

Supporting Information

Site-specific Incorporation of a Thioester Containing Amino Acid into Proteins

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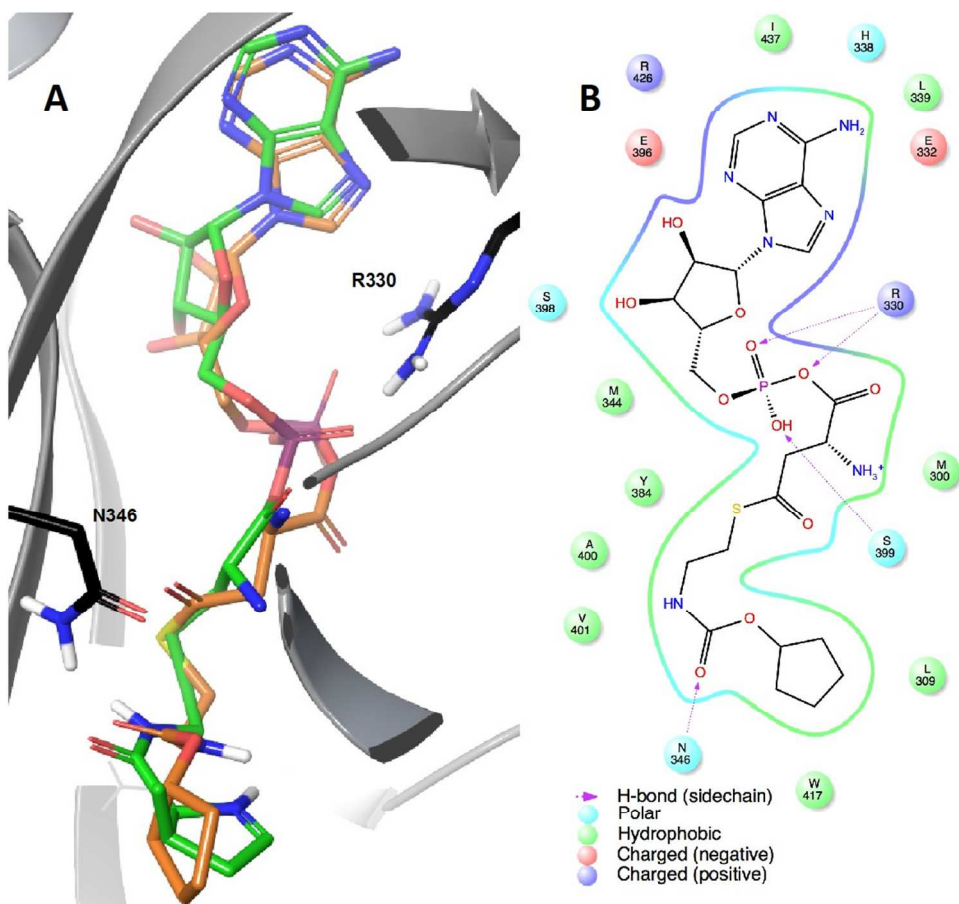
1. Materials and methods.

All moisture sensitive reactions were performed in flame dried borosilicate glassware under an inert and dry atmosphere of argon. ACS grade solvents were used for extraction and flash chromatography. ACS grade reagents were used without further purification. Anhydrous solvents and cyclopentanol were prepared by storage over 3Å molecular sieves (30-50% w/v) overnight. Sparging with argon was conducted for 5 min. Flash chromatography was performed using 40-63 µm silica gel with 60 Å porosity. The progress of reactions was monitored by analytical thin-layer chromatography (TLC, silica gel F-254 plates). Visualization of TLC plates was accomplished with 254 nm light and/or ninhydrin (0.3% w/v in 97:3 EtOH-H₂SO₄). The TLC solvent system 8:8:3:1 n-BuOH-H₂O-AcOH-acetone was prepared freshly prior to use, and amino acid standards were dissolved in basic ethanol. Melting points are uncorrected. NMR spectra were obtained at the indicated field strength with internal standards, or referencing to residual solvent peaks. All NMR spectra were recorded at ambient temperature. High resolution mass spectra were obtained using either ESI or MALDI techniques.

