Thioamides in the Collagen Triple Helix

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Page	Contents

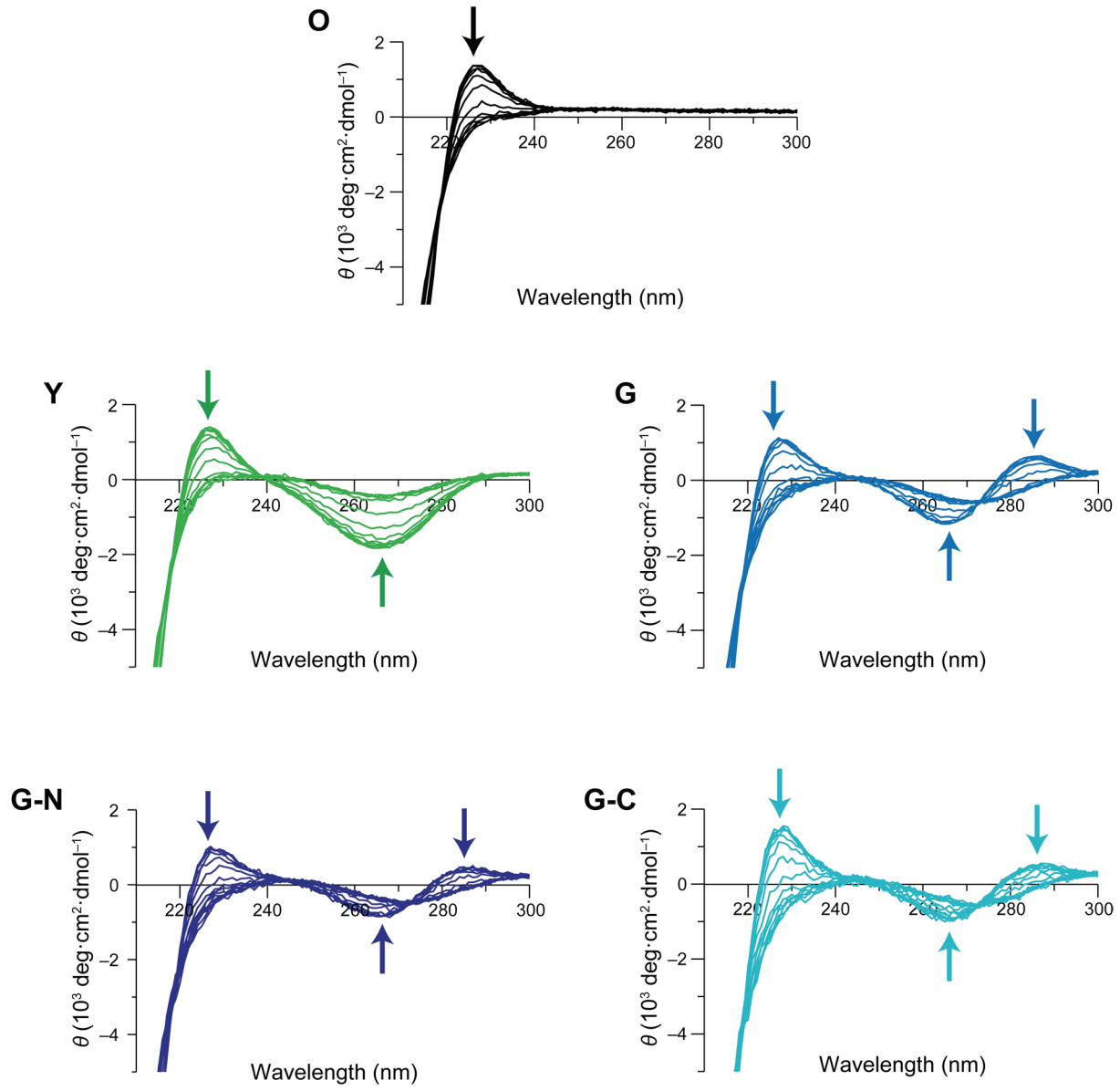


Fig. S1. Circular dichroism spectra of CMPs at various temperatures. Arrows indicate wavelengths that have large temperature-dependent changes in ellipticity.

