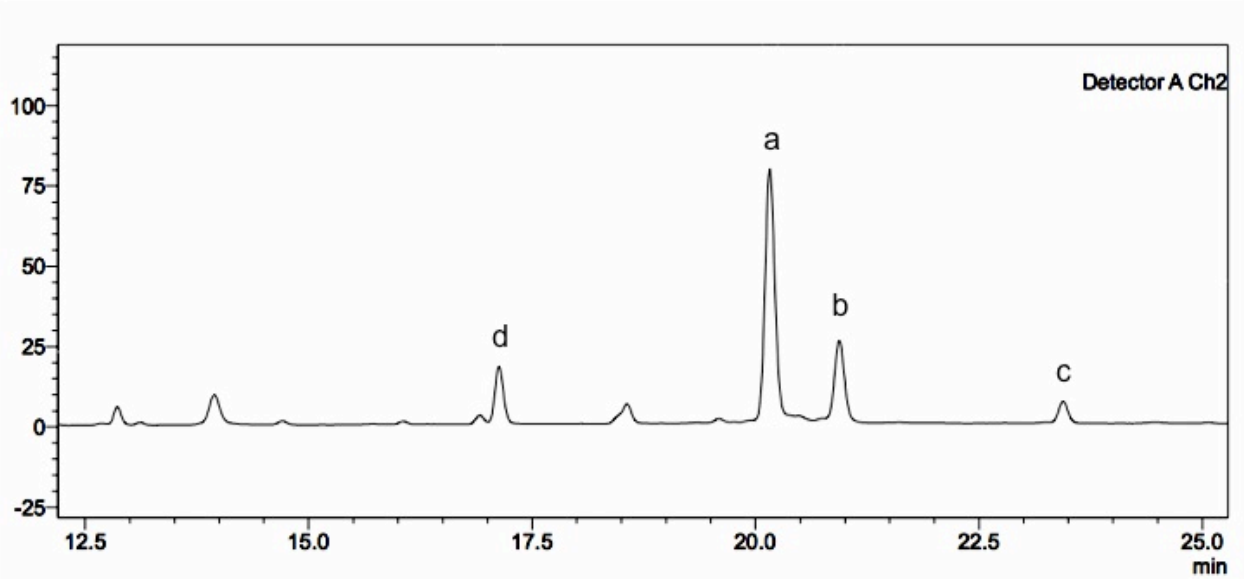
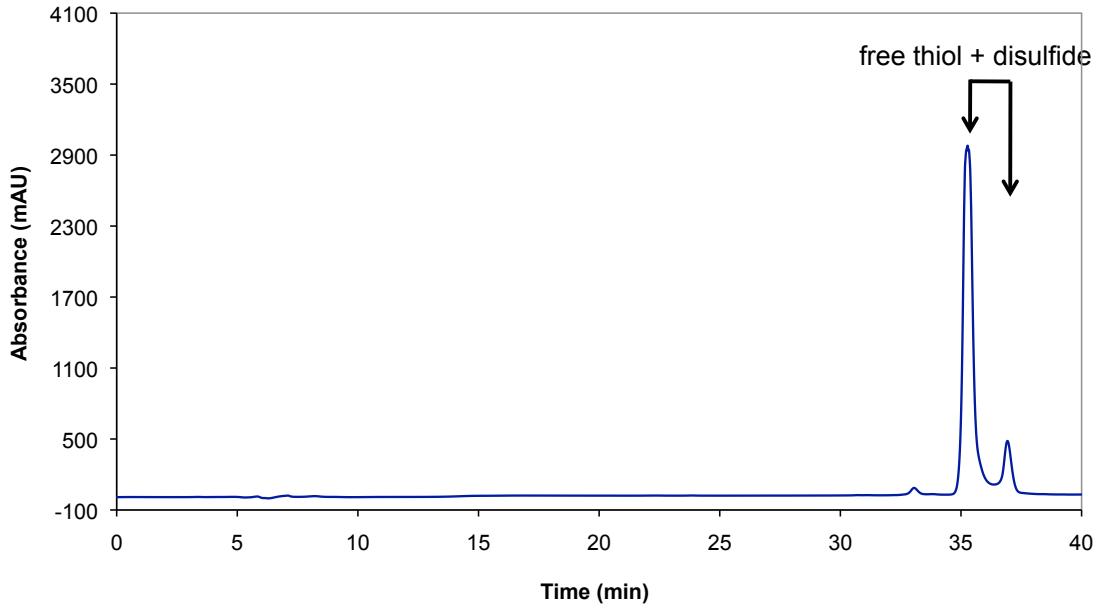
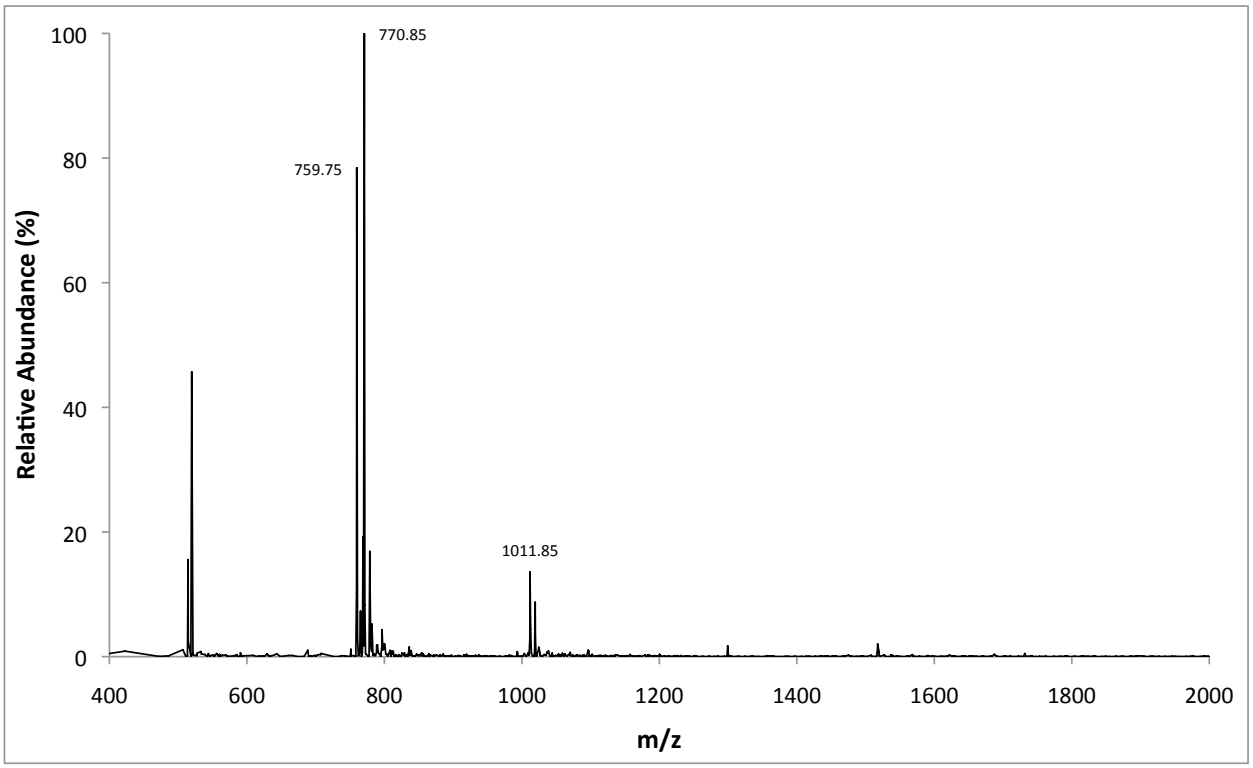


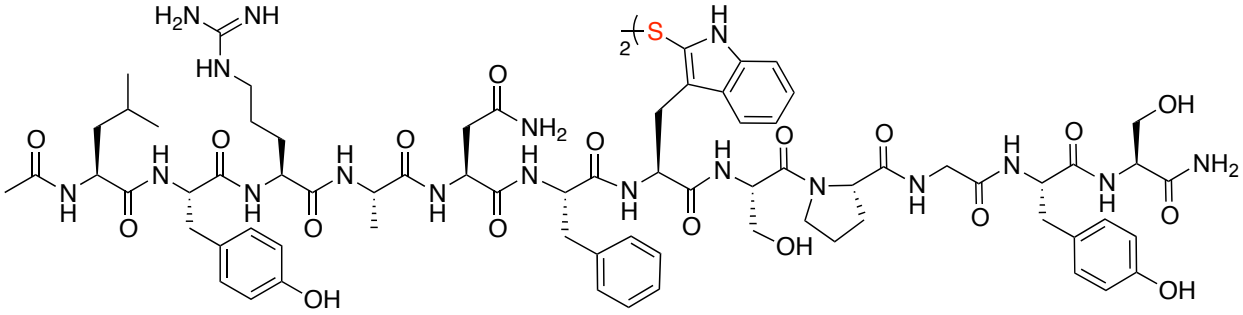
Figure S17. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 24 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANM-SPh **7**; d = Ac-LYRANM-OH.



Analytical HPLC: (free thiol + disulfide) R_t 35.2 min, 36.9 min; (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass (Disulfide) $[M+H^+]^+$: 3033.31, $[M+2H^+]^{2+}$: 1517.16, $[M+3H^+]^{3+}$: 1011.78, $[M+4H^+]^{4+}$: 759.08, $[M+3H^+ + K^+]^{4+}$: 768.57; Mass Found (ESI^+); 1011.78 $[M+3H^+]^{3+}$, 759.75 $[M+4H^+]^{4+}$, 770.85 $[M+3H^+ + K^+]^{4+}$



Ac-LYRANF(2-SH)WSPGYS-NH_2 (Disulfide)



Ligation of H-(2-SH)WSPGYS-NH_2 **4** (2.1 mg, 2.9 μmol) and Ac-LYRANF-SPh **8** (3.5 mg, 3.8 μmol) was performed as outlined in the general procedures. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (2.7 mg, 65% yield), which quickly oxidized in air to yield the disulfide dimer.

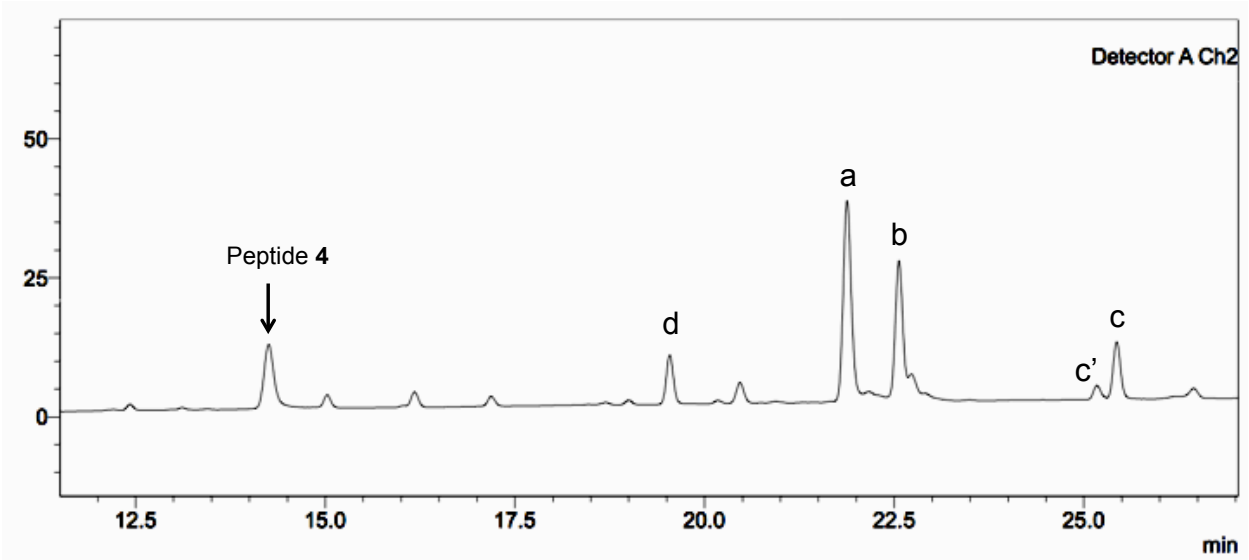


Figure S18. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 3.25 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANF-SPh **8**; c' = Ac-LYRANF-SPh (Phe epimer); d = Ac-LYRANF-OH.

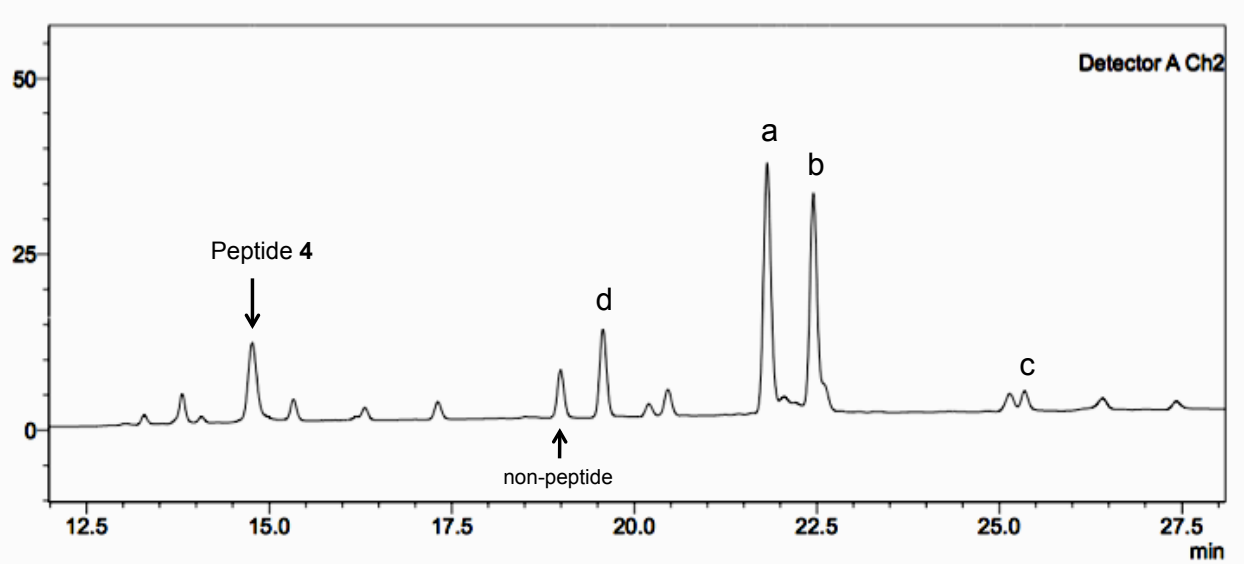
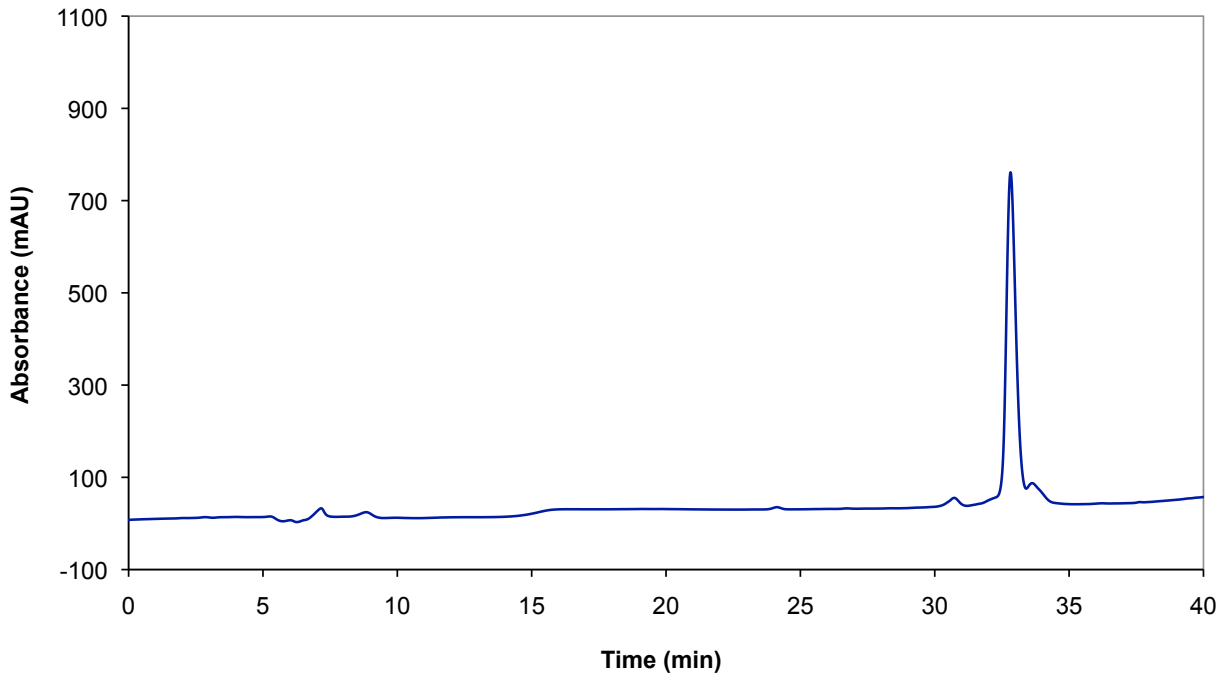
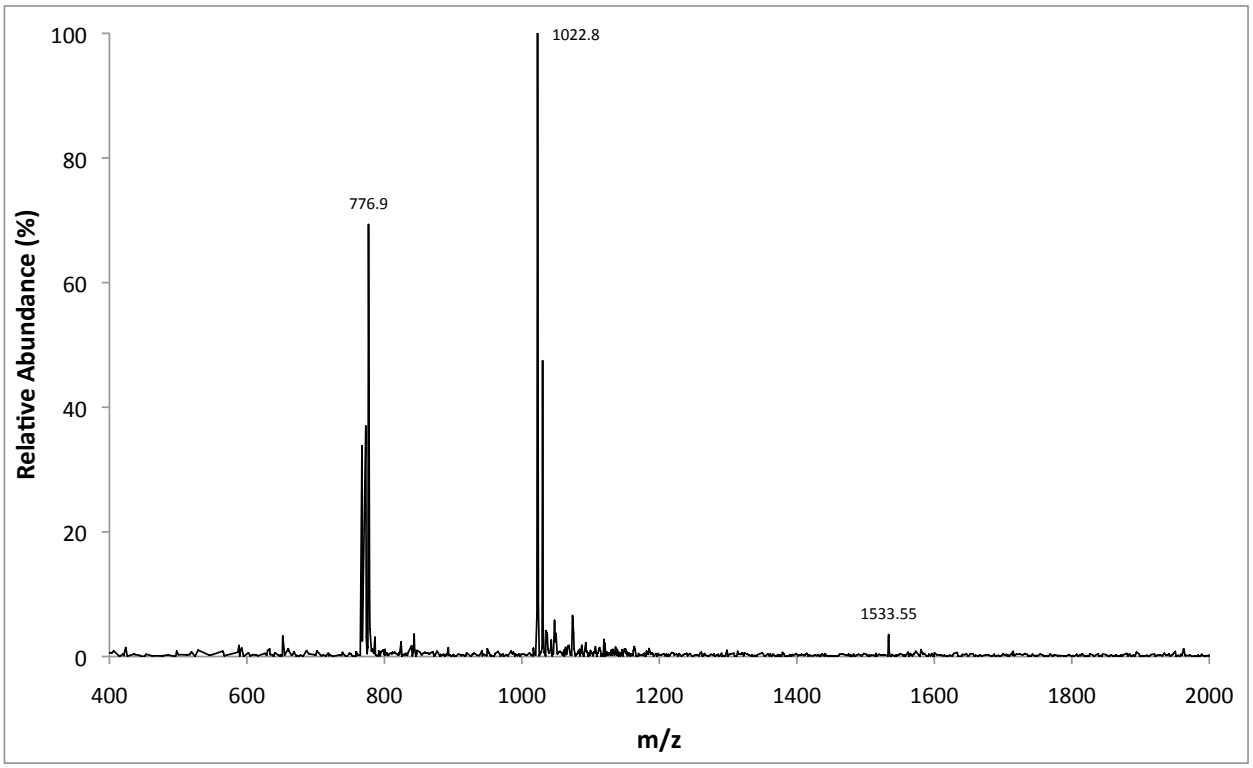


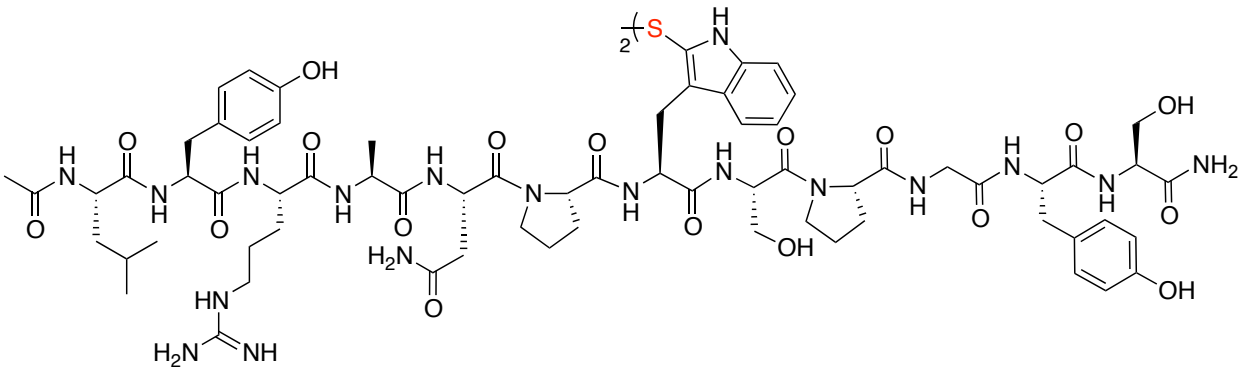
Figure S19. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 24 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANF-SPh **8**; d = Ac-LYRANF-OH.



Analytical HPLC: R_t 32.8 min (0-70% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 3065.37, $[M+2H^+]^{2+}$: 1533.19, $[M+3H^+]^{3+}$: 1022.46, $[M+3H^+{+}K^+]^{4+}$: 776.59; Mass Found (ESI^+); 1533.55 $[M+2H^+]^{2+}$, 1022.8 $[M+3H^+]^{3+}$, 776.9 $[M+3H^+{+}K^+]^{4+}$



Ac-LYRANP(2-SH)WSPGYS-NH_2 (Disulfide)



Ligation of H-(2-SH)WSPGYS-NH_2 **4** (1.1 mg, 1.5 μmol) and Ac-LYRANP-SPh **9** (2.1 mg, 2.4 μmol) was performed as outlined in the general procedures. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (1.2 mg, 58% yield), which quickly oxidized in air to yield the disulfide dimer.

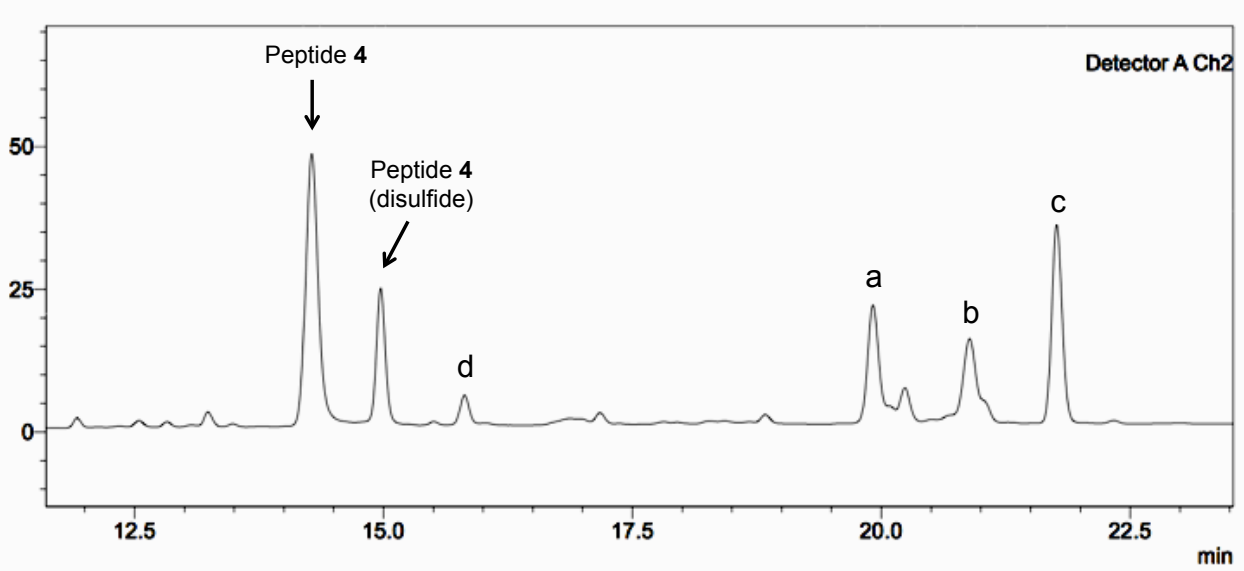


Figure S20. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 3.5 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANP-SPh **9**; d = Ac-LYRANP-OH.

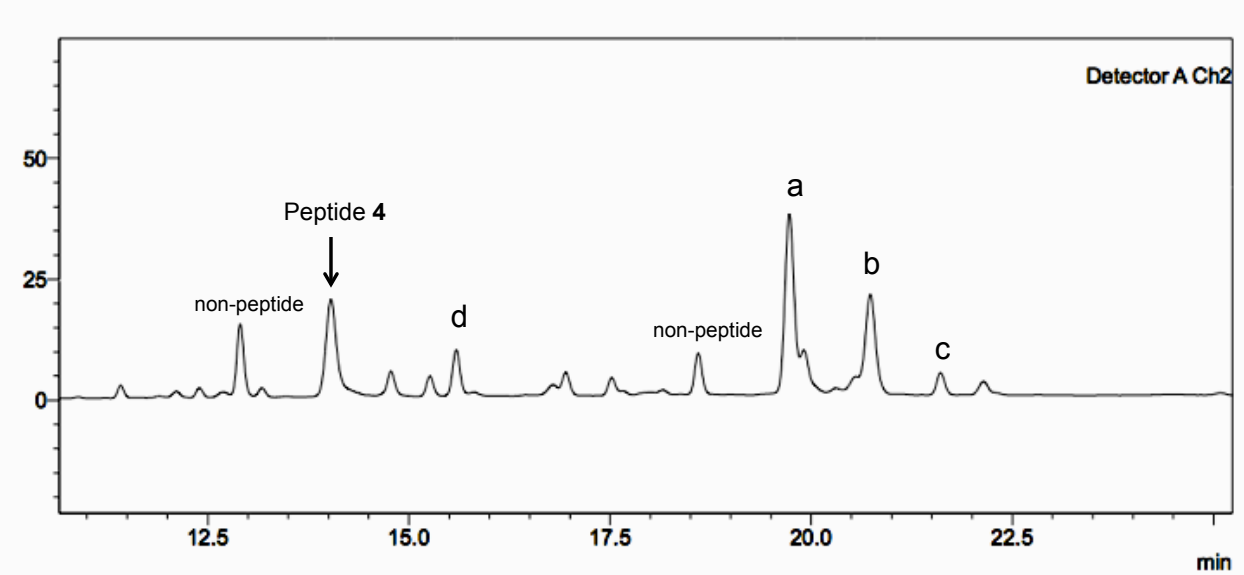
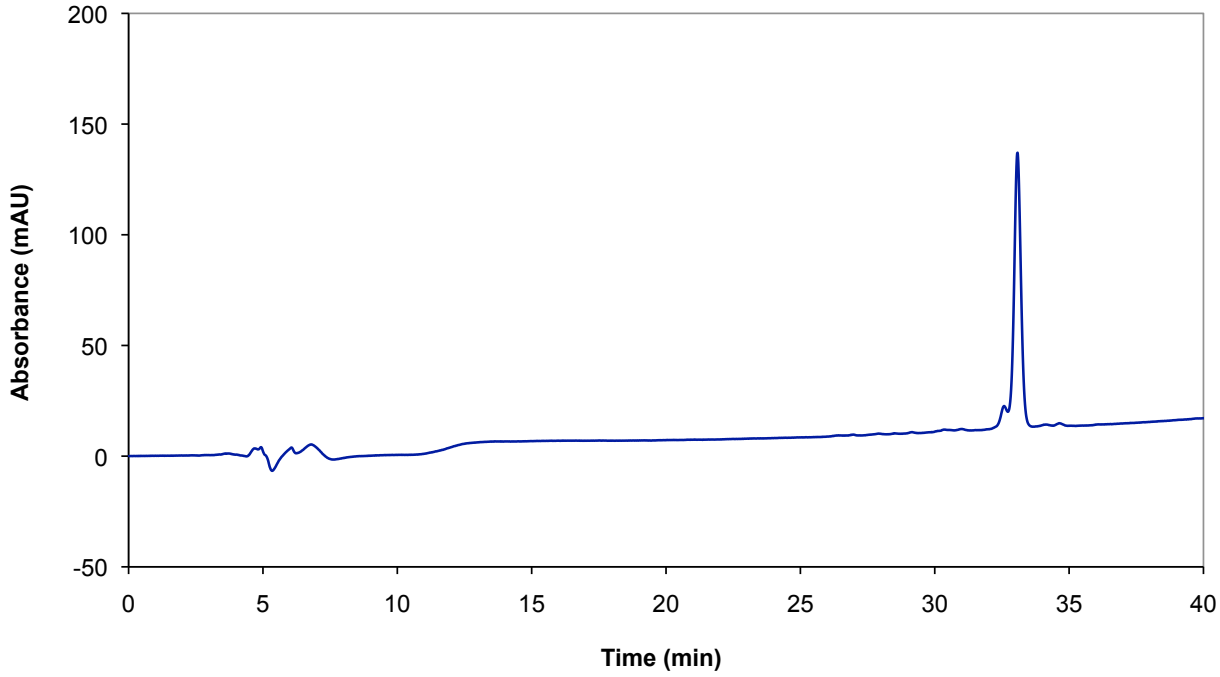
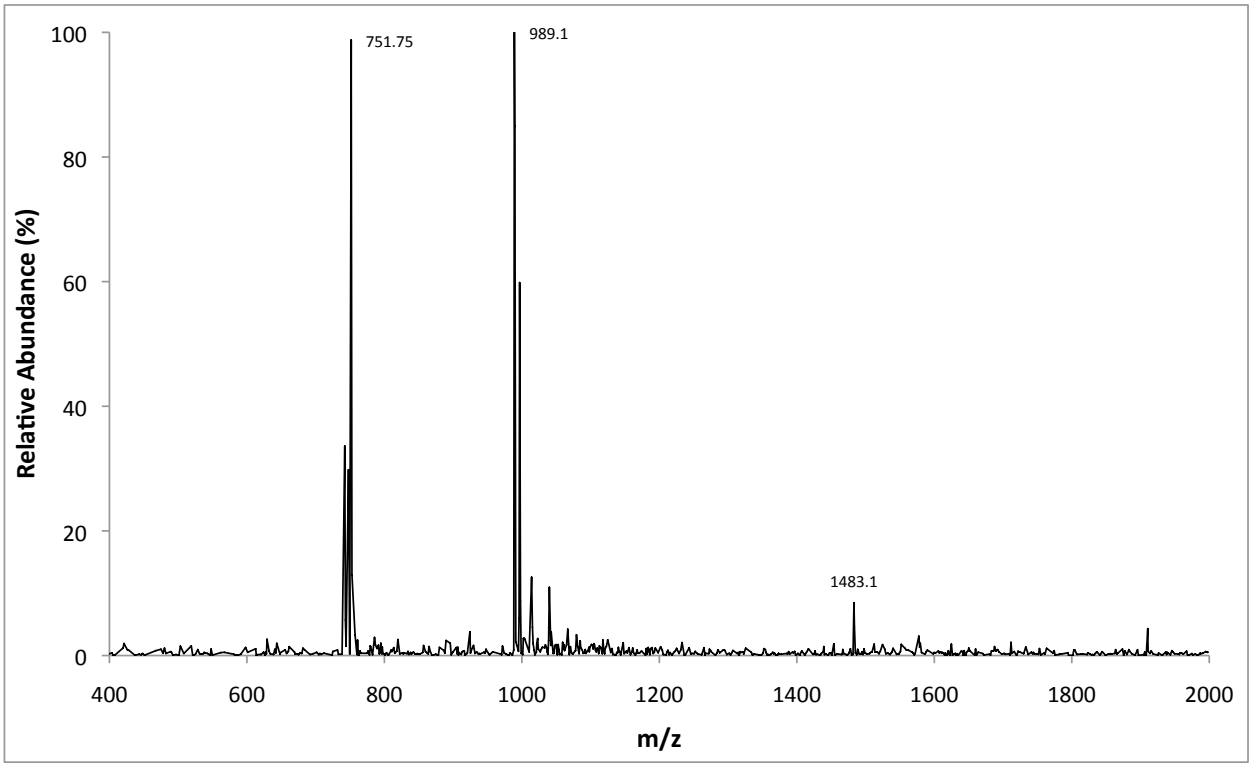


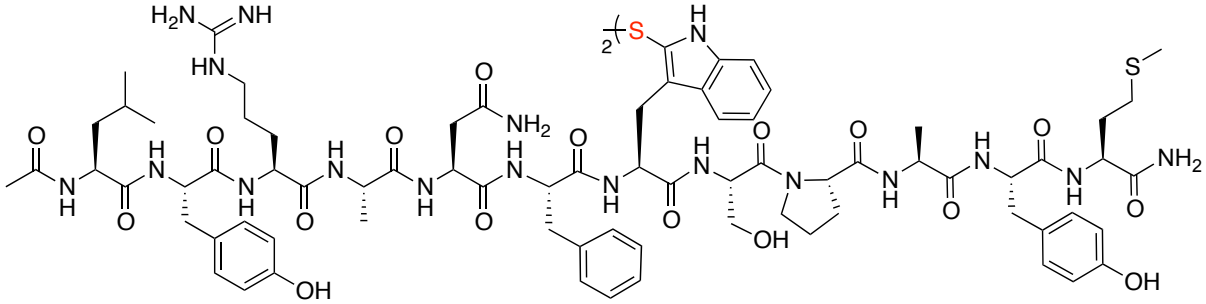
Figure S21. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 24 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANP-SPh **9**; d = Ac-LYRANP-OH.