10x PBS and 10x TAE buffer were purchased from Cellgro. LB agar and LB broth were ordered from BD Difco™. Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Anatrace, and 4-12% Bis-Tris gels for SDS-PAGE were purchased from Invitrogen. PfuUltra II Fusion HS DNA Polymerase was obtained from Agilent Technologies; dNTPs were obtained from New England Biolabs; and oligonucleotide primers were purchased from Integrated DNA Technologies. Plasmid DNA preparation was carried out with the ZR Plasmid Miniprep-Classic Kit (Zymo research). Absorbance and emission spectra were measured on a Molecular Devices SpectraMax 250 Microplate Spectrophotometer. A Fisher scientific 550 sonic disruptor was used.

2. Molecular modelling of ThioD in wt-PylRS.

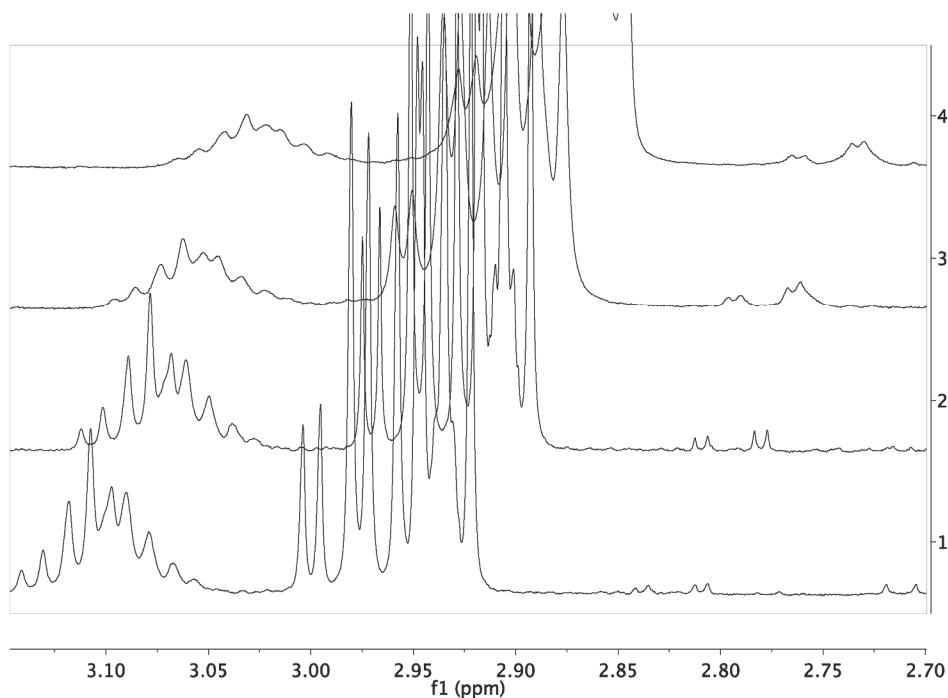
Figure S1. A) Superposition of AMP-charged ThioD (orange) and a stable Pyl-AMP analog (green) bound to wt-PylRS (PDB: 2Q7H). Docking of ThioD and wt-PylRS was performed using AutoDock Vina.¹ B) Ligand interaction diagram of AMP-charged ThioD bound to wt-PylRS with a ligand-residue cutoff distance of 3 Å. Key receptor-ligand hydrogen bonds (< 2 Å) are depicted as dashed arrows. The Maestro[®] software package was used to visualize the docking results.



