

Supporting Information

Synthesis of Thiophenylalanine-Containing Peptides via Cu(I)-Mediated Cross-Coupling

Christina R. Forbes and Neal J. Zondlo*
Department of Chemistry and Biochemistry
University of Delaware
Newark, DE 19716

Contents

Part 1

S2. Materials

S2. Peptide synthesis

S3. NMR spectroscopy

S4. Determination of pK_a for 4-thiophenylalanine

S5. Fluorescence spectroscopy

S6. Circular dichroism

S6-S22. Synthetic procedures for all peptides

S7. Solution-phase cross-coupling reaction of AcSH with Ac-T(4-I-Phe)PN-NH₂

S8. Solid-phase cross-coupling reaction of PhSH, thiobenzoic acid, and allyl mercaptan

S10. Solid-phase cross-coupling reaction of AcSH with Ac-T(4-I-Phe)PN-NH₂

S11. General procedure for solid phase synthesis of 4-thiophenylalanine-containing peptides

S23-S27. NMR spectra (amide regions) for peptides **2-7**, **17-24**

S28-S29. CD data for trp cage peptides **15** and **16**

S29. References

Part 2

S31-S39. Complete NMR spectra for Ac-TXPN-NH₂ peptides **2-7**, **17-24**

S40-S45. HPLC reinjection chromatograms for purified peptides **2-7**, **12-15**, **17-24**

S46-S71. Mass spectra for peptides **1-24**

Materials

Fmoc-L-amino acids were purchased from Novabiochem (San Diego, CA), Bachem (San Carlos, CA), or Chem-Impex (Wood Dale, IL). Rink amide MBHA resin, Fmoc-4-iodo-L-phenylalanine, and diisopropylethylamine (DIPEA) were purchased from Chem-Impex. Acetic anhydride (Ac_2O), trifluoroacetic acid (TFA), phenol, thioanisole, triisopropylsilane (TIS), sodium periodate (NaIO_4), copper(I) iodide, 1,10-phenanthroline, thiolacetic acid, methyl iodide, 2-aminoethanethiol, 2,2'-dithiodipyridine, lithium hydroxide, thiophenol, and dithiothreitol (DTT) were purchased from Acros. Ethanedithiol (EDT) was purchased from Pfaltz & Bauer (Waterbury, CT). Methyltrioxorhenium (MTO) was purchased from Strem Chemicals (Newburyport, MA). Piperidine, Hoveyda-Grubbs catalyst (second generation), allyl iodide, and 2-nitrobenzyl bromide were purchased from Aldrich. Tri(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Hampton Research (Aliso Viejo, CA). O-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Senn Chemicals (San Diego, CA). Acetonitrile (MeCN), dimethylformamide (DMF), methylene chloride (CH_2Cl_2), methanol (MeOH), ether, pyridine, *t*-amyl alcohol, toluene, acetic acid, sodium chloride, sodium borohydride, sodium nitrite, magnesium sulfate, and hydrogen peroxide were purchased from Fisher. Deionized water was purified by a Millipore Synergy 185 water purification system with a Simpapak2 cartridge. Solid-phase post-synthetic modification reactions were performed in capped disposable fritted columns (Image Molding), in disposable Eppendorf tubes (1.5 mL), or in glass vials (2 mL). All compounds were used as purchased with no additional purification.

Peptide synthesis

Peptides (0.1 or 0.25 mmol) were synthesized on a Rainin PS3 peptide synthesizer on Rink amide resin via standard Fmoc solid phase peptide synthesis using HBTU as a coupling reagent. 60 minute couplings were performed with 4 equivalents of Fmoc amino acid and HBTU. 3 equivalents were used for Fmoc-4-iodo-L-phenylalanine. All peptides were acetylated on the N-terminus and contained C-terminal amides.

Peptides were cleaved from the resin and deprotected for 2-4 hours under standard conditions (90% TFA/5% TIS/5% H_2O for TXPN, GPPXPPGY, and KKHMCX, where X is 4-iodophenylalanine or 4-thiophenylalanine derivatives; 84% TFA/4% each of

H₂O/phenol/thioanisole/ethanedithiol for trp cage peptides). TFA was removed by evaporation. Peptides were precipitated with cold ether and the precipitate was dried. The peptides were dissolved in water or buffer and then filtered using a 0.45 μm syringe filter. The peptides were purified and the conversion and purity were determined using reverse phase HPLC on a Vydac C18 semi-preparative column (250 \times 10 mm, 5-10 μm particle, 300 \AA pore) or on a Varian Microsorb MV C18 analytical column (250 \times 4.6 mm, 3-5 μm particle, 100 \AA pore) using a linear gradient of buffer B (20% water, 80% MeCN, 0.05% TFA) in buffer A (98% water, 2% MeCN, 0.06% TFA). Peptide purity was verified via reinjection on an analytical HPLC column. Peptides were characterized by ESI-MS (positive ion mode) on an LCQ Advantage (Finnigan) mass spectrometer.

Concentrations of peptides were determined by UV, where 4-iodophenylalanine $\epsilon_{280} = 280 \text{ M}^{-1} \text{ cm}^{-1}$.¹ Concentrations of 4-thiophenylalanine-containing peptides were determined via Ellman's test or by UV using measured molar extinction coefficients ($\epsilon_{280, \text{pH } 4.0} = 1440 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{280, \text{pH } 5.0} = 2230 \text{ M}^{-1} \text{ cm}^{-1}$, or $\epsilon_{280, \text{pH } 8.5} = 15,600 \text{ M}^{-1} \text{ cm}^{-1}$) (vide infra).

For reaction schemes, the prime notation (') indicates that the peptide is protected and on solid phase.

NMR spectroscopy

Peptides were dissolved in buffer containing 5 mM phosphate (pH 4.0 or as indicated), 25 mM NaCl, 90% H₂O/10% D₂O, and 100 μM TSP. 100 μM TCEP was added for thiophenylalanine-containing peptides to prevent disulfide formation. Peptide concentrations were 10 μM -200 μM . NMR spectra were collected at 298 K on a Brüker AVN 600 MHz NMR spectrometer equipped with a triple resonance cryoprobe or a TXI probe. Spectra were internally referenced with TSP. 1-D spectra were collected with a Brüker w5 watergate pulse sequence and a relaxation delay of 2-3 s. 2-D spectra were collected with a watergate TOCSY pulse sequence.

Well-resolved peaks in the NMR spectra were integrated after baseline normalization. $K_{\text{trans/cis}}$ was calculated based on the average integrated ratios of 2-3 pairs of peaks. In general, backbone amide peaks and/or Thr methyl peaks were used for integration.

Determination of pK_a for T(4-SH-Phe)PN (4)

Purified Ac-T(4-SH-Phe)PN-NH₂ (4) was dissolved in buffer containing 50 mM phosphate and 100 μ M DTT. Peptide concentrations were 45-65 μ M. Absorbance spectra were collected on a Perkin-Elmer Lambda 25 UV-Vis spectrometer in a 1 cm cell. Absorbance scans were taken from 310 nm to 220 nm with a slit width of 1 nm. After UV scans, the pH of each sample was measured with a pH electrode (Mettler Toledo). After measurements, the absence of disulfide formation was confirmed via HPLC. Data were the average of at least three independent trials. Error bars are shown and indicate standard error.

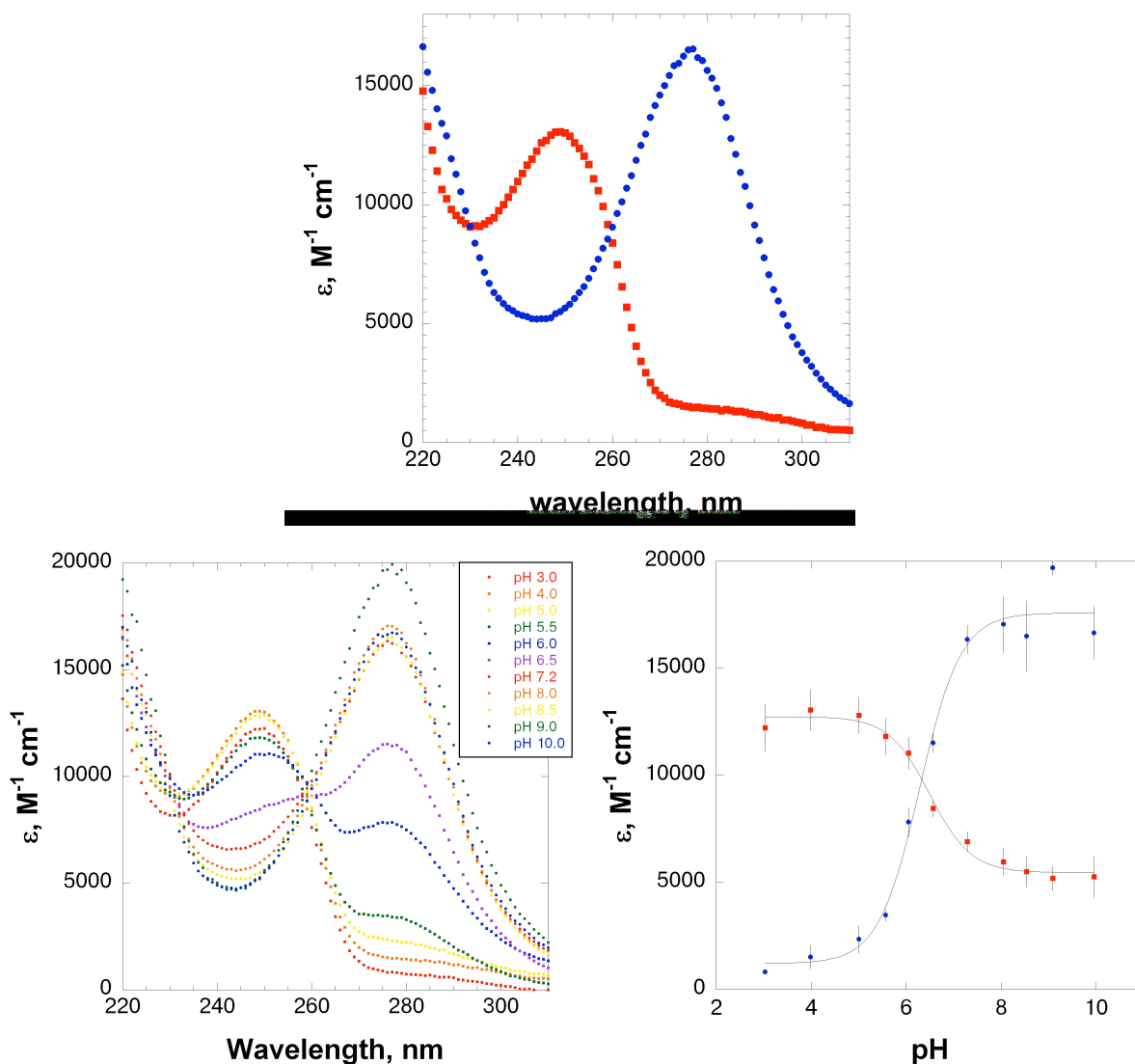


Figure S1. Top: UV-vis spectra of Ac-T(4-SH-Phe)PN-NH₂ (4) at pH 4.0 (thiol, red squares) and pH 8.5 (thiolate, blue circles). Bottom left: Absorbance spectra of Ac-T(4-SH-Phe)PN-NH₂ (4) at indicated pH values. Data represent the average of at least 3 independent trials. Bottom

right: pH dependence of the molar extinction coefficient in Ac-T(4-SH-Phe)PN-NH₂ (**4**) at 249 nm (red squares) and 276 nm (blue circles). Error bars indicate standard error.

$$\epsilon_{249} = \frac{\epsilon_{249,\text{thiolate}} 10^{(pH-pK_a)} + \epsilon_{249,\text{thiol}}}{1 + 10^{(pH-pK_a)}} \quad pK_a = 6.50 \pm 0.08$$

$$\epsilon_{276} = \frac{\epsilon_{276,\text{thiolate}} 10^{(pH-pK_a)} + \epsilon_{276,\text{thiol}}}{1 + 10^{(pH-pK_a)}} \quad pK_a = 6.28 \pm 0.09$$

Fluorescence spectroscopy

Purified Ac-T(4-SH-Phe)PN-NH₂ (**4**) was dissolved in buffer containing 50 mM phosphate (pH 4.0 or 8.5). Peptide concentrations were 100 μ M with 125 μ M DTT. Fluorescence spectra were collected on a Photon Technology International fluorescence spectrometer model QM-3/2003 with a CW source and a Hamamatsu R928 PMT. Measurements were taken in a 1 cm quartz cell (Starna). All slit widths were 3 nm. Excitation scans were taken from 250 nm to 450 nm, with emission detected at 400 nm. Emission scans were taken from 300 nm to 500 nm with excitation at 295 nm. Data were the average of at least three independent trials. Data were background-corrected but were not smoothed. Error bars are shown and indicate standard error.