Analytical HPLC: R_t 33.1 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 2965.34, $[M+2H^+]^{2+}$: 1483.17, $[M+3H^+]^{3+}$: 989.12, $[M+3H^++K^+]^{4+}$: 751.58; Mass Found (ESI^+); 1483.1 $[M+2H^+]^{2+}$, 989.1 $[M+3H^+]^{3+}$, 751.75 $[M+3H^++K^+]^{4+}$



Ac-LYRANF(2-SH)WSPAYM-NH_2 (Disulfide)



Ligation of H-(2-SH)WSPAYM-NH_2 **10** (1.1 mg, 1.3 μmol) and Ac-LYRANF-SPh **8** (1.7 mg, 1.7 μmol) was performed as outlined in the general procedures. At t = 3 h, LC-MS analysis showed complete hydrolysis of thioester, and additional Ac-LYRANF-SPh **8** (1.0 mg, 1.0 μmol) was added to the ligation reaction. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (1.7 mg, 78% yield), which quickly oxidized in air to yield the disulfide dimer.

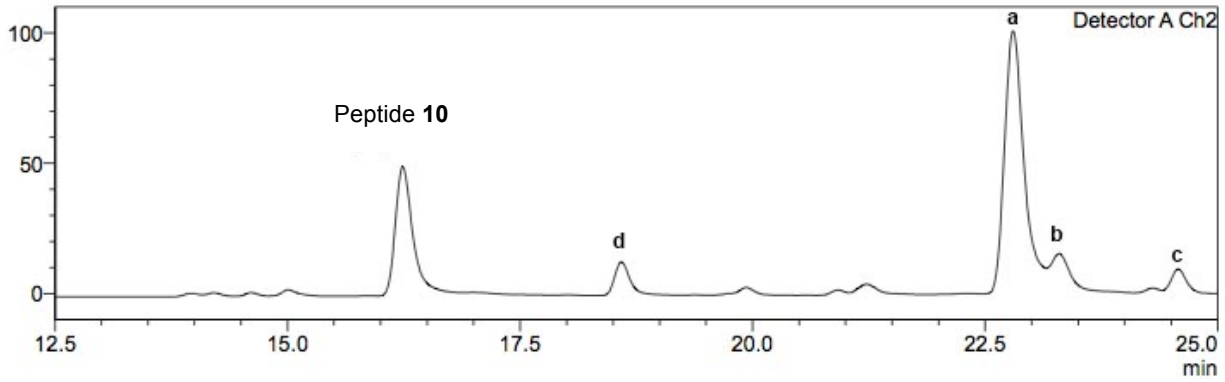


Figure S22. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 3 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANF-SPh **8**; d = Ac-LYRANF-OH.

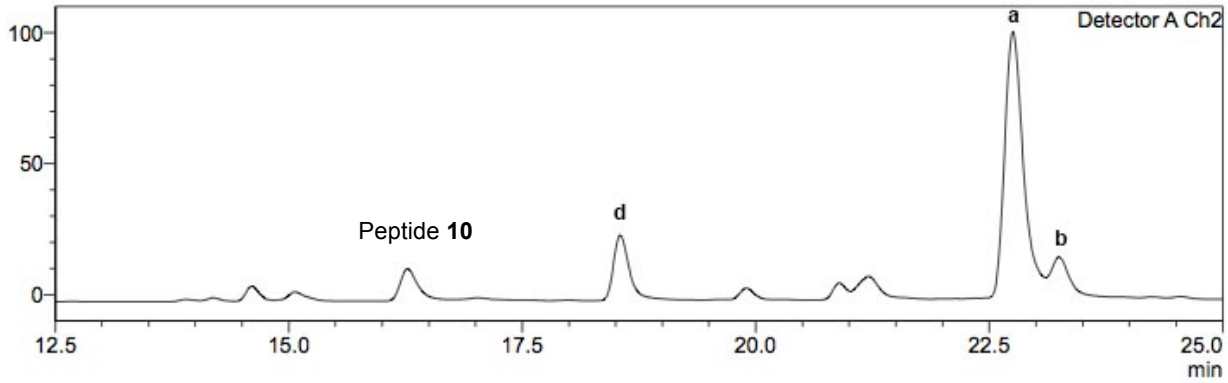
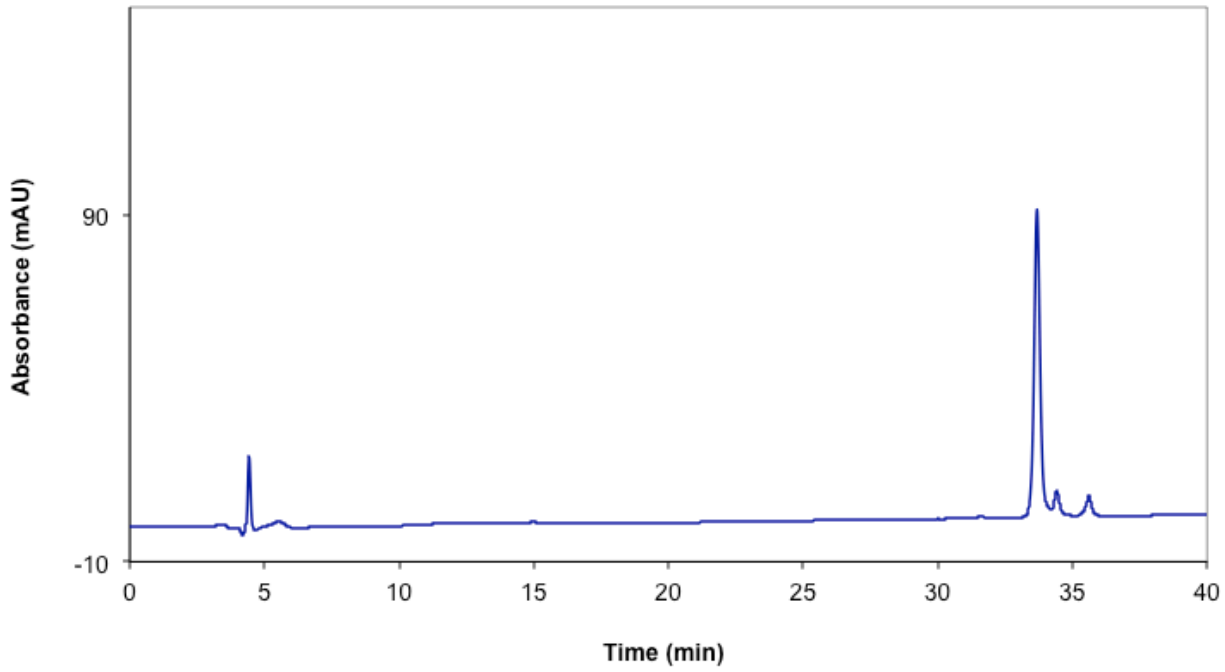
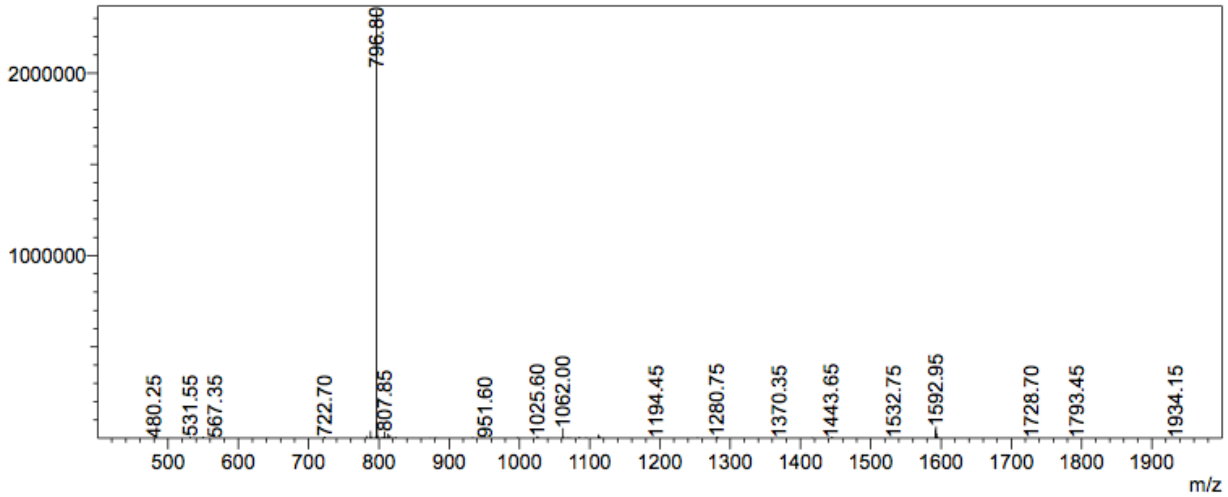


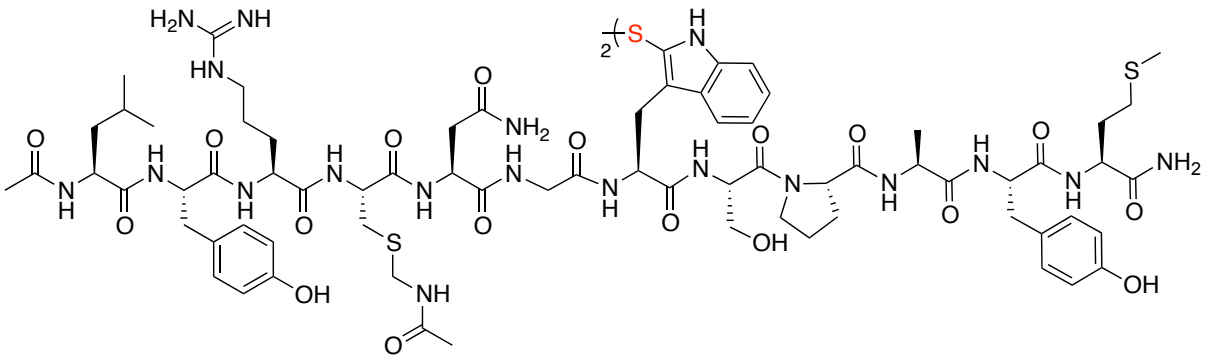
Figure S23. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 22 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRANF-SPh **8**; d = Ac-LYRANF-OH.



Analytical HPLC (free thiol): R_t 33.6 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 1591.72, $[M+2H^+]^{2+}$: 796.4; Mass Found (ESI^+); 1592.9 $[M+H^+]^+$, 796.8 $[M+2H^+]^{2+}$



Ac-LYRC(Acm)NG(2-SH)WSPAYM-NH_2 (Disulfide)



Ligation of H-(2-SH)WSPAYM-NH_2 **10** (1.9 mg, 2.1 μmol) and Ac-LYRC(Acm)NG-SPh **14** (3.5 mg, 3.4 μmol) was performed as outlined in the general procedures. At t = 3 h, LC-MS analysis showed complete hydrolysis of thioester, and additional Ac-LYRC(Acm)NG-SPh **14** (1.1 mg, 1.1 μmol) was added to the ligation reaction. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (2.2 mg, 61% yield), which quickly oxidized in air to yield the disulfide dimer.

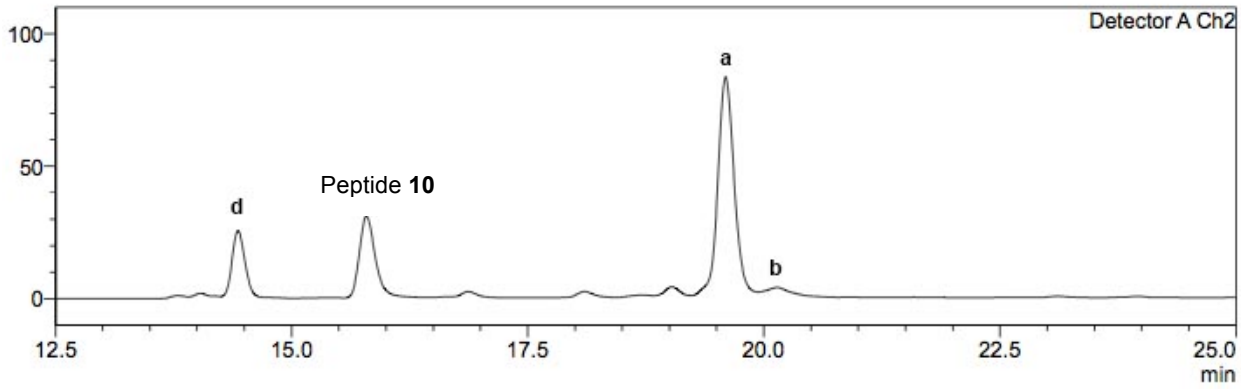


Figure S24. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 3 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRC(Acm)NG-SPh **14**; d = Ac-LYRC(Acm)NG-OH.

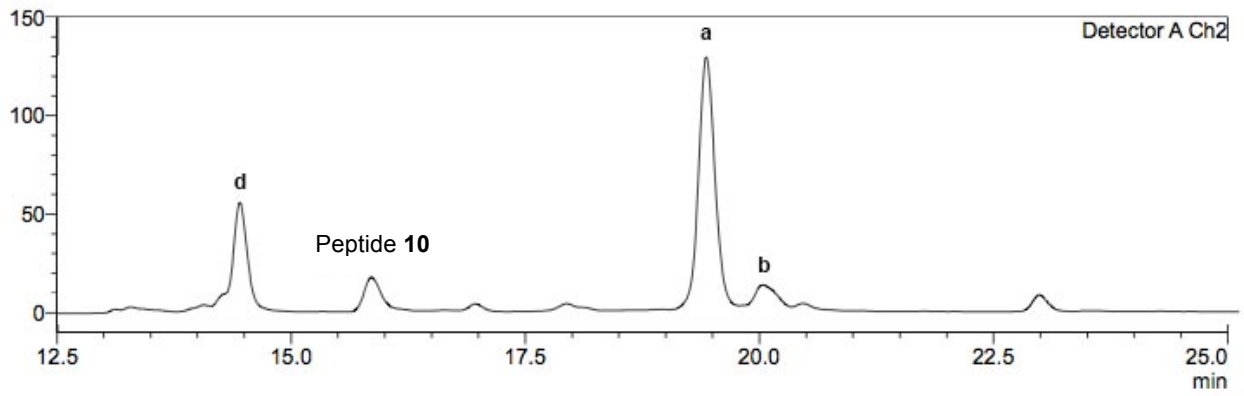
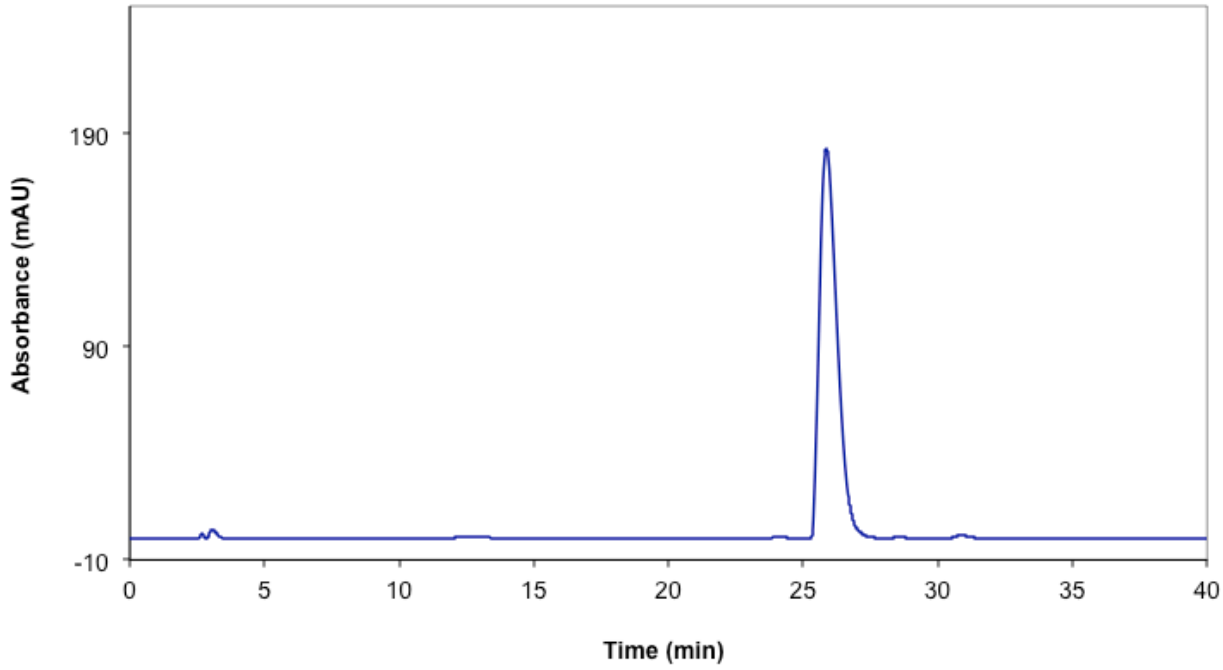
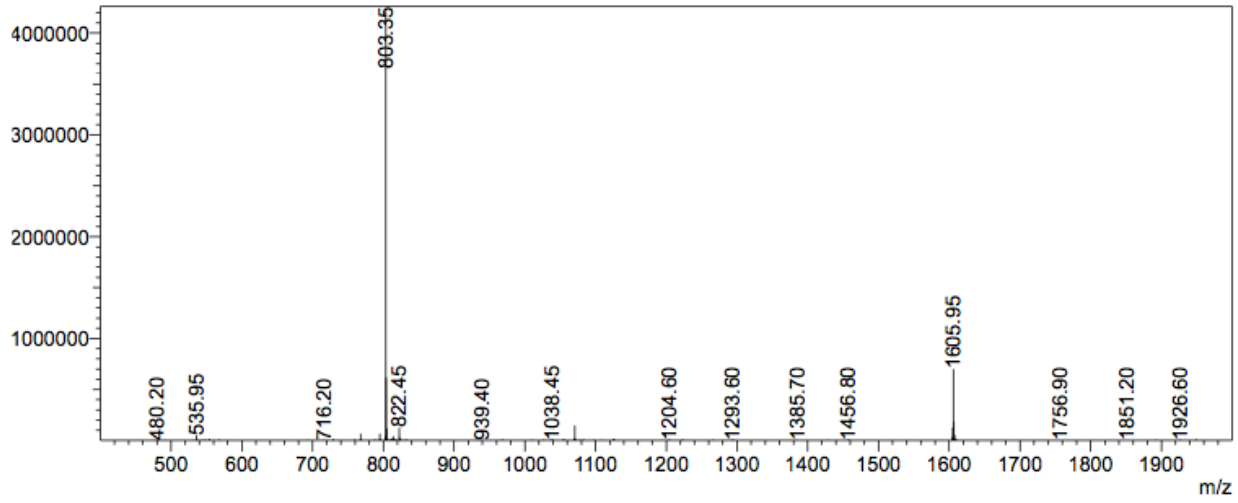


Figure S25. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude ligation mixture (t = 22 h); a = ligation product (free thiol); b = ligation product (disulfide); c = Ac-LYRC(Acm)NG-SPh **14**; d = Ac-LYRC(Acm)NG-OH.

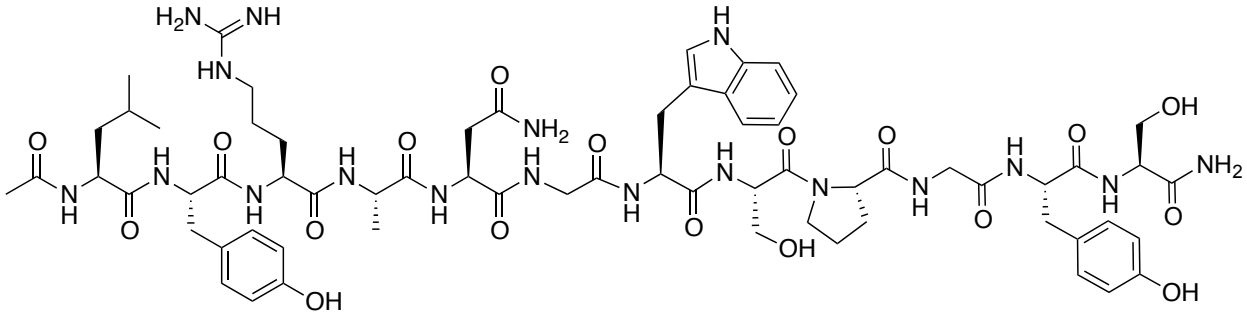


Analytical HPLC (free thiol): R_t 25.8 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 1604.68, $[M+2H^+]^{2+}$: 802.8; Mass Found (ESI^+); 1605.95 $[M+H^+]^+$, 803.35 $[M+2H^+]^{2+}$



Desulfurizations

Ac-LYRANGWSPGYS-NH_2



Desulfurization of Ac-LYRANG(2-SH)WSPGYS-NH_2 (2.9 mg, 2.0 μmol) was carried out according to the general procedures. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (2.0 mg, 71% yield).

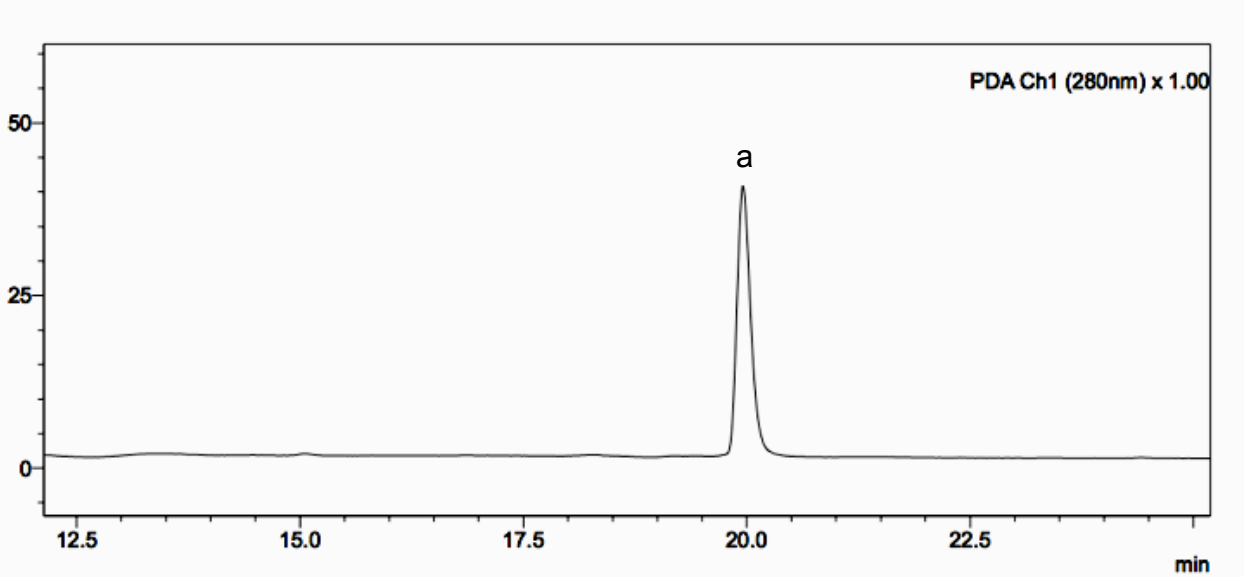
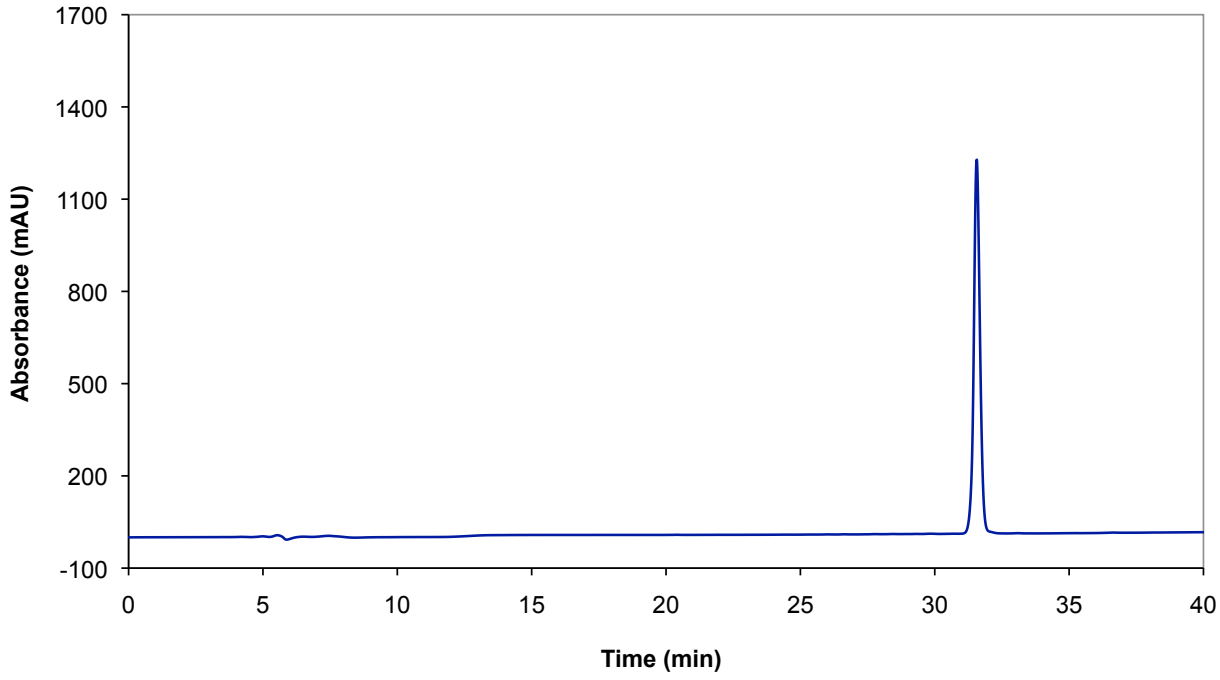
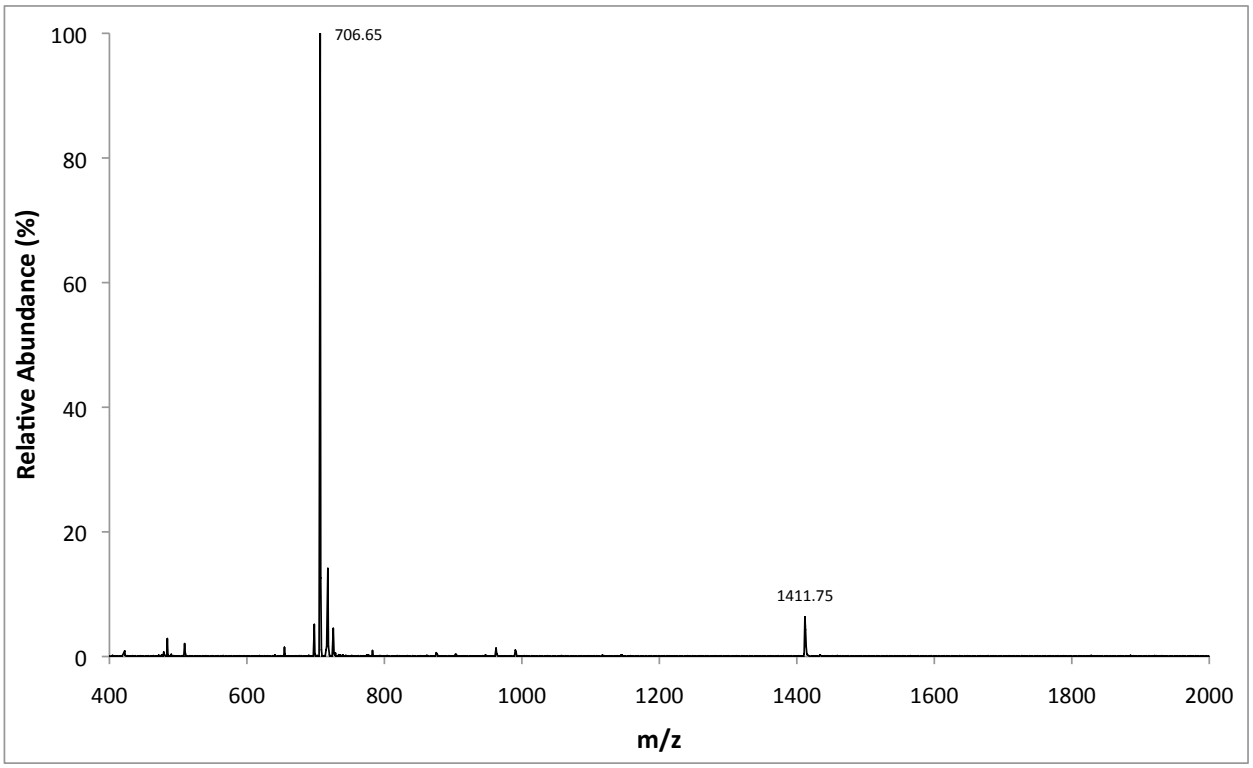


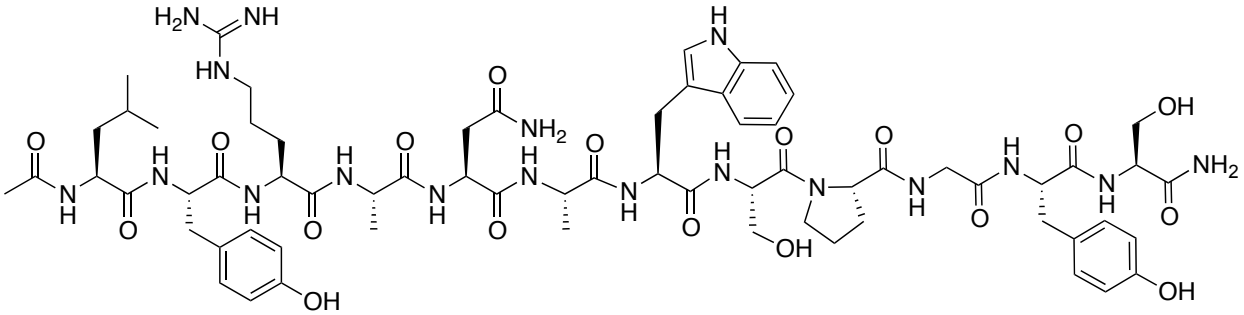
Figure S26. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude desulfurization mixture (t = 2 h); a = desulfurization product.