3. ThioD stability study.

A solution of ThioD in phosphate buffer (60 mM, 7.1 pD) and a solution of glutathione (60 mM) in a phosphate buffer (30 mM, 7.2 pD) were prepared. 2,2-Dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) was used as an internal standard. The solutions were sparged, mixed together, and filtered through PTFE (0.2 μm) into a NMR tube filled with Ar. Gradual precipitation was observed and confirmed to be 11: ^1H NMR (600 MHz, D_2O) δ 5.16 – 4.93 (m, 1H), 3.30 (t, $J = 6.5$ Hz, 2H), 2.65 (t, $J = 6.5$ Hz, 2H), 1.96 – 1.33 (m, 8H); ThioD (8 mM) was dissolved in phosphate buffer (pD 7.2): ^1H NMR (600 MHz, D_2O) δ 5.02 (m, 1H), 4.05 (dd, $J = 7.2, 4.6$ Hz, 1H), 3.31 (m, 4H), 3.10 (m, 2H), 1.97 – 1.53 (m, 8H); Glutathione (34 mM) dissolved in phosphate buffer (7.1 pD): ^1H NMR (400 MHz, D_2O) δ 4.54 (dd, $J = 7.1, 5.2$ Hz, 1H), 3.83 – 3.61 (m, 4H), 3.28 (dd, $J = 14.3, 4.5$ Hz, 2H), 2.93 (m, 3H), 2.59 – 2.44 (m, 2H), 2.14 (td, $J = 7.6, 6.3$ Hz, 2H). Aspartate (19 mM) was dissolved in phosphate buffer (7.1 pD); ^1H NMR (400 MHz, D_2O) δ 3.90 (dd, $J = 8.9, 3.8$ Hz, 1H), 2.74 (dd, $J = 8.9, 3.7$ Hz, 2H). $t_{1/2} = 48 \pm 1$ h, $n = 41$; error in the half life was calculated as the combined uncertainty of the measurements.

Figure S2. Selected spectra from the stability study of ThioD: 1) $t_0 + 5$ min, 2) $t_0 + 1$ h, 3) $t_0 + 23$ h, and 4) $t_0 + 36$ h. HSQC confirmed that the multiplet at 3.10 ppm comprised only ThioD. The doublet of doublets at 2.82 ppm corresponds to L-aspartate.



4. Direct evolution of PylRS mutants that selectively recognize ThioD.

The PylRS library inserts were constructed by overlapping PCR, digested with NdeI and PstI, and then inserted into pBK vector. The primers used are listed in Table S1.

The library encoded in the pBK vector (Kan^r) was transformed into *E. coli* DH10B cells (Life Technologies) harboring a positive selection plasmid (pRep-Pyl, Tet^r) that encodes both chloramphenicol acetyltransferase (with an Asp112TAG mutation) and tRNA^{Pyl}. Over 1.0 X 10⁸ transformants were obtained to cover an expected library size of ~3.0 X 10⁷ members. Positive selection was performed on LB agar plates containing 50 µg/mL kanamycin, 25 µg/mL tetracycline, 50 µg/mL chloramphenicol and 2 mM ThioD for 48 h at 37 °C. The pBK plasmid was isolated from surviving colonies and transformed into *E. coli* DH10B cells harboring a negative selection plasmid (pNeg-Pyl, Amp^r) that contains tRNA^{Pyl} and a toxic barnase gene with two amber mutations (Gln2TAG and Asp44TAG) under control of the araBAD promoter. The negative selection was carried out on LB agar containing 100 µg/mL ampicillin, 50 µg/mL kanamycin and 0.2% L-arabinose for 12 h at 37 °C. The pBK plasmid was isolated from surviving cells, and a second round of positive selection was carried out as described above in the presence 60 µg/mL chloramphenicol. After 48 h growth at 37 °C, 28 single colonies were picked and re-grown on LB plates with 50 µg/mL chloramphenicol in the presence or absence of 2 mM ThioD. One colony showed obvious growth advantage in the presence of ThioD, and the corresponding pBK plasmid was separated and sequenced.

Table S1.