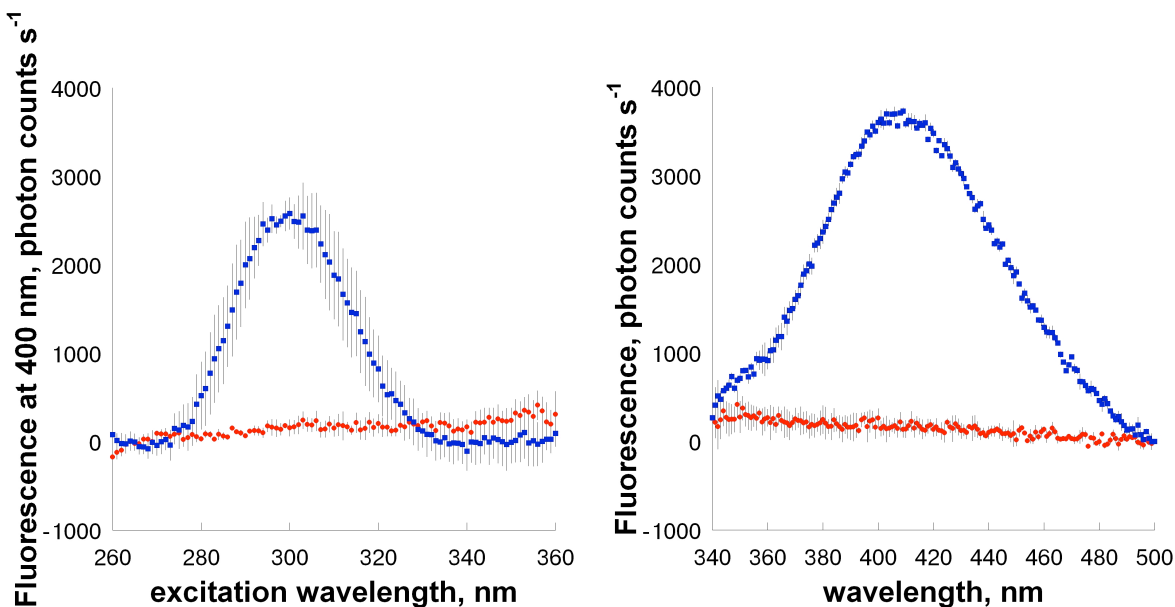


Figure S2. Left: Excitation scans of the peptide Ac-T(4-SH-Phe)PN-NH₂ (**4**) in 50 mM aqueous phosphate at pH 4.0 (red circles) and at 8.5 (blue squares). Fluorescence was detected at 400 nm. Right: Emission scans of the peptide Ac-T(4-SH-Phe)PN-NH₂ (**4**) in 50 mM aqueous phosphate at pH 4.0 (red circles) and at 8.5 (blue squares) excitation at 295 nm. Data represent the average of at least 3 independent trials. Error bars indicate standard error.

Circular dichroism

Spectra were collected at 4 °C and 25 °C on a Jasco model J-810 Spectropolarimeter in a 1 mm cell with 20-65 μ M peptide in 15 mM sodium phosphate buffer (pH 4.0, 7.0, or 8.5). TCEP (0.56 mM) was added to solutions of peptides containing 4-thiophenylalanine. The absence of disulfide formation was determined via HPLC after CD analysis. Individual spectra were collected every nm with an averaging time of 4 s and three accumulations. Data were background-corrected but were not smoothed. Data were the average of at least three independent trials. Error bars are shown and indicate standard error.

Thermal denaturation experiments

CD experiments were conducted using solution of peptides 20-65 μ M in 15 mM sodium phosphate buffer (pH 4.0, 7.0, or 8.5). TCEP (0.56 mM) was added to solutions of trp cage peptides containing 4-thiophenylalanine to prevent disulfide formation. After the CD experiments, the absence of disulfides was confirmed via HPLC. CD spectra were recorded in a 1 mm pathlength cell on a Jasco model J-810 spectropolarimeter. Thermal denaturation data were collected at 222 nm using a bandwidth of 4 nm. The temperature was increased in 1 °C intervals from 2 °C to 90 °C over 1.5 h. Data were collected with a 4 s response time. T_m data were smoothed using a 5-data-point smoothing window (KaleidaGraph 4.0); the smoothed data are shown in Figure 3 of the manuscript. Data shown in the Supporting Information are the non-smoothed data. In the Supporting Information, error bars are shown and indicate standard error.

T_m temperatures were determined using a slope for the 100% unfolded baseline ($d[\theta]_{222}/dT$) of $-8.83 \text{ deg cm}^2 \text{ dmol}^{-1}/^\circ\text{C}$; and a slope for the 100% folded baseline ($d[\theta]_{222}/dT$) of $+63.5 \text{ deg cm}^2 \text{ dmol}^{-1}/^\circ\text{C}$, based on prior work on the trp cage miniprotein.²

Ac-T(4-I-Phe)PN-NH₂ (1)

Peptide **1** was purified using a linear gradient of 0-45% buffer B in buffer A over 60 minutes: t_R 43.1 min, exp. 644.2, obs. 667.1 (M + Na)⁺.

Ac-GPP(4-I-Phe)PPGY-NH₂ (8)

Peptide **8** was purified using a linear gradient of 0-55% buffer B in buffer A over 80 minutes: t_R 54.6 min, exp. 997.3, obs. 998.0 (M + H)⁺.

Ac-KKHMC(4-I-Phe)-NH₂ (9)

Peptide **9** was purified using a linear gradient of 0-70% buffer B in buffer A over 60 minutes: t_R 36.3 min, exp. 959.3, obs. 480.8 (M + 2H)²⁺.

Ac-KKHMC(*t*Bu)(4-I-Phe)-NH₂ (10)

Peptide **10** was purified using a linear gradient of 0-75% buffer B in buffer A over 60 minutes: t_R 37.1 min, exp. 1015.4, obs. 508.9 (M + 2H)²⁺.

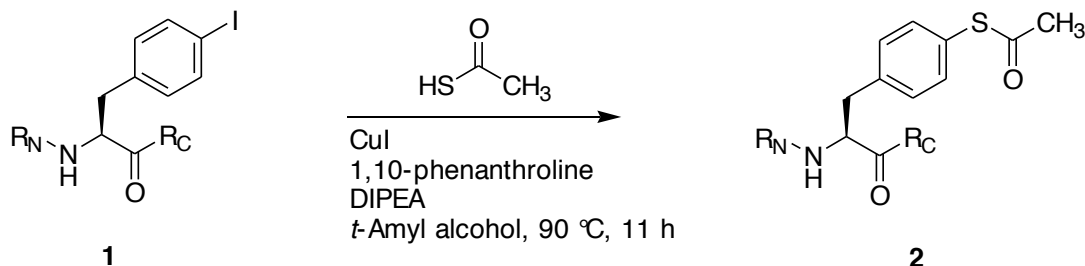
Y3(4-I-Phe) trp cage (11)

Peptide **11** was purified using a linear gradient of 0-70% buffer B in buffer A over 80 minutes: t_R 67.1 min, exp. 2319.0, obs. 1160.9 (M + 2H)²⁺.

trp cage (16)

Peptide **16** was purified using a linear gradient of 0-70% buffer B in buffer A over 80 minutes: t_R 51.5 min, exp. 2209.1, obs. 1106.2 (M + 2H)²⁺.

Solution-phase synthesis of Ac-T(4-thioacetyl-Phe)PN-NH₂ (2)



The purified 4-iodophenylalanine-containing peptide (**1**) (1 μ mol) was lyophilized and a mixture of *t*-amyl alcohol (200 μ L) and DIPEA (33 μ L, 0.2 mmol) was added. Copper(I) iodide (1.8 mg, 10 μ mol), 1,10-phenanthroline (3.6 mg, 20 μ mol), and thioacetic acid were added (8.6 μ L, 0.12 mmol). The mixture was incubated on a heating block at 90 °C for 11 h. The reaction was allowed to cool to room temperature. Water was added to the mixture (200 μ L). The aqueous layer was washed with ether, then filtered on a 0.45 μ m syringe filter to produce the peptide Ac-T(4-thioacetyl-Phe)PN-NH₂ (**2**) in 29% yield, as determined by integration of the HPLC chromatogram. Peptide **2** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: t_R 41.0 min, exp. 593.2, obs. 615.2 (M + Na)⁺. Additional peaks at t_R

46.9 min (see Figure S83) and t_R 51.7 min (see Figure S84) indicated overacetylation of the peptide in the solution phase reaction based on the observed masses. NMR data suggest the possibility of the acetylation of unprotected Thr under the solution reaction conditions.

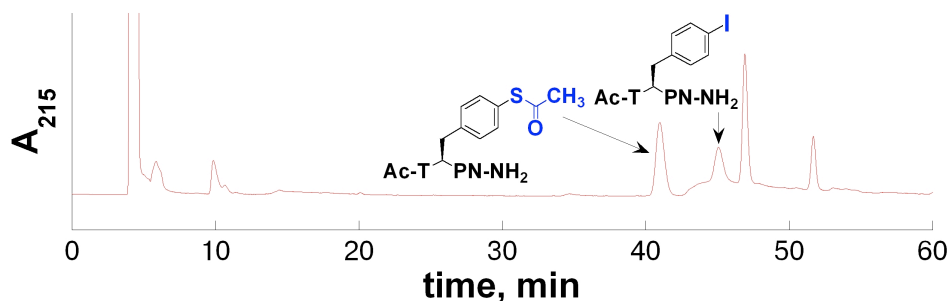
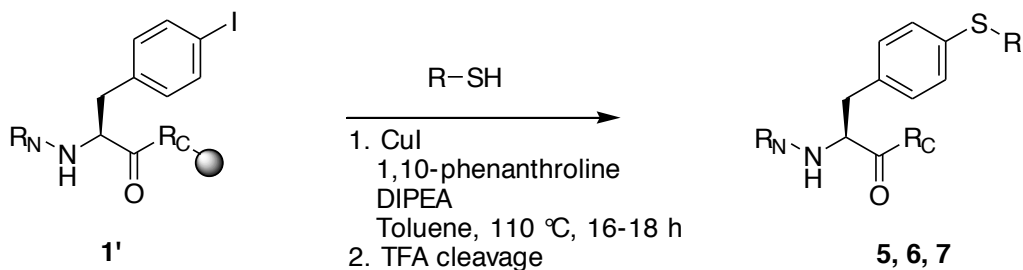


Figure S3. Crude HPLC chromatogram of solution phase cross-coupling to produce the peptide Ac-T(4-thioacetyl-Phe)PN-NH₂ (**2**).

Solid-phase synthesis of Ac-T(4-thiophenyl-Phe)PN-NH₂ (5**), Ac-T(4-S-allyl-Phe)PN-NH₂ (**6**), and Ac-T(4-thiobenzoyl-Phe)PN-NH₂ (**7**)**



Resin with the 4-iodophenylalanine-containing peptide (**1'**) (10-15 mg, 7-10 μ mol) was placed in a glass vial, and toluene (200 μ L) and DIPEA (33 μ L, 0.2 mmol) were added. Copper(I) iodide (1.8 mg, 10 μ mol); 1,10-phenanthroline (3.6 mg, 20 μ mol); and thiophenol (12.3 μ L, 0.12 mmol), thiobenzoic acid (14.1 μ L, 0.12 mmol), or allyl mercaptan (9.6 μ L, 0.12 mmol) were added. The vial was capped and the mixture was stirred in an oil bath set to 110 $^{\circ}$ C for 16-18 h. In the case of the peptide **7**, the reaction temperature was set to 100 $^{\circ}$ C. The resin was washed with DMF (4 \times 4 mL), CH₂Cl₂ (2 \times 4 mL), and MeOH (2 \times 4 mL) and dried with ether. The resin was cleaved and the peptide deprotected under standard cleavage/deprotection conditions to produce the peptide Ac-T(4-thiophenyl-Phe)PN-NH₂ (**5**) in 90% yield, Ac-T(4-S-allyl-Phe)PN-NH₂ (**6**) in 2% yield, or Ac-T(4-thiobenzoyl-Phe)PN-NH₂ (**7**) in 21% yield, as determined by integration of the respective HPLC chromatograms. Peptide **5** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: t_R 52.6 min, exp. 626.3,

obs. 627.1 ($M + H$)⁺. Peptide **6** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: t_R 44.4 min, exp. 590.3, obs. 591.0 ($M + H$)⁺. Peptide **7** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: t_R 53.5 min, exp. 654.3, obs. 677.3 ($M + Na$)⁺.

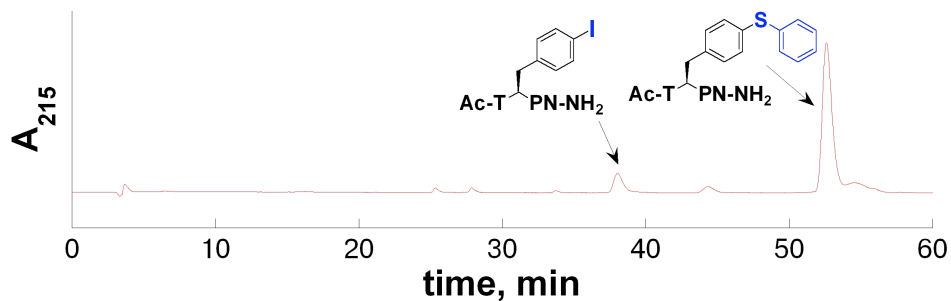


Figure S4. Crude HPLC chromatogram of solid phase cross-coupling reaction to produce the peptide Ac-T(4-thiophenyl-Phe)PN-NH₂ (**5**).

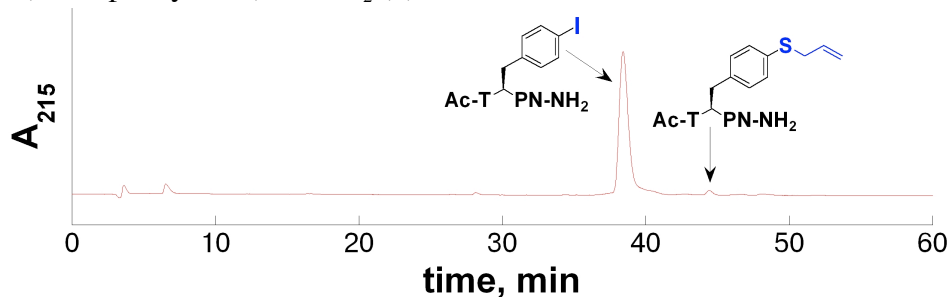


Figure S5. Crude HPLC chromatogram of solid phase cross-coupling PN reaction to produce the peptide Ac-T(4-S-allyl-Phe)PN-NH₂ (**6**).