Analytical HPLC: R_t 31.6 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 1411.68, $[M+2H^+]^{2+}$: 706.34; Mass Found (ESI^+); 1411.75 $[M+H^+]^+$, 706.65 $[M+2H^+]^{2+}$



Ac-LYRANAWSPGYS-NH_2



Desulfurization of Ac-LYRANA(2-SH)WSPGYS-NH_2 (3.0 mg, 2.1 μmol) was carried out according to the general procedures. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (2.6 mg, 89% yield).

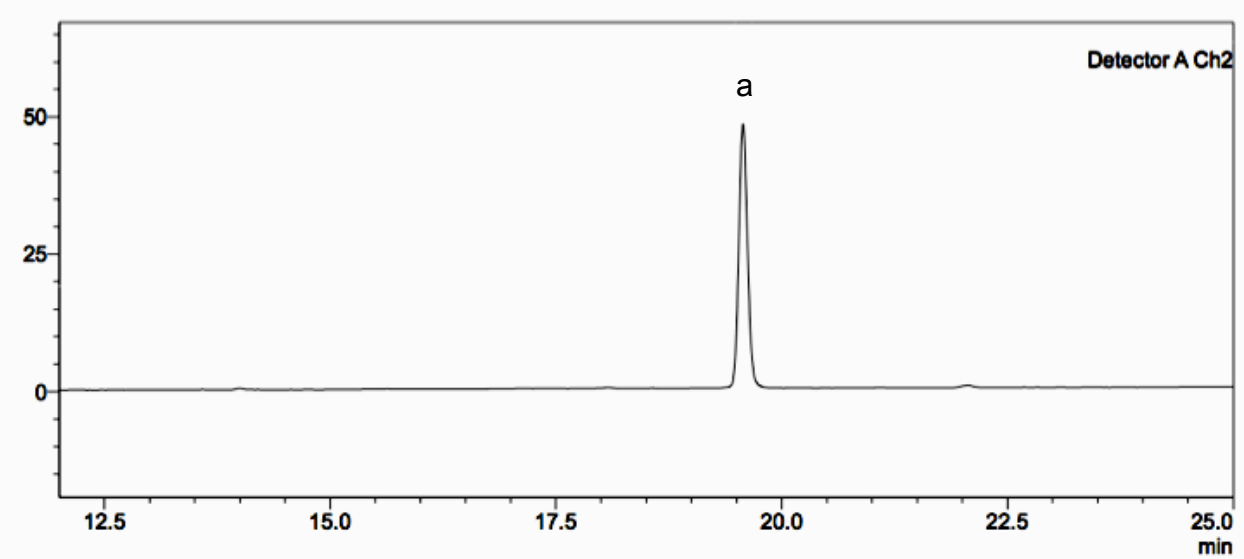
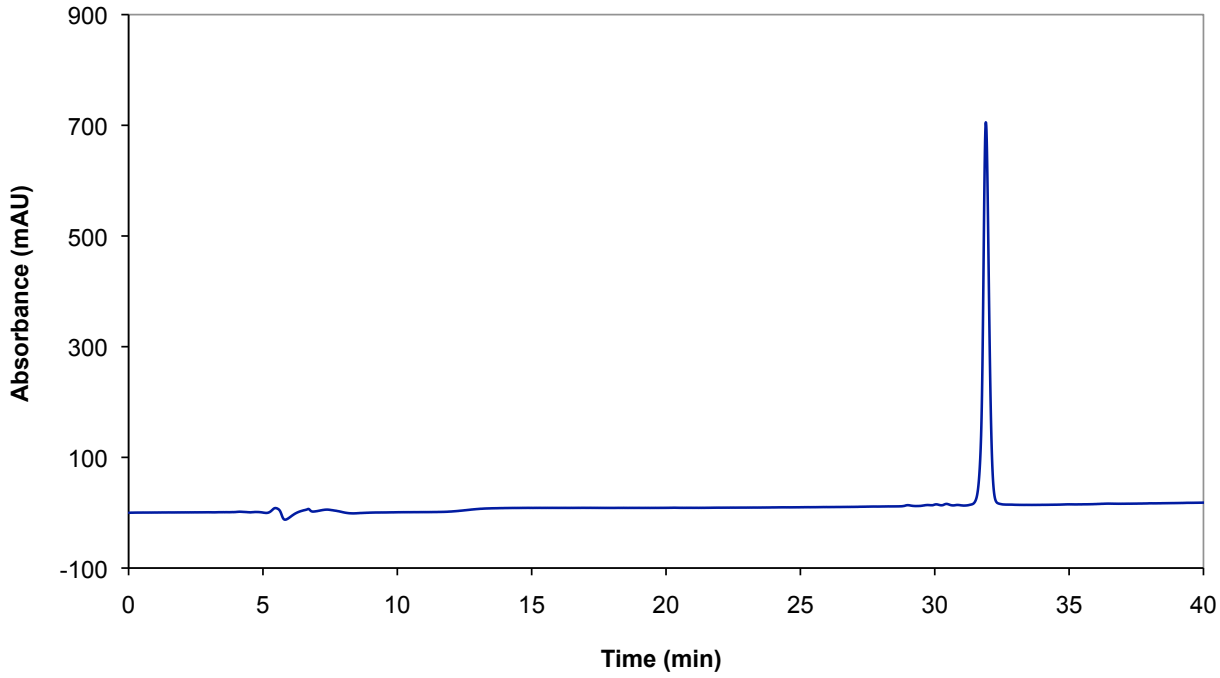
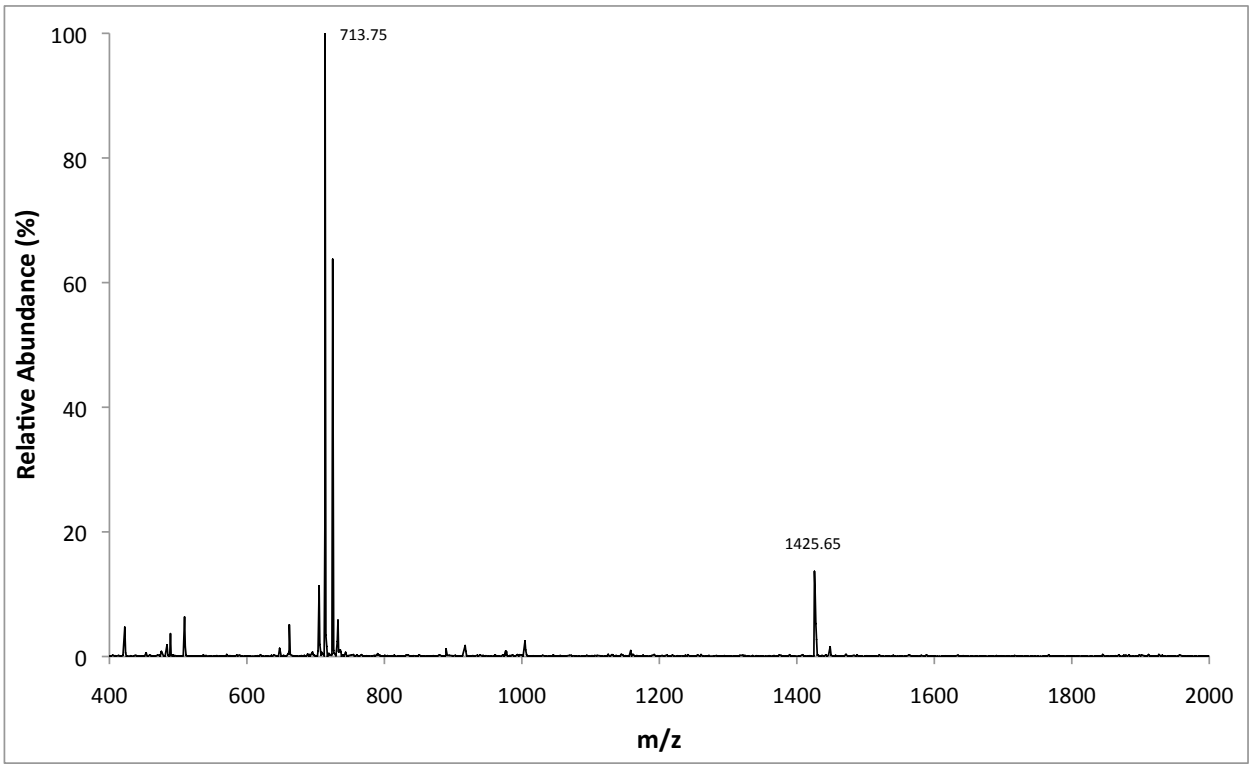


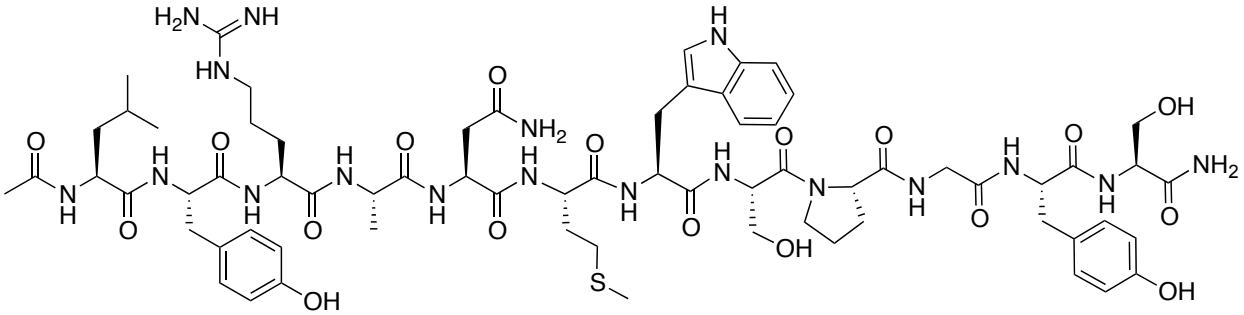
Figure S27. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude desulfurization mixture (t = 2 h); a = desulfurization product.



Analytical HPLC: R_t 31.8 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 1425.69, $[M+2H^+]^{2+}$: 713.35; Mass Found (ESI^+); 1425.65 $[M+H^+]^+$, 713.75 $[M+2H^+]^{2+}$



Ac-LYRANMWSPGYS-NH_2



Desulfurization of Ac-LYRANM(2-SH)WSPGYS-NH_2 (2.2 mg, 1.4 μmol) was carried out according to the general procedures. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (1.3 mg, 61% yield).

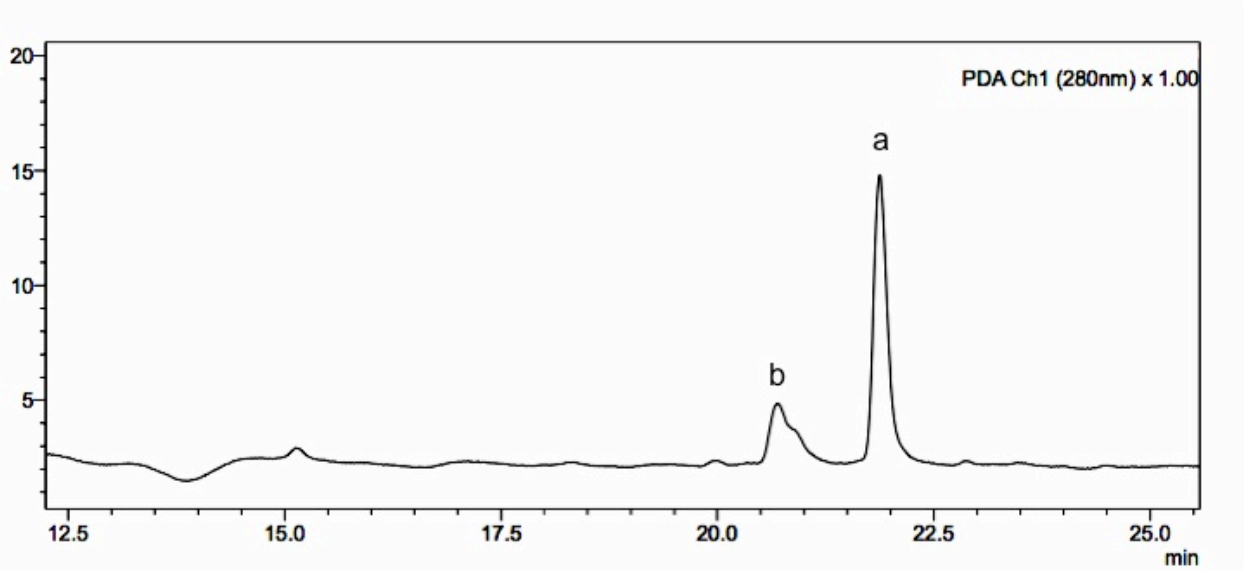
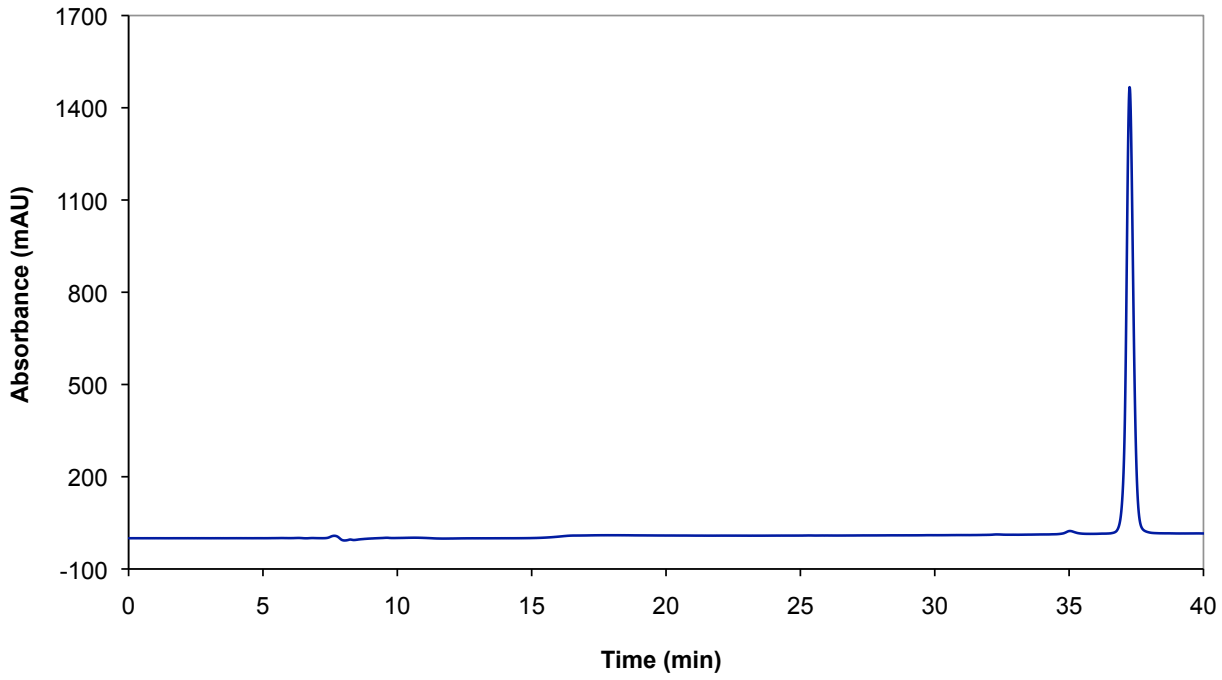
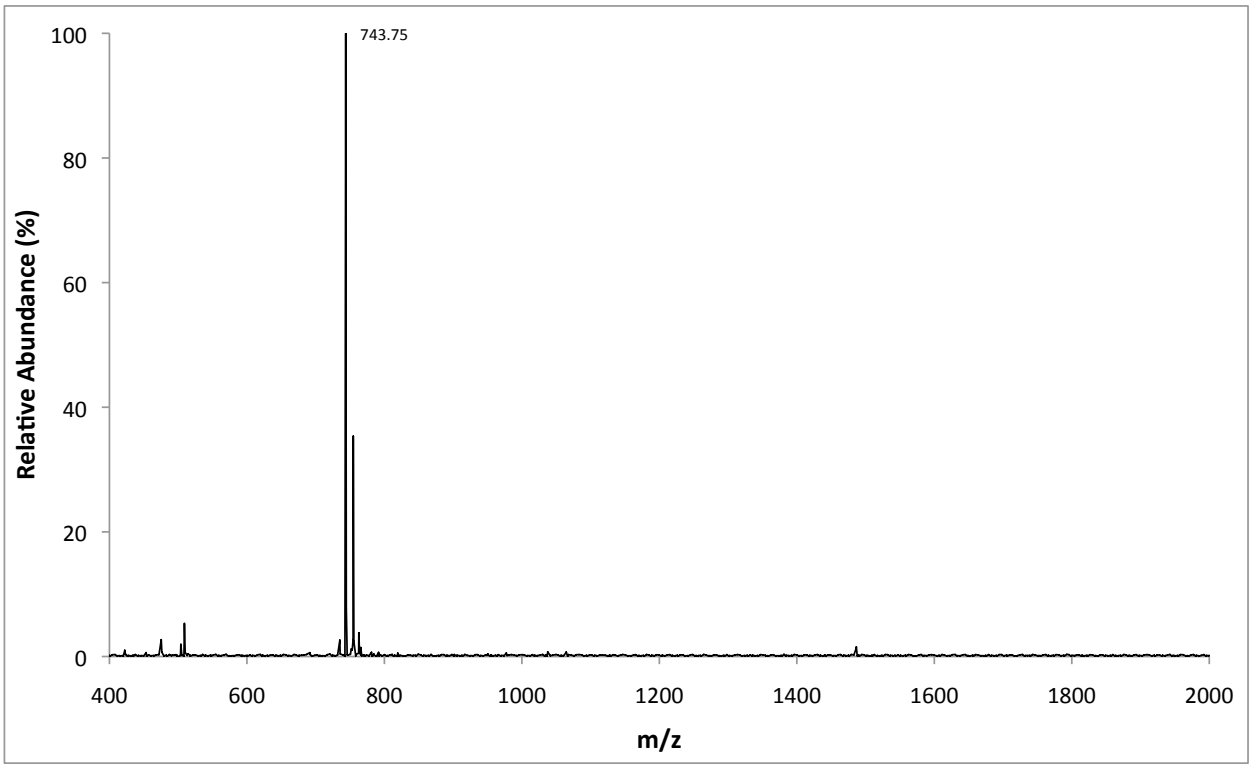


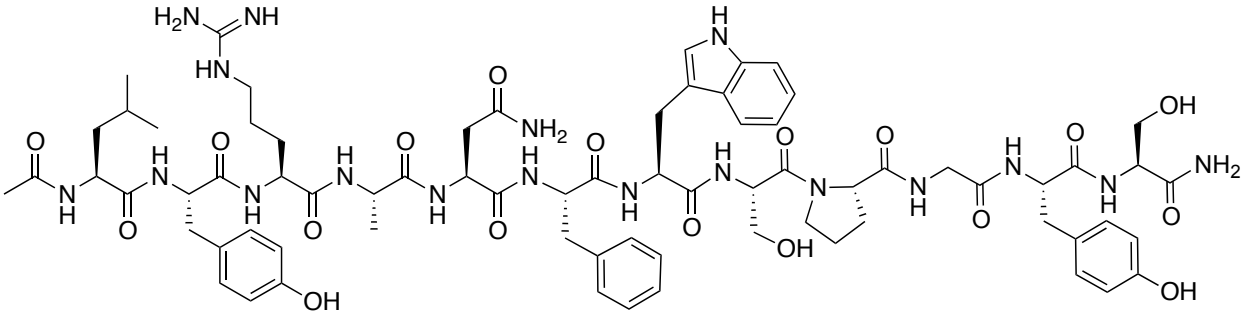
Figure S28. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude desulfurization mixture (t = 2 h); a = desulfurization product; b = desulfurization product bound to Pd.



Analytical HPLC: R_t 37.2 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 1485.69, $[M+2H^+]^{2+}$: 743.35; Mass Found (ESI^+); 743.75 $[M+2H^+]^{2+}$



Ac-LYRANFWSPGYS-NH_2



Desulfurization of Ac-LYRANF(2-SH)WSPGYS-NH_2 (2.7 mg, 1.8 μmol) was carried out according to the general procedures. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (1.9 mg, 72% yield).

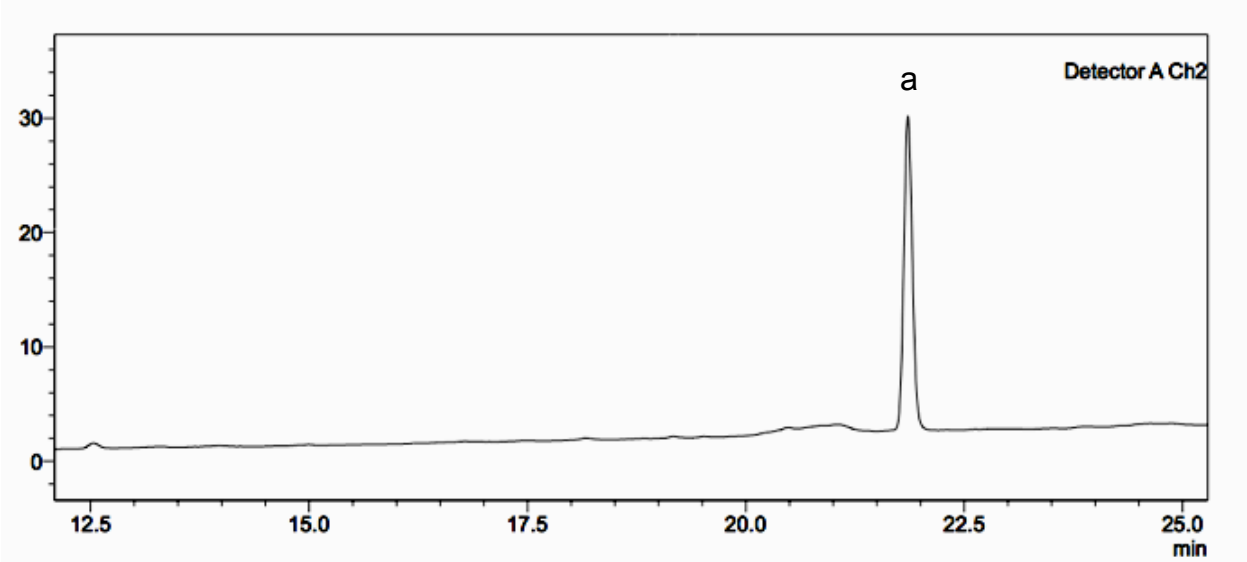
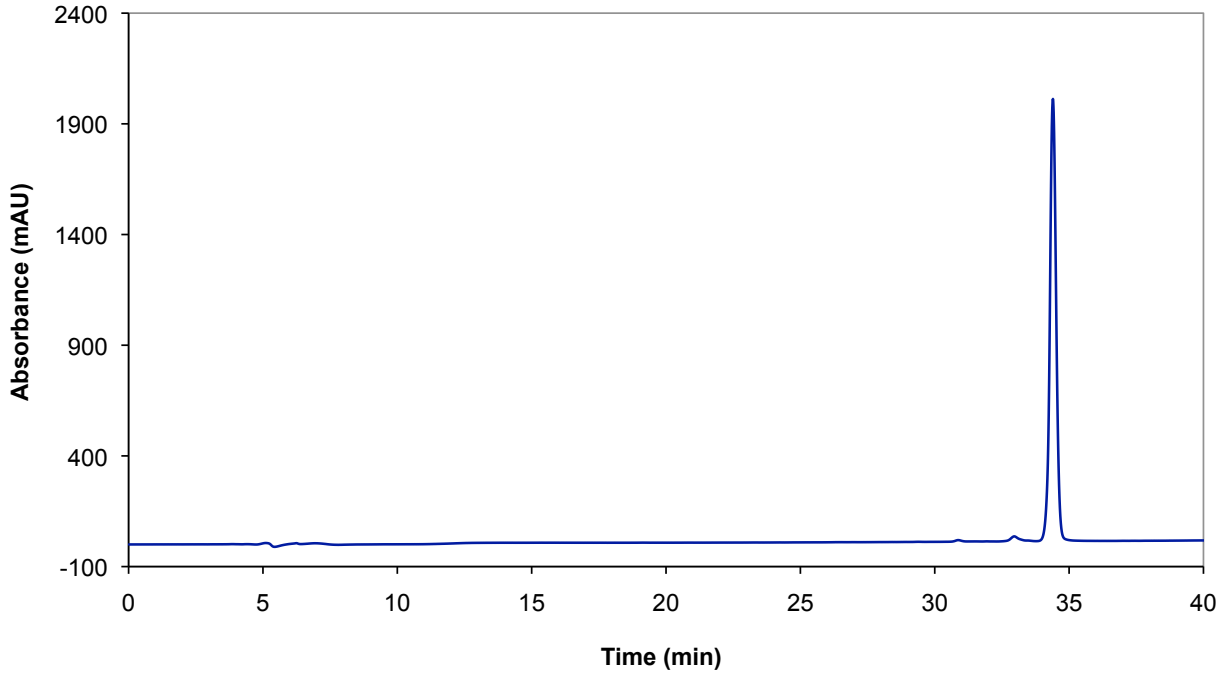
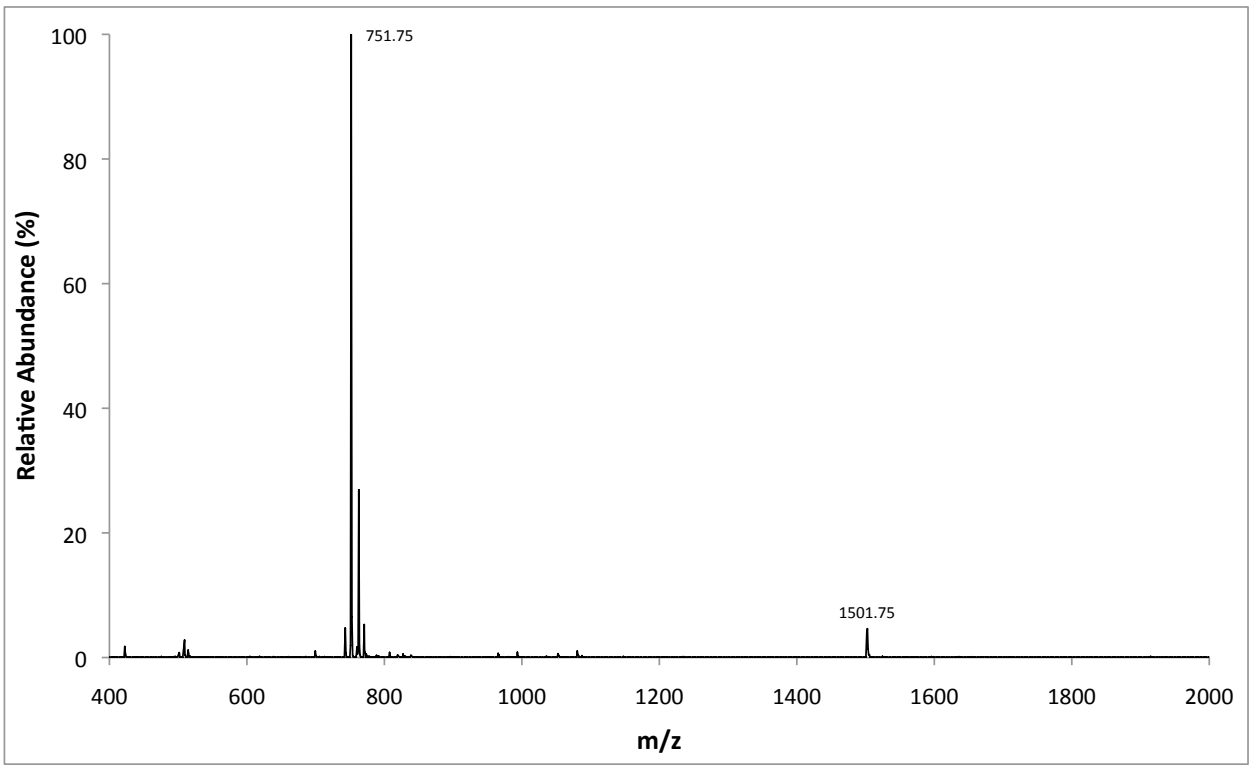


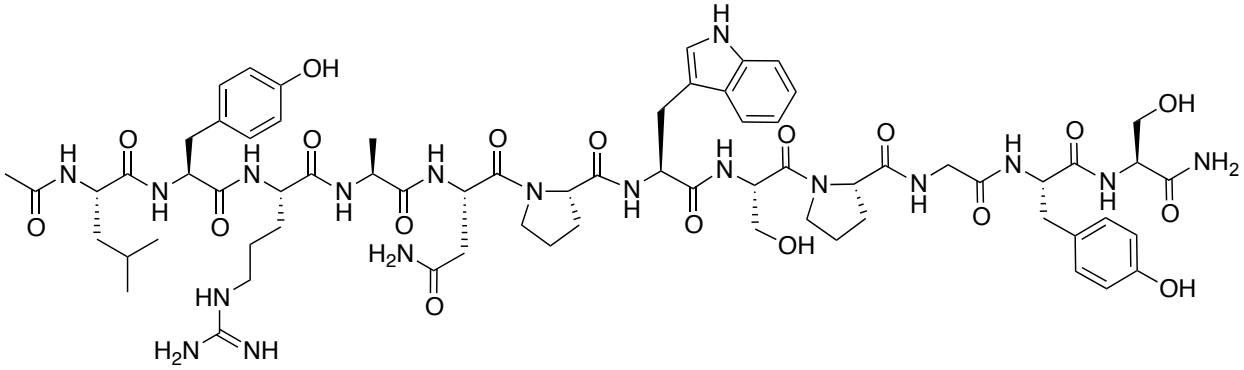
Figure S29. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude desulfurization mixture (t = 2 h); a = desulfurization product.