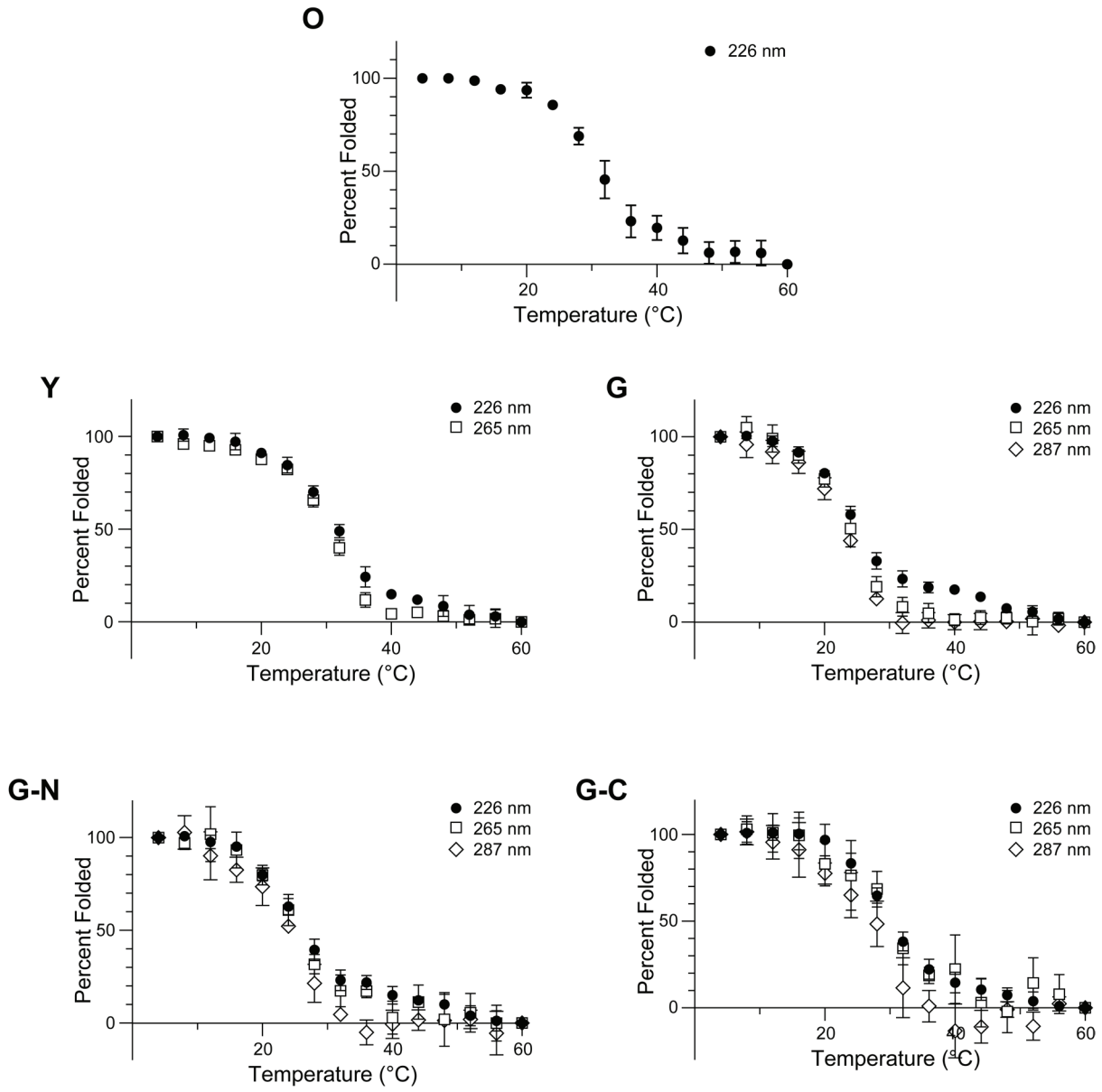
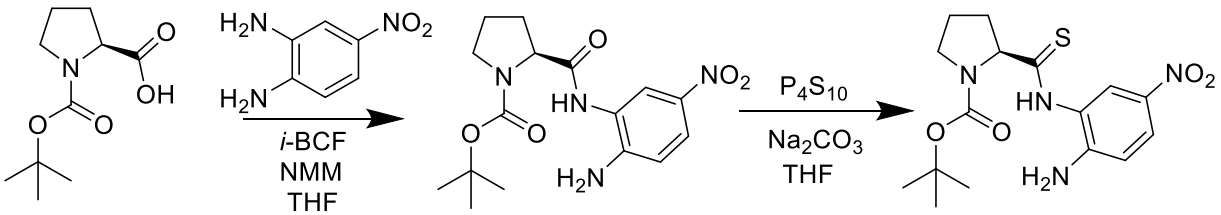


Fig. S2. Thermal denaturation data for triple-helical CMPs as monitored at various wavelengths.

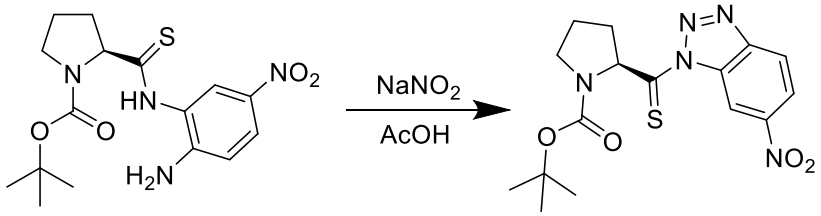
General Experimental. Commercial chemicals were of reagent grade or better, and were used without further purification. Proline starting materials were obtained from Chem-Impex (Wood Dale, IL). All other chemicals were obtained from Sigma–Aldrich. Anhydrous THF and DMF were obtained from CYCLE-TAINER solvent delivery systems (J. T. Baker, Phillipsburg, NJ). Reactions were monitored by thin-layer chromatography with visualization by UV light or staining with $KMnO_4$. Flash chromatography was performed with columns of silica gel 60, 230–400 mesh (Silicycle, Québec City, Canada). The removal of solvents and other volatile materials "under reduced pressure" refers to the use of a rotary evaporator at water-aspirator pressure (<20 torr) and a water bath of <45 °C. All yields are unoptimized.

Instrumentation. NMR spectra were acquired at ambient temperature with a Bruker Avance III 500 MHz spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz) in the National Magnetic Resonance Facility at Madison (NMRFAM). ^{13}C spectra were proton-decoupled. Peptide synthesis was performed wtih a Protein Technologies Prelude automated synthesizer in the University of Wisconsin–Madison Biotechnology Center. Peptide purification was accomplished on a Shimadzu LC-20 HPLC. ESI mass spectrometry was performed with a Micromass LCT instrument in the Mass Spectrometry Facility of the Department of Chemistry at the University of Wisconsin–Madison or on a Shimazdu LCMS 2020. MALDI mass spectrometry was performed on a Voyager DE-Pro MALDI–TOF mass spectrometer in the Biophysics Instrumentation Facility at the University of Wisconsin–Madison. Circular dichroism spectra were collected using an AVIV Model 420 circular dichroism spectrometer in the Biophysics Instrumentation Facility at the University of Wisconsin–Madison.