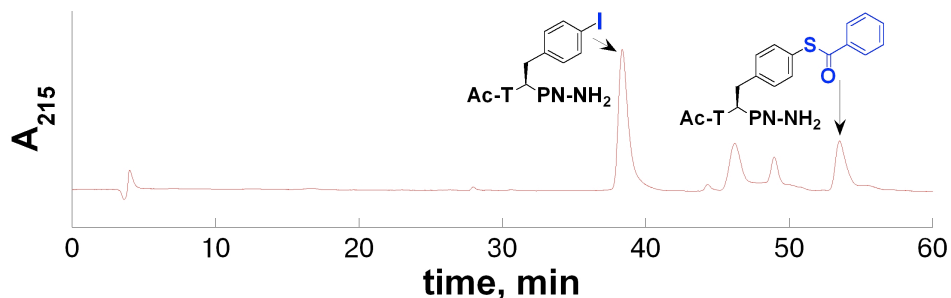
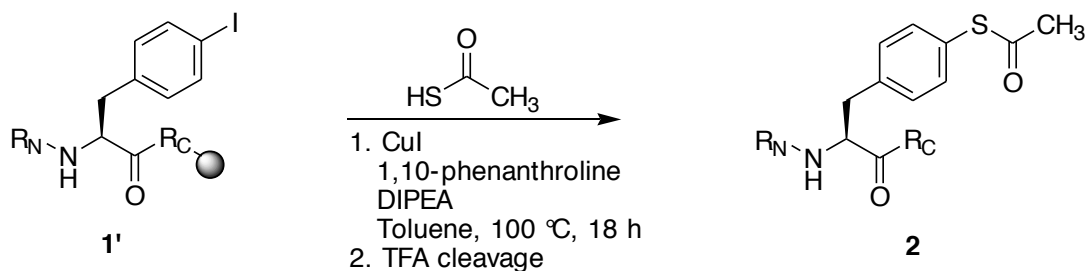


Figure S6. Crude HPLC chromatogram of solid phase cross-coupling reaction to produce the peptide Ac-T(4-thiobenzoyl-Phe)PN-NH₂ (**7**).

Solid-phase synthesis of Ac-T(4-thioacetyl-Phe)PN-NH₂ (2)



Resin with the 4-iodophenylalanine-containing peptide (**1'**) (10-15 mg, 7-10 μ mol) was placed in a glass vial, and toluene (200 μ L) and DIPEA (33 μ L, 0.2 mmol) were added. Copper(I) iodide (1.8 mg, 10 μ mol), 1,10-phenanthroline (3.6 mg, 20 μ mol), and thiolacetic acid were added (8.6 μ L, 0.12 mmol). The vial was capped and the mixture was stirred in an oil bath set to 100 °C for 18 h. The reaction was allowed to cool to room temperature. The resin was washed with DMF (4 \times 4 mL), CH₂Cl₂ (2 \times 4 mL), and MeOH (2 \times 4 mL) and dried with ether. The resin was cleaved and the peptide deprotected under standard cleavage/deprotection conditions to produce peptide **2** in 57% yield, as determined by integration of the HPLC chromatogram. Peptide **2** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: t_R 41.0 min, exp. 593.2, obs. 615.2 (M + Na)⁺.

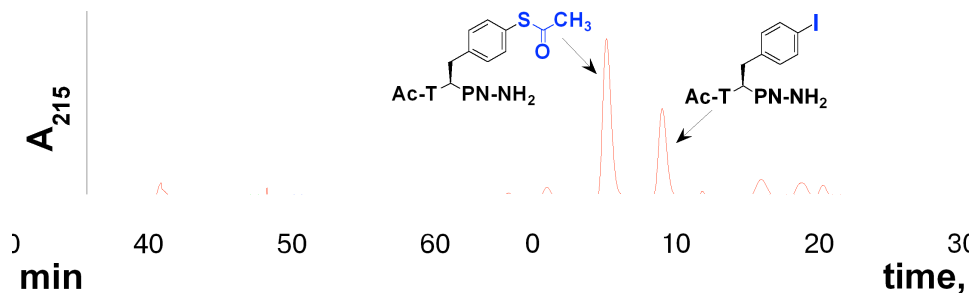
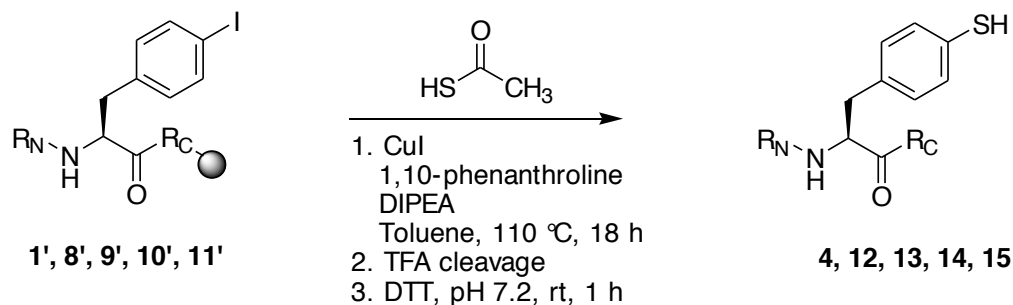


Figure S7. Crude HPLC chromatogram of solid phase cross-coupling reaction to produce the peptide Ac-T(4-thiophenyl-Phe)PN-NH₂ (**2**) under conditions to optimize the yield of thioacetyl product.

Solid-phase synthesis of Ac-T(4-SH-Phe)PN-NH₂ (4**), Ac-GPP(4-SH-Phe)PPGY-NH₂ (**12**), Ac-KKHMC(4-SH-Phe)-NH₂ (**13**), Ac-KKHMC(*t*-Bu)(4-SH-Phe)-NH₂ (**14**), and Y3(4-SH-Phe) trp cage (**15**)**



Resin with the 4-iodophenylalanine-containing peptide (**1'**, **8'**, **9'**, **10'**, **11'**) (10-15 mg, 7-10 μmol) was placed in a glass vial, and toluene (200 μL) and DIPEA (33 μL , 0.2 mmol) were added. Copper(I) iodide (1.8 mg, 10 μmol), 1,10-phenanthroline (3.6 mg, 20 μmol), and thioacetic acid were added (8.6 μL , 0.12 mmol). The vial was capped and the mixture was stirred in an oil bath set to 110 $^\circ\text{C}$ for 16-18 h (in the case of Ac-KKHMCX-NH₂, the reaction was run for 24 h). The resin was washed with DMF (4 \times 4 mL), CH₂Cl₂ (2 \times 4 mL), and MeOH (2 \times 4 mL) and dried with ether. The resin was cleaved and the peptide deprotected under standard cleavage/deprotection conditions to produce the peptide Ac-T(4-thioacetyl-Phe)PN-NH₂ (**2**), Ac-GPP(4-thioacetyl-Phe)PPGY-NH₂, Y3(4-thioacetyl-Phe) trp cage, Ac-KKHMC(4-thioacetyl-Phe)-NH₂, or Ac-KKHMC(*t*-Bu)(4-thioacetyl-Phe)-NH₂, along with the corresponding disulfides. To obtain the 4-thiophenylalanine-containing peptide, the crude, precipitated peptide was dissolved in 1 M phosphate buffer (pH 7.2, 50 μL). DTT was then added (50 μL , 100 mM solution) and the mixture was incubated at room temperature for 1 h to produce the peptide Ac-T(4-SH-Phe)PN-NH₂ (**4**) in 99% yield, Ac-GPP(4-SH-Phe)PPGY-NH₂ (**12**) in 98% yield, Ac-KKHMC(4-SH-Phe)-NH₂ (**13**) in greater than 90% yield, Ac-KKHMC(*t*-Bu)(4-SH-Phe)-NH₂ (**14**) in greater than 90% yield, or Y3(4-SH-Phe) trp cage (**15**) in 67% yield. Peptide **4** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: t_R 30.1 min, exp. 550.2, obs. 573.1 (M + Na)⁺. Peptide **12** was purified via HPLC using a gradient of 0-55% buffer B in buffer A over 80 minutes: t_R 49.0 min, exp. 903.4, obs. 904.1 (M + H)⁺. Peptide **13** was purified via HPLC using a gradient of 0-70% buffer B in buffer A over 60 minutes: t_R 31.9 min, exp. 865.4, obs. 866.4 (M + H)⁺. Peptide **14** was purified via HPLC using a gradient of 0-75% buffer B in buffer A over 60 minutes: t_R 39.4 min, exp. 921.4, obs. 461.9 (M + 2H)²⁺.

Peptide **15** was purified via HPLC using a gradient of 0-70% buffer B in buffer A over 80 minutes: t_R 60.7 min, exp 2225.1, obs. 1113.9 ($M + 2H$)²⁺. All yields were determined by integration of the respective HPLC chromatograms.

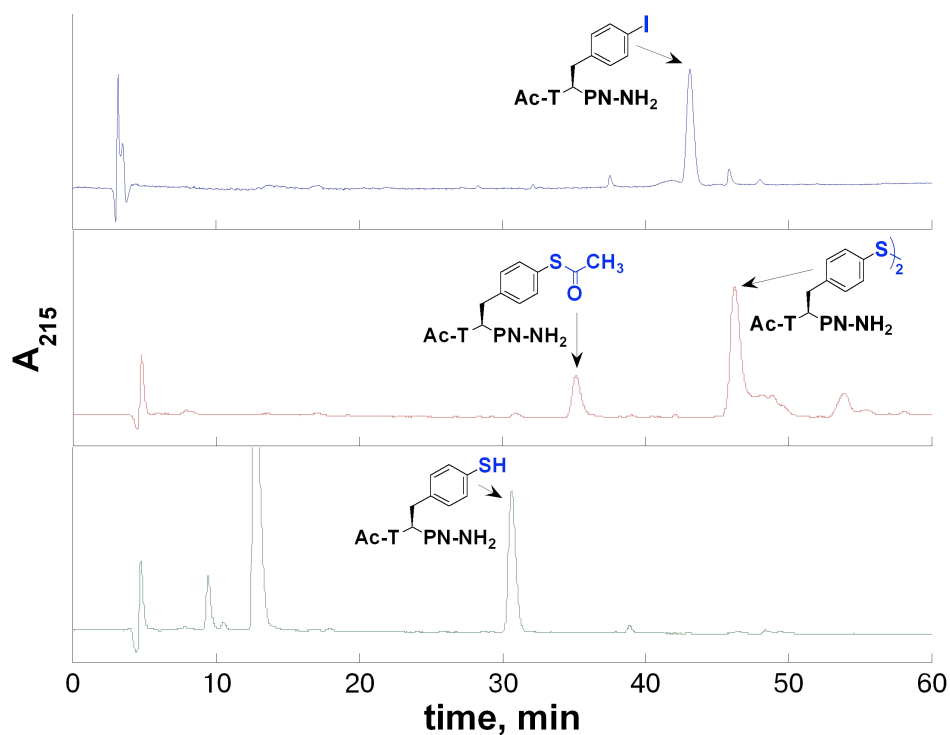


Figure S8. Crude HPLC chromatograms of solid phase cross-coupling reaction: starting material Ac-T(4-I-Phe)PN-NH₂ (**1**) (top); crude products of the cross-coupling reaction in toluene (middle), and crude products after deacetylation and reduction with DTT (**4**) (bottom).

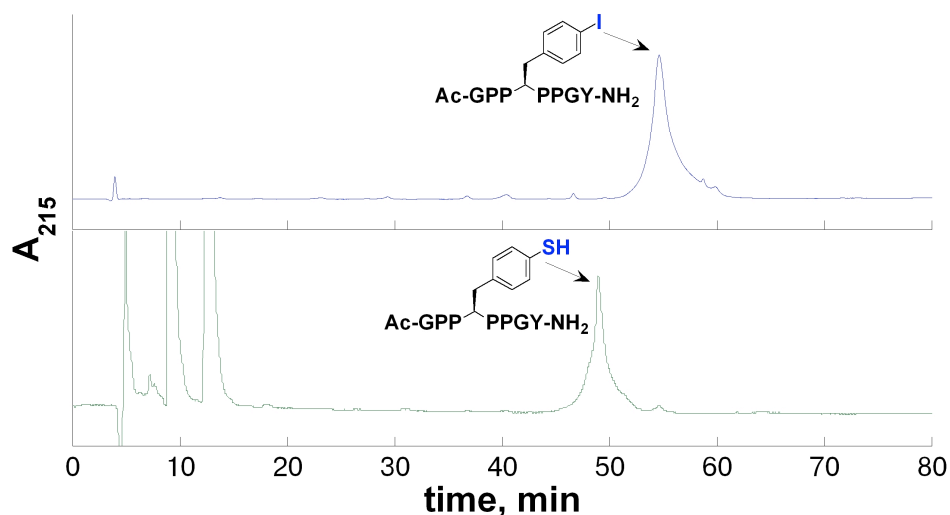


Figure S9. Crude HPLC chromatograms of solid phase cross-coupling reaction: starting material Ac-GPP(4-I-Phe)PPGY-NH₂ (**8**) (top); crude products of cross-coupling reaction after deacetylation and reduction with DTT (**12**) (bottom).

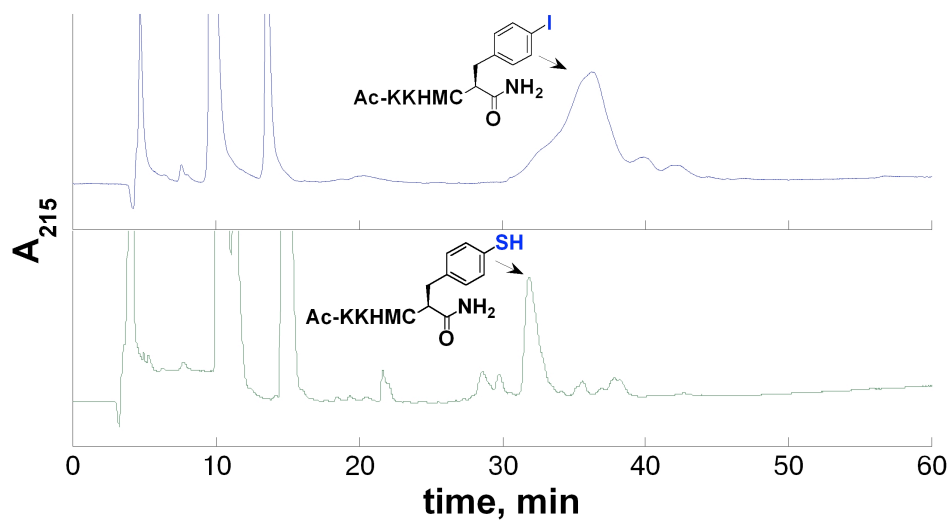


Figure S10. Crude HPLC chromatograms of solid phase cross-coupling reaction: starting material Ac-KKHMC(4-I-Phe)-NH₂ (**9**) after reduction with DTT (top); crude products of cross-coupling reaction after deacetylation and reduction with DTT (**13**) (bottom).