Analytical HPLC: R_t 34.4 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 1501.72, $[M+2H^+]^{2+}$: 751.36; Mass Found (ESI^+); 1501.75 $[M+H^+]^+$, 751.75 $[M+2H^+]^{2+}$



Ac-LYRANPWSPGYS-NH_2



Desulfurization of Ac-LYRANP(2-SH)WSPGYS-NH_2 (1.1 mg, 0.74 μmol) was carried out according to the general procedures. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (0.8 mg, 82% yield).

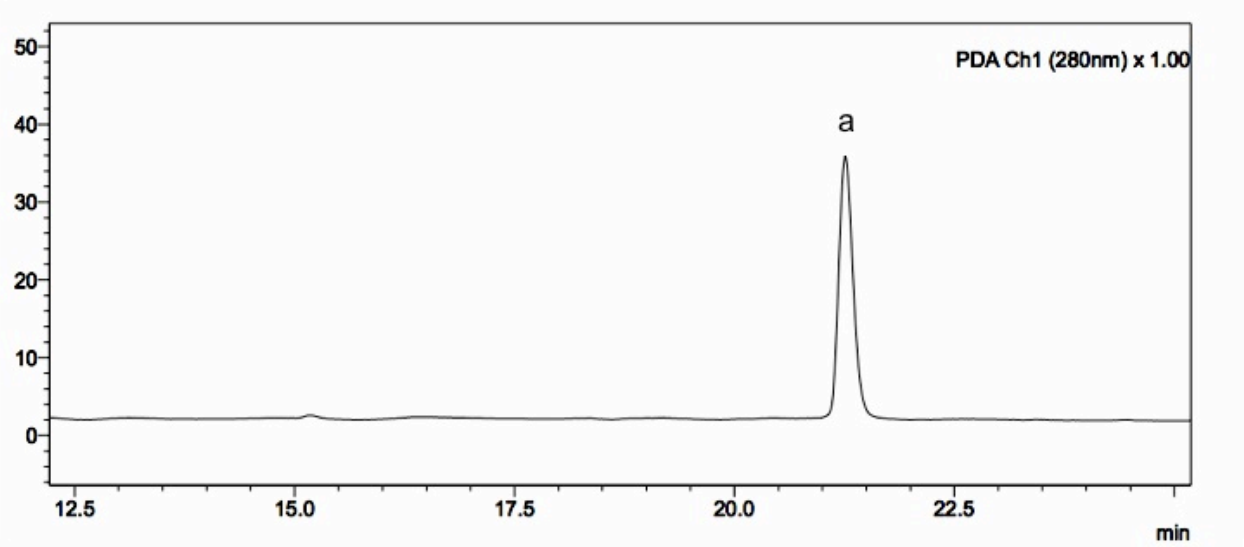
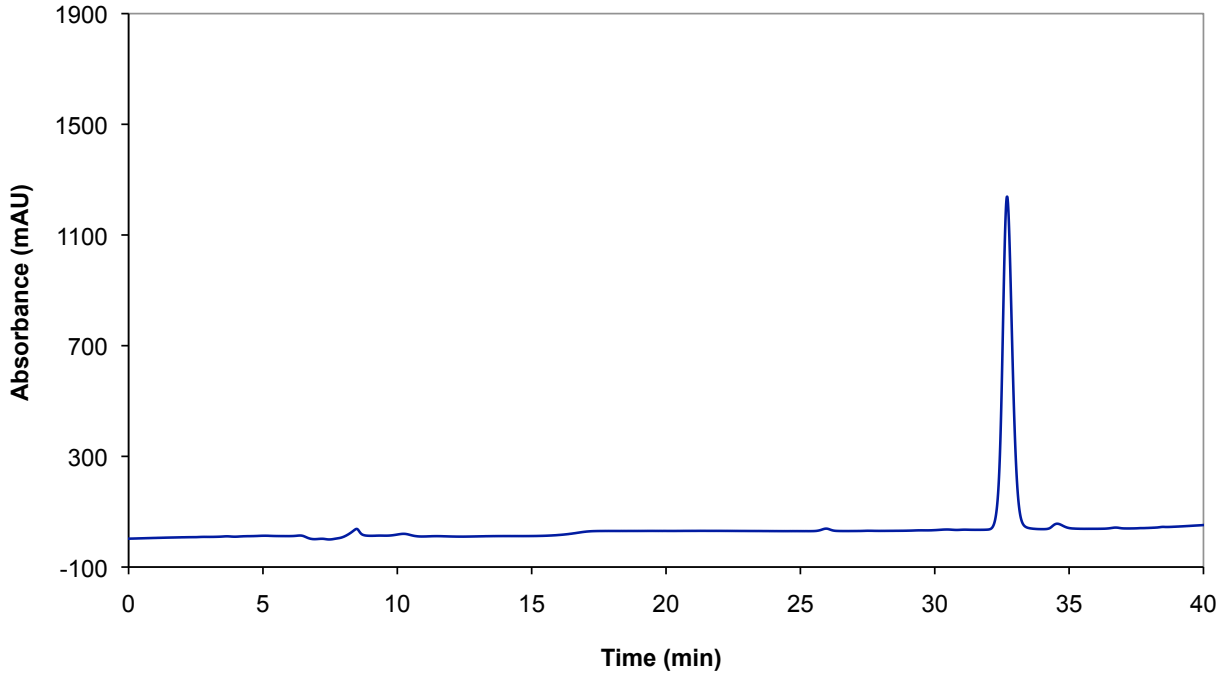
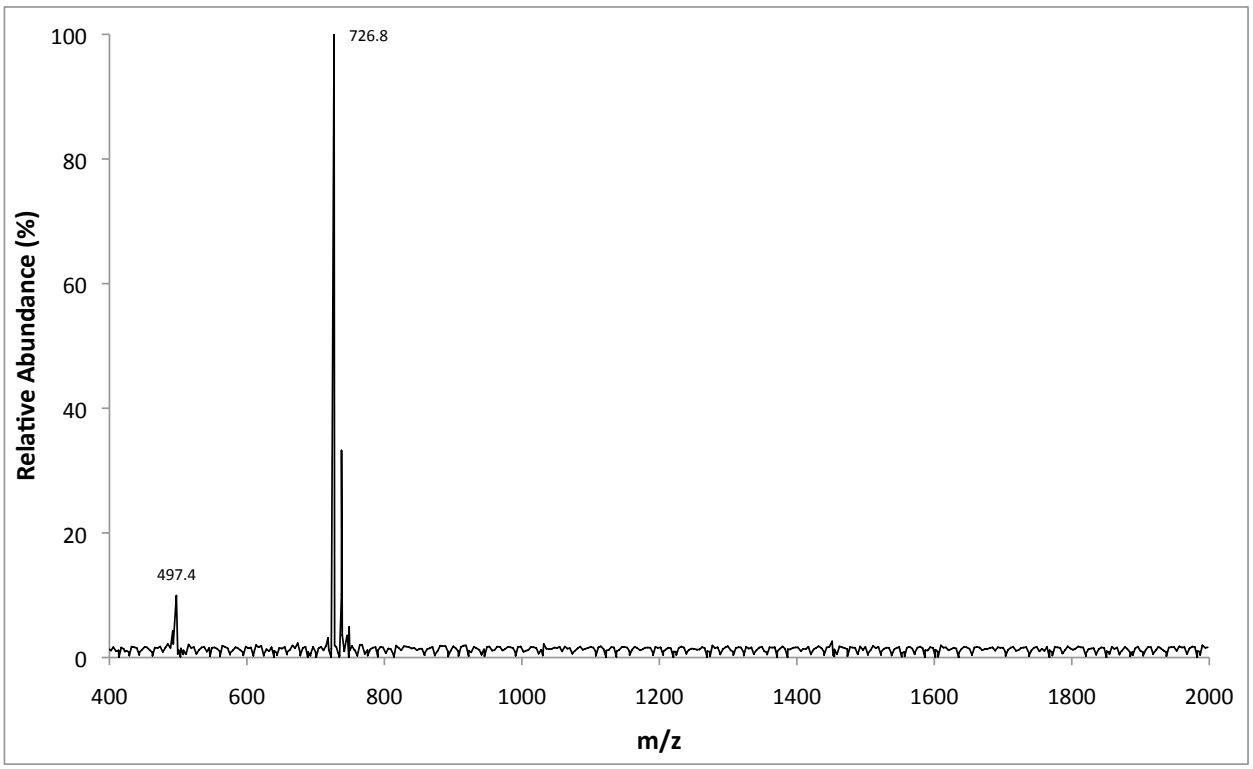


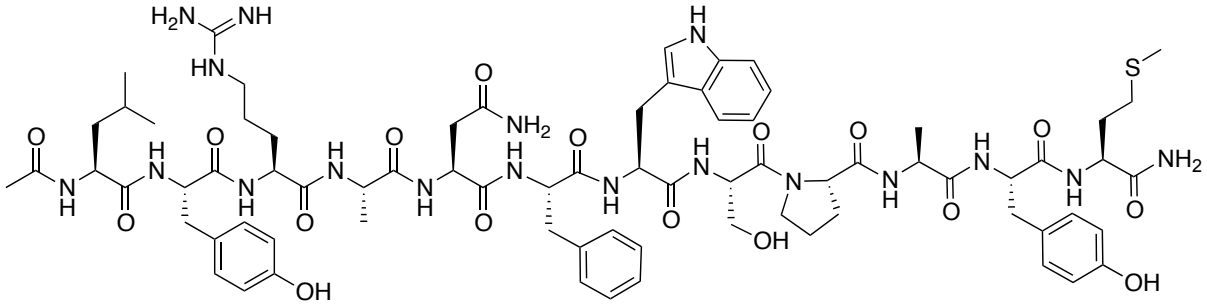
Figure S30. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude desulfurization mixture (t = 2 h); a = desulfurization product.



Analytical HPLC: R_t 32.7 min (0-70% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 1451.71, $[M+2H^+]^{2+}$: 726.36, $[M+2H^++K^+]^{2+}$: 497.23; Mass Found (ESI^+); 726.8 $[M+2H^+]^{2+}$, 497.4 $[M+2H^++K^+]^{3+}$



Ac-LYRANFWSPAYM-NH_2



Desulfurization of Ac-LYRANF(2-SH)WSPAYM-NH_2 (1.2 mg, 0.70 μmol) was carried out according to the general procedures. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (1.1 mg, 99% yield).

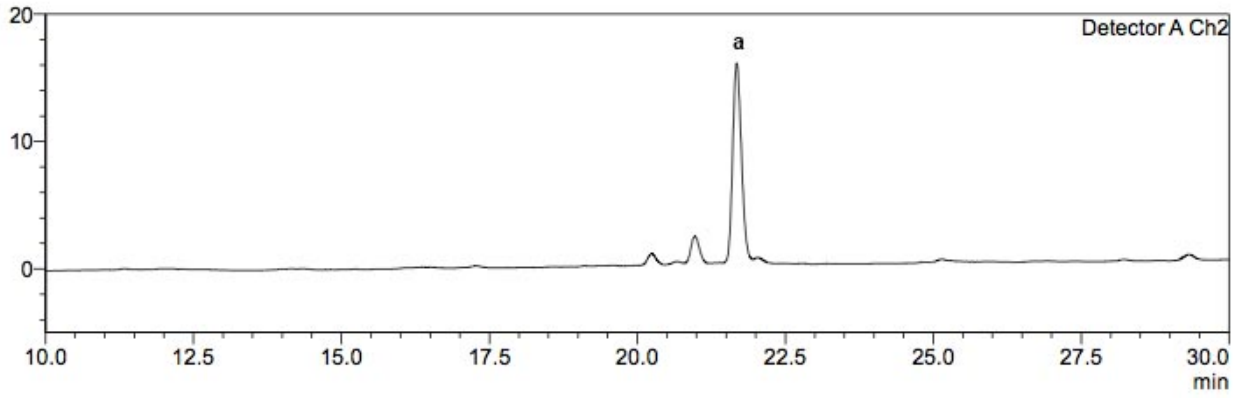
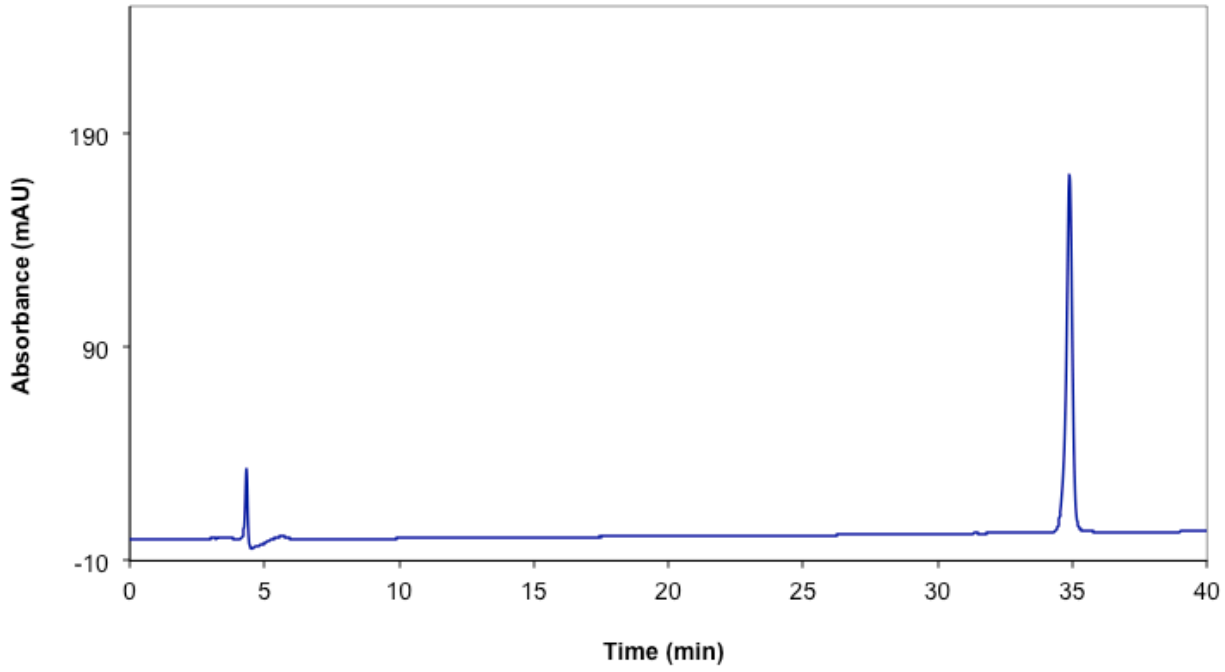
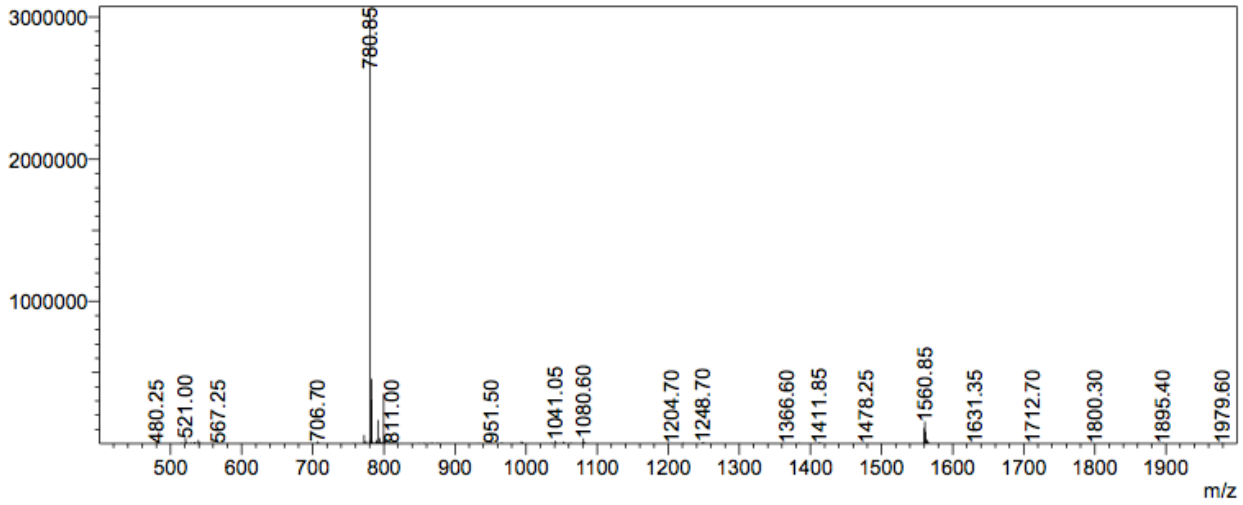


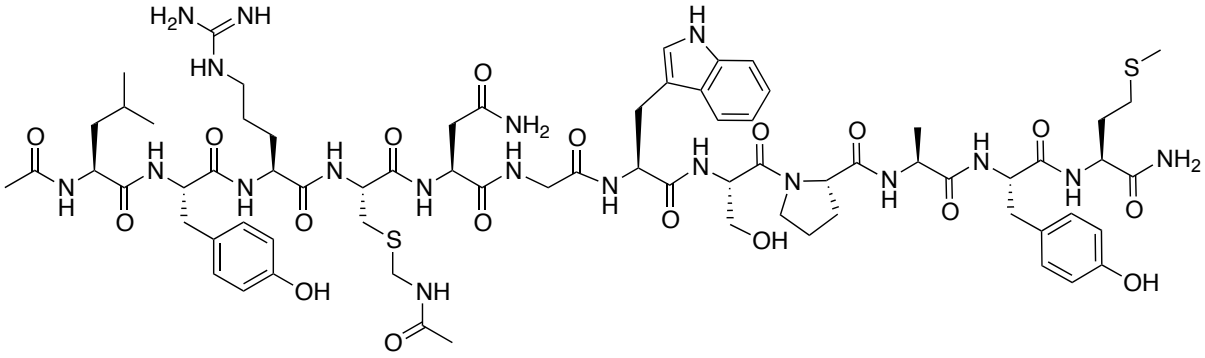
Figure S31. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude desulfurization mixture (t = 2 h); a = desulfurization product.



Analytical HPLC: R_t 34.8 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 1559.75, $[M+2H^+]^{2+}$: 780.4; Mass Found (ESI^+); 1560.85 $[M+H^+]^+$, 780.85 $[M+2H^+]^{2+}$



Ac-LYRC(Acm)NGWSPAYM-NH_2



Desulfurization of Ac-LYRC(Acm)NG(2-SH)WSPAYM-NH_2 (1.2 mg, 0.72 μmol) was carried out according to the general procedures. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded the title compound as a white solid (0.8 mg, 66% yield).

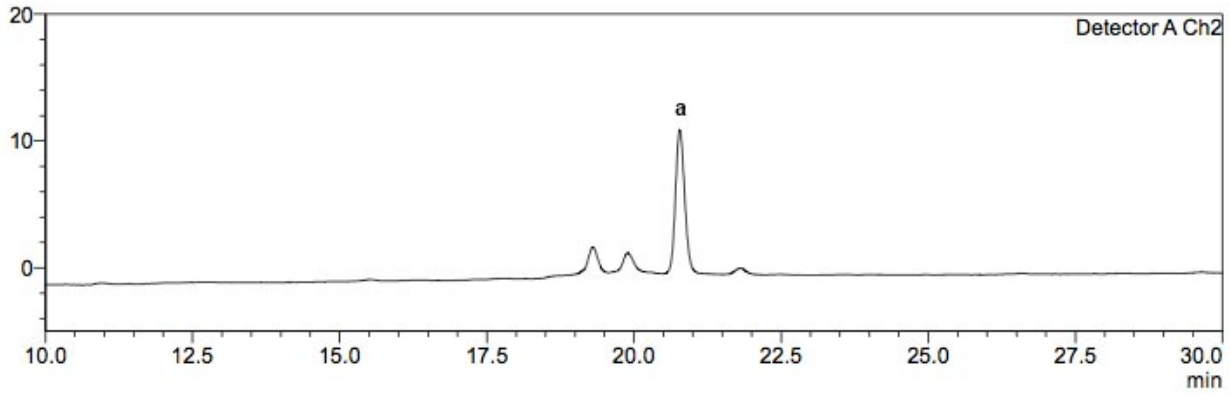
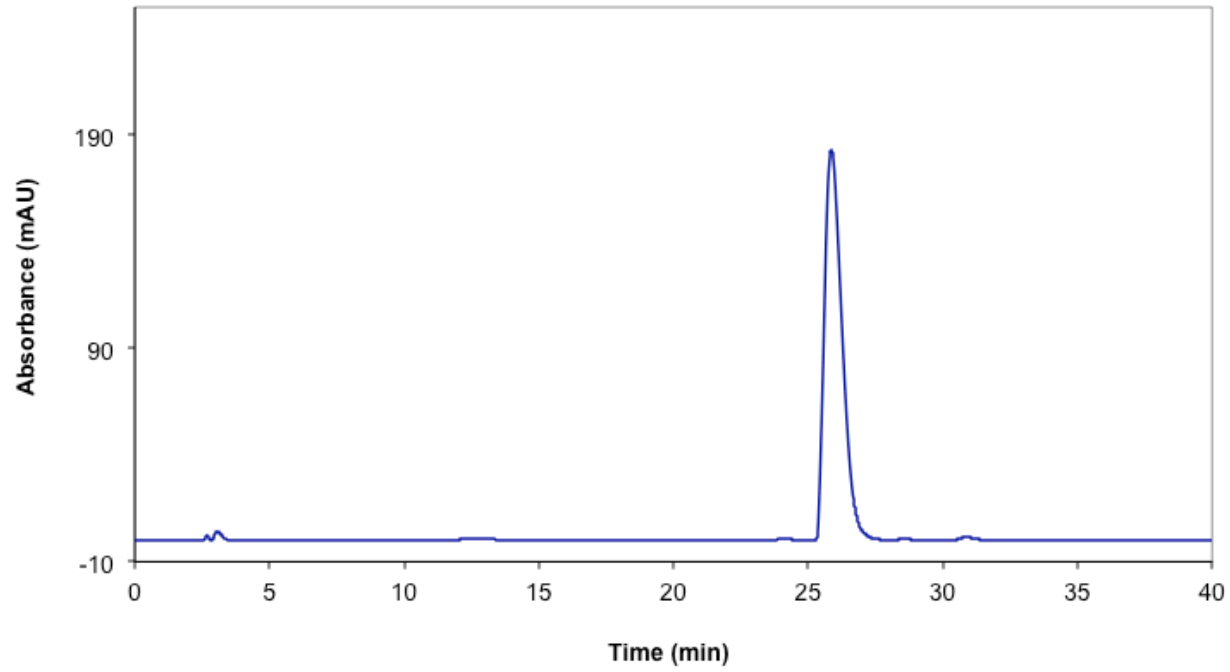
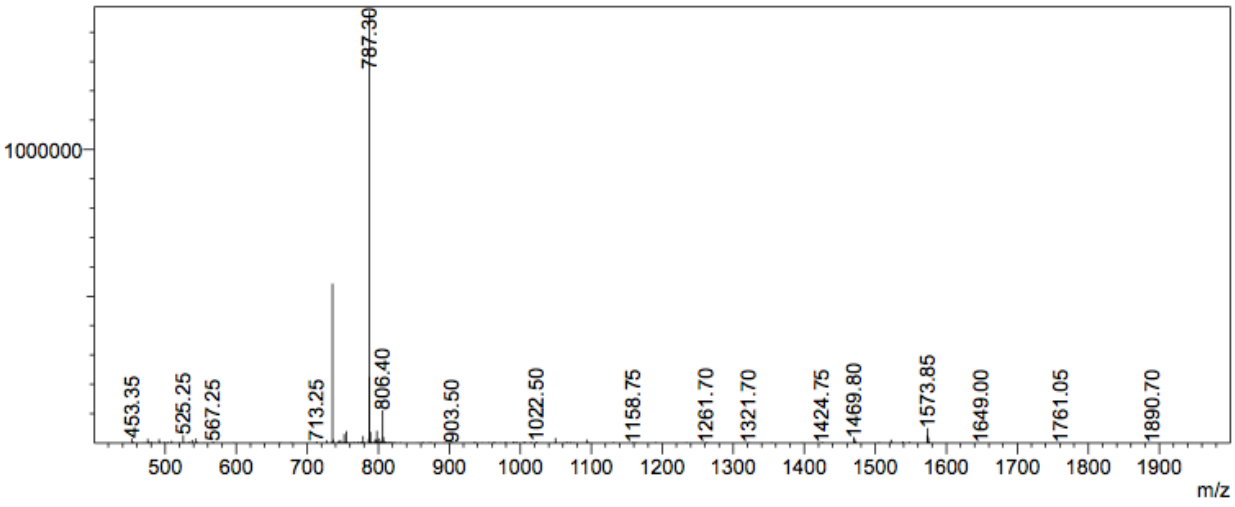


Figure S32. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of crude desulfurization mixture (t = 2 h); a = desulfurization product.

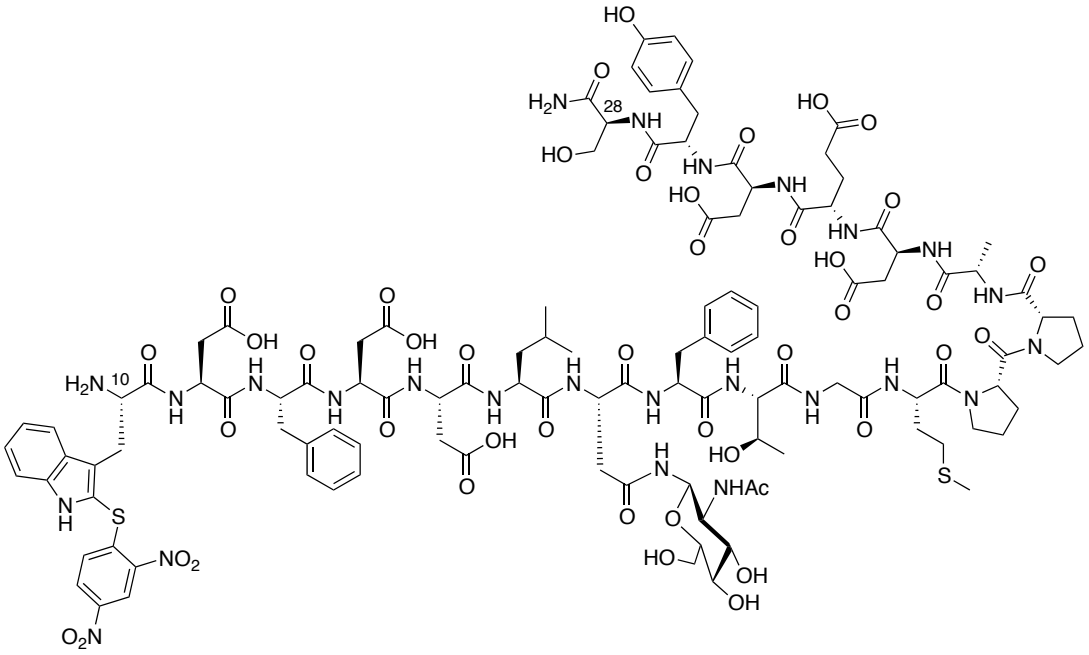


Analytical HPLC: R_t 25.7 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+H^+]^+$: 1572.71, $[M+2H^+]^{2+}$: 786.86; Mass Found (ESI^+); 1573.85 $[M+H^+]^+$, 787.30 $[M+2H^+]^{2+}$



Preparation of CXCR1 (1-28)

CXCR1 (10-28) Trp(DNPS) 20



CXCR1 (17-28) was prepared on Rink amide resin (100 μmol) *via* commercial automated Fmoc-SPPS.[3] Elongation of the peptide using standard manual Fmoc-SPPS, including incorporation of the fully acetylated glycosylamino acid Fmoc-Asn(GlcNAc)-OH **21**, was performed as outlined in the general procedures to generate the Rink amide-bound peptide CXCR1 (10-28). Following

SPPS, the peptide was *O*-deacetylated on-resin, according to the conditions in the general procedures.

Rink amide bound H_2N-WDFDDLN(GlcNAc)FTGMPPADEDYS (25 μmol) was subjected to solid-phase sulfenylation in accord with the general procedures. Following cleavage from the resin (85:5:5:5 v/v/v/v TFA/*i*Pr_3SiH/H_2O/thioanisole) and purification *via* preparative reverse phase HPLC (10% to 70% B over 40 min, 0.1% TFA), Peptide H-(2-DNPS)WDFDDLN(GlcNAc)FTGMPPADEDYS-NH_2 **20** was obtained as a yellow solid after lyophilization (6.0 mg, 9% yield based on the original resin loading).

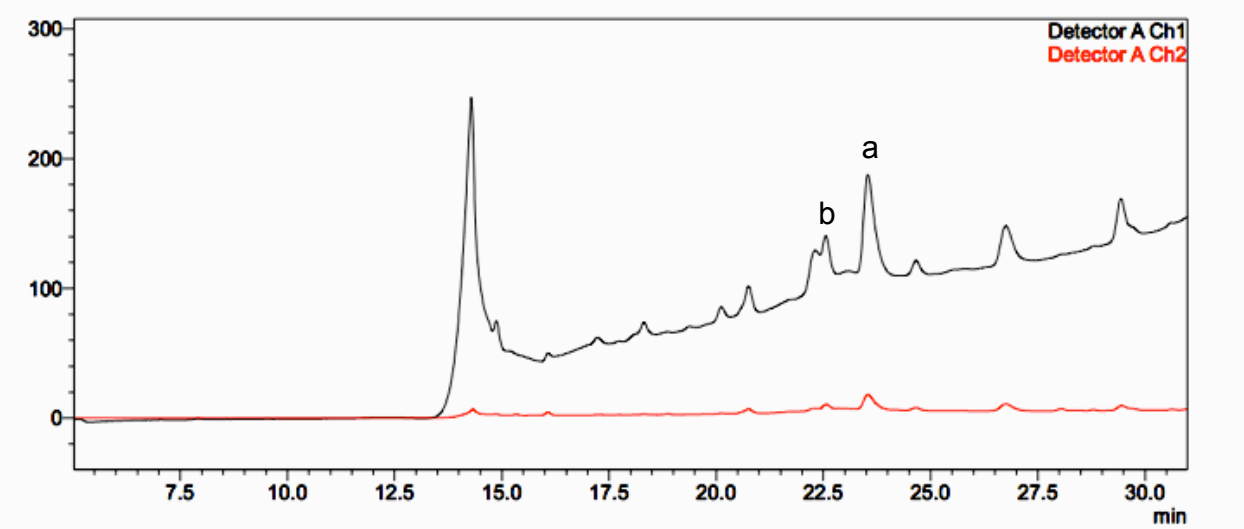


Figure S33a. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230, 254 nm) of a test-cleavage (90:5:5 v/v/v TFA/*i*Pr_3SiH/H_2O, 2 h) of the crude glycopeptide following on-resin *O*-deacetylation and *prior* to solid-phase sulfenylation; a = glycopeptide product; b = glycopeptide product bearing a single oxidized Met residue.