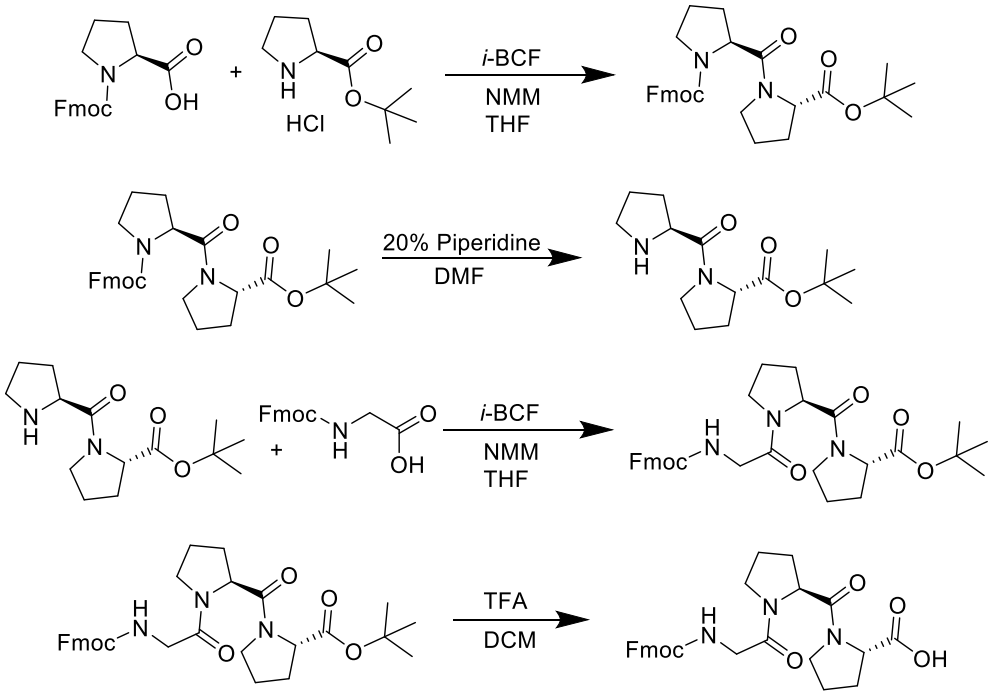


***N*-Boc-L-proline 2-amino-5-nitrothioanilide.** *N*-Methylmorpholine (4.1 mL, 37.2 mmol) was added to a solution of *N*-Boc-L-proline (4.0 g, 18.6 mmol) in THF (100 mL) at –20 °C. Isobutyl chloroformate (2.4 mL, 18.6 mmol) was added dropwise and stirred for 10 min. 4-Nitro-1,2-phenylenediamine (2.85 g, 18.6 mmol) was added, and the reaction mixture was stirred at –20 °C for 3 h before warming to room temperature and stirring overnight. THF was removed under reduced pressure, and the resulting yellow solid was dissolved in EtOAc. This solution was washed with 1 M NaH_2PO_4 and saturated aqueous $NaHCO_3$. The organic portion was dried over anhydrous Na_2SO_4(s) and concentrated under reduced pressure. The resulting yellow oil was dissolved in THF, and this solution was added to a solution of P_4S_{10} (4.13 g, 9.3 mmol) and Na_2CO_3 (1.00 g, 9.3 mmol) in THF at 0 °C. The reaction mixture was allowed to warm to room temperature and stir overnight. The solvent was removed under reduced pressure. The residue was dissolved in EtOAc and washed with saturated aqueous $NaHCO_3$. The aqueous portion was back-extracted with EtOAc. The organic portions were combined, dried over anhydrous Na_2SO_4(s), and concentrated under reduced pressure. The resulting yellow oil was purified by chromatography on silica gel with an eluent of 4% v/v MeOH in DCM, affording a yellow solid (5.75 g, 85%). ^{1}H NMR (500 MHz, DMSO, mixture of two rotamers, δ): 11.22/11.09 (s, 1H),

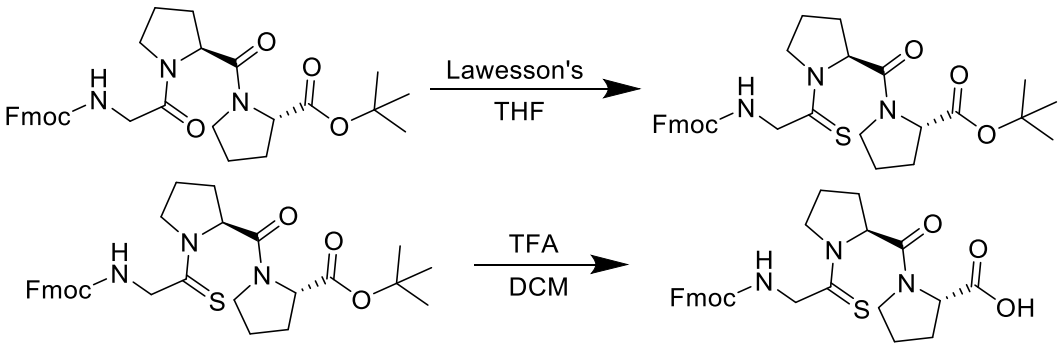
8.01/7.83 (d, J = 2.5 Hz, 1H), 7.97 (dd, J = 9.1, 2.5 Hz, 1H), 6.85/6.78 (d, J = 9.1 Hz, 1H), 6.49/6.23 (s, 2H), 4.70/4.65 (dd, J = 8.4, 4.5 Hz, 1H), 3.52/3.42 (m, 2H), 2.32 (m, 1H), 2.06 (m, 2H), 1.84 (m, 1H), 1.41/1.39 (s, 9H); ^{13}C NMR (125 MHz, DMSO, δ): 207.7, 207.1, 154.4, 154.2, 151.0, 150.3, 135.5, 135.2, 125.2, 124.9, 124.8, 124.7, 122.6, 122.4, 114.5, 113.6, 79.5, 79.0, 47.3, 47.11, 33.9, 32.7, 28.2, 28.1, 24.0, 23.1; ESI-MS: $[M + H]^+$ calcd 367.1435, found 367.1432.