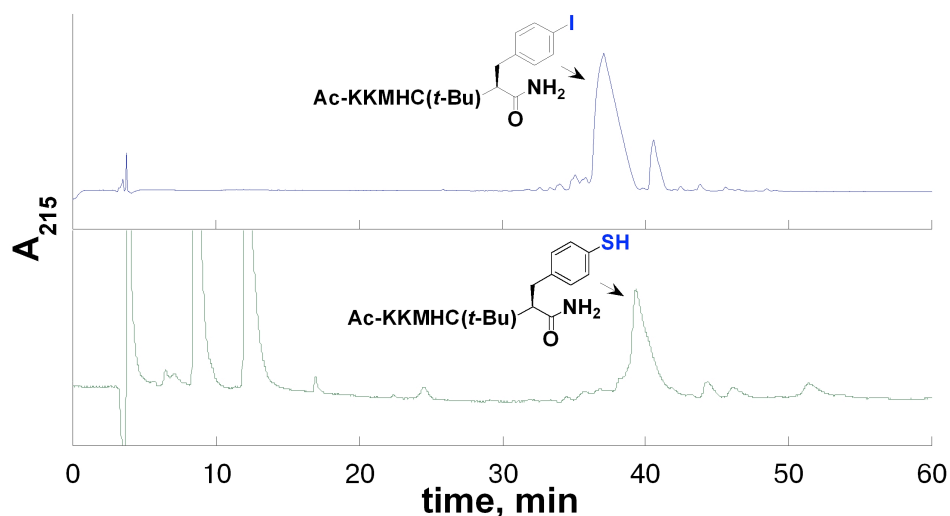


Figure S11. Crude HPLC chromatograms of solid phase cross-coupling reaction: starting material Ac-KKMHC(t-Bu)(4-I-Phe)-NH₂ (**10**) (top); crude products of cross-coupling reaction after deacetylation and reduction with DTT (**14**) (bottom).

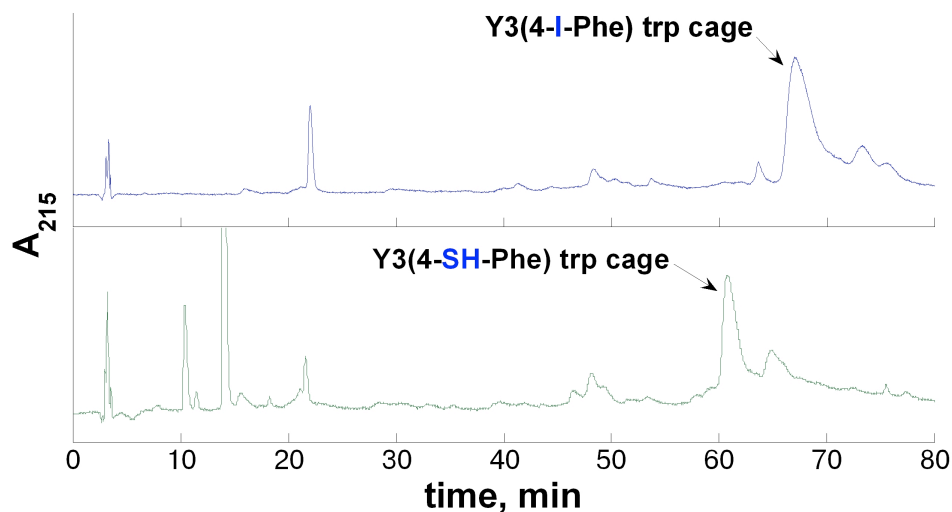
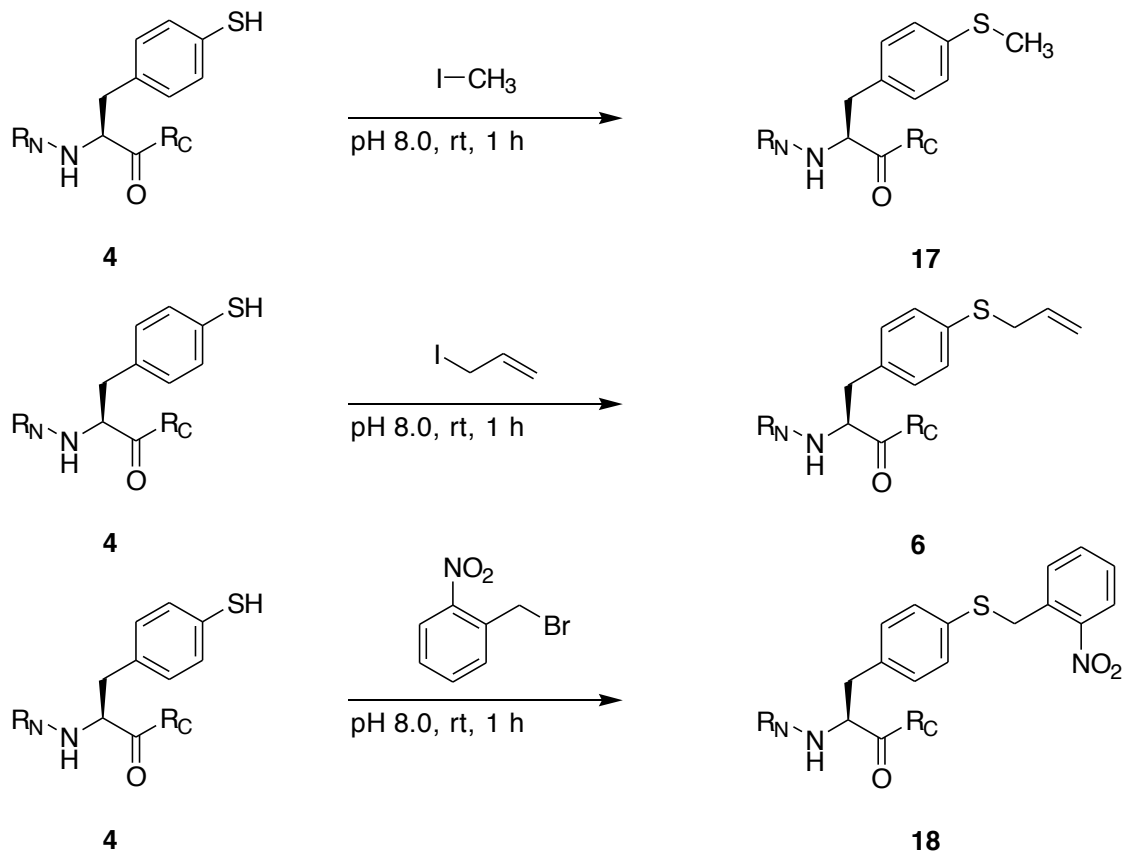


Figure S12. Crude HPLC chromatograms of solid phase cross-coupling reaction: starting material Y3(4-I-Phe) trp cage (**11**) (top); crude products of cross-coupling reaction after deacetylation and reduction with DTT (**15**) (bottom).

Solution-phase synthesis of Ac-T(4-SMe-Phe)PN-NH₂ (17**), Ac-T(4-S-allyl-Phe)PN-NH₂ (**6**), and Ac-T(4-S(2-nitrobenzyl)-Phe)PN-NH₂ (**18**)**



The purified and lyophilized peptide containing 4-thiophenylalanine (**4**) (0.3-0.8 μmol) was dissolved in 100 mM phosphate buffer (pH 8.0, 100 μL). Methyl iodide (1 μL , 16 μmol), allyl iodide (1 μL , 11 μmol), or a solution of 2-nitrobenzyl bromide (5 μL , 2.3 mg/mL in MeCN) was added and the mixture was incubated at room temperature for 1 h to produce the peptide Ac-T(4-SMe-Phe)PN-NH₂ (**17**) in 89% yield, Ac-T(4-S-allyl-Phe)PN-NH₂ (**6**) in 90% yield, or Ac-T(4-S-(2-nitrobenzyl)-Phe)PN-NH₂ (**18**) in 99% yield, respectively. Excess 2-aminoethanethiol (approximately 5 mg) was then added to quench residual alkyl halide. The peptide **17** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: t_{R} 34.7 min, exp. 564.2, obs. 587.2 (M + Na)⁺. The peptide **6** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: t_{R} 43.0 min, exp. 590.3, obs. 613.2 (M + Na)⁺. The peptide **18** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: t_{R} 58.9 min, exp. 685.3, obs. 708.2 (M + Na)⁺.

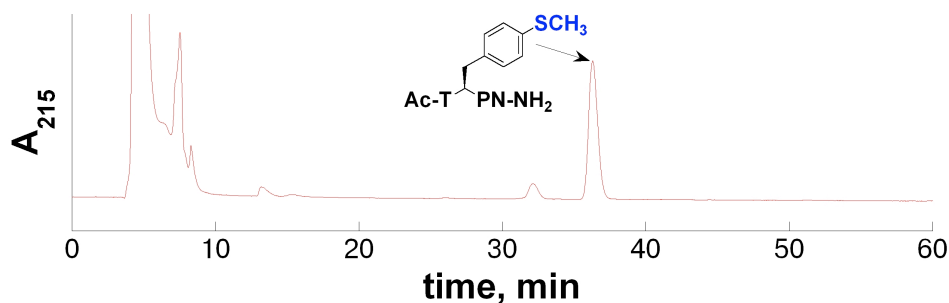


Figure S13. Crude HPLC chromatogram of solution phase reaction to produce the peptide Ac-T(4-SMe-Phe)PN-NH₂ (**17**).

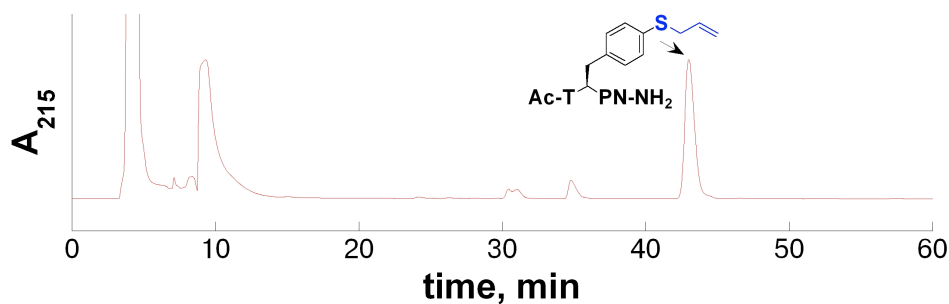


Figure S14. Crude HPLC chromatogram of solution phase reaction to produce the peptide Ac-T(4-S-allyl-Phe)PN-NH₂ (**6**).

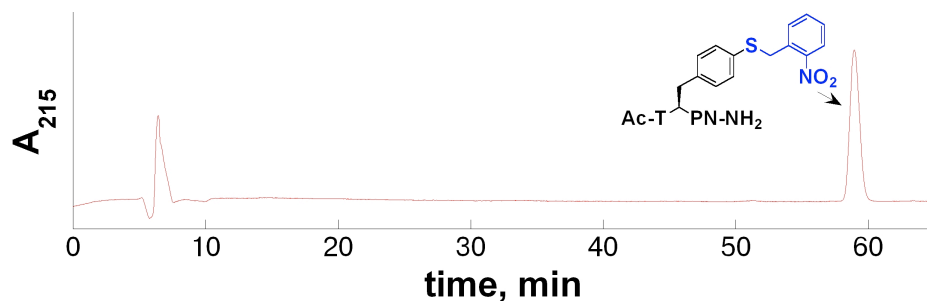
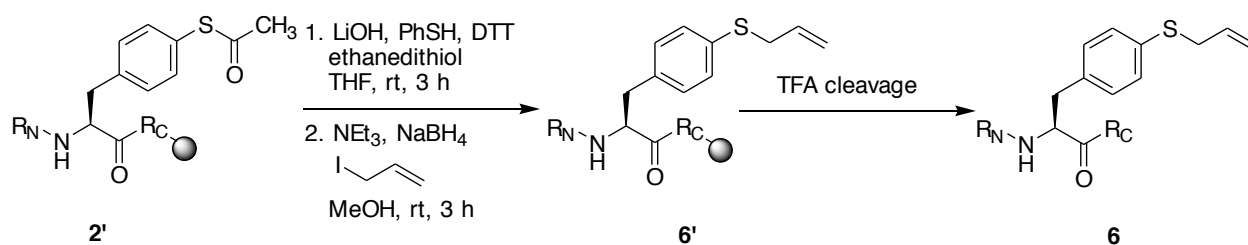


Figure S15. Crude HPLC chromatogram of solution phase reaction to produce the peptide Ac-T(4-S-(2-nitrobenzyl)-Phe)PN-NH₂ (**18**).

Solid-phase synthesis of Ac-T(4-S-allyl-Phe)PN-NH₂ (**6**)



The 4-thioacetylphenylalanine-containing peptide on resin (**2'**) (5-10 mg, 3-7 μ mol) was placed in a capped disposable fritted column and THF (3 mL) was added. LiOH (7.2 mg, 300 mmol), DTT (23.1 mg, 150 mmol), ethanedithiol (25 μ L, 300 mmol), and thiophenol (31 μ L, 300 mmol) were added. The resin was mixed at room temperature for 3 h. The resin was washed with MeOH (3 \times 3 mL). MeOH (3 mL), NaBH₄ (28.4 mg, 750 mmol), triethylamine (200 μ L, 1.43 mmol), and allyl iodide (69 μ L, 750 mmol) were added to the resin and the reaction was stirred for 3 h at room temperature. The resin was washed with MeOH (3 \times 3 mL) and dried with ether. The resin was cleaved and the peptide **6'** deprotected using standard cleavage/deprotection conditions. The peptide **6** was produced with 82% yield as determined by integration of the HPLC chromatogram.