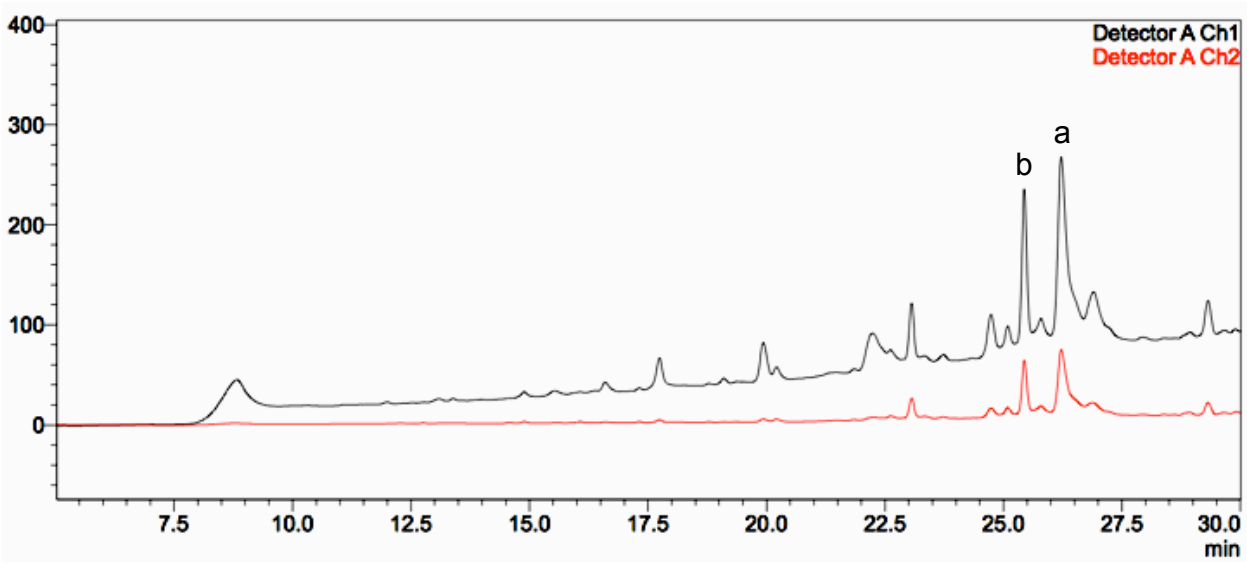


Figure S33b. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230, 254 nm) of a test-cleavage (90:5:5 v/v/v TFA/*i*Pr_3SiH/H_2O, 2 h) of the crude glycopeptide *following* solid-phase sulfenylation; a = sulfenylation product **20**; b = sulfenylation product **20** bearing a single oxidized Met residue. It should be noted that Met oxidation could be substantially reduced through incorporation of thioanisole into the peptide cleavage solution.

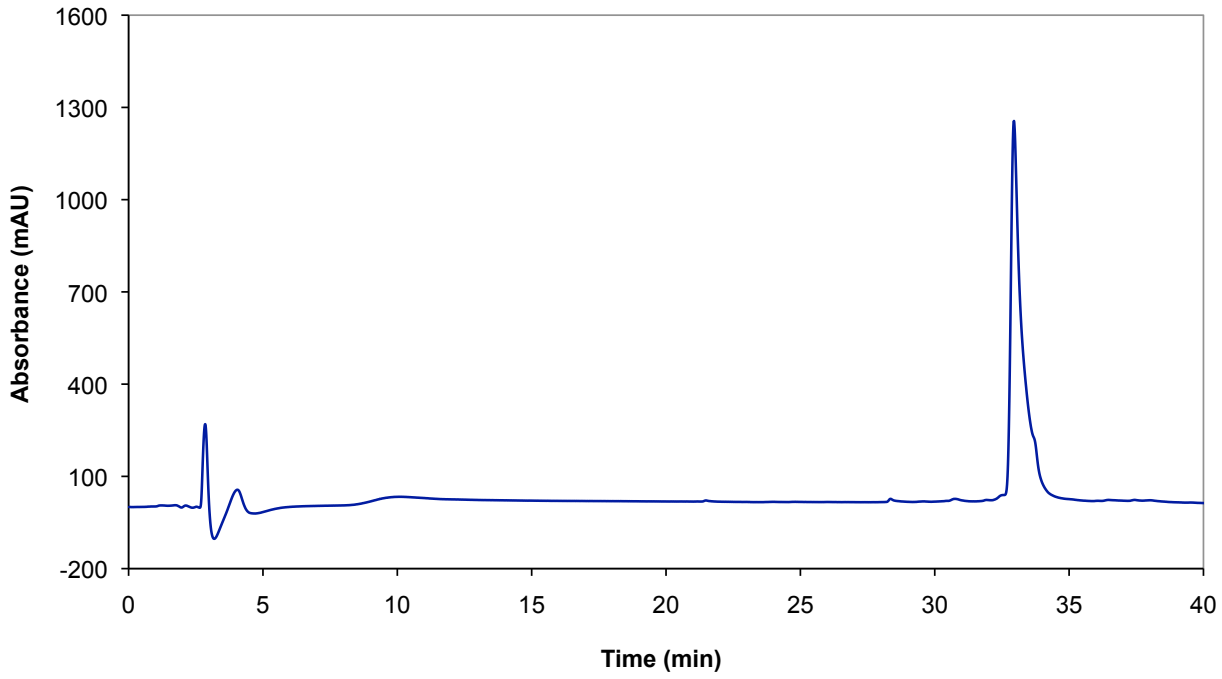
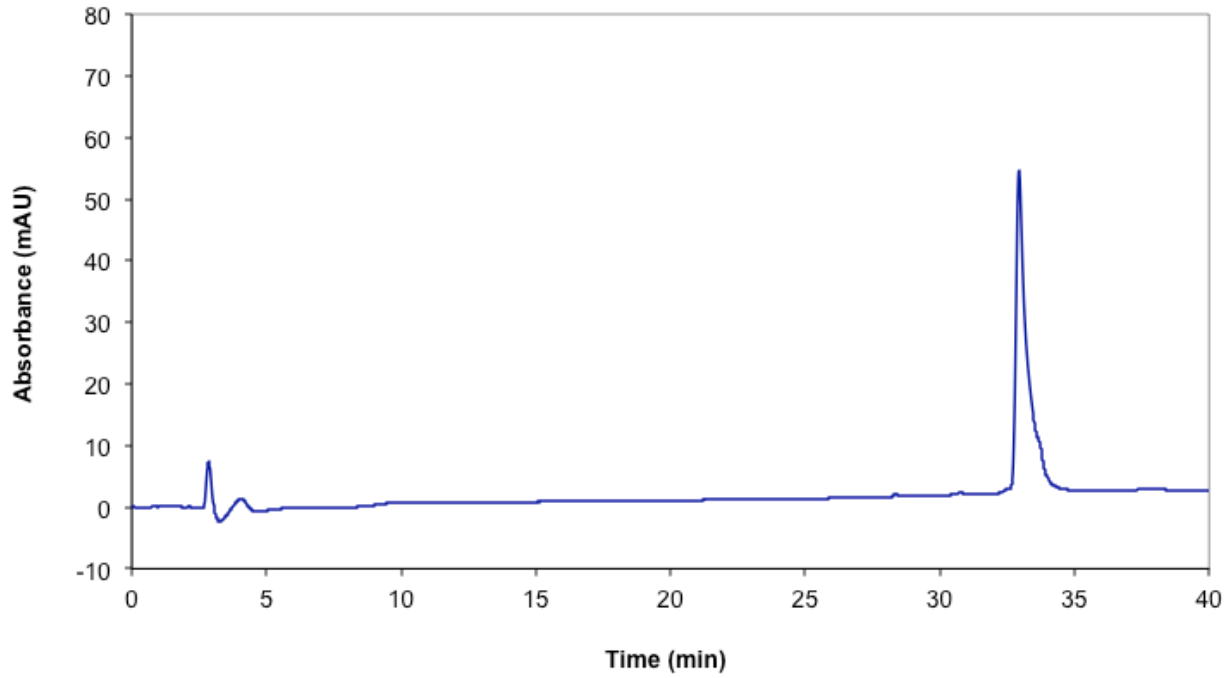
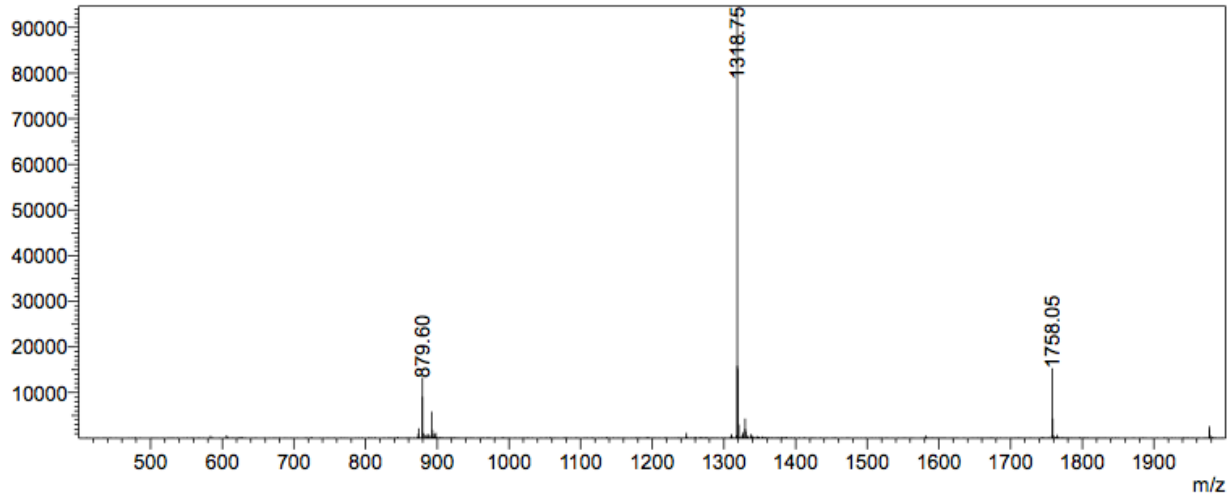


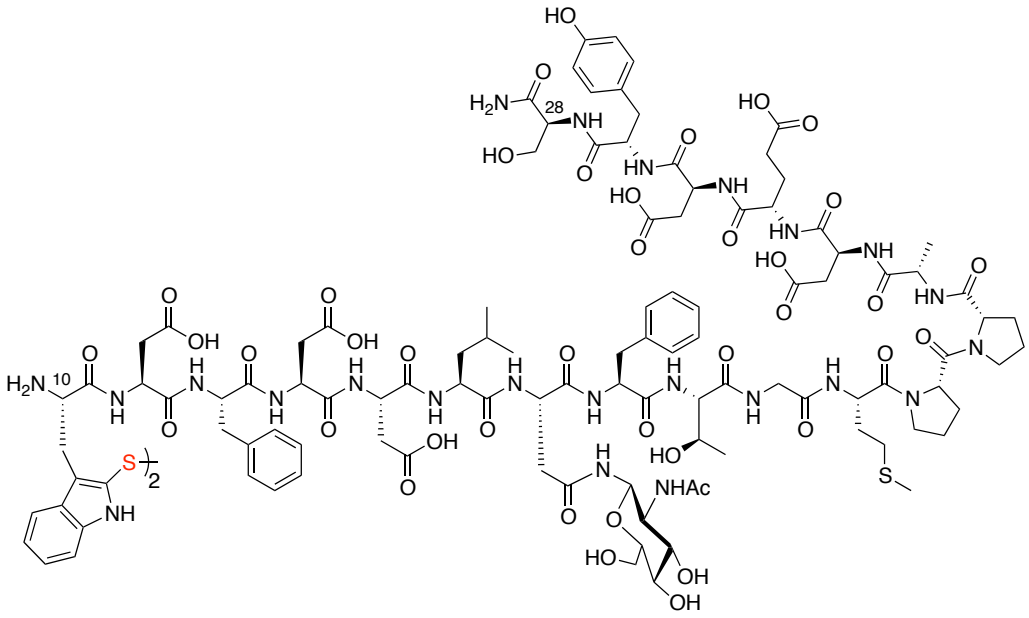
Figure S33c. Analytical HPLC: R_t 32.9 min (0-50% B over 40 min, 0.1% TFA, λ = 210 nm).



Analytical HPLC: R_t 32.9 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+2H^+]^{2+}$: 1318.0, $[M+3H^+]^{3+}$: 879.0; Mass Found (ESI^+); 1318.8 $[M+2H^+]^{2+}$, 879.6 $[M+3H^+]^{3+}$



CXCR1 (10-28) Trp(SH) 18



Peptide **18** was prepared from the thiolysis of H-(2-DNPS)WDFDDL(GlcNAc)NFTGMPPADEDYS-NH_2 **20** (5.0 mg, 1.9 μmol) with thiophenol as outlined in the general procedures. The crude peptide was treated with TCEP to reduce disulfides and immediately purified by preparative reverse phase HPLC (10% to 70% B over 40 min, 0.1% TFA). The purified product oxidized spontaneously in air to afford the target compound (disulfide) as a white solid following lyophilization (2.8 mg, 60% yield).

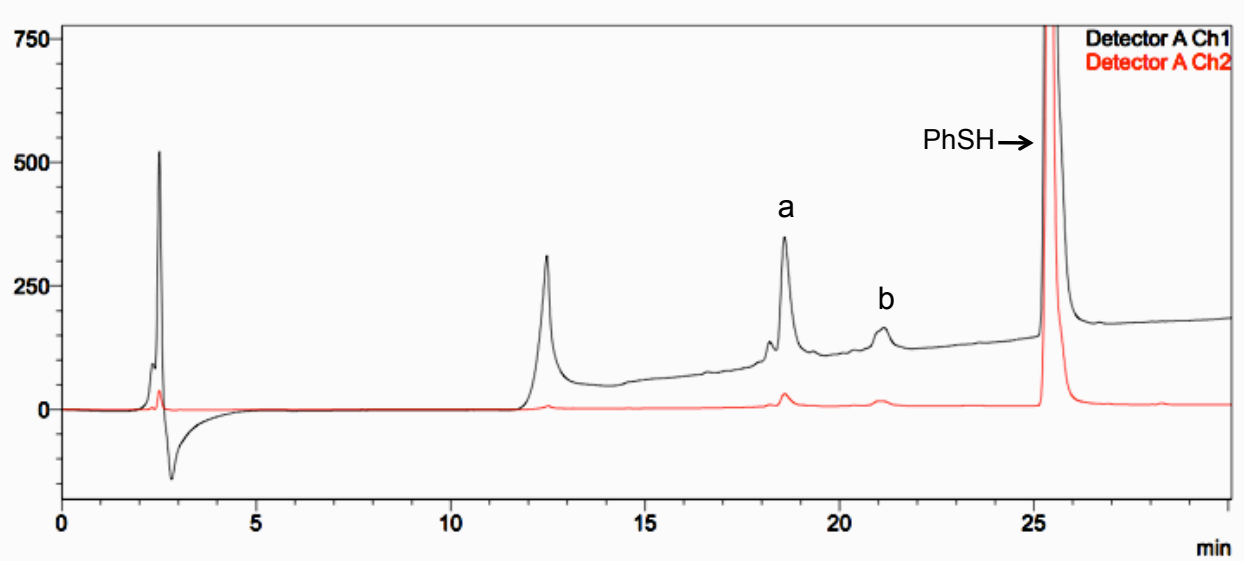
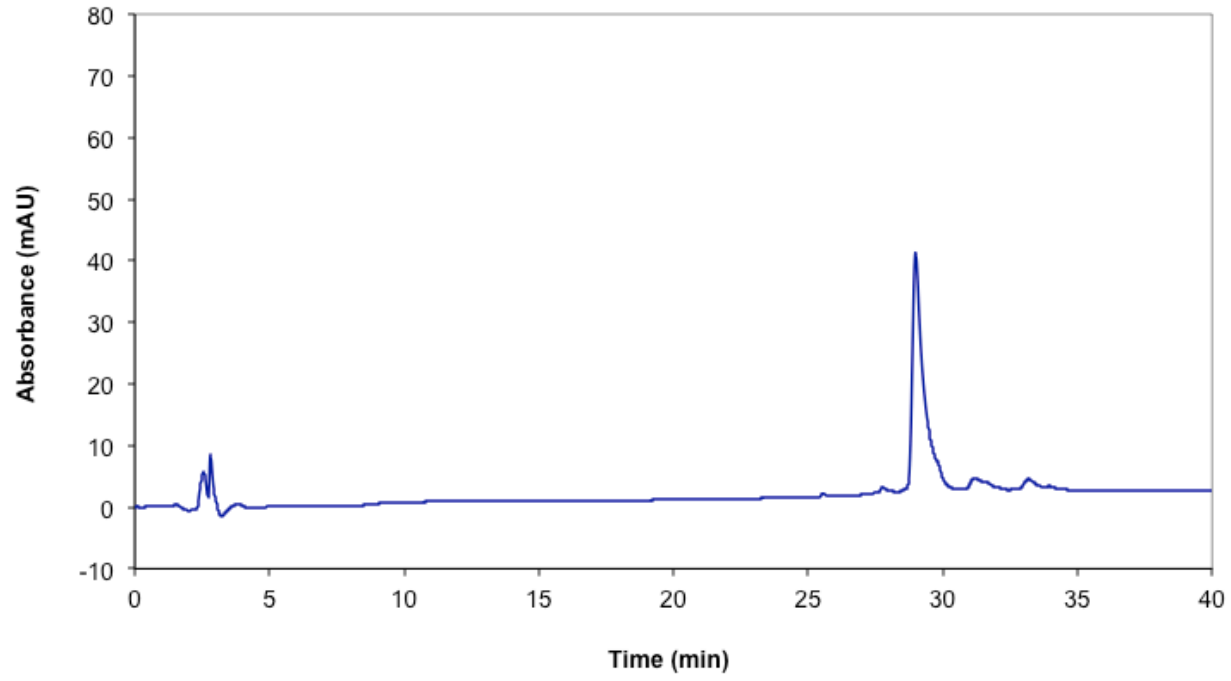
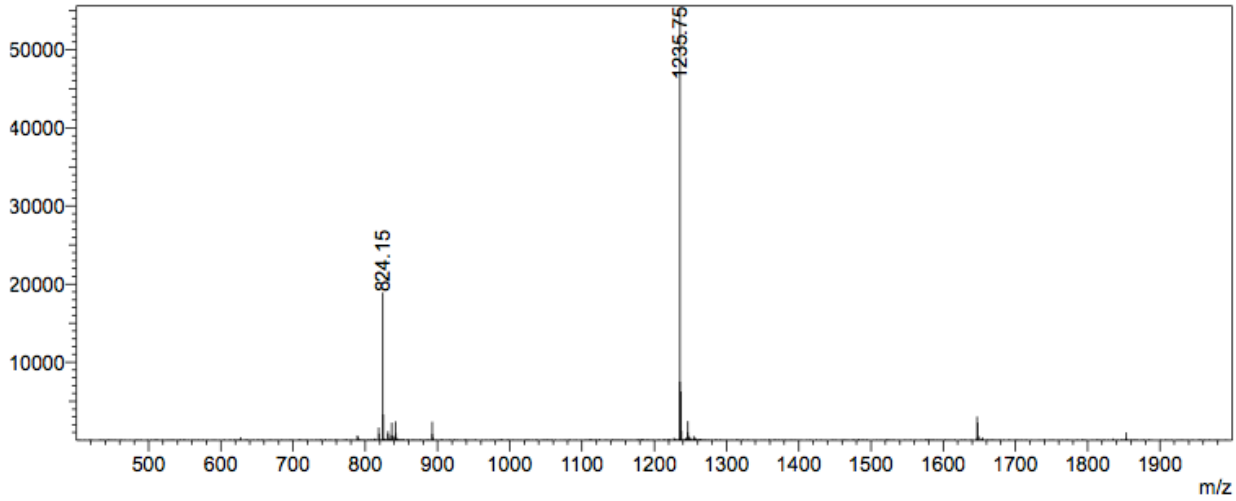


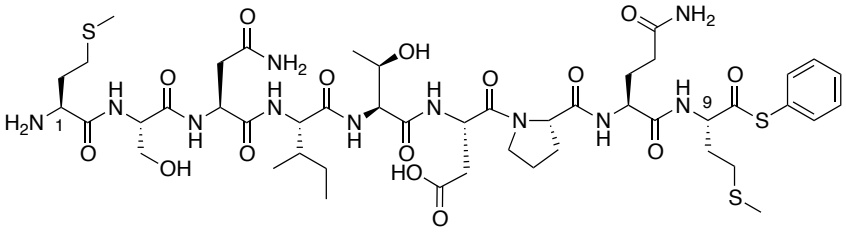
Figure S34. LC-MS trace (0 to 70% B over 30 min, 0.1% formic acid, λ = 230, 254 nm) of crude thiolysis reaction (t = 1.5 h); a = peptide **18**; b = peptide **18**-thiophenol asymmetric disulfide.



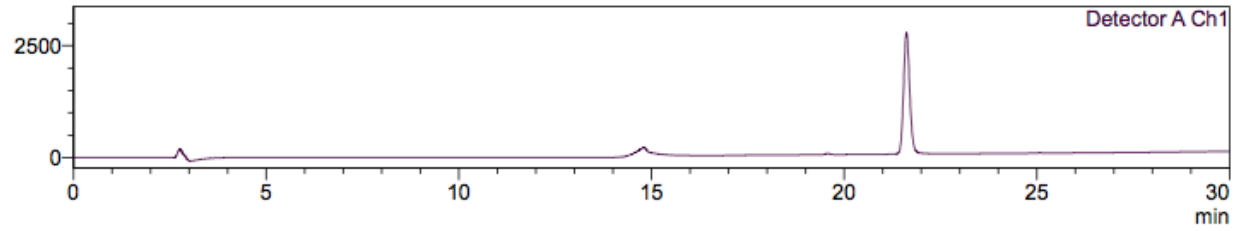
Analytical HPLC: R_t 29.2 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+2H^+]^{2+}$: 1235.0, $[M+3H^+]^{3+}$: 823.6; Mass Found (ESI^+); 1235.8 $[M+2H^+]^{2+}$, 824.2 $[M+3H^+]^{3+}$



CXCR1 (1-9) thiophenyl thioester 19

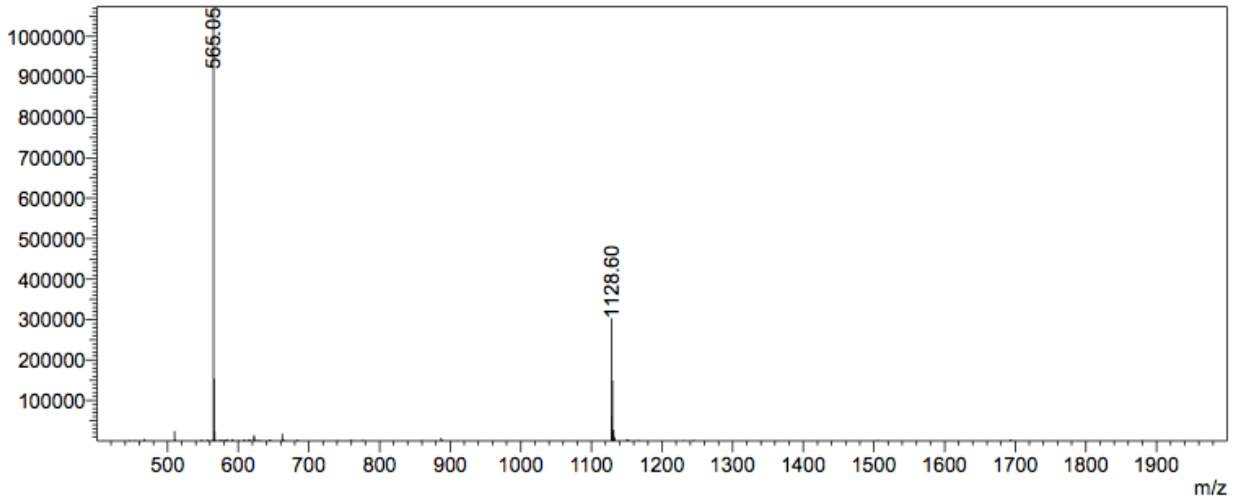


CXCR1 (1-9) thiophenyl thioester **19** was prepared from the corresponding alkyl thioester as outlined in the general procedures. HPLC purification afforded the thiophenyl thioester **19** (54%) and recovered alkyl thioester (27%) as white solids following lyophilization.

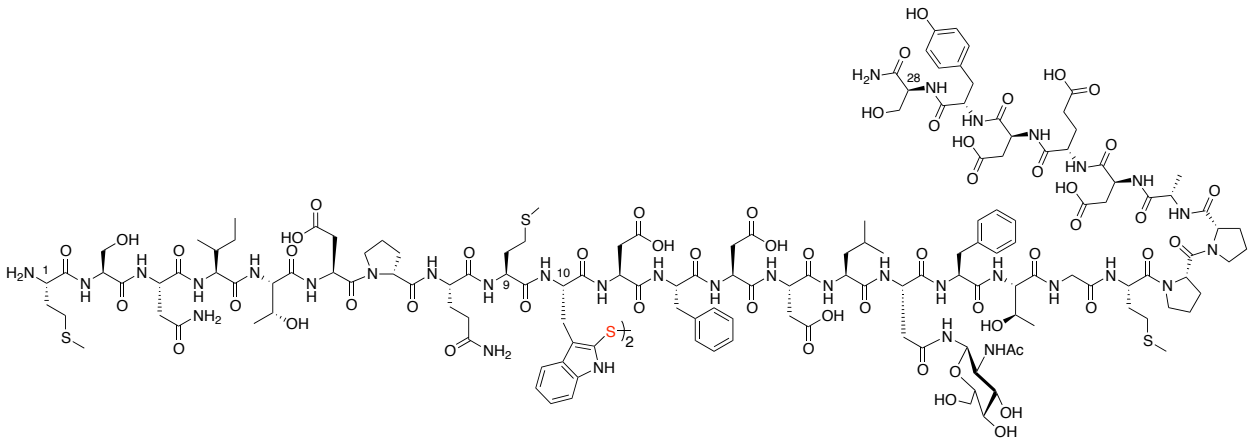


LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of purified H_2N-MSNITDPQM-SPh (R_t 22.1 min)

Calculated Mass $[M+H^+]^+$: 1128.4, $[M+2H^+]^{2+}$: 564.7; Mass Found (ESI^+); 1128.6 $[M+H^+]^+$, 565.1 $[M+2H^+]^{2+}$



CXCR1 (1-28) 2-Thiol Trp peptide 22 (ligation of peptide 18 and 19)



Ligation of H-(2-SH)WDFDDLN(GlcNAc)FTGMPPADEDYS-NH_2 **18** (1.7 mg, 0.68 μmol) and H_2N-MSNITDPQM-SPh **19** (2.2 mg, 1.9 μmol, 2.8 equiv.) was performed as outlined in the general procedures. LC-MS analysis at t = 4 h indicated complete consumption of peptide **18** and formation of ligation product **22**. Purification *via* semi-preparative reverse phase HPLC (0 to 50% B over 40 min, 0.1% TFA) followed by lyophilization afforded **22** as a white solid (2.1 mg, 91% yield), which quickly oxidized in air to yield the disulfide dimer.

It should be noted that an initial ligation was performed for extended reaction times (t = 16 h), which led to the formation of a byproduct consistent with the oxidation of a single Met residue (see Figure S36: reaction at t = 3 h showing no oxidation, and Figure S37: reaction at t = 16 h, where peak e = oxidized ligation product).

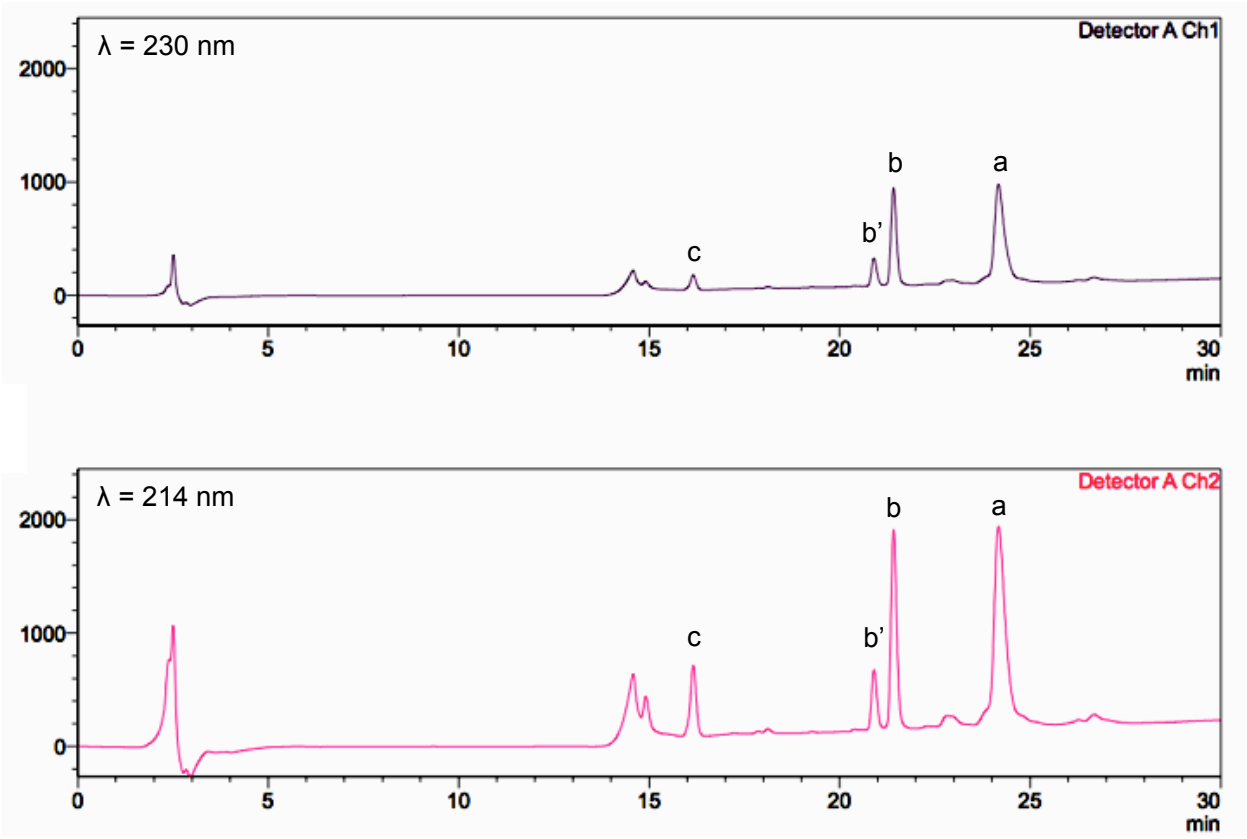


Figure S35. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm, 214 nm) of crude ligation mixture (t = 4 h); a = ligation product **22**; b = H_2N-MSNITDPQM-SPh **19**; b' = H_2N-MSNITDPQM-SPh (C-terminal Met epimer); c = H_2N-MSNITDPQM-OH.