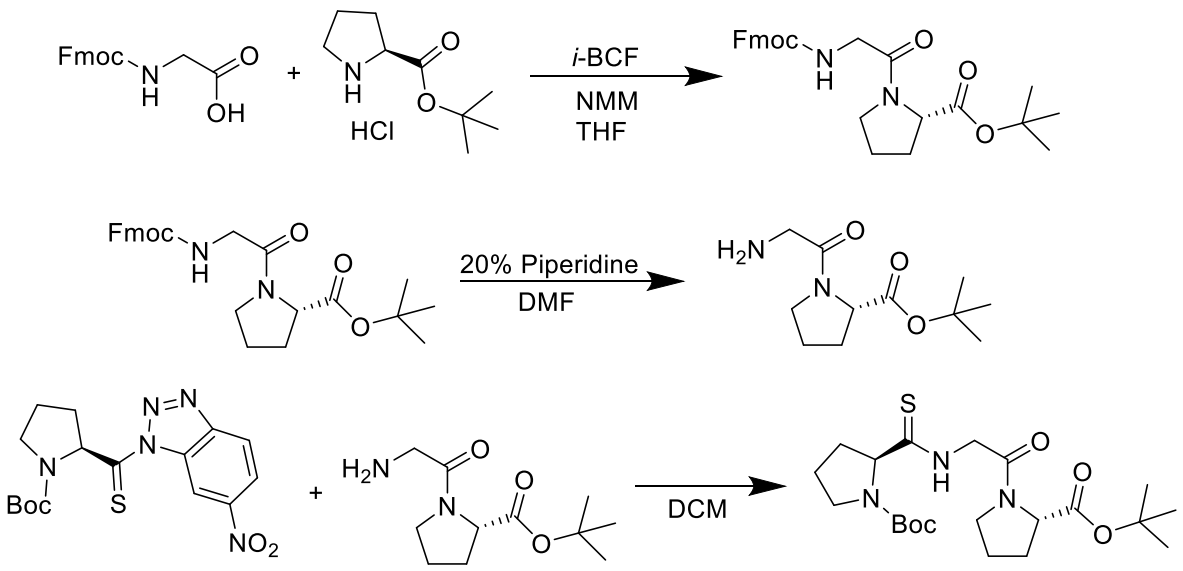
1-(*N*-Boc-L-thioprolyl)-6-nitrobenzotriazole. *N*-Boc-L-proline 2-amino-5-nitrothioanilide (5.75 g, 15.7 mmol) was dissolved in glacial acetic acid diluted with 5% v/v H_2O, and the resulting solution was cooled to 0 °C. $NaNO_2$ (1.63 g, 23.6 mmol) was added portionwise over 5 min, and the reaction mixture was stirred for an additional 30 min at 0 °C. The solution was diluted with water and extracted twice with DCM. The organic portions were washed with saturated aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4(s), and evaporated to dryness to yield an orange solid (5.00 g, 84%). ^{1}H NMR (500 MHz, $CDCl_3$, mixture of two rotamers, δ): 9.74 (d, J = 1.9 Hz, 1H), 8.48/8.45 (dd, J = 6.9, 1.9 Hz, 1H), 8.35/8.30 (d, J = 6.9 Hz, 1H), 6.22 (m, 1H), 3.71 (m, 2H), 2.67 (m, 1H), 2.12 (m, 3H), 1.48/1.24 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$, δ): 209.5, 208.0, 154.2, 153.3, 149.6, 148.7, 132.1, 131.9, 122.2, 122.0, 121.5, 121.3, 113.1, 112.8, 80.2., 80.2, 67.8, 67.6, 47.2, 46.9, 34.5, 33.5, 28.5, 28.2, 23.8, 23.1; ESI-MS: $[M + H]^+$ calcd 378.1231, found 378.1224.



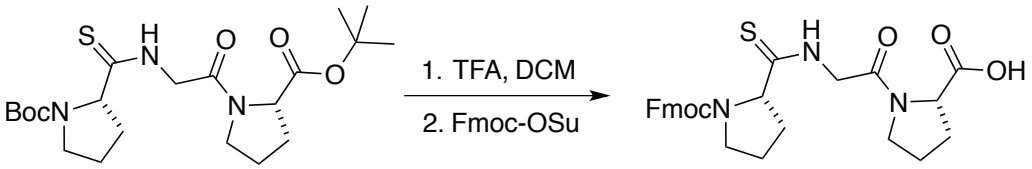
Fmoc-glycyl-prolyl-proline. Fmoc-proline (4.0 g, 11.8 mmol) was dissolved in 100 mL of anhydrous THF containing *N*-methylmorpholine (3.9 mL, 23.6 mmol). The solution was cooled to –20 °C before isobutyl chloroformate (1.54 mL, 11.8 mmol) was added dropwise. The solution was stirred for 10 min before the addition of proline *tert*-butyl ester hydrochloride (2.46 g, 11.8 mmol). The reaction mixture was stirred overnight and then allowed to warm to room temperature. The solvent was removed under reduced pressure, and the resulting yellow solid was dissolved in EtOAc and washed with 1 M NaH_2PO_4 and saturated aqueous $NaHCO_3$. The organic portion was dried over anhydrous Na_2SO_4(s) and concentrated under reduced pressure. The resulting solid (5.25 g, 10.7 mmol, 91% yield) was dissolved in 20 mL of 20% v/v piperidine in DMF and stirred for 1 h at room temperature before the solvent was removed under reduced pressure. Meanwhile, Fmoc-glycine (3.19, 10.7 mmol) dissolved in 100 mL anhydrous THF with *N*-methylmorpholine (3.9 mL, 21.4 mmol). The solution was cooled to –20 °C before isobutyl chloroformate (1.54 mL, 10.7 mmol) was added dropwise. The reaction stirred for ten minutes before the addition of prolyl-proline *tert*-butyl ester. The reaction was stirred overnight and allowed to warm to room temperature. THF was removed under reduced pressure, and the resulting yellow solid was dissolved in EtOAc and washed with 1 M NaH_2PO_4 and saturated aqueous $NaHCO_3$. The organic portion was dried over anhydrous Na_2SO_4(s), and solvent was removed under reduced pressure. Purification of the resulting solid by chromatography on silica gel in 4% v/v MeOH in DCM afforded Fmoc-glycyl-prolyl-proline *tert*-butyl ester as a crystalline solid, which was then dissolved in 1:1 TFA/DCM for 1 h. Removal of solvent under reduced pressure afforded Fmoc-glycyl-prolyl-proline as an off-white solid (3.15 g, 6.4 mmol, 55% overall yield).



Fmoc-thioglycyl-prolyl-proline. Fmoc-glycyl-prolyl-proline *tert*-butyl ester (2.0 g, 3.6 mmol) and Lawesson's reagent (0.74 g, 1.8 mmol) were dissolved in 100 mL THF, and the resulting solution was heated to reflux overnight. Solvent was removed under reduced pressure, and the residue was purified by chromatography on silica gel in 7:3 EtOAc/hexanes to afford Fmoc-thioglycyl-prolyl-proline *tert*-butyl ester as a crystalline solid (1.0 g, 1.8 mmol, 50% yield). Fmoc-thioglycyl-prolyl-proline *tert*-butyl ester (1.0 g, 1.8 mmol) was dissolved in 1:1 TFA/DCM for 1 h. Removal of solvent under reduced pressure afforded Fmoc-thioglycyl-prolyl-proline as an off-white solid (0.9 g, 1.8 mol, quantitative yield).