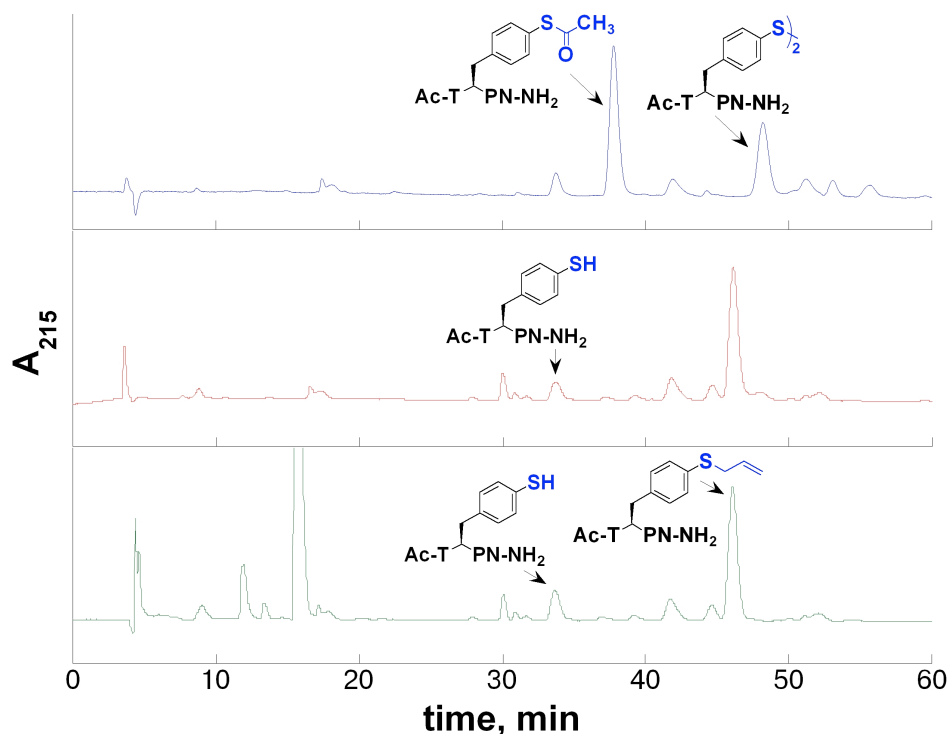
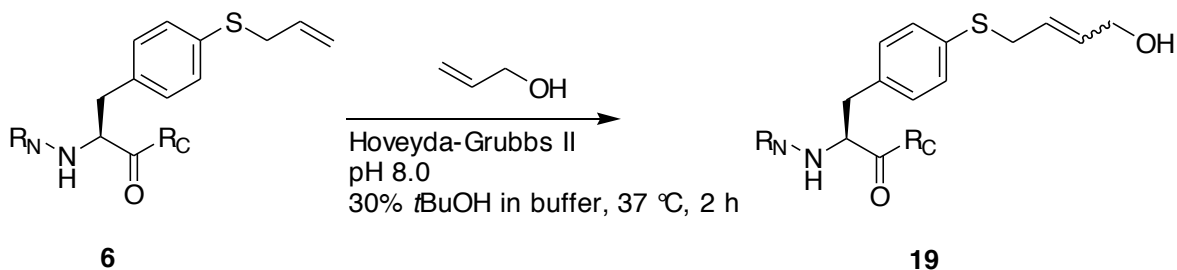


Figure S16. Crude HPLC chromatograms of products of the solid phase alkylation reaction to produce the peptide Ac-T(4-S-allyl-Phe)PN-NH₂ (**6**) on resin: Crude products of the cross-coupled starting material (top); crude products of the solid phase reaction (middle); crude products from above after reduction of disulfides with DTT (bottom).

Solution-phase synthesis of Ac-T(4-(SCH₂CH=CHCH₂OH)-Phe)PN-NH₂ (**19**)



The purified and lyophilized 4-S-allylthiophenylalanine-containing peptide (**6**) (0.3-0.8 μmol) was dissolved in phosphate buffer (50 mM, pH 8.0, 200 μL). Hoveyda-Grubbs II catalyst (2.6 mg, 4.2 μmol) was dissolved in *t*-butanol (338 μL) with gentle warming and vortexing. The Hoveyda-Grubbs catalyst solution (86 μL) was added to the peptide. The mixture was vortexed and sparged with nitrogen gas. Allyl alcohol (3.6 μL , 53 μmol) was added and the mixture was then incubated for 2 h to produce peptide Ac-T(4-(SCH₂CH=CHCH₂OH)-Phe)PN-NH₂ (**19**) as an inseparable mixture of *E* and *Z* isomers. The reaction mixture was then washed with ether. In the absence of MgCl₂, the peptide **19** was produced in 69% yield at room temperature and in

61% yield at 37 °C. With MgCl₂ added (MgCl₂·6H₂O, 10 mg, 4.2 μmol), the peptide **19** was produced in 70% yield at room temperature and in 64% yield at 37 °C. Peptide **19** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: *t*_R 34.2 min, exp. 620.3, obs. 621.0 (M + H)⁺.

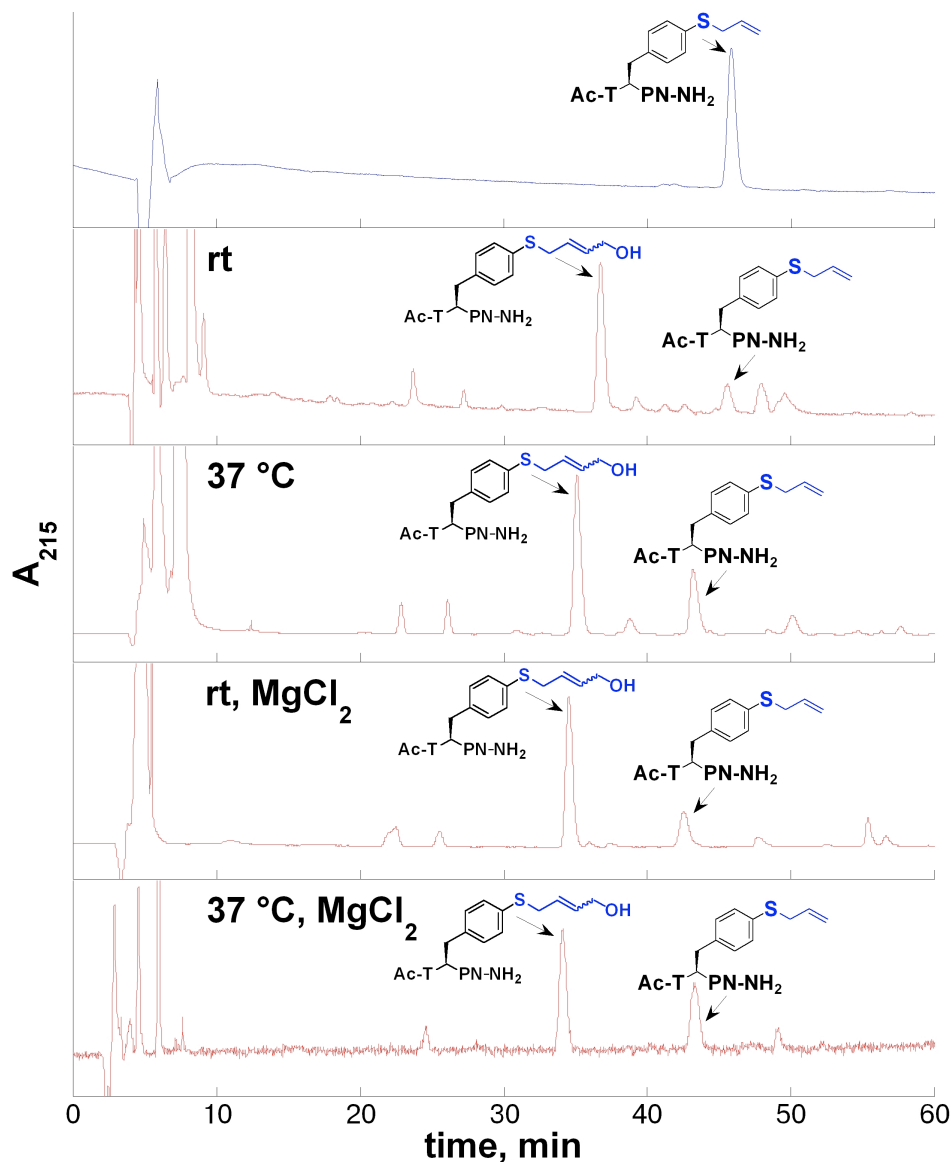
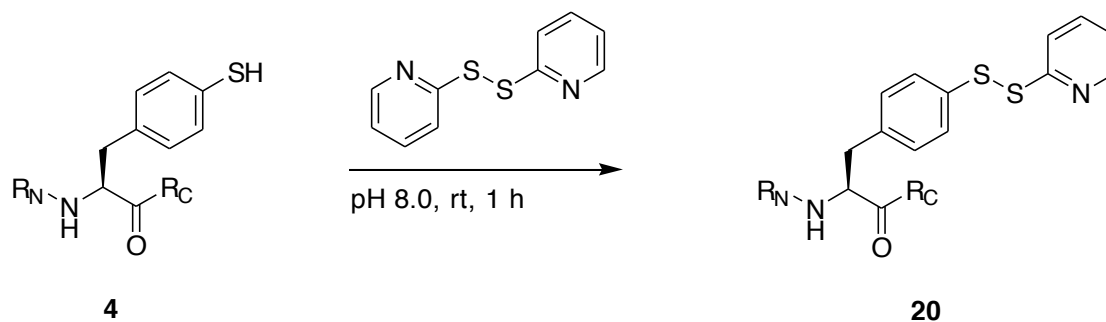


Figure S17. Crude HPLC chromatograms of solution phase cross-metathesis reactions to produce the peptide Ac-T(4-(S(CH₂CH=CHCH₂OH)-Phe)PN-NH₂) (**19**): starting material Ac-T(4-S-allyl-Phe)PN-NH₂ (**6**) (top); reaction at room temperature (second); reaction at 37 °C (third); reaction at room temperature with 180 mM MgCl₂ added (fourth); and reaction 37 °C with 180 mM MgCl₂ added (bottom). Peaks at 25.9 and 23.0 minutes were the sulfoxides of the starting material and product, respectively.

Solution-phase synthesis of Ac-T(4-S-SPy-Phe)PN-NH₂ (**20**)



The purified peptide containing 4-thiophenylalanine (**4**) (0.3-0.8 μmol) was dissolved in phosphate buffer (pH 8.0, 100 mM, 100 μL). A solution of 2,2'-dithiodipyridine (100 mM in MeCN, 20 μL) was added. The mixture was incubated at room temperature for 2 h to produce the peptide Ac-T(4-S-SPy-Phe)PN-NH₂ (**20**) in 99% yield. The peptide **20** was purified via HPLC using a gradient of 0-45% buffer B in buffer A over 60 minutes: t_R 46.5 min, exp. 659.2, obs. 660.1 (M + H)⁺.

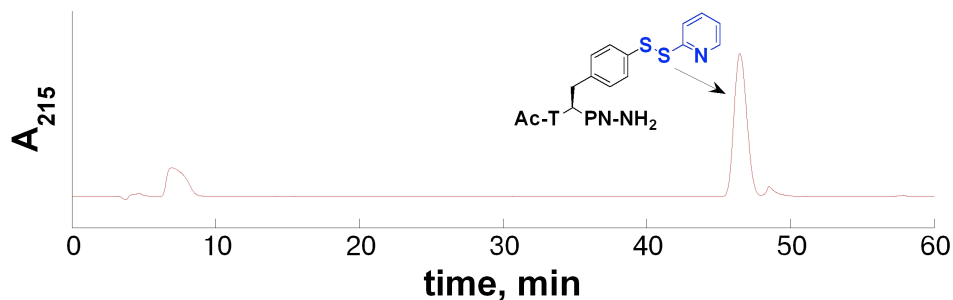
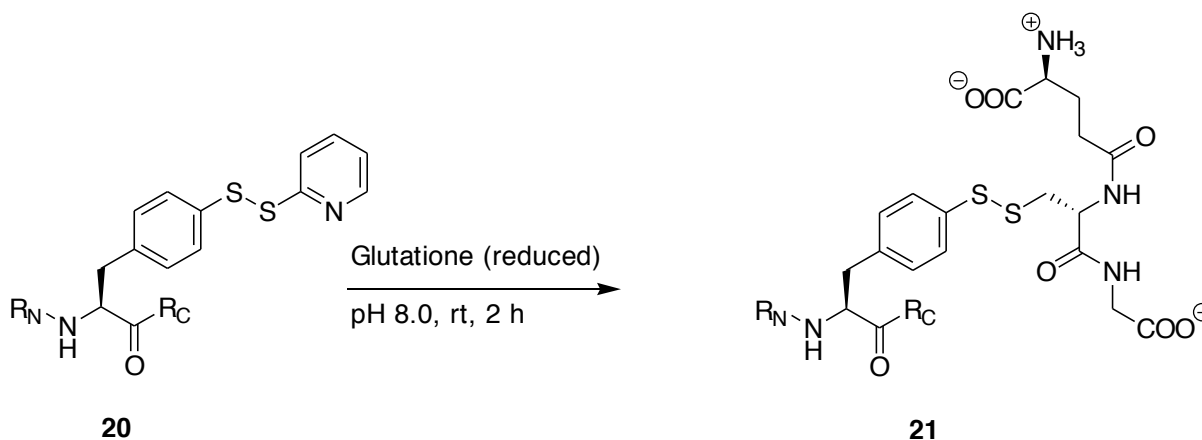


Figure S18. Crude HPLC chromatogram of solution phase reaction to produce the peptide Ac-T(4-S-SPy-Phe)PN-NH₂ (**20**).

Solution-phase synthesis of Ac-T(4-(glutathione disulfide)thiophenylalanine)PN-NH₂ (**21**)



The purified peptide containing 4-(thiopyridyl disulfide)phenylalanine (**20**) (0.3-0.8 μmol) was dissolved in phosphate buffer (pH 8.0, 100 mM, 200 μL). A solution of glutathione (reduced, 100 mM in water, 20 μL) was added to the peptide. The mixture was incubated at room temperature for 2 h to produce the peptide Ac-T(4-(glutathione disulfide)S-Phe)PN-NH₂ (**21**) in 95% yield. The peptide **21** was purified via HPLC using a gradient of 0-50% buffer B in buffer A over 60 minutes: t_{R} 26.5 min, exp. 855.3, obs. 856.1 (M + H)⁺.