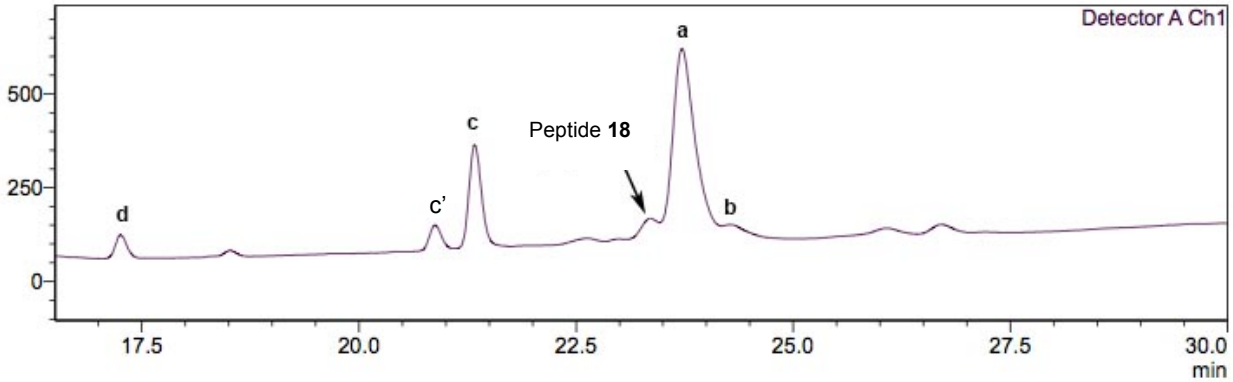


Figure S36. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of crude ligation mixture (t = 3 h); a = ligation product (free thiol); b = ligation product (disulfide); c = H_2N-MSNITDPQM-SPh; c' = H_2N-MSNITDPQM-SPh (C-terminal Met epimer) d = H_2N-MSNITDPQM-OH.

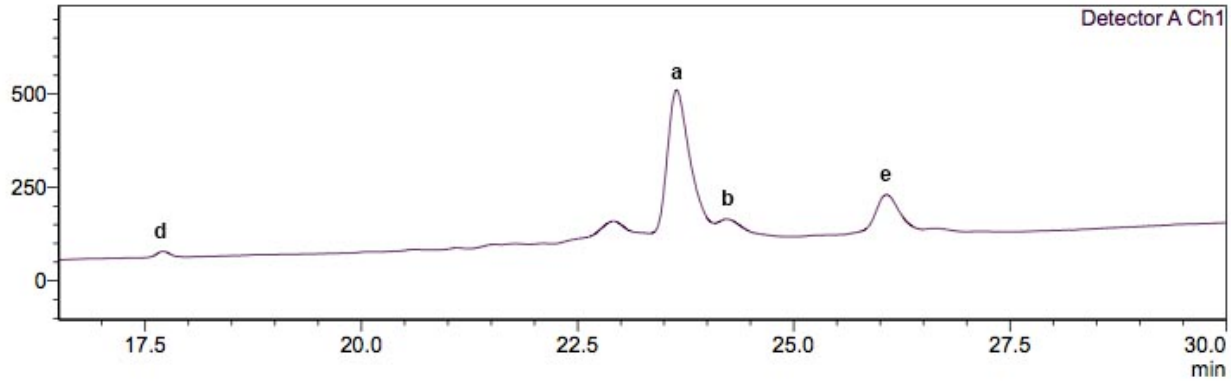
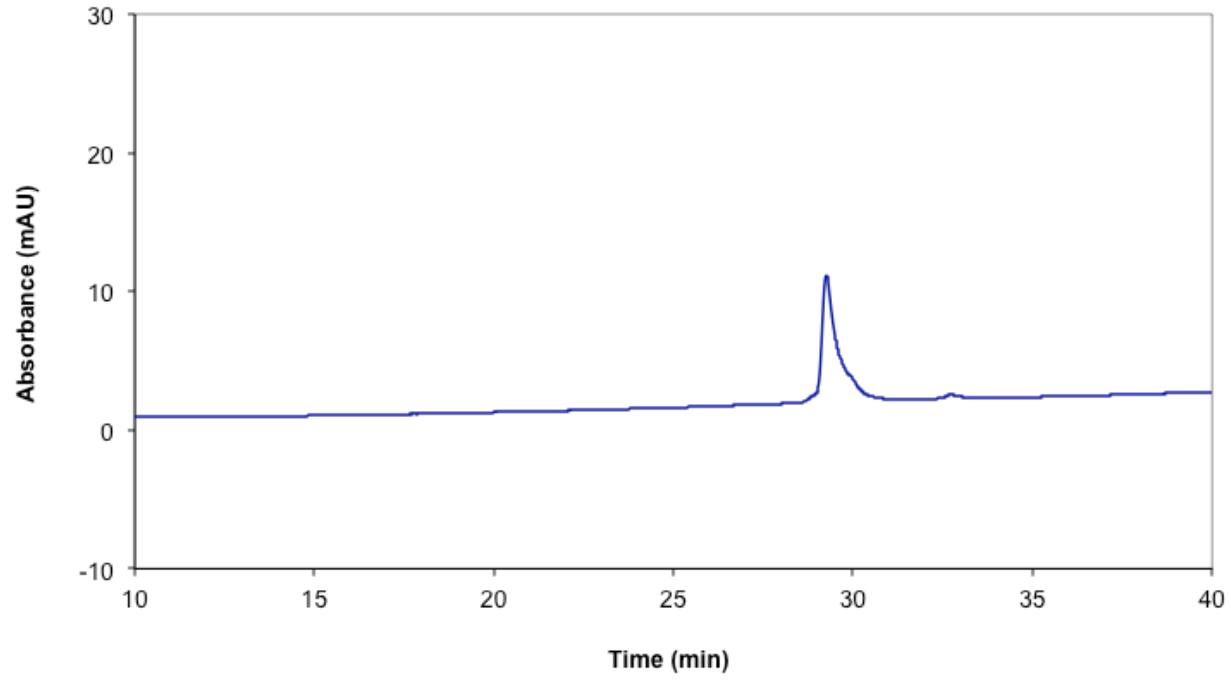
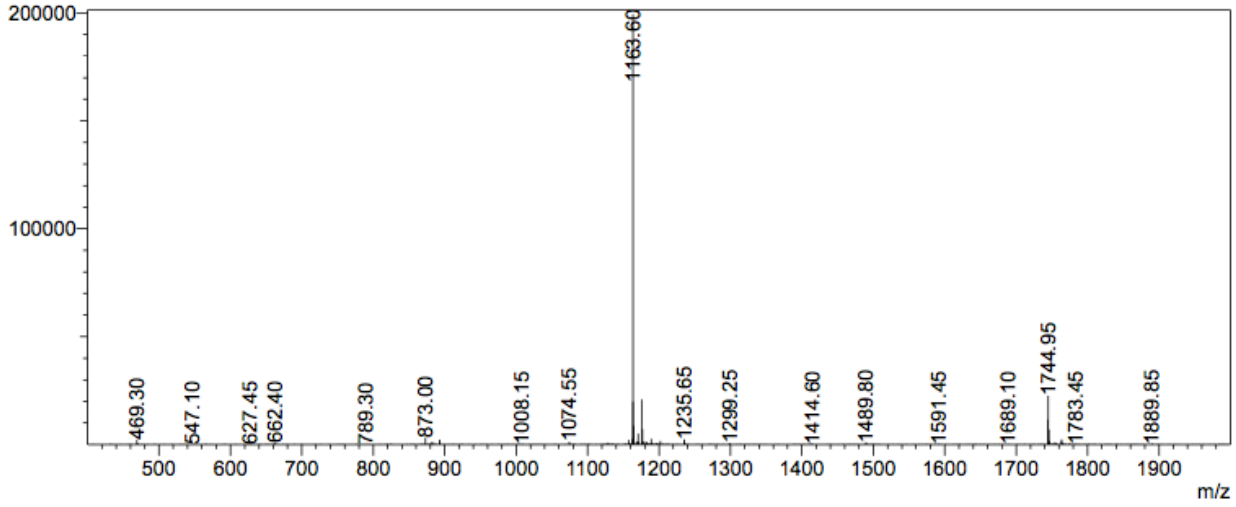


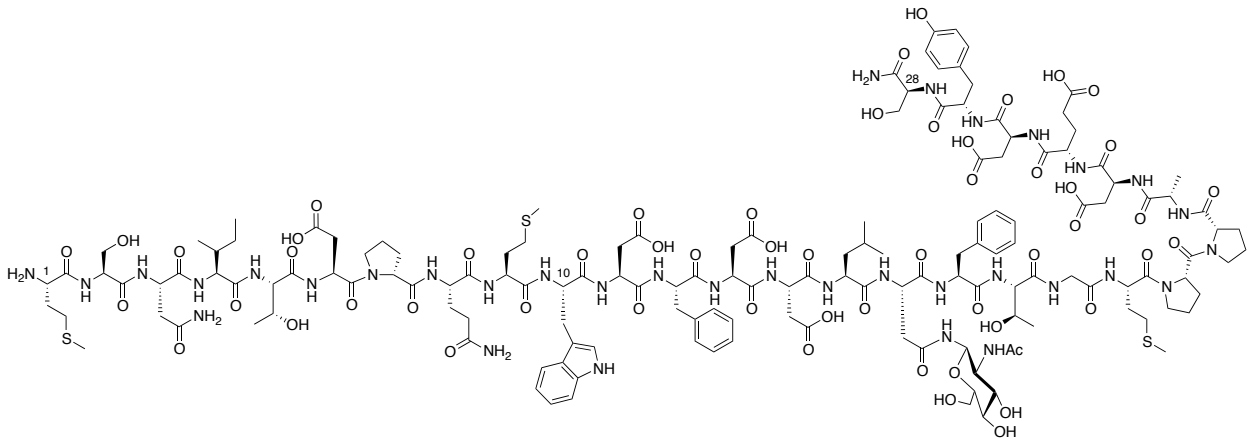
Figure S37. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of crude ligation mixture (t = 16 h); a = ligation product (free thiol); b = ligation product (disulfide); c = H_2N-MSNITDPQM-SPh; d = H_2N-MSNITDPQM-OH; e = oxidized ligation product.



Analytical HPLC (+ TCEP) (free thiol): R_t 29.2 min (0-50% B over 40 min, 0.1% TFA, λ = 230 nm); Calculated Mass $[M+2H^+]^{2+}$: 1743.7, $[M+3H^+]^{3+}$: 1162.8; Mass Found (ESI^+); 1745.0 $[M+2H^+]^{2+}$, 1163.6 $[M+2H^+]^{2+}$.



Desulfurization of peptide 22 to afford CXCR1 (1-28)



Desulfurization of H_2N-MSNITDPQM(2-SH)WDFDDLN(GlcNAc)FTGMPPADEDYS-NH_2 **22** (1.7 mg, 0.49 μmol) was set up according to the general procedures, and reacted for a period of 5 minutes at 0 ^oC. The reaction proceeded quantitatively as determined by analytical HPLC (see Figure S38). Following centrifugation to remove the solid Pd catalyst, the crude mixture was treated with excess thiourea and purified by semi-preparative HPLC (0 to 50% B over 40 min, 0.1% formic acid) to provide CXCR1 (1-28) **17** as a white solid (1.3 mg, 79% yield).

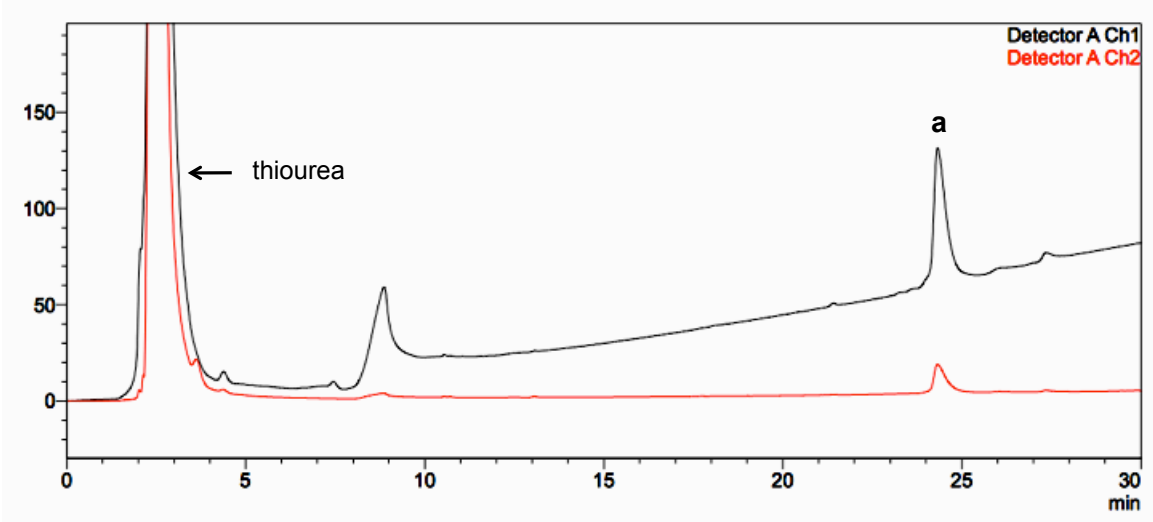


Figure S38. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm, 254 nm) of crude desulfurization mixture (t = 5 min) after the addition of thiourea; a = desulfurization product.

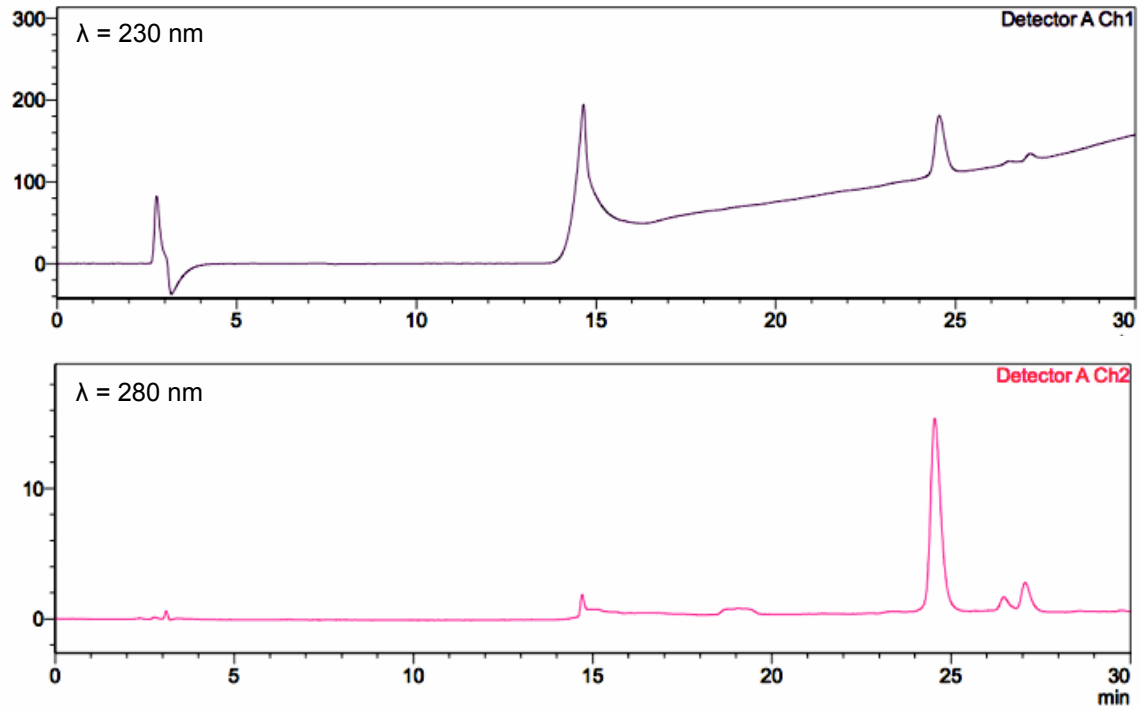
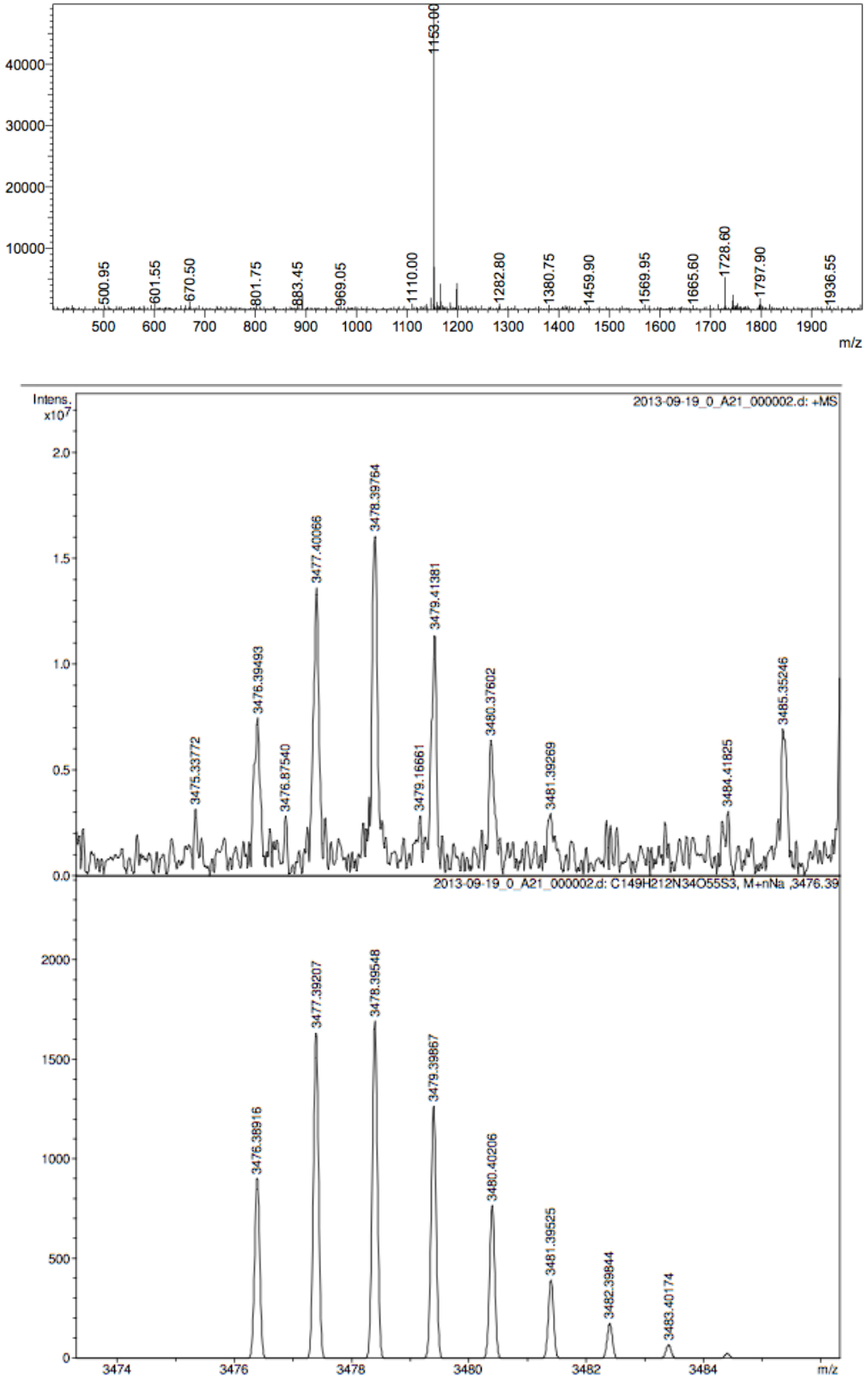


Figure S39. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm, 280 nm) of purified H-H_2N-MSNITDPQMWDFDDL(GlcNAc)NFTGMPPADEDYS-NH_2 **17** (R_t 24.5 min).

Calculated Mass $[M+2H^+]^{2+}$: 1727.7, $[M+3H^+]^{3+}$: 1152.1; Mass Found (ESI^+); 1728.6 $[M+2H^+]^{2+}$, 1153.0 $[M+3H^+]^{3+}$



MALDI-TOF HRMS: Calcd. for $C_{149}H_{212}N_{34}O_{55}S_3$ ($M+Na^+$) 3478.3976, found 3478.3955.

Analysis of thioester epimerization in ligation buffer

An aliquot of purified, pre-formed thiophenyl thioester was dissolved in ligation buffer (6 M Gn•HCl/0.1 M Na_2HPO_4, with or without 50 mM TCEP, pH = 7.0). At t = 0 h, 1 h, and 3 h, 5 μL samples were removed and quenched with 50 μL water + 0.1% TFA. The resulting samples were analyzed *via* LC-MS. Thioester hydrolysis and epimerization of the C-terminal residue were monitored over time. Representative studies using the activated thioesters Ac-LYRANA-SPh **6** (Figures S40 and S42) and Ac-LYRANV-SPh (Figure S44) are detailed below.

Ac-LYRANA-SPh (pH = 7.0):

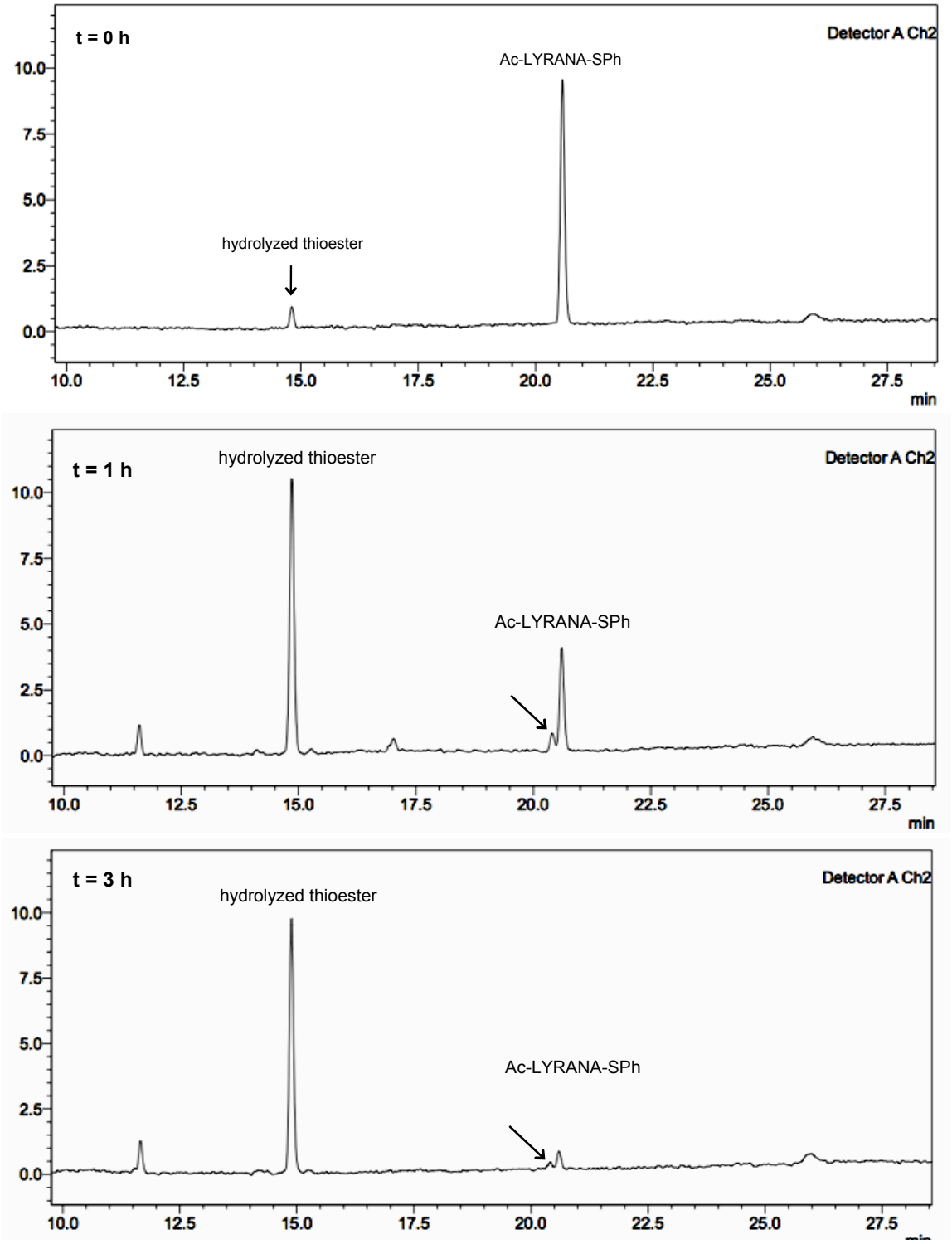


Figure S40. LC-MS traces (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of Ac-LYRANA-SPh **6** incubated in ligation buffer (pH = 7.0) at t = 0 h, t = 1 h, and t = 3 h; Arrow depicts presence of epimerized thiophenyl thioester.

Integration results, t = 1 h:

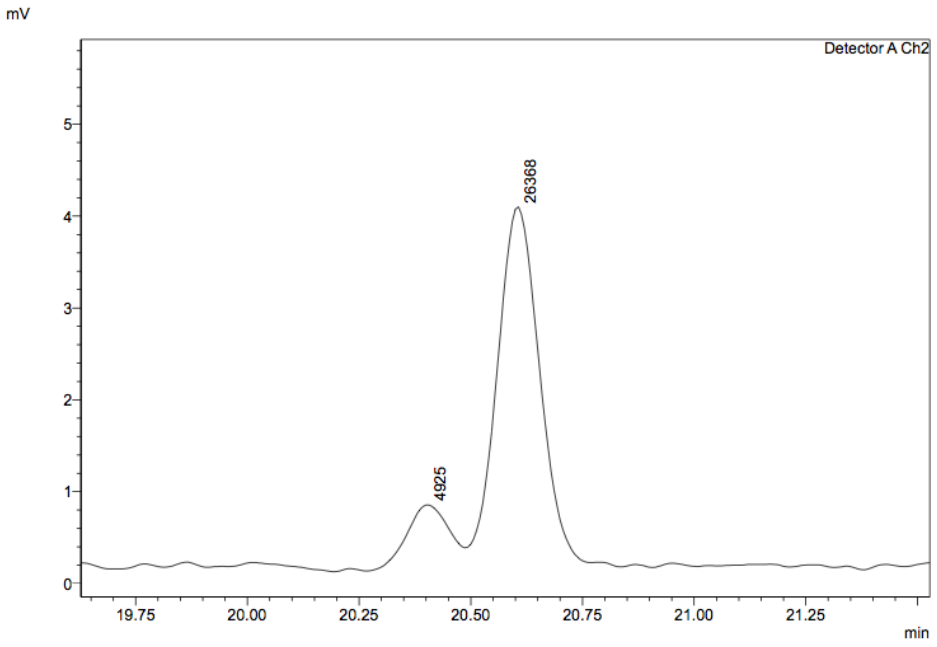


Figure S41. Integration results (λ = 280 nm) for Ac-LYRANA-SPh **6** incubation experiment (t = 1 h) in ligation buffer (pH = 7.0); integrals correspond to 15.7% epimerization at t = 1 h.

Ac-LYRANA-SPh (pH = 7.0, no TCEP added):

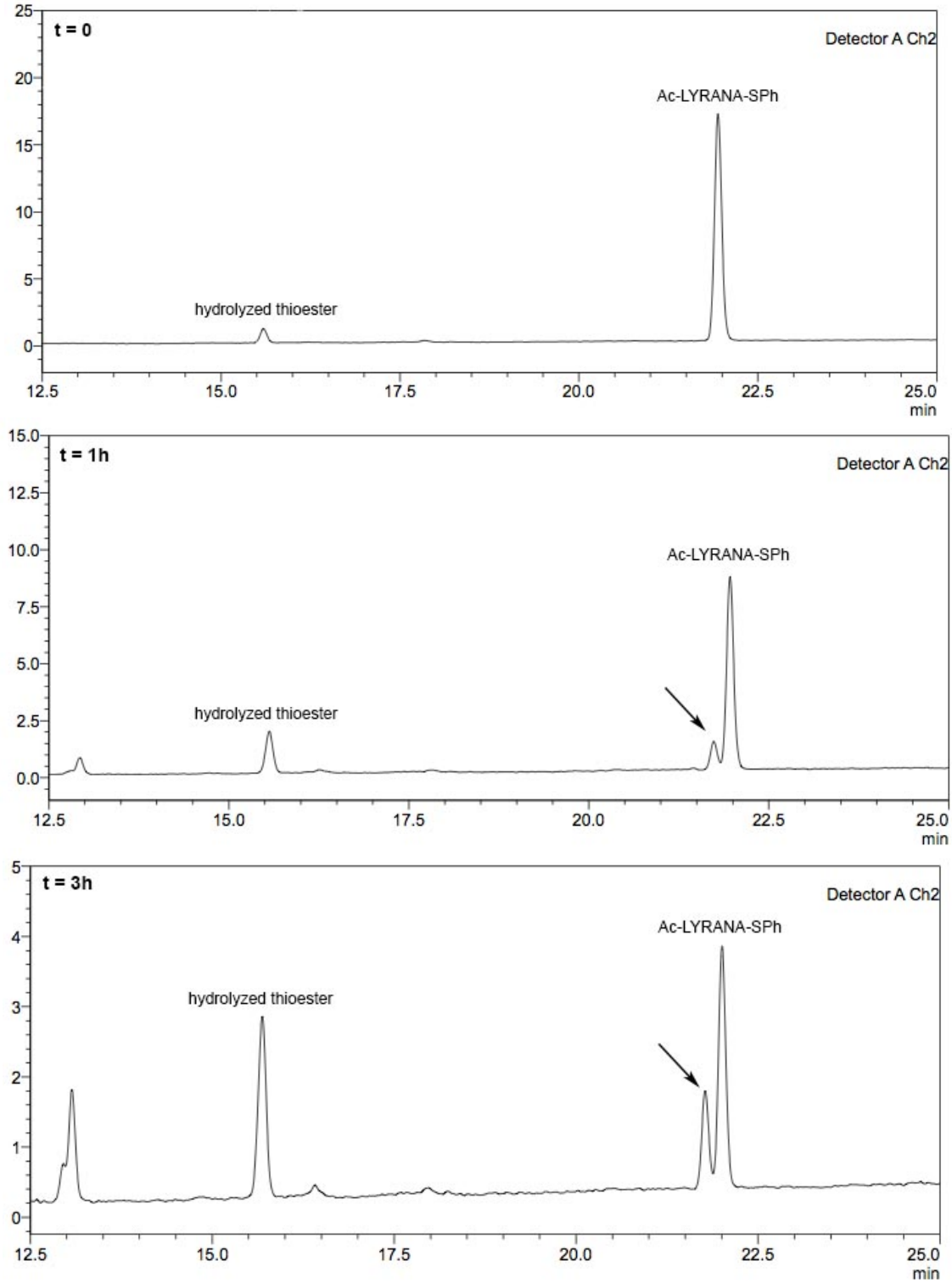


Figure S42. LC-MS traces (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of Ac-LYRANA-SPh incubated in ligation buffer without TCEP (pH = 7.0) at t = 0 h, t = 1 h, and t = 3 h; Arrow depicts presence of epimerized thiophenyl thioester.