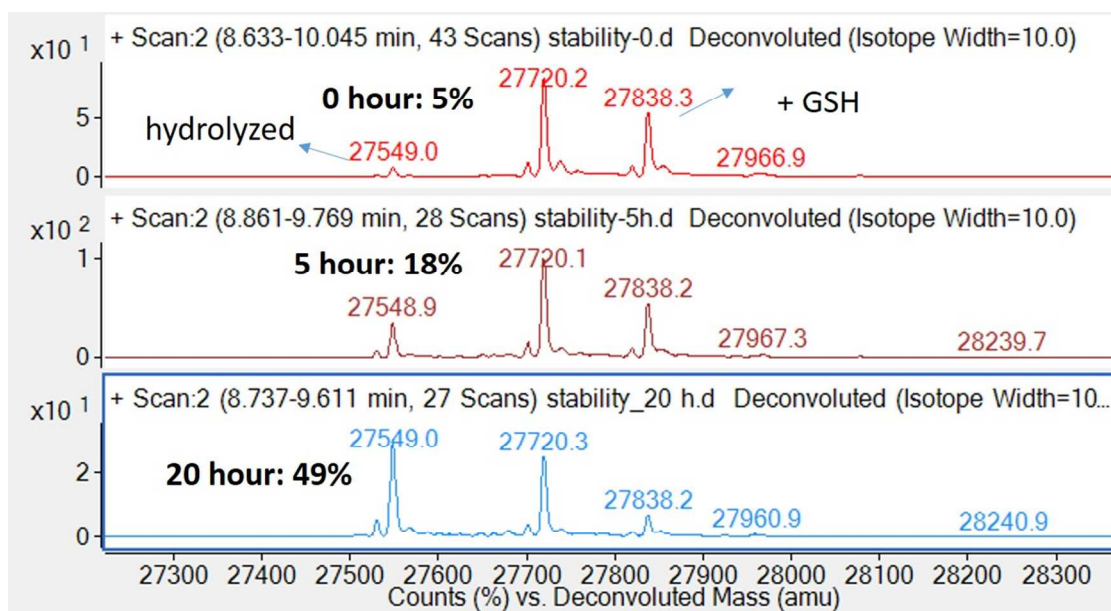
oligonucleotide	Sequence (5'-3')
ThioDlib-F1-f	gaggaatcccatatggataaaaaacc
ThioDlib-F1-r	AAGCATTGGCCTCAAGCAGAGATTTT
ThioDlib-F2-f	tctctgcttgaggccaatgcttNNKccgactCTTNNKAACTATCTGcgaaaac tcgatag
ThioDlib-F2-r	CACCATAGTAAATTCTTCCAGGTGCTC
ThioDlib-F3-f	GCACCTGGAAGAATTTACTATGGTGNNKTTTCNNKAGATGGGTT CGGGATGTACTCGGGA
ThioDlib-F3-r	GACCATACAGGAATCTCCTACGATTTT
ThioDlib-F4-f	aaatcgtaggagattcctgtatggctNNKggggataactcttgatataatgcacgg
ThioDlib-F4-r	TTTAGCGTTTGAAACTGCAGTTATAGA

5. Protein (sfGFP-Y151ThioD) expression and purification by Ni²⁺ affinity chromatography.

E. coli DH10B was co-transformed with pUltra-ThioDRS and pET22b-T5-sfGFP-Y151TAG. A single colony was picked and inoculated into 5 mL LB, and grown overnight. The saturated culture was diluted 200-fold (125 µL to 25 mL) with LB and grown at 37 °C. When the OD reached 0.6, powdery ThioD was directly added to the culture to a final concentration of 2 mM, and the culture was grown for another 30 min at 25 °C before induction with 1 mM IPTG. The induced cells were grown an additional 6 h at 25 °C. The cells were pelleted and lysed with a sonic disruptor in the presence of protease inhibitors, and insoluble protein and cell debris were removed by centrifugation. The protein was purified by Ni²⁺ affinity chromatography, and finally buffer exchanged with an Amicon centrifugal filter. Protein concentration was determined with a NanoDrop™ 1000 Spectrophotometer (according to the manufacture's protocol).

6. Investigation of incorporated ThioD stability on protein sfGFP-Y151ThioD.

Figure S3. The purified sfGFP-Y151ThioD at 0.1 mg/mL was incubated in pH 7.4 DPBS at 37 °C for 5 h or 20 h, and analyzed by ESI-QTOF mass spectrometry. The percentage of hydrolyzed protein was based on the integrated peak areas.