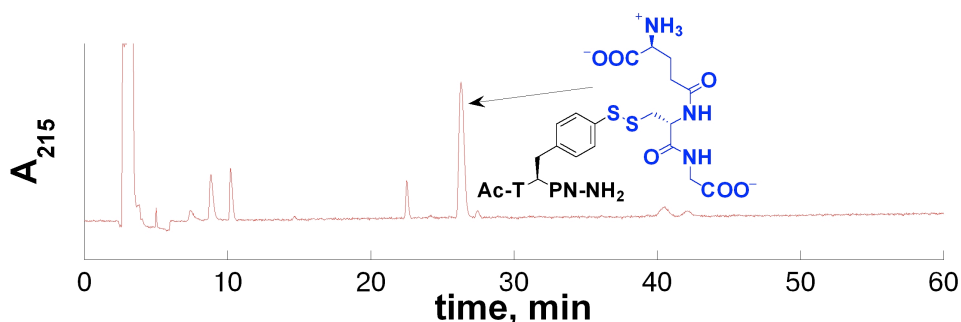
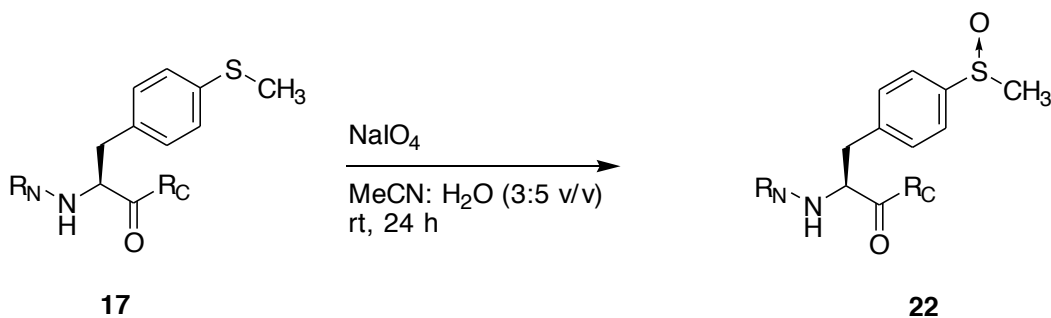


Figure S19. Crude HPLC chromatogram of solution phase reaction to produce the peptide Ac-T(4-(glutathione disulfide)S-Phe)PN-NH₂ (**21**).

Solution-phase synthesis of Ac-T(4-S(O)Me-Phe)PN-NH₂ (**22**)



To the purified peptide containing 4-methylthiophenylalanine (**17**) (0.3-0.8 μmol), a mixture of MeCN:H₂O (3:5 v/v, 80 μL) was added. NaIO₄ was then added (2.4 μL , 12.3 mg/mL in water). The mixture was stirred at room temperature for 24 h to produce the peptide Ac-T(4-S(O)Me-Phe)PN-NH₂ (**22**) as an inseparable mixture of diastereomers in 89% overall yield. The peptide **22** was then purified via HPLC using a gradient of 0-20% buffer B in buffer A over 60 minutes: t_{R} 27.7 min, exp. 580.2, obs. 603.1 (M + Na)⁺.

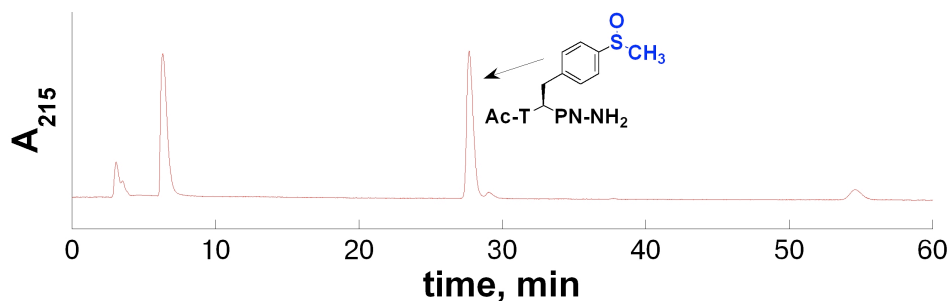
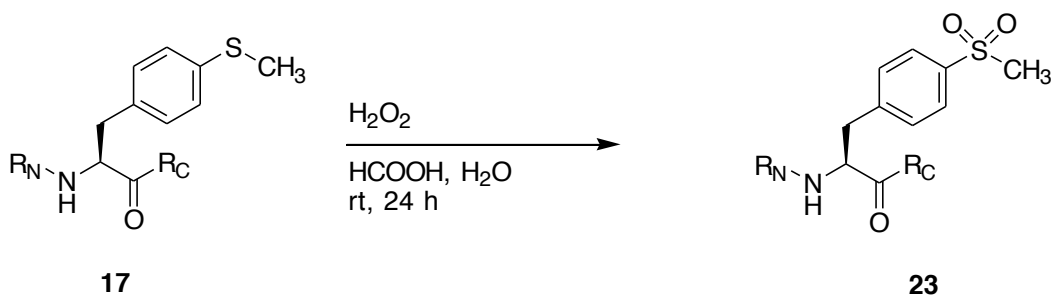


Figure S20. Crude HPLC chromatogram of solution phase reaction to produce the peptide Ac-T(4-S(O)Me-Phe)PN-NH₂ (**22**).

Solution-phase synthesis of Ac-T(4-SO₂Me-Phe)PN-NH₂ (23**)**



To the purified peptide containing 4-thiomethylphenylalanine (**17**) (0.3-0.8 μmol), a mixture of formic acid (90% in H₂O, 1 mL) was added. H₂O₂ was then added (30% in H₂O, 200 μL) and the mixture was stirred at room temperature for 24 h to produce the peptide Ac-T(4-SO₂Me-Phe)PN-NH₂ (**23**) in 68% yield. The peptide **23** was purified via HPLC using a gradient of 0-15% buffer B in buffer A over 60 minutes: t_R 36.3 min, exp. 596.2, obs. 619.1 (M + Na)⁺.

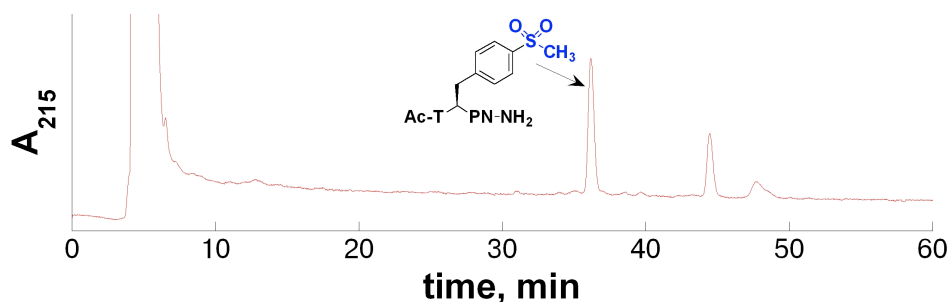
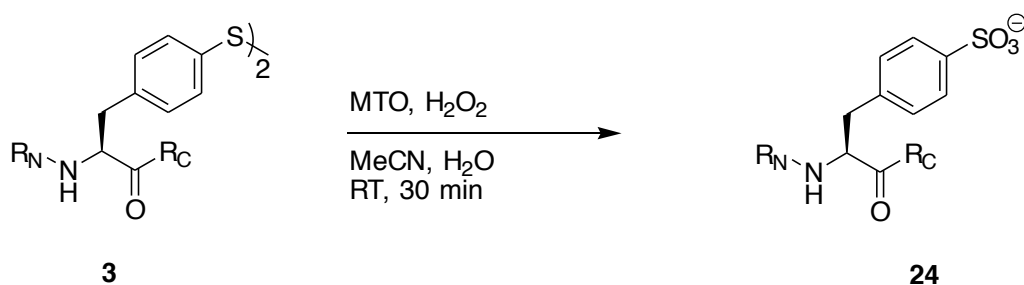


Figure S21. Crude HPLC chromatogram of solution phase reaction to produce the peptide Ac-T(4-SO₂Me-Phe)PN-NH₂ (**23**).

Solution-phase synthesis of Ac-T(4-SO₃⁻-Phe)PN-NH₂ (**24**)



Peptide containing 4-thiophenylalanine disulfide (**3**) was obtained either from cross-coupled crude products (described previously), or by dissolving peptide containing 4-thiophenylalanine (**4**) (0.3-0.8 μ mol) in phosphate buffer (pH 8.0, 100 mM, 100 μ L) with iodine (0.5 mg) and incubating at room temperature for 1 h. Purified disulfide peptide (**3**) was obtained using a gradient of 0-45% buffer B in buffer A over 60 minutes (see Figure S8): t_R 46.5 min, exp. 1098.4, obs. 1121.3 (M + Na)⁺. To a solution of H₂O₂ (30% in water, 150 μ L) was added a solution of methyltrioxorhenium (MTO, 100 μ L, 1.5 mg/mL in MeCN). The mixture was vortexed and incubated at ambient temperature for 1 min. Purified 4-thiophenylalanine disulfide peptide dissolved in MeCN (250 μ L) was then added to the MTO mixture. The MTO reaction was incubated at ambient temperature for 30 min to produce peptide Ac-T(4-SO₃⁻-Phe)PN-NH₂ (**24**). The peroxide was quenched with excess NaHSO₄ (approximately 2 mg) and incubated at room temperature for 30 min. The reaction volume was reduced by evaporating the MeCN with nitrogen gas to approximately 100 μ L, and then 100 μ L of either phosphate buffer (100 mM, pH 4.0) or water was added. The peptide **24** was purified via HPLC using isocratic buffer A over 20 minutes followed by a gradient of 0-45% buffer B in buffer A over 40 minutes: t_R 9.2 min, exp. 598.2, obs. 599.1 (M + H)⁺.

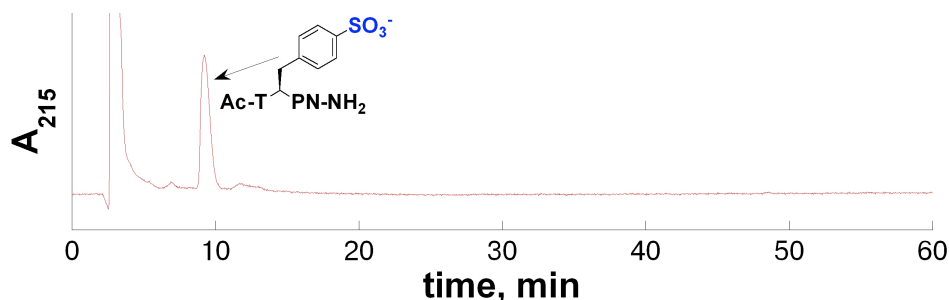
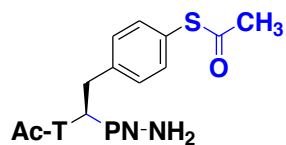
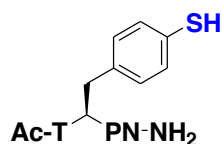
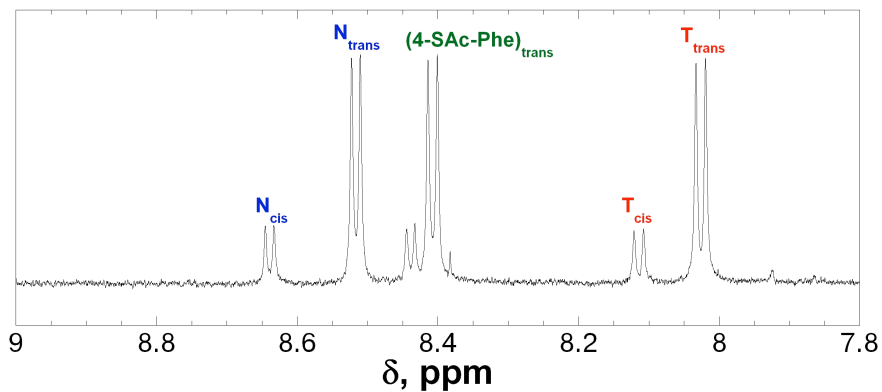


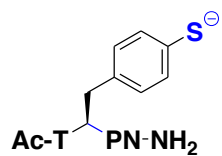
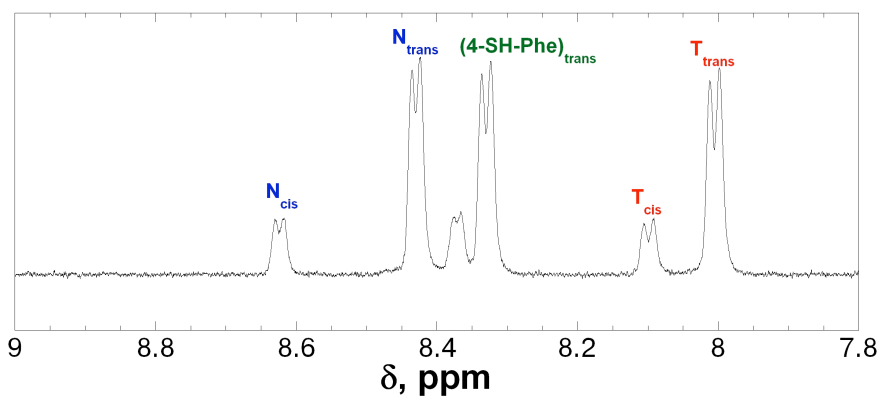
Figure S22. Crude HPLC chromatogram of solution phase reaction to produce the peptide Ac-T(4-SO₃⁻-Phe)PN-NH₂ (**24**).