Integration results, t = 1 h:

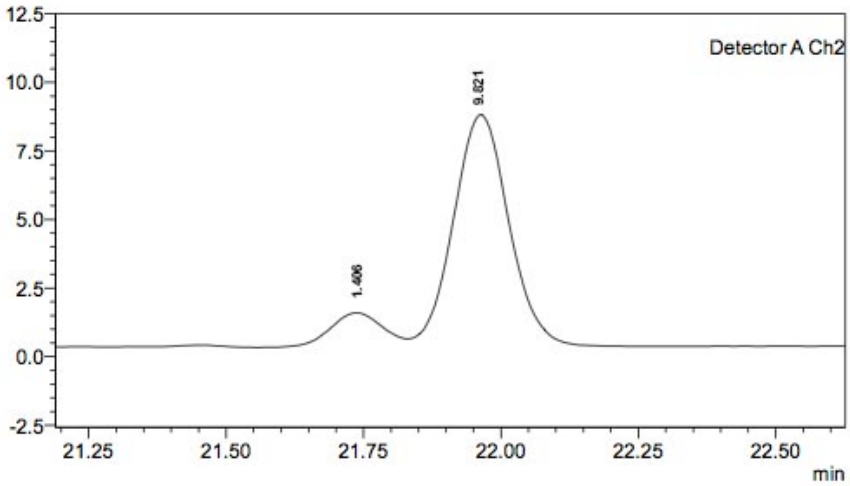


Figure S43. Integration results (λ = 280 nm) for Ac-LYRANA-SPh **6** incubation experiment (t = 1 h) in ligation buffer (pH = 7.0, no TCEP added); integrals correspond to 13% epimerization at t = 1 h.

Ac-LYRANV-SPh (pH = 7.0):

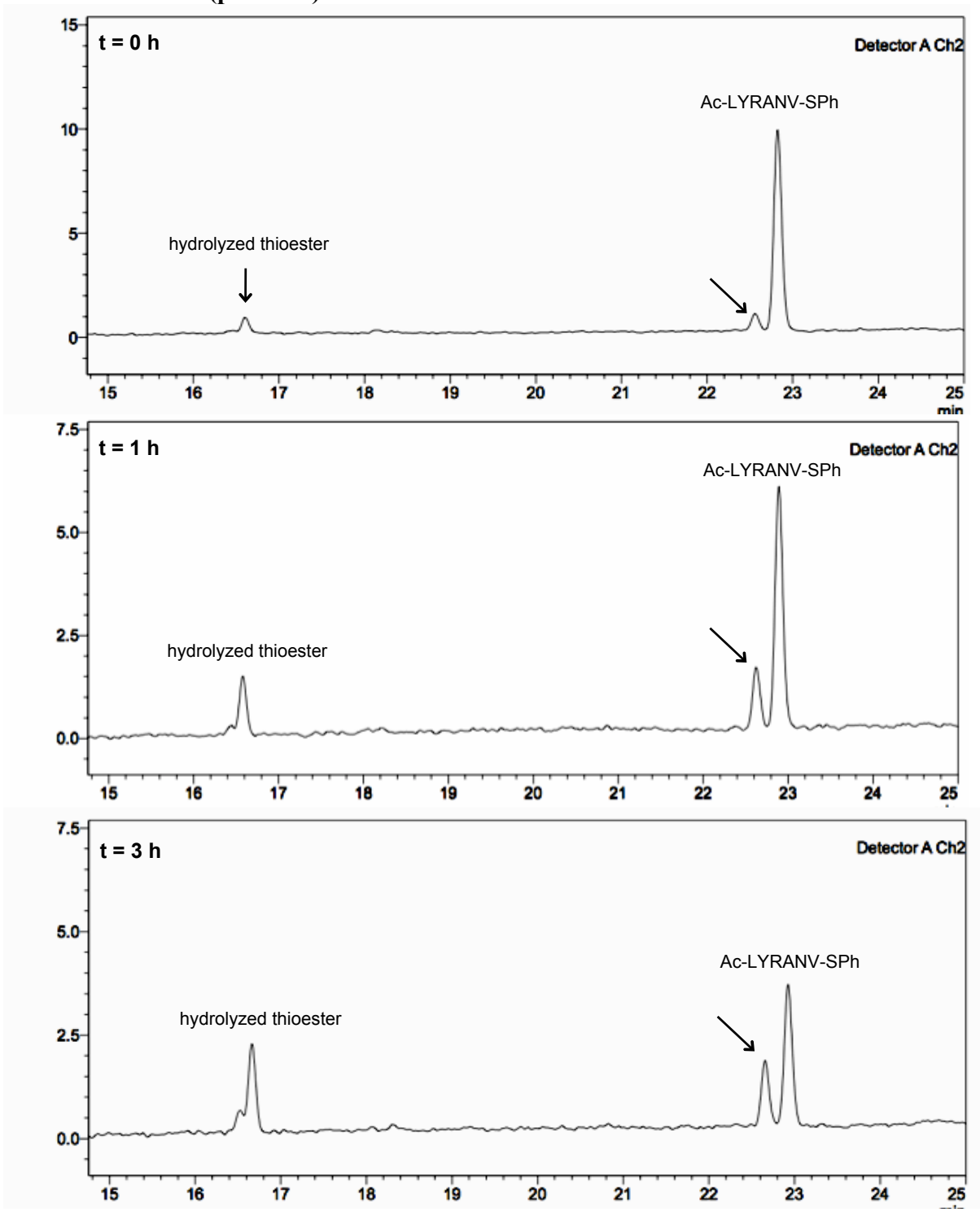


Figure S44. LC-MS traces (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of Ac-LYRANV-SPh incubated in ligation buffer (pH = 7.0) at t = 0 h, t = 1 h, and t = 3 h; Arrow depicts presence of epimerized thiophenyl thioester.

Additional activated Ala-thioesters were prepared by thiol exchange reaction with Ac-LYRANA-S$(CH_2)_2CO_2$Et, in an analogous manner to that described for the synthesis of the thiphenyl thioesters in the general procedures section (treatment with 2 vol.% external thiol in 6 M guanidine hydrochloride, 100 mM Na_2HPO_4, pH 6.8) Each thioester was analyzed for epimerization during the thiol exchange reaction or *via* incubation of the purified, activated thioester in ligation buffer as described previously.

Ac-LYRANA-SMPAA

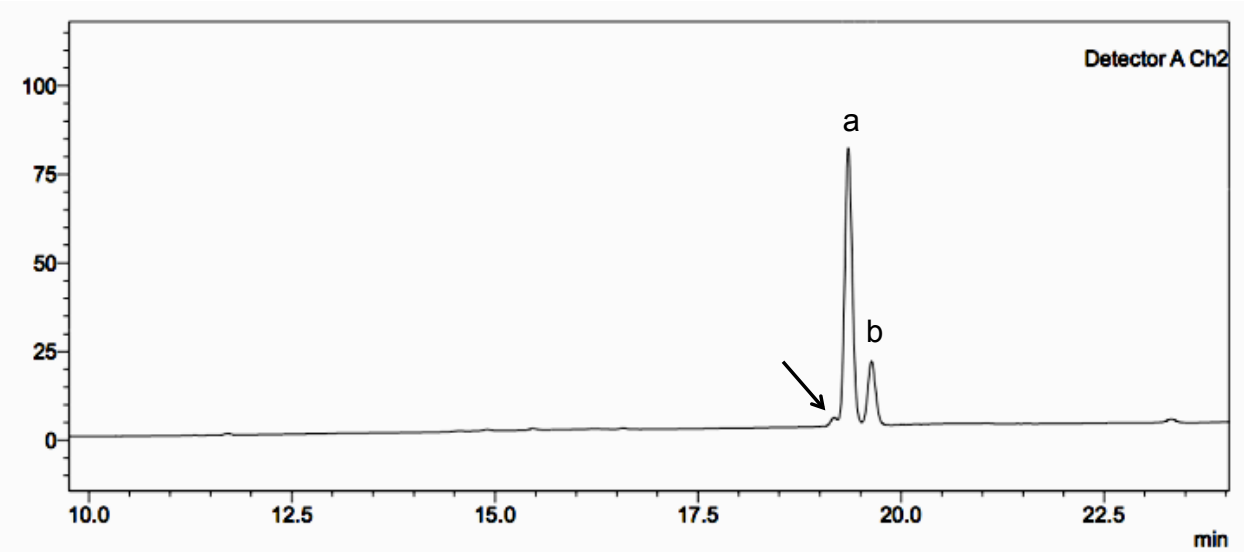


Figure S45. Crude LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of the formation of Ac-LYRANA-SMPAA from the corresponding alkyl thioester (t = 16 h); a = Ac-LYRANA-SMPAA; b = Ac-LYRANA-S$(CH_2)_2CO_2$Et starting material; arrow depicts formation of the Ac-LYRANA-SMPAA epimer.

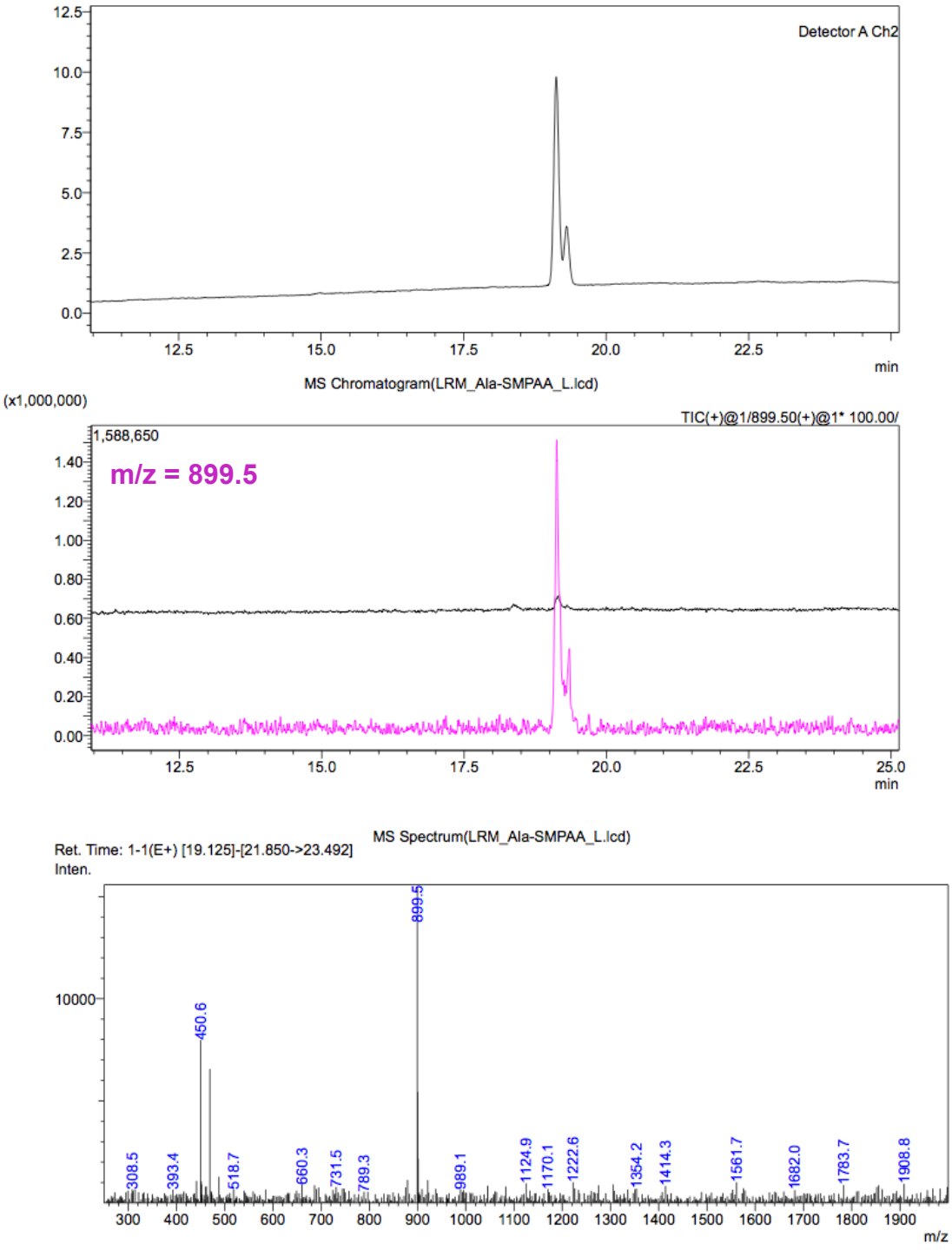


Figure S46. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of HPLC fraction following purification of Ac-LYRANA-SMPAA; both peaks have mass = 899.5, corresponding to Ac-LYRANA-SMPAA and an Ac-LYRANA-SMPAA epimer.

Ac-LYRANA-SMESNa (pH = 7.0)

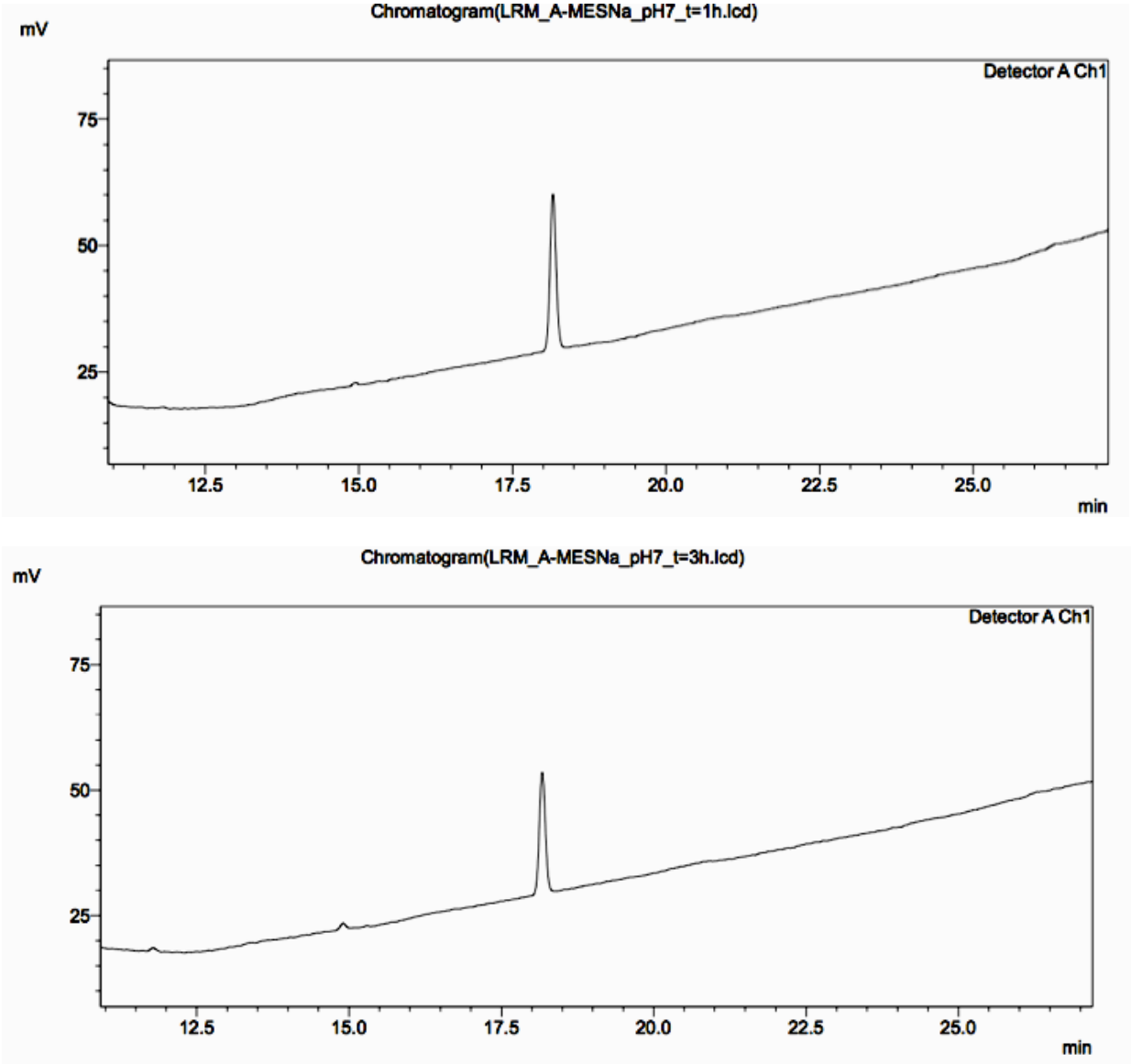


Figure S47. LC-MS trace (0 to 50% B over 30 min, 0.1% formic acid, λ = 230 nm) of of Ac-LYRANA-SMESNa incubated in ligation buffer (pH = 7.0) at t = 1 h and t = 3 h.

Ac-LYRANA-(2-hydroxy)thiophenol (pH = 6.6)

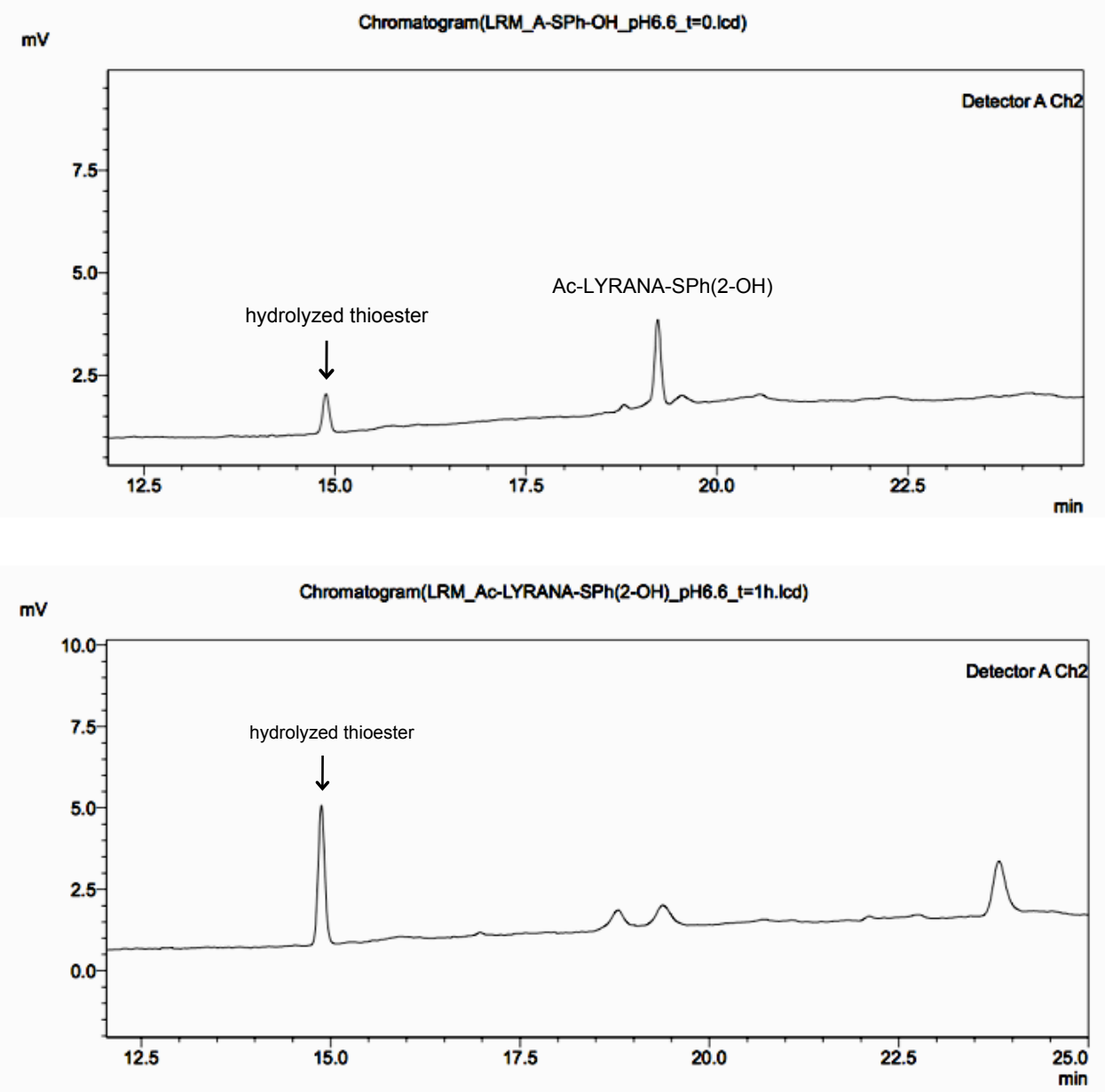


Figure S48. LC-MS traces (0 to 50% B over 30 min, 0.1% formic acid, λ = 280 nm) of of Ac-LYRANA-SPh(2-OH) incubated in ligation buffer (pH = 6.6) at t = 0 h and t = 1 h.

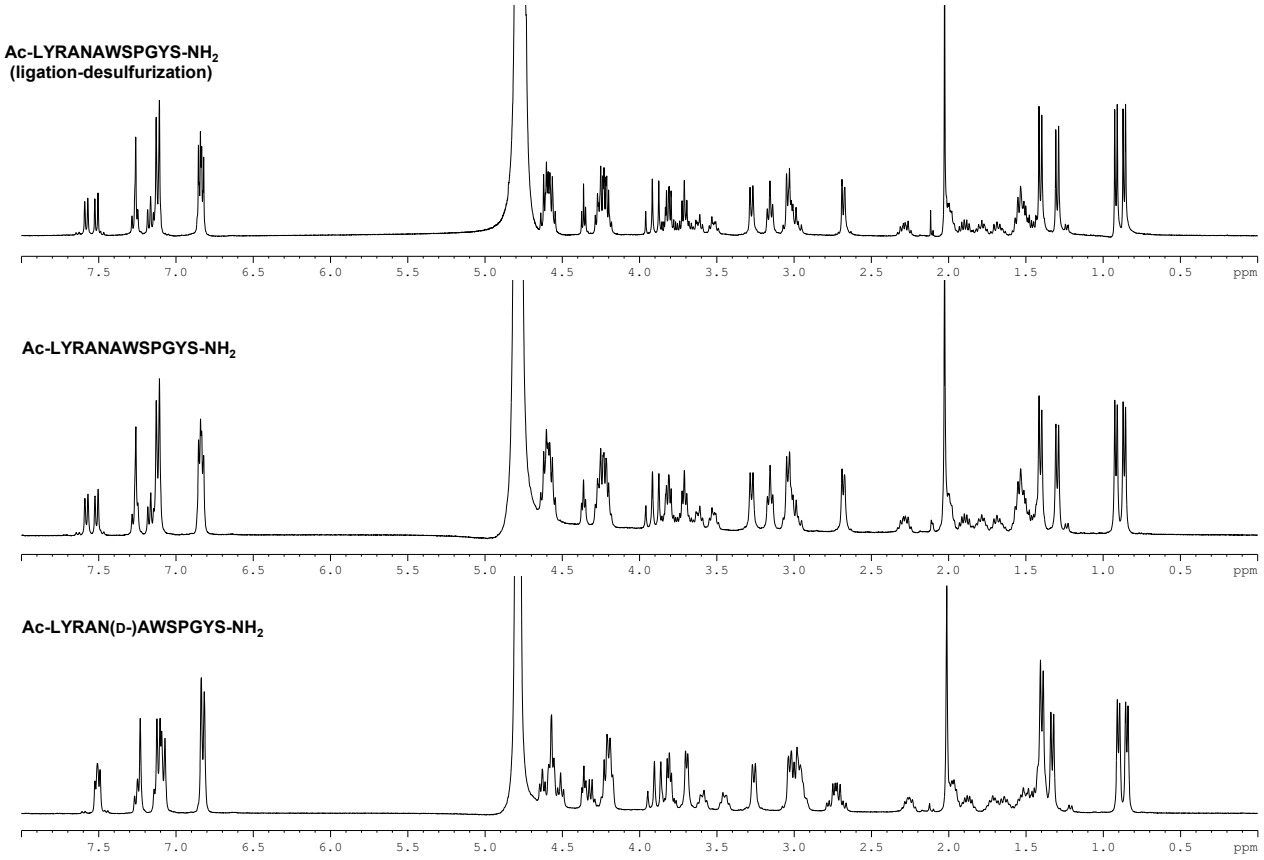
Analytical standards

Analytical standards Ac-LYRANXWPSGYS-NH_2 (X = Ala, D-Ala, Phe, D-Phe, and Met) were prepared using standard Fmoc-SPPS on Rink amide resin (12.5 μmol scale, see general procedures) and purified *via* reverse-phase preparative HPLC (0 to 50% B over 40 min, 0.1% TFA, λ = 230 nm). Analytical standards were analyzed *via* ^{1}H NMR and analytical HPLC and compared to the corresponding synthetic peptides accessed by ligation-desulfurization chemistry at 2-thiol Trp.

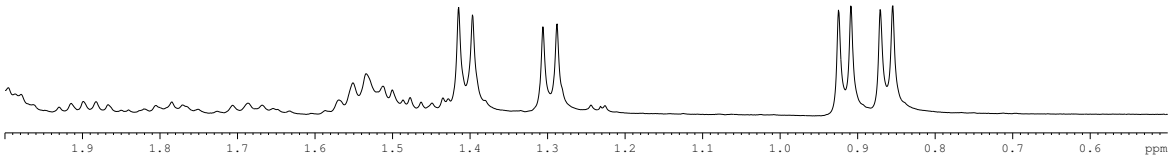
Epimerization study

A) NMR comparisons

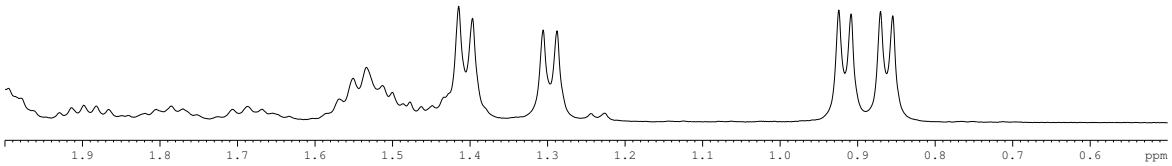
NMR spectra were obtained in D_2O (Ac-LYRANXWPSGYS-NH_2, X = A, M) or 1:1 D_2O/MeOD (Ac-LYRANXWPSGYS-NH_2, X = F). Results indicate agreement between the L-amino acid standards and the synthetic peptides accessed *via* ligation-desulfurization chemistry.



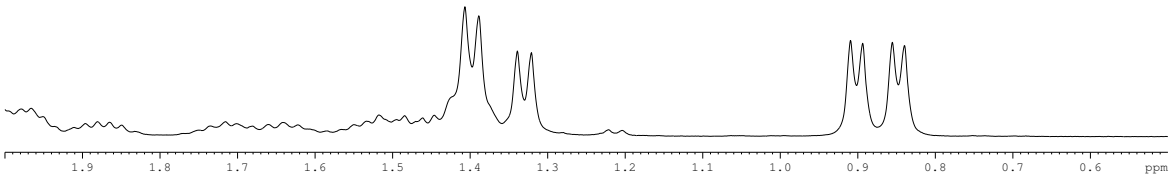
Ac-LYRANAWSPGYS-NH_2
(ligation-desulfurization)



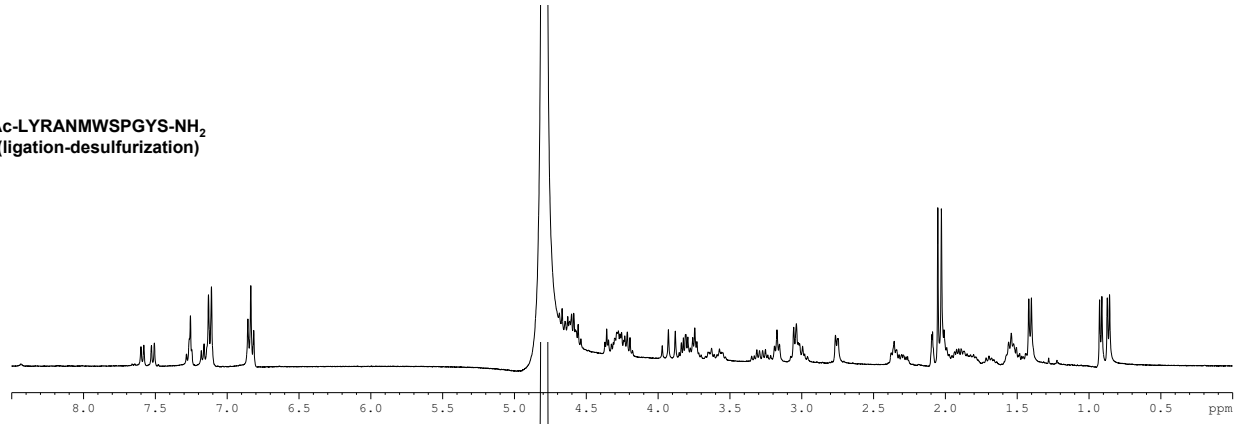
Ac-LYRANAWSPGYS-NH_2



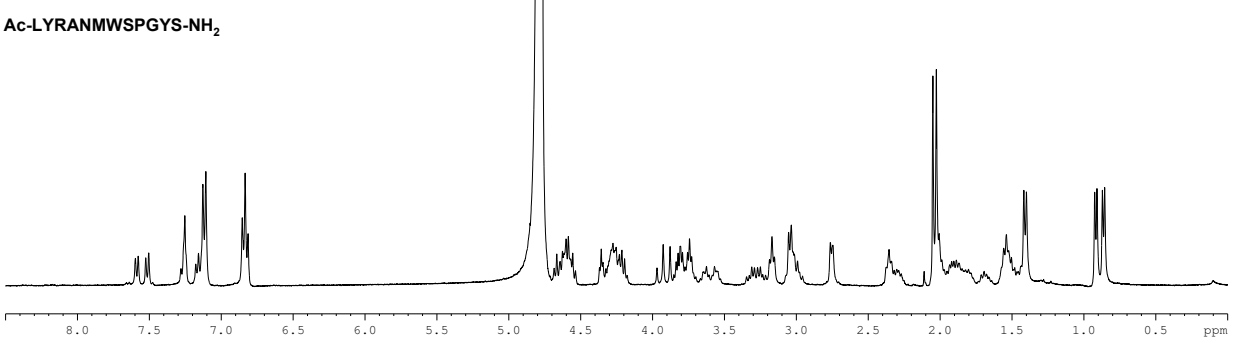
Ac-LYRAN(D-)AWSPGYS-NH_2

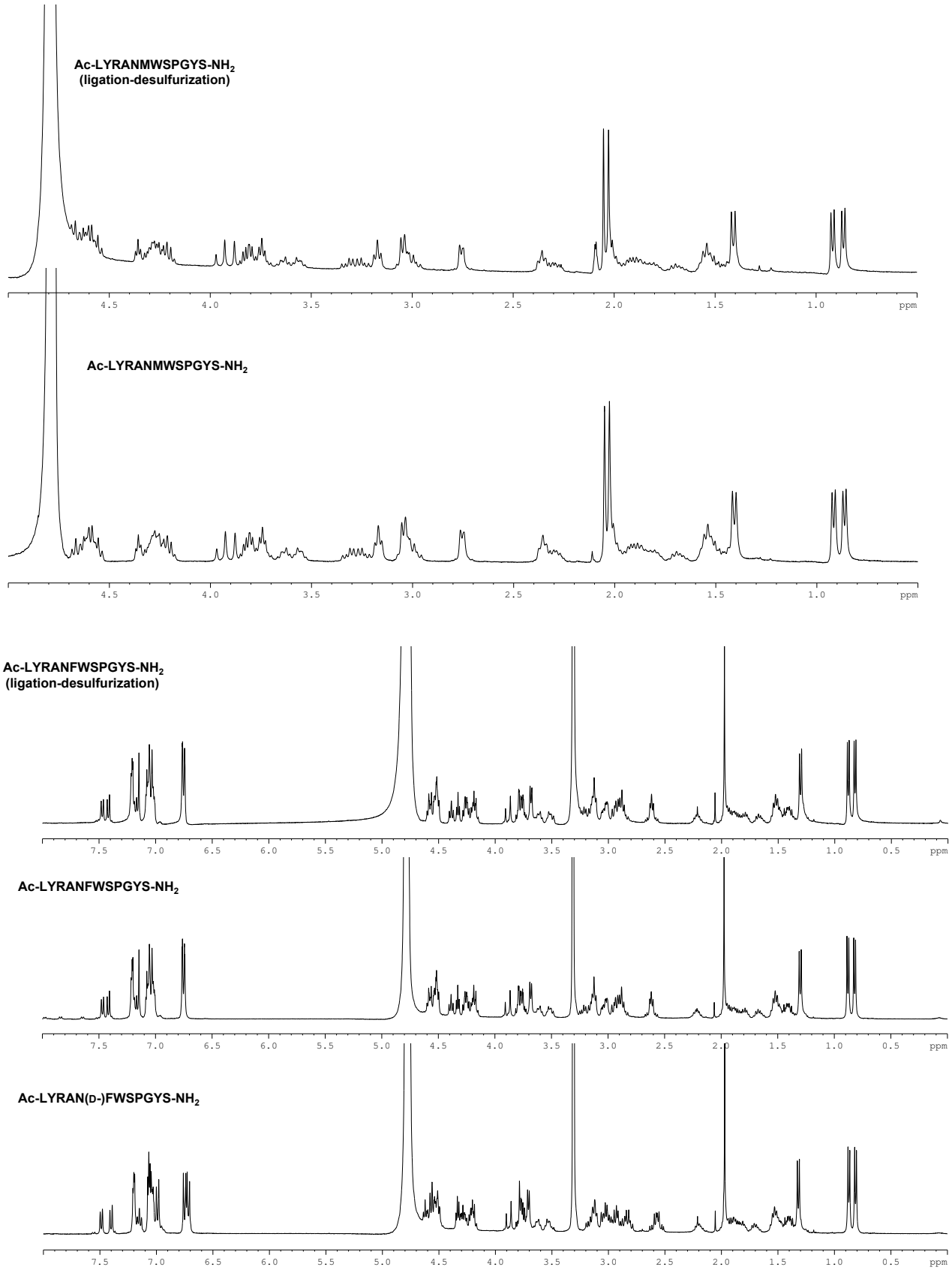


Ac-LYRANMWSPGYS-NH_2
(ligation-desulfurization)