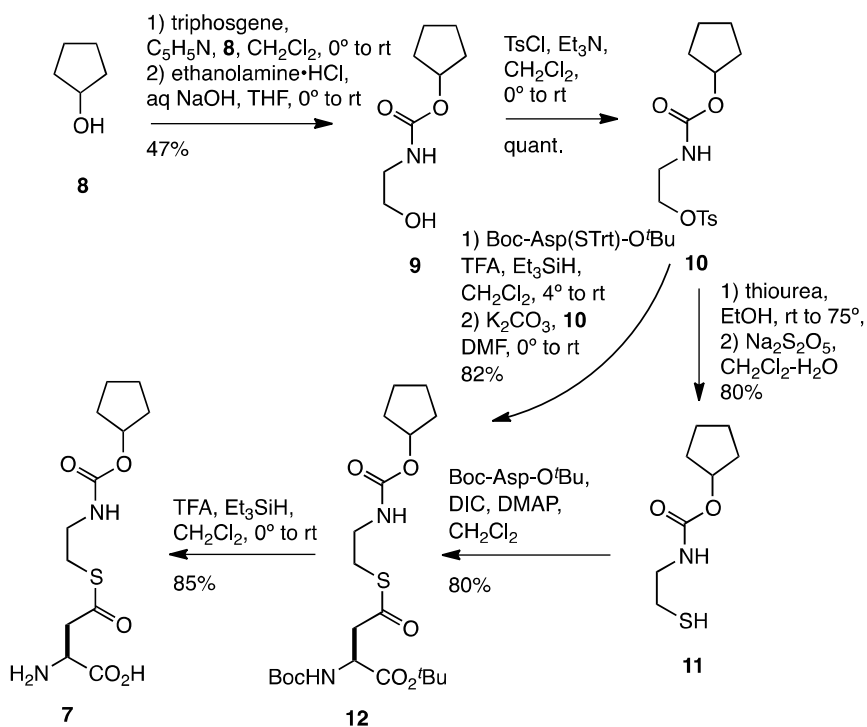


7. Condition used in Labelling of sfGFP-Y151ThioD

sfGFP-Y151ThioD (3.7 μ M, 0.1 mg/mL) was treated with FI-cys (100 equiv.) in 50 mM pH 7.4 phosphate buffer containing 25 mM TCEP at 37 °C for 20 h. For the alkylation reaction, the above labelling product was subject to buffer exchange against DPBS to remove excessive amount of FI-cys, and then the labelling product at 0.1 mg/mL was incubated with 15 mM iodoacetamide in pH 8 phosphate buffer containing 10 mM TCEP at room temperature for 1 h.

8. Synthesis of ThioD.

Scheme S1. Synthesis of nAA 7 (ThioD). TsCl = tosyl chloride, TFA = trifluoroacetic acid, DIC = diisopropylcarbodiimide, DMAP = *p*-dimethylaminopyridine, and DMF = dimethylformamide.



Alcohol 9: A procedure for the preparation of cyclohexylchloroformate was adapted.² To a 1.35 M solution of triphosgene (9.00 g, 36.4 mmol) in dry CH_2Cl_2 , was added dry **8** (9.9 mL, 0.11 mol) dropwise over 7 min with stirring in an ice bath. A solution of dry pyridine (8.8 mL, 0.11 mol) in dry CH_2Cl_2 (5.4 mL) was added dropwise to the mixture over 35 min, and stirring continued for 2 h as the ice melted. The mixture was diluted with CH_2Cl_2 (50 mL), and washed with ice cold H_2O (3 x 25 mL). The organic phase was dried with $CaCl_2$ and concentrated to give crude cyclopentylchloroformate that was used without further purification. Ethanolamine hydrochloride (40 g, 0.41 mol) and NaOH (16 g, 0.41 mol) were dissolved in H_2O (180 mL) and THF (100 mL) in a 2-neck RBF (500 mL) fitted with an egg shaped stir bar (3 x 1.5² cm). The solution was chilled in a brine/ice bath for 5 min. A solution of the cyclopentylchloroformate in THF (80 mL) was placed into an addition funnel and added dropwise to the

ethanolamine for over 35 min with 500 RPM stirring. The solution was left to stir in the brine-ice bath for an additional 2 h, and then taken out of the ice bath and stirred for 1 h. EtOAc (300 mL) was added and the mixture was washed with 2N HCl (2 x 50 mL), H₂O (3 x 50 mL), and the combined aqueous phases extracted with CH₂Cl₂ (4 x 50 mL). The combined organic phases were dried with MgSO₄ and concentrated to give the crude product. This material was dry loaded with Celite[®] 545 (25 g), and flash chromatographed on silica gel (19 x 4.5 cm) eluting with 100 mL each of 10%, 20%, 35%, 50%, 70%, and 90% EtOAc/hexanes, taking 50 mL fractions. Fractions 12 - 19 afforded 9 as a solid (9.3 g, 47%). *R_f* = 0.20 (60% EtOAc/hexanes, ninhydrin); mp 40 - 42 °C; ¹H NMR (300 MHz, CDCl₃) δ 5.15 – 5.04 (m, 1H), 5.04 – 4.88 (m, 1H), 3.71 (q, *J* = 5.1 Hz, 2H), 3.33 (q, *J* = 5.1 Hz, 2H), 2.20 (s, 1H), 1.99 – 1.29 (m, 8H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 156.2, 75.9, 60.0, 43.0, 32.4, 23.3; HRMS (MALDI) [M+H⁺]_{calc} = 174.1130, [M+H⁺]_{obs} = 174.1124.