Boc-thioprolyl-glycyl-proline *tert*-butyl ester. Fmoc-glycine (2.97 g, 10.0 mmol) was dissolved in 100 mL anhydrous THF containing *N*-methylmorpholine (3.3 mL, 20.0 mmol). The solution was cooled to –20 °C before isobutyl chloroformate (1.30 mL, 10.0 mmol) was added dropwise. The solution was stirred for 10 min before the addition of proline *tert*-butyl ester hydrochloride (2.07 g, 10.0 mmol). The reaction mixtue was stirred overnight and then allowed to warm to room temperature. Solvent was removed under reduced pressure, and the resulting yellow solid was dissolved in EtOAc and washed with 1 M NaH_2PO_4 and saturated aqueous $NaHCO_3$. The organic portion was dried over anhydrous Na_2SO_4(s) and evaporated to dryness. The resulting solid was dissolved in 20 mL 20% v/v piperidine in DMF and stirred for 1 h at room temperature before the solvent was removed under reduced pressure. The resulting solid was dissolved in 100 mL DCM and 1-(*N*-Boc-L-thioprolyl)-6-nitrobenzotriazole (3.9 g, 10.0 mmol) was added. The reaction mixture was stirred at room temperature overnight. Following removal of solvent under reduced pressure, purification by chromatography on silica gel in 2% v/v MeOH in DCM afforded Boc-thioprolyl-glycyl-proline *tert*-butyl ester as a yellow solid (3.09 g, 7.0 mmol, 70% overall yield).



Fmoc-thioprolyl-glycyl-proline. Boc-thioprolyl-glycyl-proline *tert*-butyl ester (1.3 g, 2.94 mmol) was dissolved in 20 mL DCM and 20 mL TFA (2.5% H_2O, 1% TIPSH), and the resulting solution was stirred for 1 h at room temperature. Solvent was removed under reduced pressure and the residue was dissolved in 100 mL DCM. Fmoc-NHS ester (1 g, 3.2 mmol) and DIEA (3 mL, 17 mmol) were added, and the reaction mixture was stirred for 90 min at room temperature. Saturated aqueous $NaHCO_3$ was added, and the mixture was stirred for 90 min at room temperature. The solution was acidified and washed with 1 M HCl, and the organic layer was dried over $MgSO_4$(s) and concentrated under reduced pressure. The residue was purified by

chromatography on silica gel in 99:1 EtOAc/AcOH, to give Fmoc-thioprolyl-glycyl-proline (81%).

Peptide Synthesis. Peptide synthesis was accomplished on a 25-μmol scale using standard Fmoc chemistry protocols on NovaSyn Fmoc-Gly TGT resin from EMD Millipore (Darmstadt, Germany). Briefly, peptide bond formation was accomplished by treatment of deprotected resin with 4 equiv each of protected amino acid and HATU, and with 8 equiv of NMM. Cleavage was performed for 1 h in TFA containing 2.5% v/v H_2O and 1% v/v TIPSH. Peptides were precipitated with diethyl ether, isolated, and dissolved in acetonitrile/water before purification by reverse-phase HPLC on a preparative NucleoSil C18 column from Macherey–Nagel (Düren, Germany) using 0.1% v/v TFA in H_2O (A) and 0.1% v/v TFA in MeCN (B) as eluents. Lyophilization afforded white solids. Analytical HPLC was performed with an analytical NucleoSil C18 column from Macherey–Nagel with a 5–95% B gradient over 25 min.

Circular Dichroism Spectroscopy. Peptide samples were dissolved in 50 mM sodium phosphate buffer, pH 7.0, to a final concentration of 200 μM and allowed to equilibrate overnight at 4 °C. Circular dichroism spectra were collected between 200 and 300 nm (1-nm bandwidth, 3-s averaging time) every 4 °C from 4 °C to 60 °C with a 5-min equilibration between temperature steps. Denaturation experiments were performed in triplicate. The mean residue ellipticity as a function of temperature was fitted to a two-state model to extract the value of T_m, which is the temperature at the midpoint of the thermal transition between the triple-helical and single-stranded states.