2



4



4

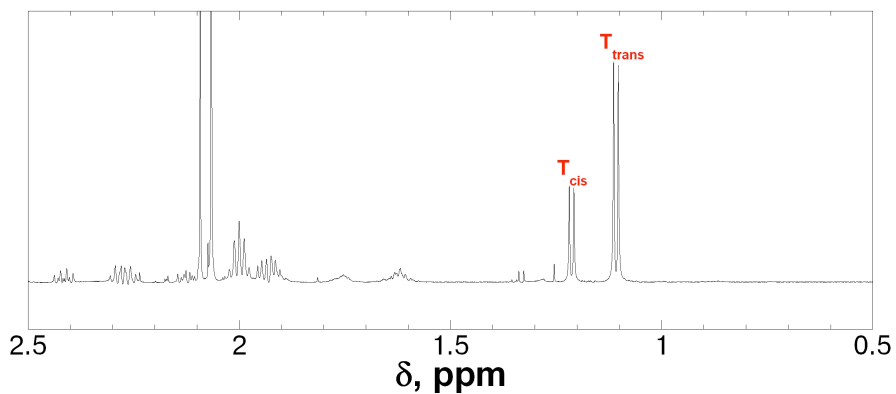


Figure S23. NMR spectra of amide regions for peptide **2** at pH 4.0, and peptides **4** at pH 4.0 and 8.5. For the thiolate, threonine methyl peaks are shown instead of the amide region, due to rapid amide exchange at pH 8.5. All samples contained 5 mM phosphate and 25 mM NaCl.

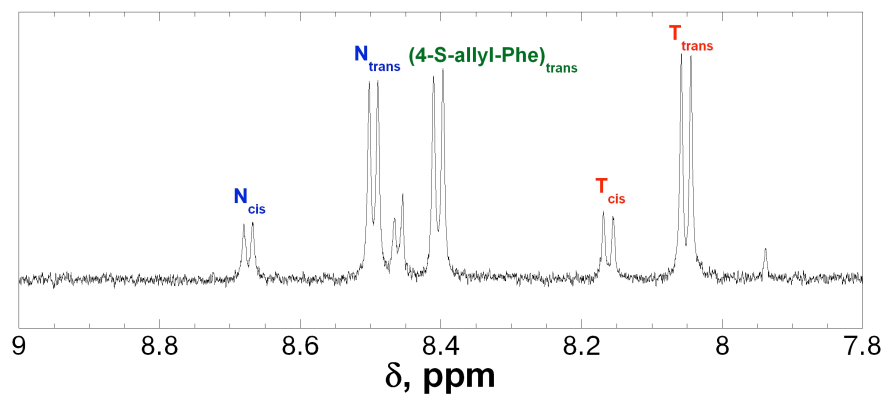
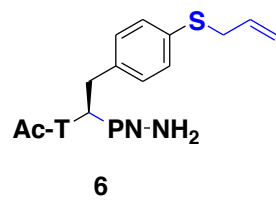
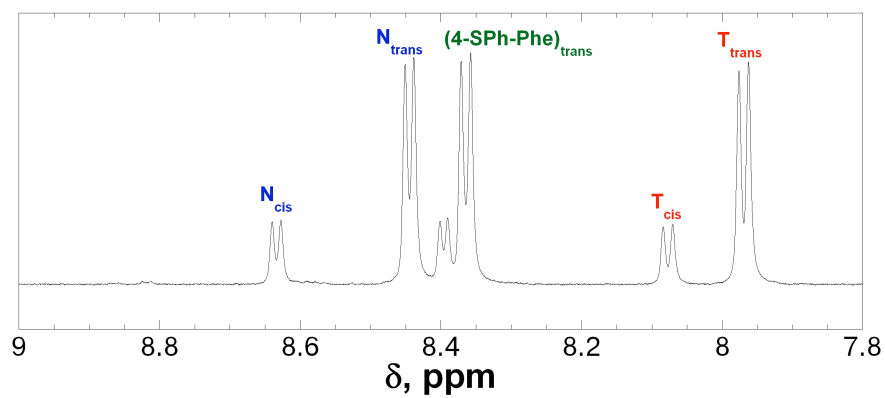
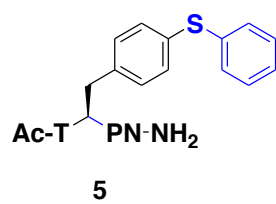
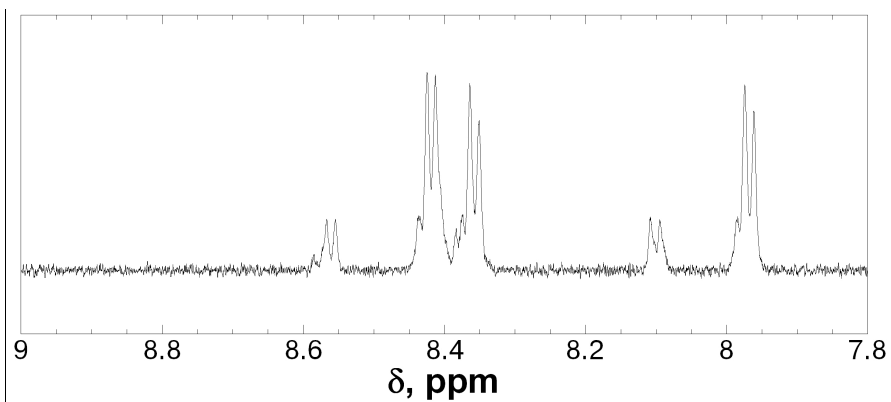
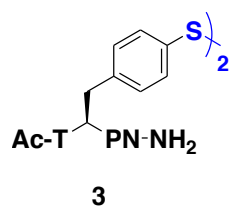


Figure S24. NMR spectra of amide regions for peptides **3**, **5**, and **6**. All samples contained 5 mM phosphate and 25 mM NaCl at pH 4.0.

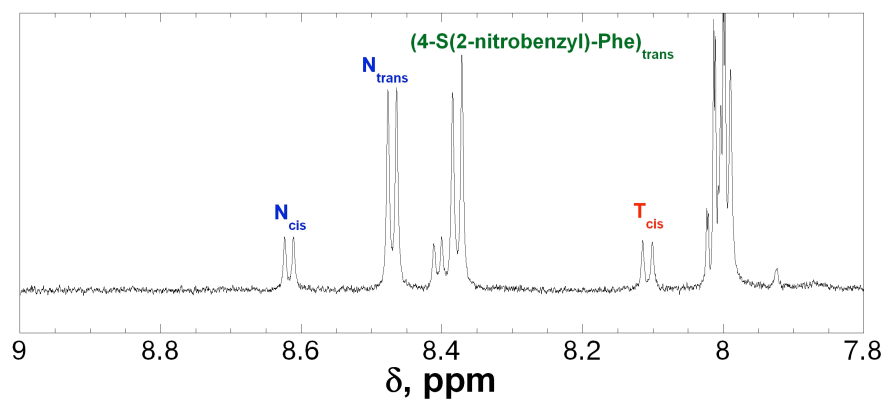
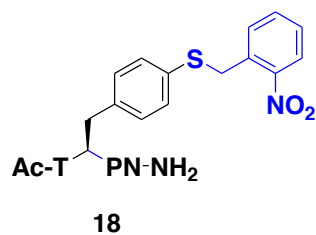
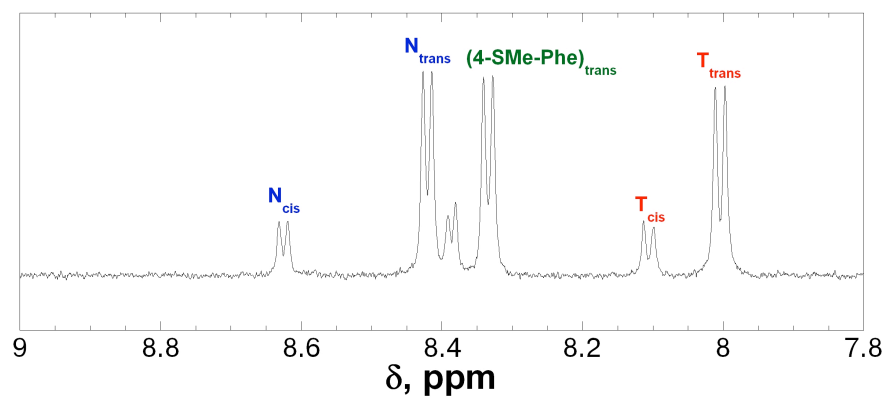
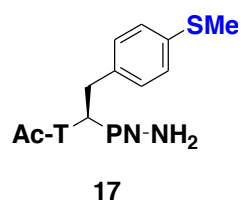
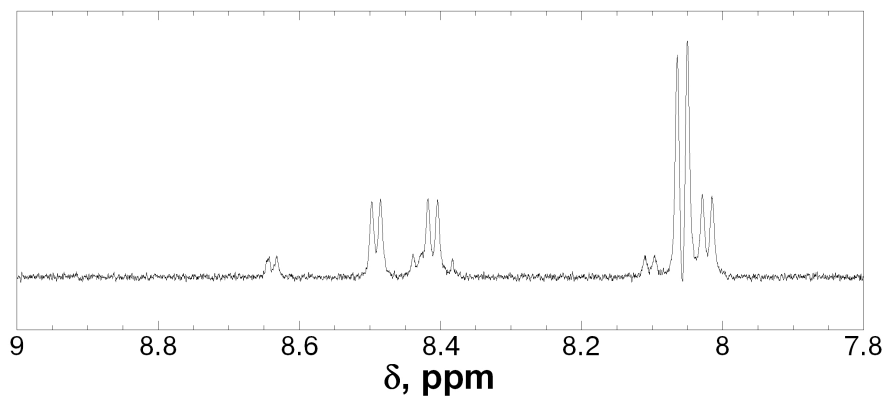
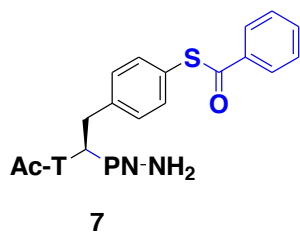


Figure S25. NMR spectra of amide regions for peptides **7**, **17**, and **18**. All samples contained 5 mM phosphate and 25 mM NaCl at pH 4.0.

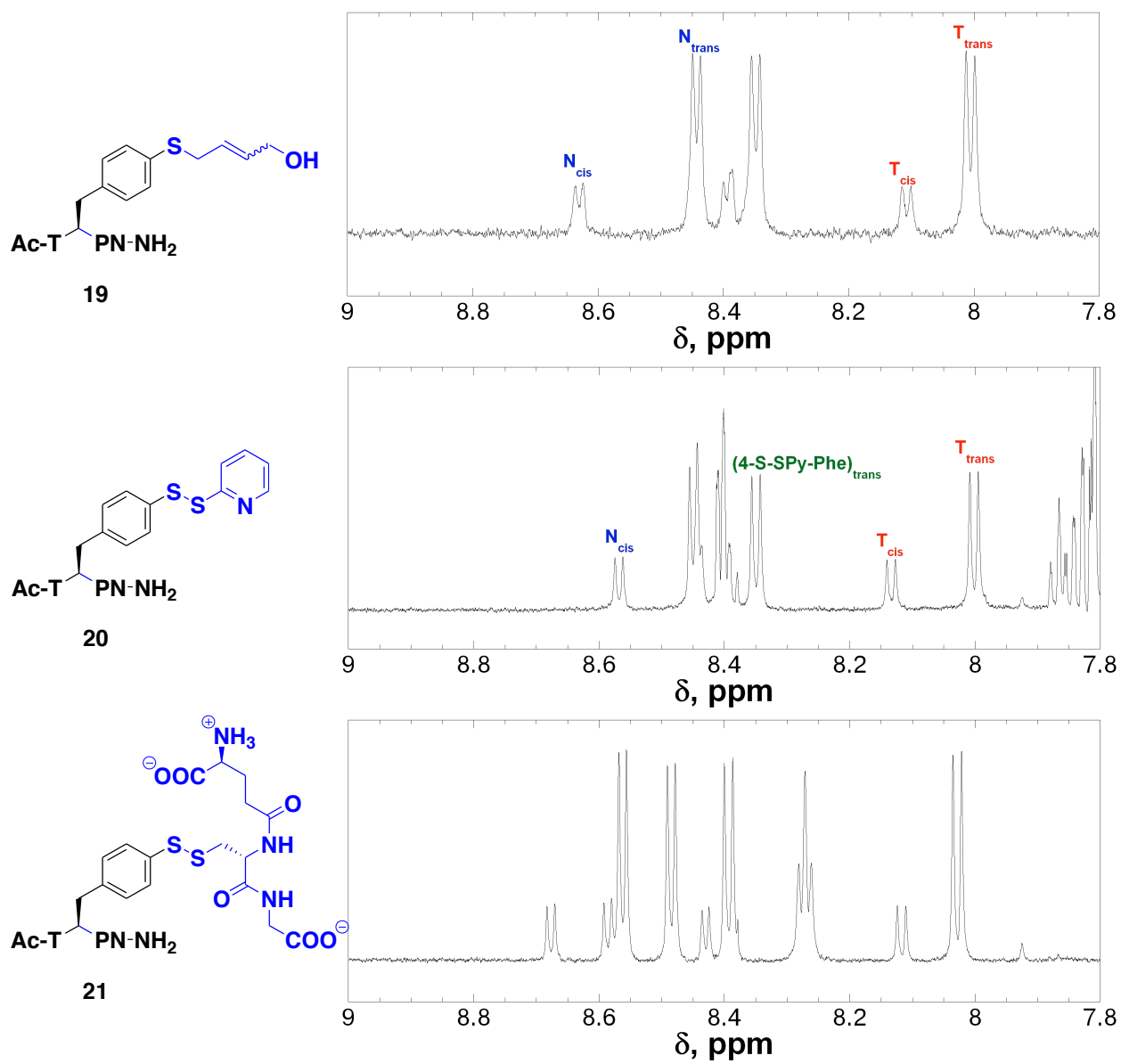


Figure S26. NMR spectra of amide regions for peptides **19**, **20**, and **21**. All contained 5 mM phosphate and 25 mM NaCl at pH 4.0.