Tosylate 10: A procedure for the tosylation of BocNHCH₂CH₂OH was adapted.³ To a stirred ice cold solution of 9 (5.4 g, 31 mmol) in CH₂Cl₂ (57 mL) was added Et₃N (9.8 mL, 70 mmol) in one portion. Tosyl chloride (7.3 g, 38 mmol) in CH₂Cl₂ (46 mL) was then added. The dark brown solution continued to stir for 29 h at ambient temperature. CH₂Cl₂ (30 mL) was added, and the organic layer was washed with citric acid chilled for 5 min on ice (10% w/v, 3 x 18 mL). The organic phase was washed with brine (10 mL), dried with MgSO₄, and concentrated. Tosylate 10 was obtained as a brown oil and used without further purification (10 g, quant.). A portion of 10 was purified by flash chromatography to furnish an analytical sample (2D TLC indicated decomposition, 82% recovered). *R_f* = 0.45 (30% EtOAc/hexanes); ¹H NMR (600 MHz, CDCl₃) δ 7.79 (d, *J* = 8.4 Hz, 2H), 7.36 (d, *J* = 8.6 Hz, 2H), 5.04 - 4.99 (m, 1H), 4.99 – 4.71 (m, 1H), 4.08 (m, 2H), 3.42 (m, 2H), 2.45 (s, 3H), 1.87 – 1.52 (m, 8H); ¹³C NMR (101 MHz, CDCl₃) δ 156.1, 144.7, 146.2, 132.3, 129.6, 127.5, 77.3, 68.9, 39.6, 32.4, 23.3, 21.3; HRMS (MALDI) [M+Na⁺]_{calc} = 350.1038, [M+Na⁺]_{obs} = 305.1028.

Thiol 11: To a solution of 10 (1.20 g, 3.66 mmol) in anhydrous EtOH (6 mL) was added thiourea (0.462 g, 6.07 mmol) and the flask was heated to 75 °C with stirring for 18 h. EtOH removal afforded a light yellow oil. The oil was dissolved in CH₂Cl₂ (14.5 mL) and to this was added a 1.0 M solution of Na₂S₂O₅ (11 mL).⁴ The mixture was refluxed for 22 h with vigorous stirring. After cooling, the mixture was partitioned between CH₂Cl₂ (10 mL) and brine (5 mL), and the aqueous layer was extracted with CH₂Cl₂ (2 x 10 mL). The combined organic layers were dried with MgSO₄, and concentrated. Flash chromatography (17 x 4.5 cm) was performed eluting with 100 mL each of 5% (3 x), 10% (2 x), 15% (5 x), 30% EtOAc/hexanes, and 100% EtOAc taking 20 mL fractions. Fractions 34 - 47 afforded 11 as an oil (0.555 g, 80%). *R_f* = 0.45 (30% EtOAc/hexanes). ¹H NMR (600 MHz, CDCl₃) δ 5.18 – 4.86 (m, 2H), 3.30 (q, *J* = 6.5 Hz, 2H), 2.61 (q, *J* = 6.9 Hz, 2H), 1.85 – 1.46 (m, 8H), 1.32 (t, *J* = 8.4 Hz, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 156.3, 77.5, 43.8, 32.7, 25.0, 23.6; HRMS (MALDI) [M+Na⁺]_{calc} = 212.0722, [M+Na⁺]_{obs} = 212.0718.