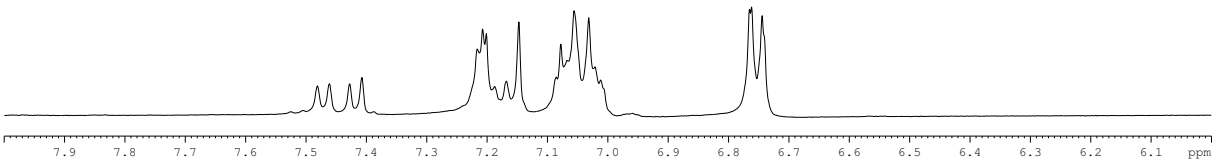


Ac-LYRANMWSPGYS-NH_2

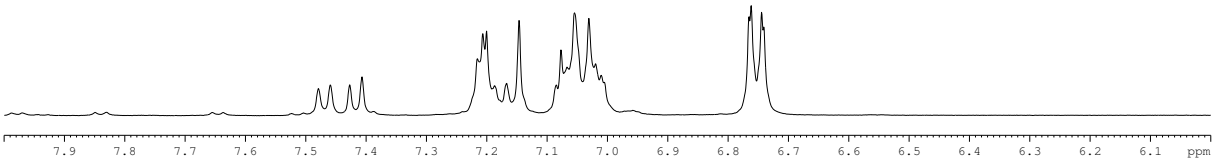




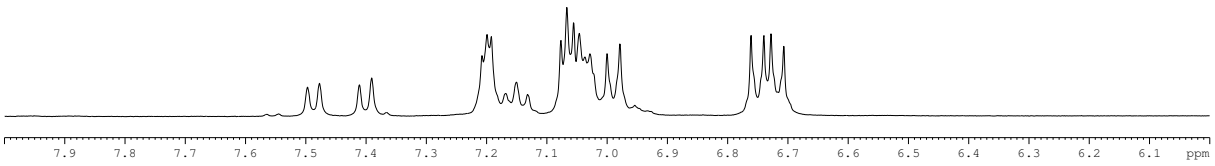
Ac-LYRANFWSPGYS-NH_2
(ligation-desulfurization)



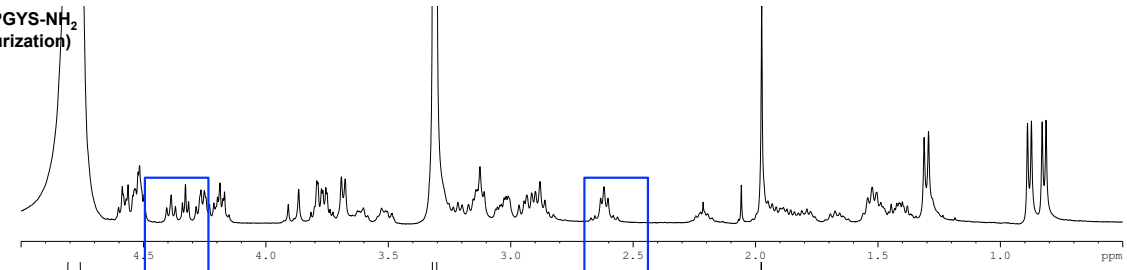
Ac-LYRANFWSPGYS-NH_2



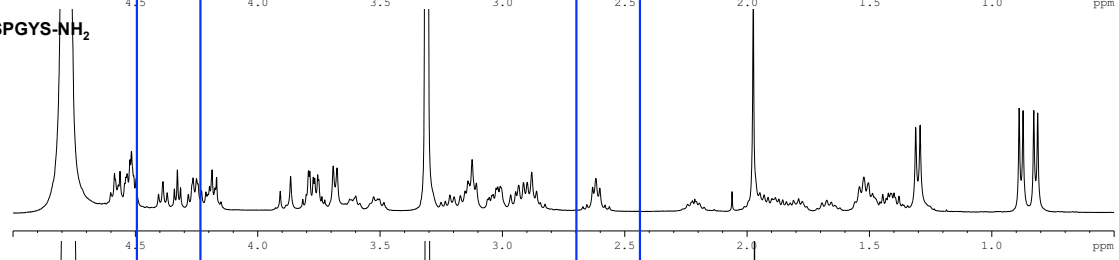
Ac-LYRAN(D-)FWSPGYS-NH_2



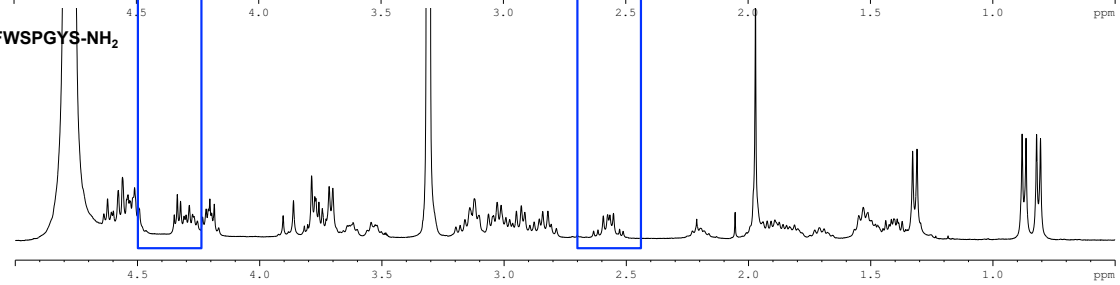
Ac-LYRANFWSPGYS-NH_2
(ligation-desulfurization)



Ac-LYRANFWSPGYS-NH_2



Ac-LYRAN(D-)FWSPGYS-NH_2

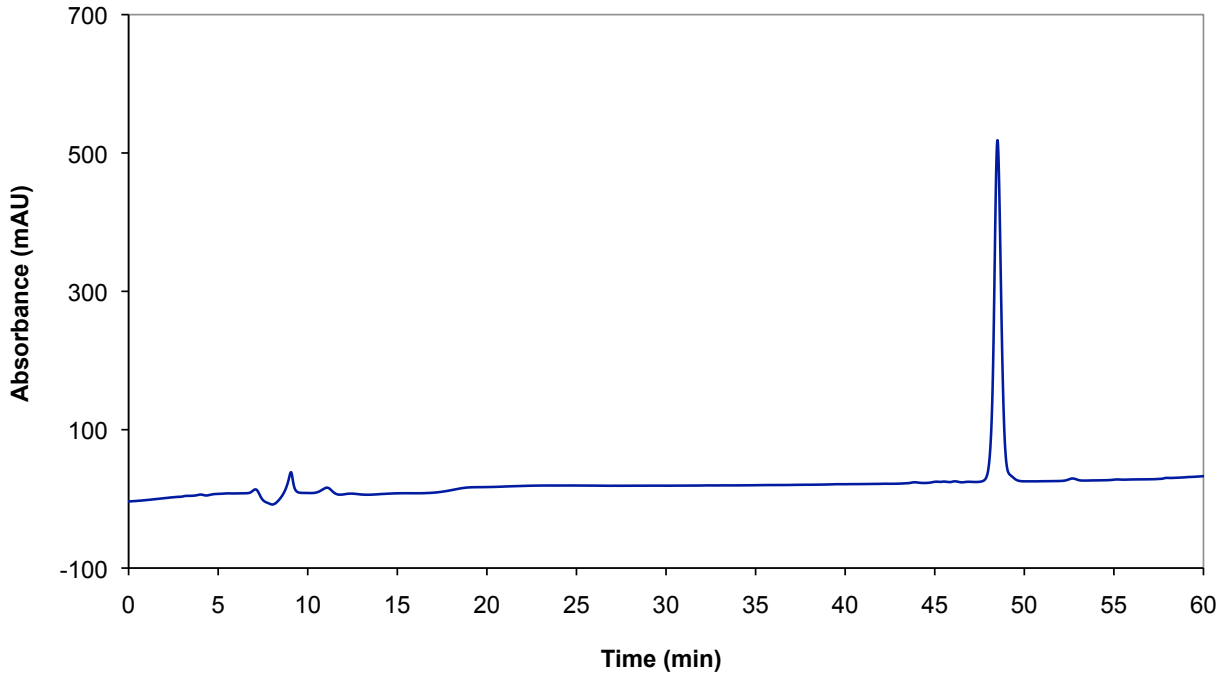


B) Analytical HPLC co-injections

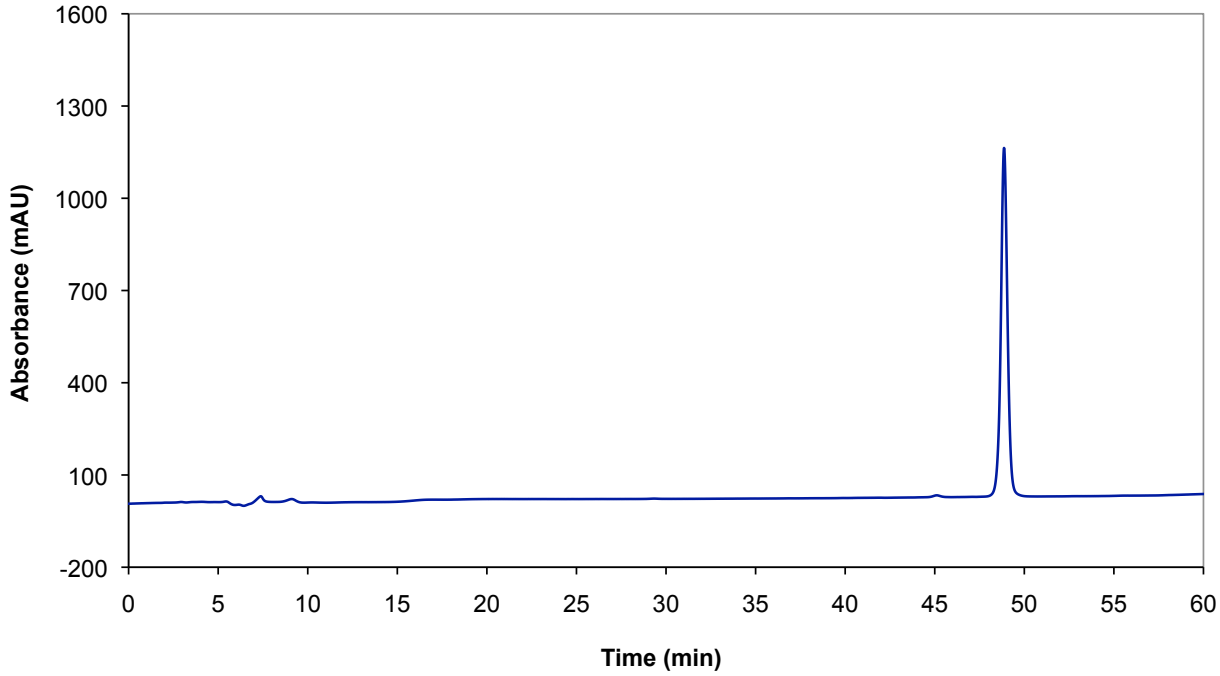
Analytical standards were coinjected with purified ligation-desulfurization products and analyzed *via* analytical reverse-phase HPLC (0 to 50% B over 60 min, 0.1% TFA, λ = 230 nm). 2-thiol Trp ligation-desulfurization products were found to co-elute with authentic peptide standards.

Co-injections:

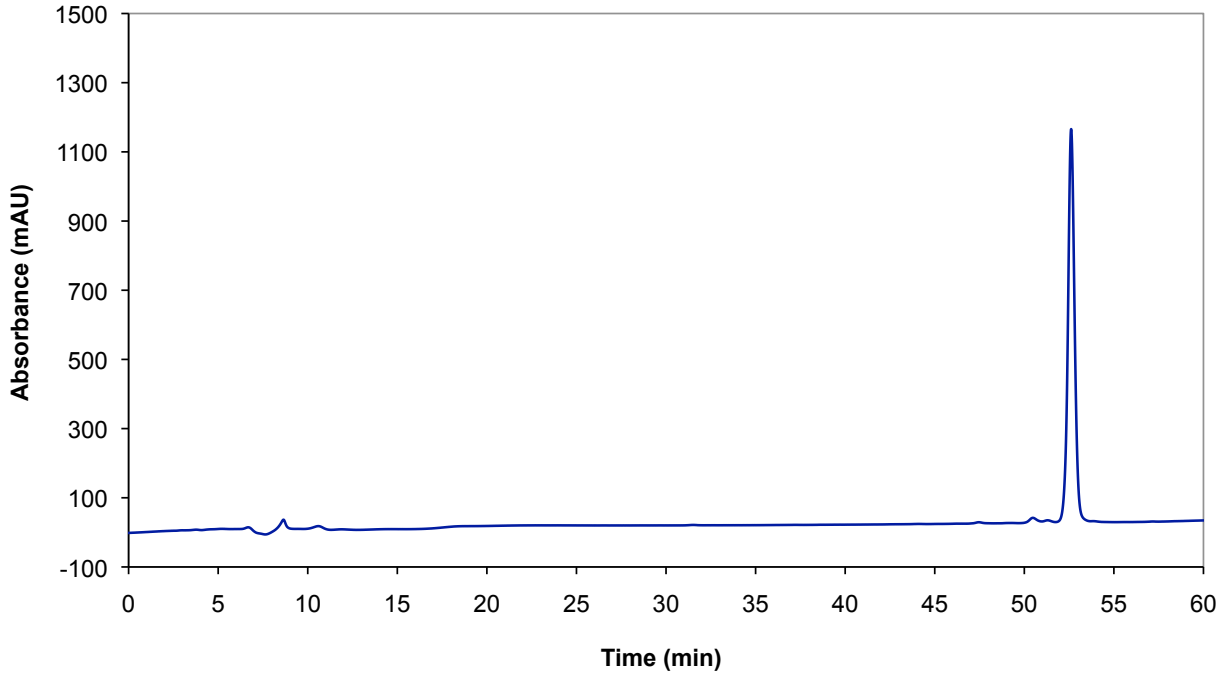
1. Ac-LYRANAWPSGYS-NH_2 standard and Ac-LYRANAWPSGYS-NH_2 ligation-desulfurization product



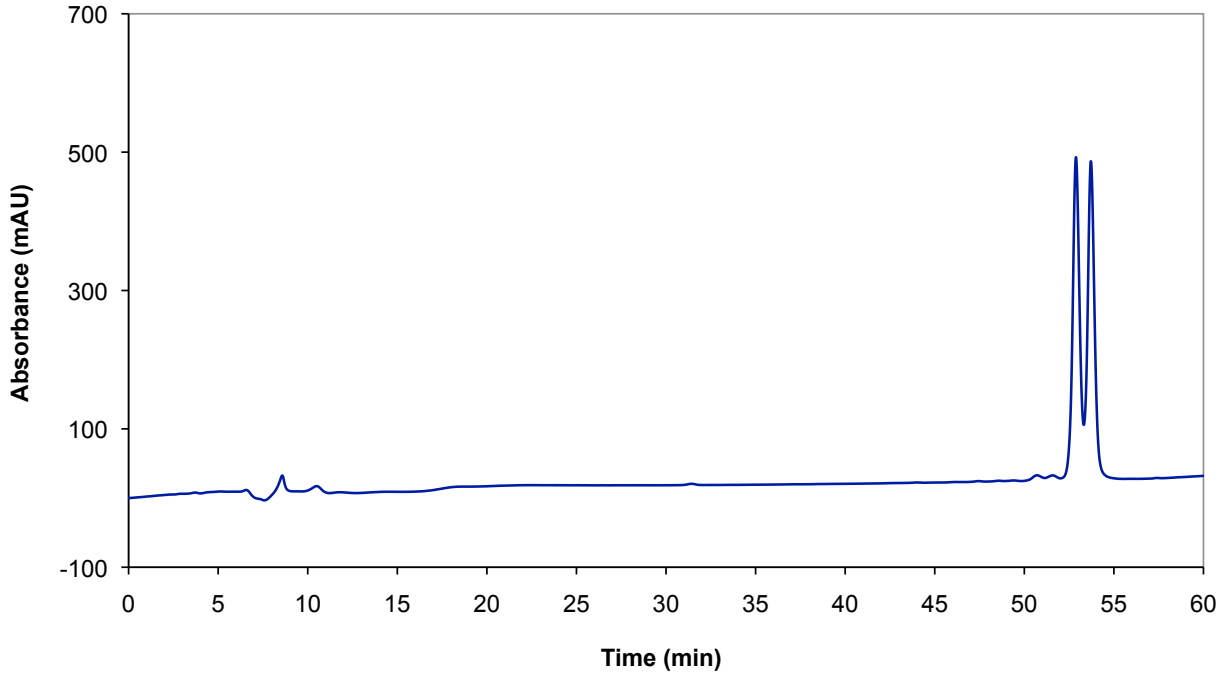
2. Ac-LYRANMWPSGYS-NH_2 standard and Ac-LYRANMWPSGYS-NH_2 ligation-desulfurization product



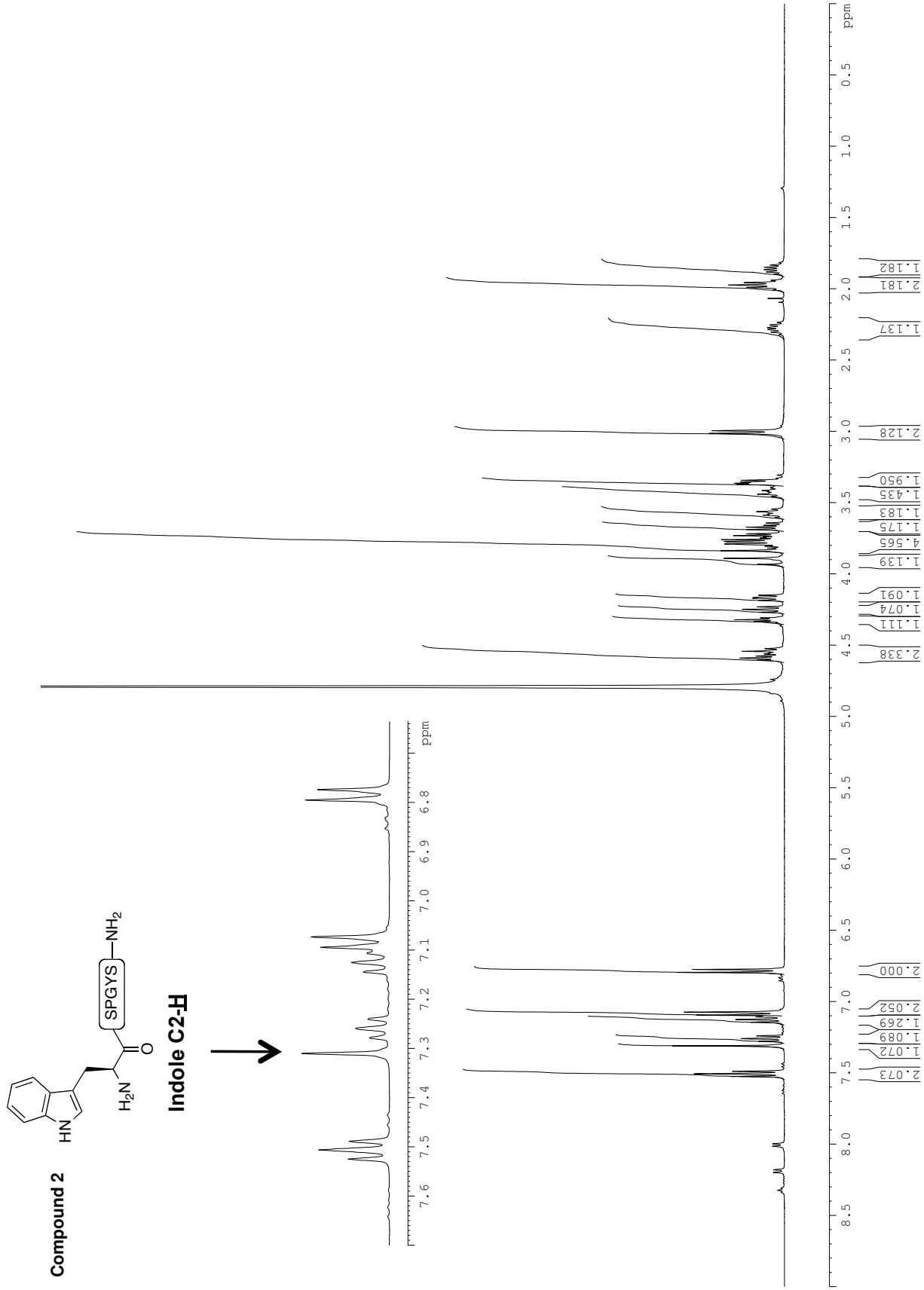
3. Ac-LYRANFWPSGYS-NH_2 standard and Ac-LYRANFWPSGYS-NH_2 ligation-desulfurization product



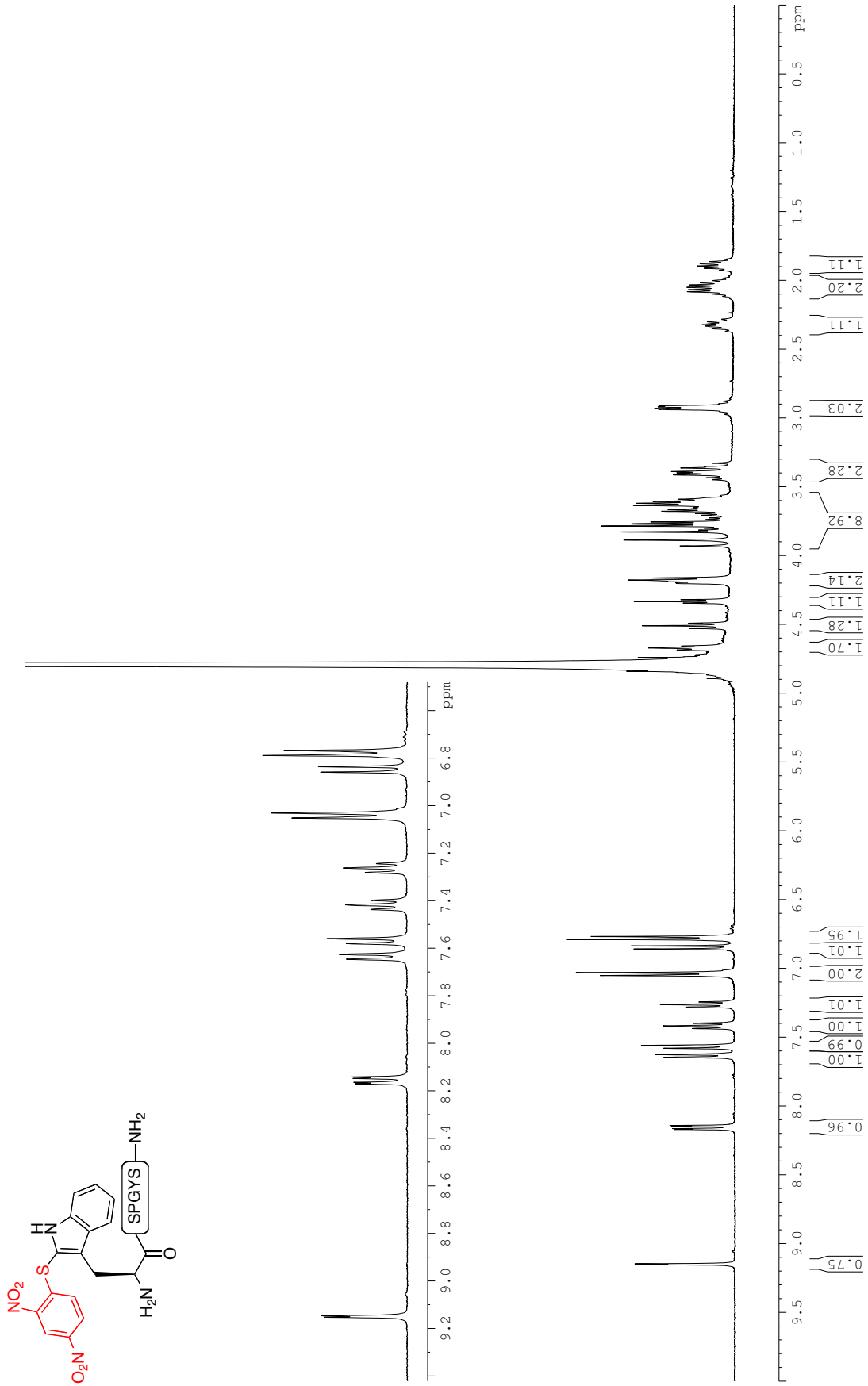
4. Ac-LYRAN(D-)FWPSGYS-NH_2 standard and Ac-LYRANFWPSGYS-NH_2 standard (for comparison)

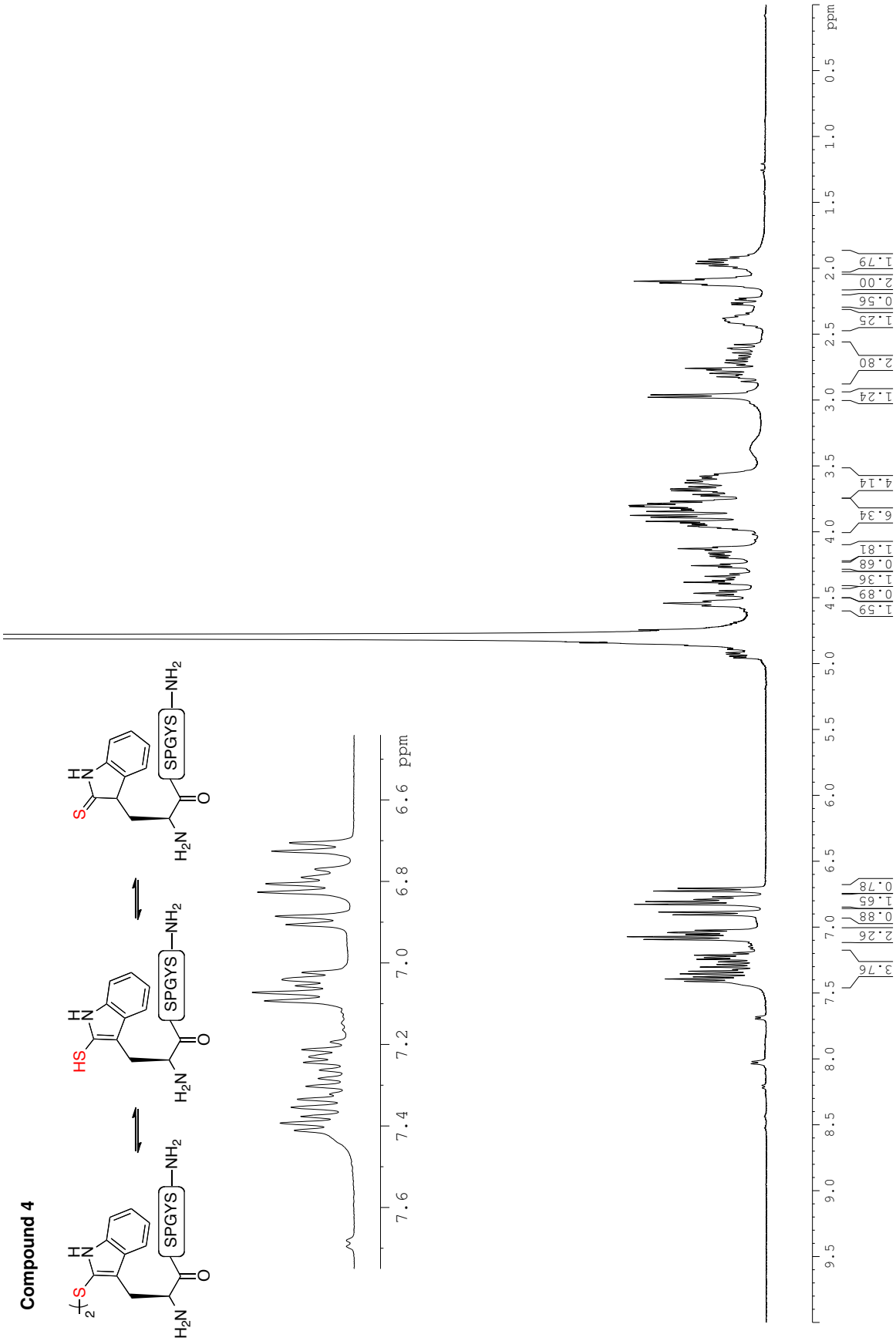


Additional NMR Spectra



Compound 3





References

1. Y. Kajihara, A. Yoshihara, K. Hirano and N. Yamamoto, *Carbohydr. Res.*, 2006, **341**, 1333-1340.
2. D. Bang, B. L. Pentelute and S. B. Kent, *Angew. Chem. Int. Ed.*, 2006, **45**, 3985-3988.
3. Synthesis performed by Mimotopes Pty Ltd and peptide supplied on-resin.