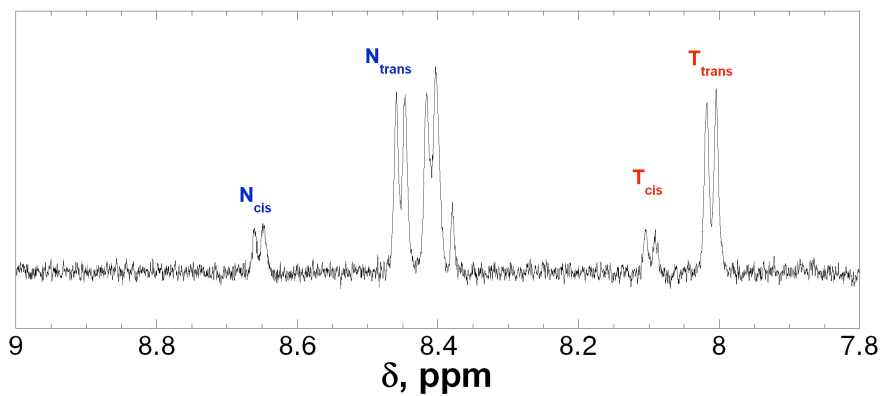
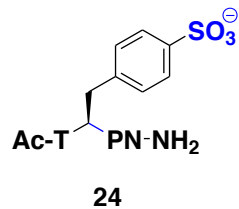
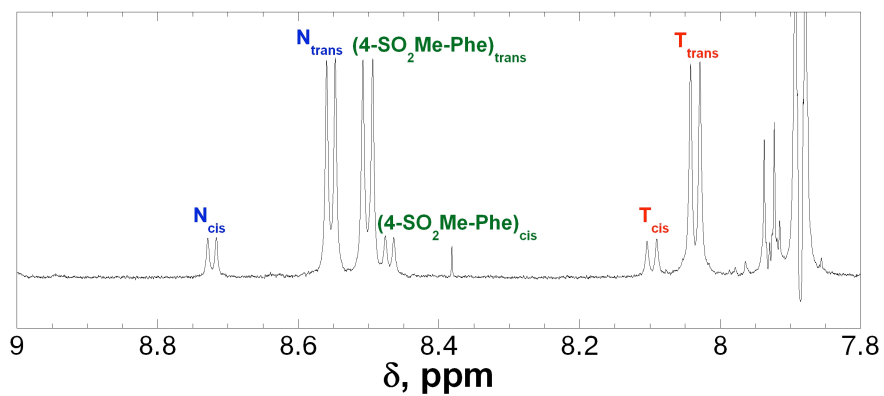
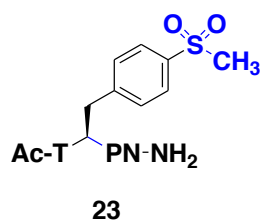
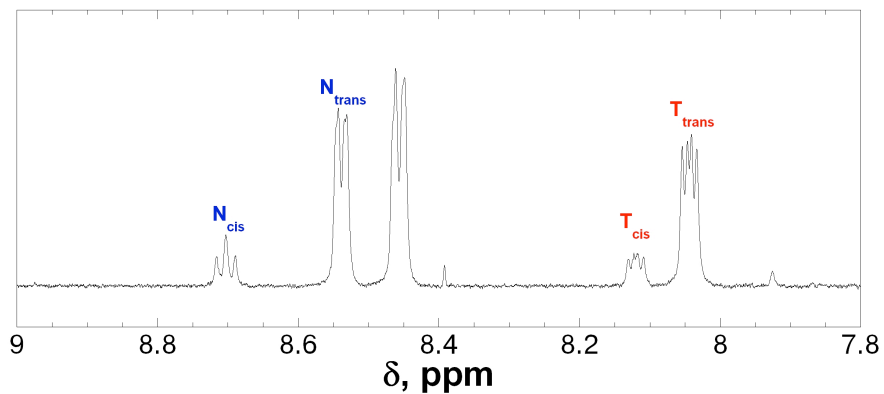
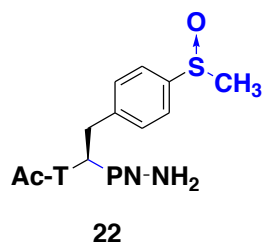


Figure S27. NMR spectra of amide regions for peptides **22**, **23** and **24**. Peptide **22** contains a mixture of sulfoxide diastereomers. All contained 5 mM phosphate and 25 mM NaCl at pH 4.0.

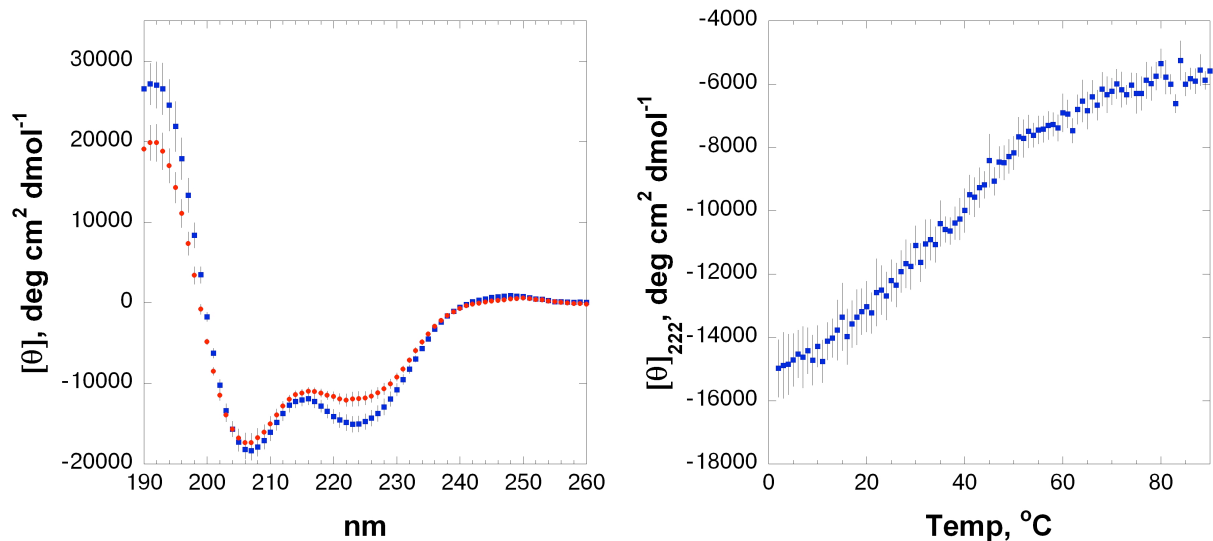


Figure S28. Left: CD spectra of Y3(4-SH-F) trp cage (**15**) in 15 mM aqueous phosphate (pH 4.0) and 0.56 mM TCEP at 4 °C (blue squares) and at 25 °C (red circles): mean residue ellipticity versus wavelength. Right: thermal denaturation experiment in 15 mM aqueous phosphate (pH 4.0). Data represent the average of at least 3 independent trials. Error bars indicate standard error.

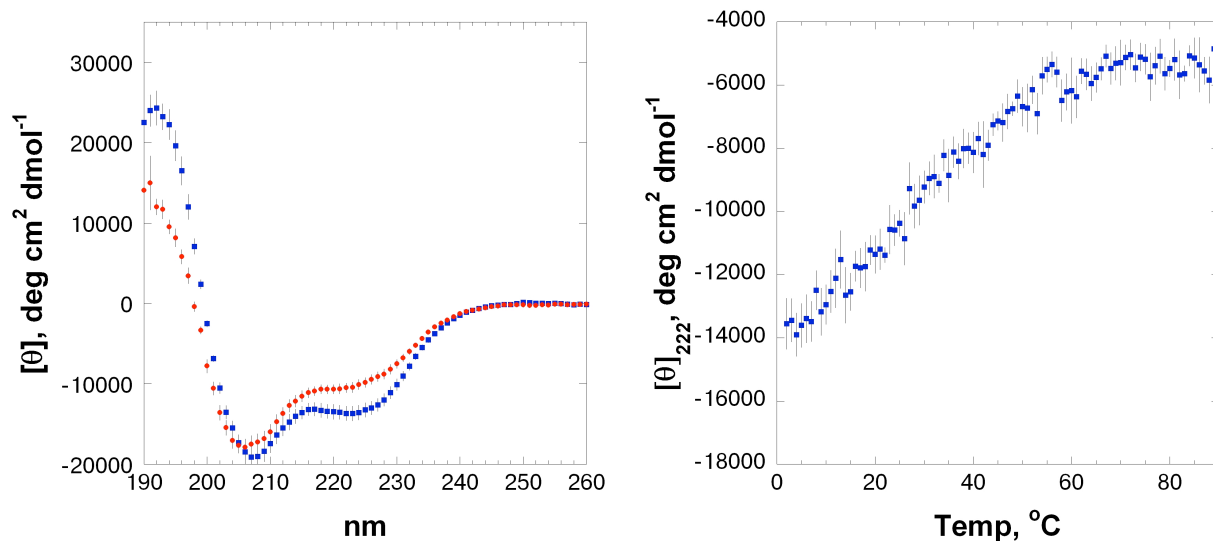


Figure S29. Left: CD spectra of Y3(4-SH-F) trp cage (**15**) in 15 mM aqueous phosphate (pH 8.5) at 4 °C (blue squares) and at 25 °C (red circles) and 0.56 mM TCEP. Right: thermal denaturation experiment in 15 mM aqueous phosphate (pH 8.5). Data represent the average of at least 3 independent trials. Error bars indicate standard error.

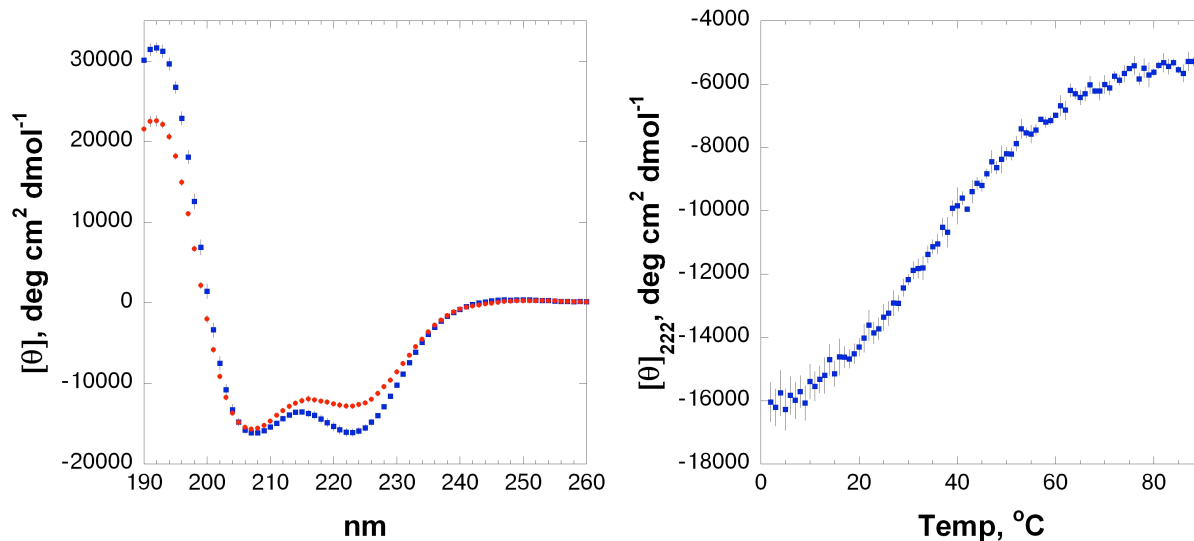


Figure S30. Left: CD spectra of trp cage (**16**) in 15 mM aqueous phosphate (pH 4.0) at 4 °C (blue squares) and at 25 °C (red circles). Right: thermal denaturation experiment in 15 mM aqueous phosphate (pH 4.0). Data represent the average of at least 3 independent trials. Error bars indicate standard error. Data on the trp cage peptide at pH 7.0 are in the Supporting Information of reference 2.

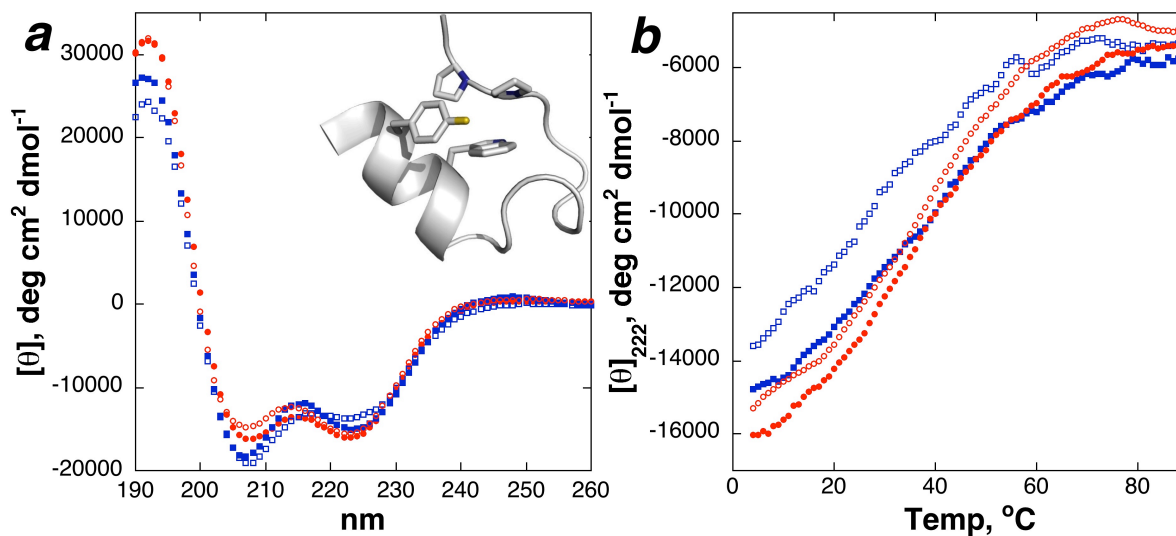


Figure S31. Circular dichroism of native (**16**, red circles) and Y3(4-SH-Phe) (**15**, blue squares) trp cage at pH 4.0 (closed) and 7.0 or 8.5 (open). (a) CD data at 4 °C. (b) Thermal denaturation.

References

1. De Filippis, V.; Colombo, G.; Russo, I.; Spadari, B.; Fontana, A. *Biochemistry* **2002**, *41*, 13556-13569.
2. Naduthambi, D.; Zondlo, N. J. *J. Am. Chem. Soc.* **2006**, *128*, 12430-12431.