Thioacid route to 12: The reported detritylation was modified by substituting triethylsilane for triisopropylsilane.⁵ Boc-Asp(STrt)-OtBu (1.02 g, 1.78 mmol) was dissolved in dry CH₂Cl₂ (39 mL), and the solution was sparged. With stirring, sparged Et₃SiH (0.86 mL, 5.3 mmol) was added in one portion. The mixture was submerged in an ice bath and sparged TFA (1.40 mL) was added dropwise over 3 min. The

ice bath was removed once the addition was complete, and the yellow solution stirred 5 additional min before co-evaporation with sparged toluene (4 mL). The yellow cation disappeared after 7 min. The resulting white solid was pumped for a period of 1.3 h at 200 mTorr, and sparged dry DMF (2.0 mL) was added after the flask was submerged in an ice bath for 5 min. K_2CO_3 (4.85 g, 35.1 mmol) was calcined at 160 °C, purged with Ar (3 x), and added in one portion; effervescence was observed. Crude 10 (1.29 g, 3.96 mmol) in dry DMF (0.75 mL) was added dropwise over 2 min. The slurry was allowed to warm as the ice bath melted, and stirred for 16 h. DMF was removed by evaporation with n-heptane (2 x 5 mL). The remaining mixture was partitioned between EtOAc (80 mL) and H_2O (30 mL). The organic layer was washed with 2N H_2SO_4 (5 mL), H_2O (5 mL), and brine (5 mL). The solution was dried with $MgSO_4$ and reduced. Flash chromatography (16.5 x 3.5 cm) eluting with 200 mL of each 5% (2 x), 10% (2 x), 20% (3.5 x), and 30% EtOAc/hexanes, taking 10 mL fractions. Fractions 30 - 47 afforded 12 as a viscous oil (0.67 g, 82%). $R_f=0.24$ (20% EtOAc/hexanes); 1H NMR (600 MHz, $CDCl_3$) δ 5.50 – 5.25 (m, 1H), 5.13 – 5.03 (m, 1H), 4.98 - 4.90 (m, 1H), 4.81 – 4.76 (m, 1H), 4.47 – 4.38 (m, 1H), 3.40 – 3.25 (m, 2H), 3.16 – 2.97 (m, 4H), 1.87 – 1.58 (m, 8H), 1.43 (d, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 196.6, 169.5, 156.3, 155.2, 82.5, 80.0, 77.4, 51.0, 45.7, 40.3, 32.7, 29.2, 28.3, 28.2, 27.8, 23.6; HRMS (MALDI) $[M+Na^+]_{calc} = 483.2141$, $[M+Na^+]_{obs} = 483.2136$.