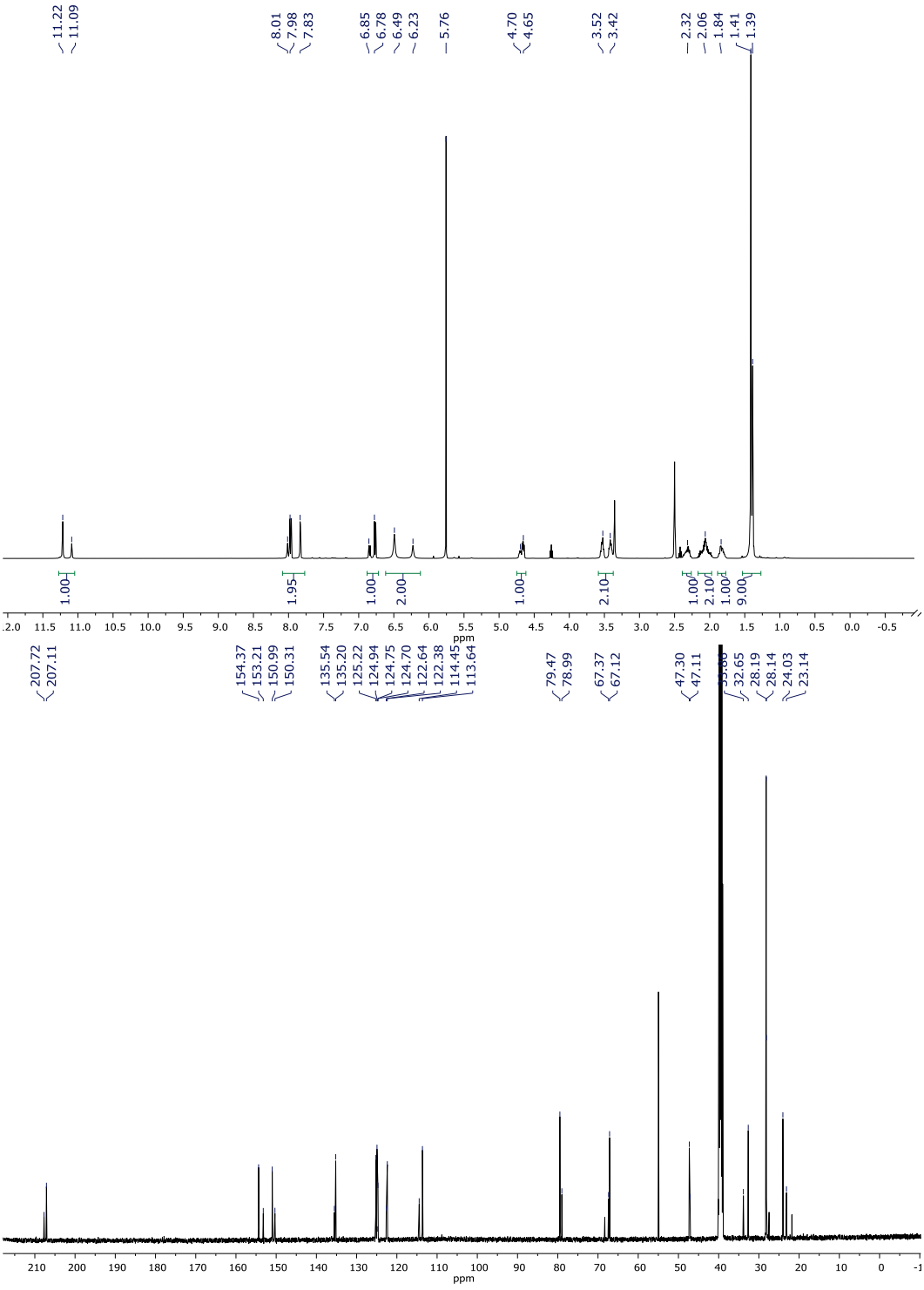


Fig. S3. ^{1}H and ^{13}C NMR spectra of *N*-Boc-L-proline-2-amino-5-nitrothioanilide in DMSO/DCM.

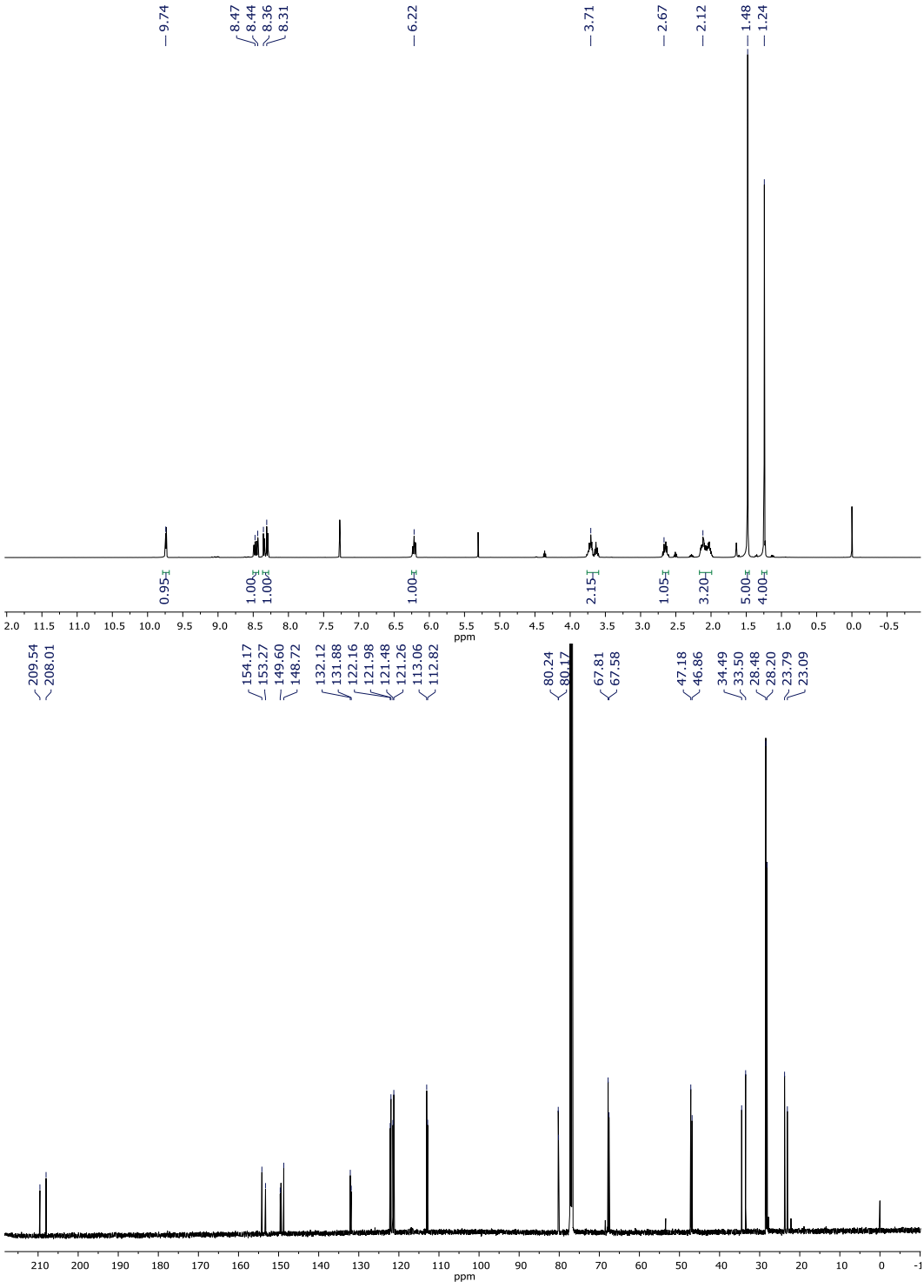


Fig. S4. ^{1}H and ^{13}C NMR spectra of 1-(*N*-Boc-L-thioprolyl)-6-nitrobenzotriazole in $CDCl_3$/DCM.

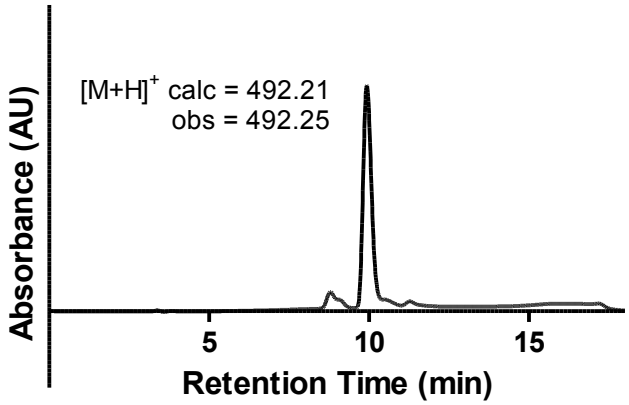


Fig. S5. LC-MS analysis of Fmoc-glycyl-prolyl-proline

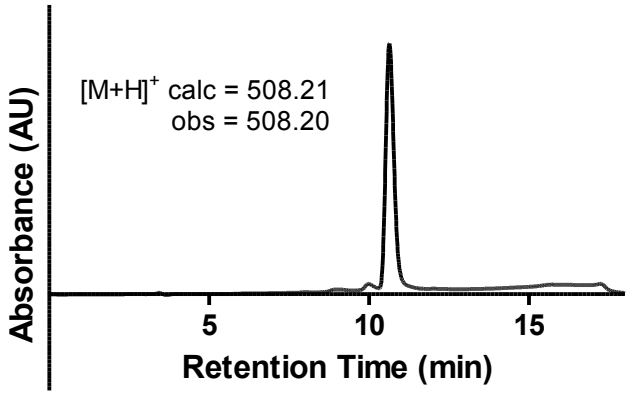


Fig. S6. LC-MS analysis of Fmoc-thioglycyl-prolyl-proline

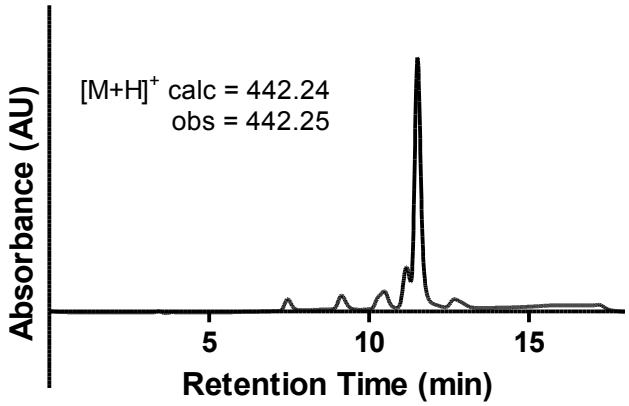


Fig. S7. LC-MS analysis of Boc-thioprolyl-glycyl-proline *tert*-butyl ester

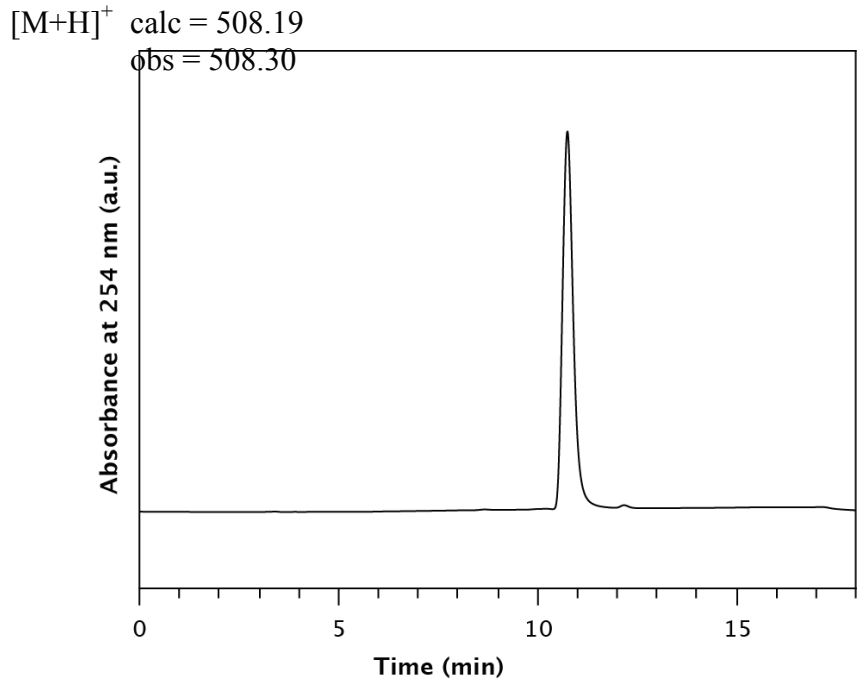


Fig. S8. LC-MS analysis of Fmoc-thioprolyl-glycyl-proline

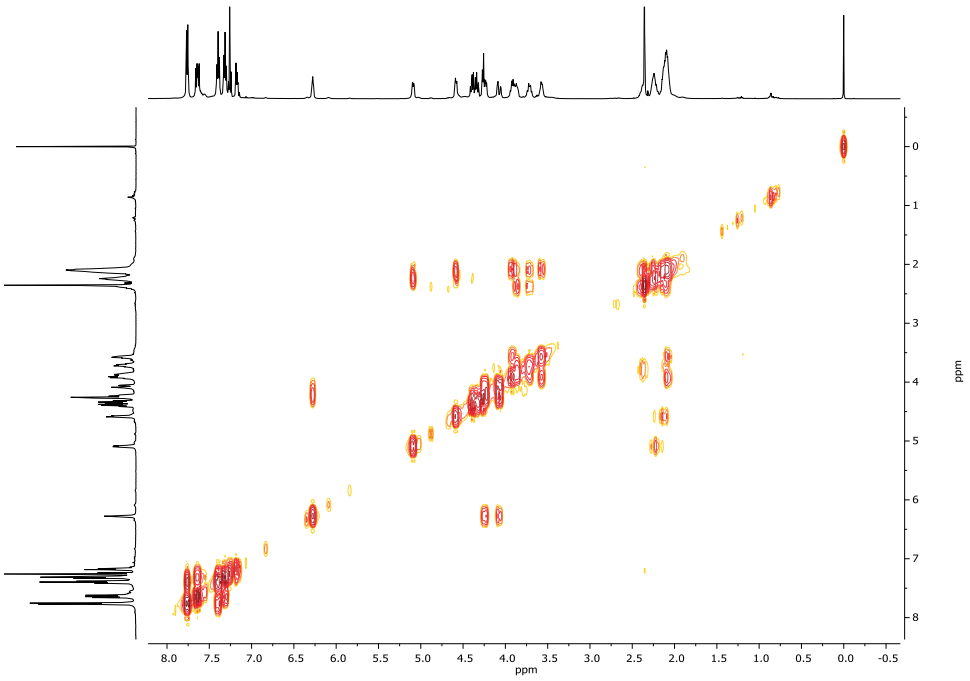


Fig. S9. ^{1}H,^{1}H-COSY spectrum of Fmoc-thioglycyl-prolyl-proline in $CDCl_3$ at an ^{1}H frequency of 500 MHz. Coupling to the carbamate proton (6.3 ppm) identifies multiplets at 4.0-4.3 ppm as glycine methylene protons.

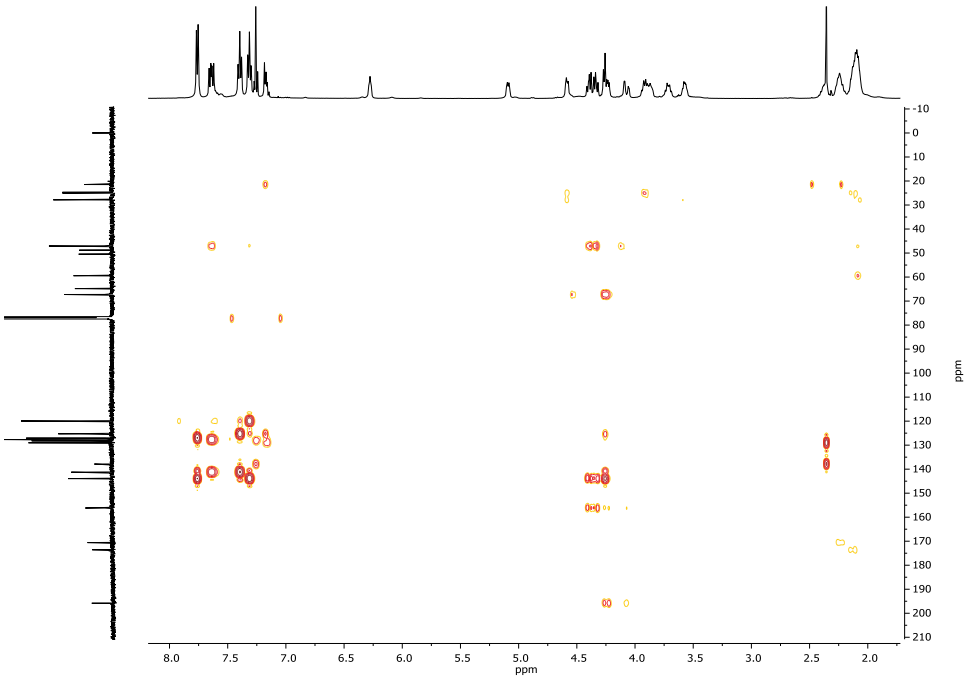


Fig. S10. ^{1}H,^{13}C-HMBC spectrum of Fmoc-thioglycyl-prolyl-proline in $CDCl_3$ at an ^{1}H frequency of 500 MHz. Coupling of the thiocarbonyl carbon (195.8 ppm) to the glycine methylene protons (4.0–4.3 ppm) confirms thionation regiochemistry. Note also the absence of coupling of the thiocarbonyl carbon to the proline α-protons (5.0 and 4.5 ppm).

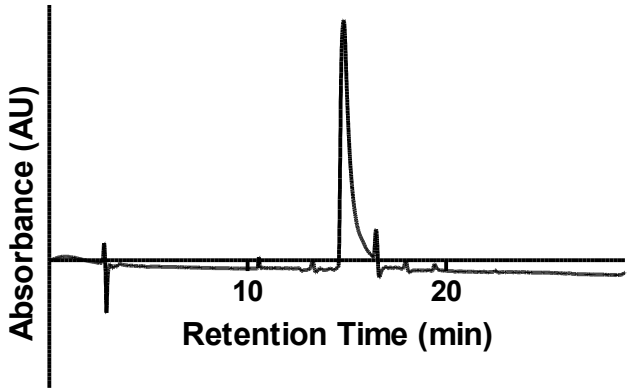


Fig. S11. Analytical HPLC trace of purified thiopeptide **Y**. MALDI-MS: $[M+H]^+$ calc = 2546.3, obs = 2547.2.

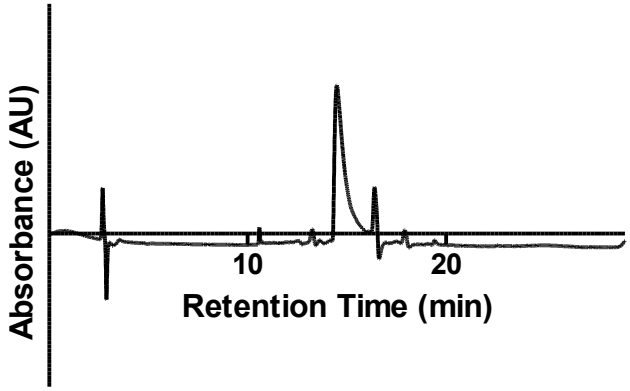


Fig. S12. Analytical HPLC trace of purified thiopeptide **G**. MALDI-MS: $[M+H]^+$ calc = 2546.3, obs = 2546.7.

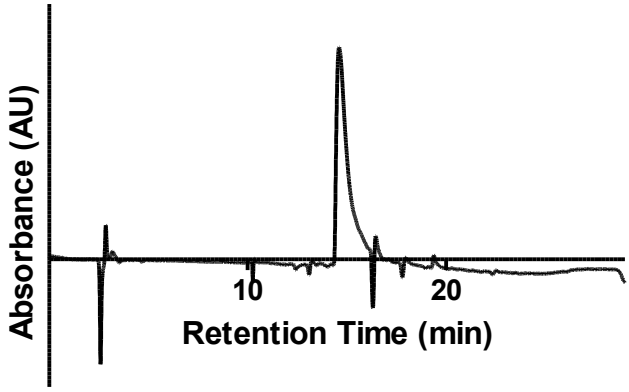


Fig. S13. Analytical HPLC trace of purified thiopeptide **G-N**. MALDI-MS: $[M+H]^+$ calc = 2546.3, obs = 2546.9.

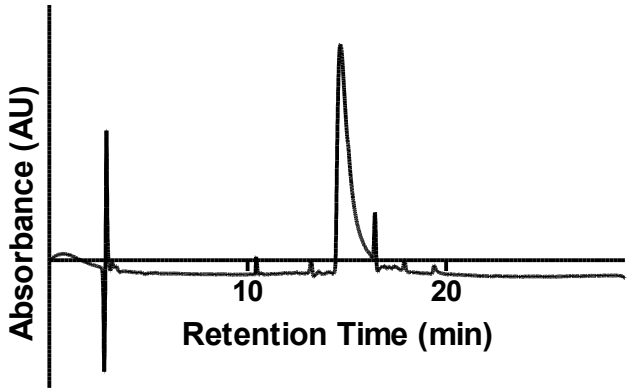


Fig. S14. Analytical HPLC trace of purified thiopeptide **G-C**. MALDI-MS: $[M+H]^+$ calc = 2546.3, obs = 2547.0.