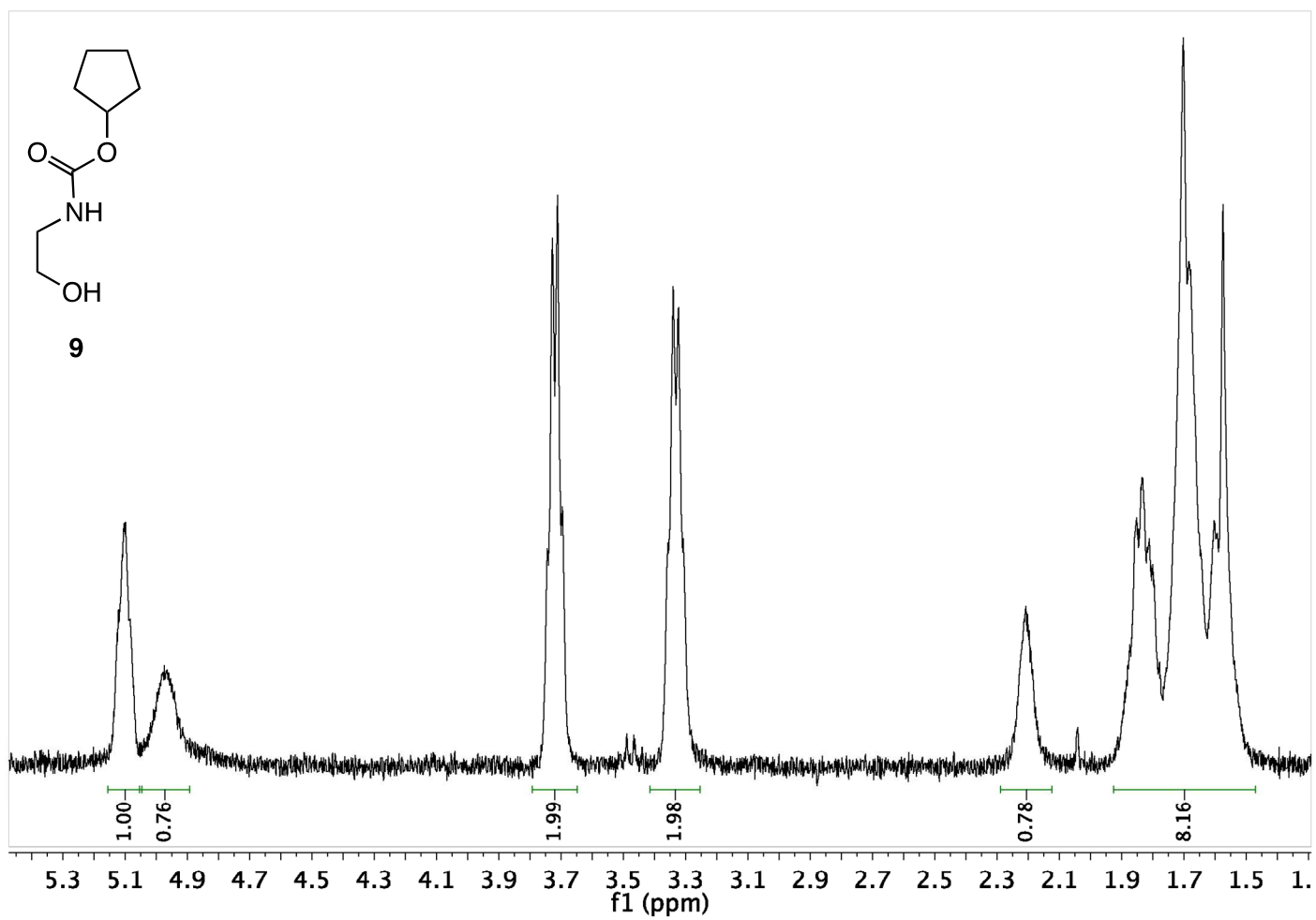
Steglich thioesterification route to 12: To a solution of 11 (0.555 g, 2.93 mmol), Boc-Asp(OH)-OtBu (1.05g, 3.20 mmol) in CH_2Cl_2 (11 mL) was added DMAP (35.5 mg, 2.91 mmol) and the mixture was azeotropically dried by evaporation of CH_2Cl_2 . Dry CH_2Cl_2 (5.8 mL) was added to the mixture, followed by DIC (0.80 mL, 5.1 mmol) over 3 min at ambient temperature. After 4.5 h, the yellow solution was concentrated. The resulting residue was dissolved in 4:1 Et_2O -hexanes (50 mL), and TEA (4 mL) was added to the ice-cold solution. The organic layer was extracted with H_2O (2 x 20 mL), 2N HCl (2 x 10 mL), and brine (30 mL). The organic layer was dried with $MgSO_4$ and concentrated. Repetitive flash chromatography afforded 12 as a viscous oil (1.09 g, 80%). $R_f= 0.24$ (20% EtOAc/hexanes); 1H NMR (600 MHz, $CDCl_3$) δ 5.50 – 5.25 (m, 1H), 5.13 – 5.03 (m, 1H), 4.98 - 4.90 (m, 1H), 4.81 – 4.76 (m, 1H), 4.47 – 4.38 (m, 1H), 3.40 – 3.25 (m, 2H), 3.16 – 2.97 (m, 4H), 1.87 – 1.58 (m, 8H), 1.43 (d, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 196.6, 169.5, 156.3, 155.2, 82.5, 80.0, 77.4, 51.0, 45.7, 40.3, 32.7, 29.2, 28.3, 28.2, 27.8, 23.6; HRMS (MALDI) $[M+Na^+]_{calc} = 483.2141$, $[M+Na^+]_{obs} = 483.2136$.

ThioD 7: To a stirred, ice-cold solution of 12 (0.780 g, 1.69 mmol) in dry CH_2Cl_2 (12.6 mL) was added Et_3SiH (0.56 mL, 3.5 mmol, 2.1 equiv) in one portion, followed by the dropwise addition of TFA (4.2 mL) over 4 min. The solution was removed from the ice bath and allowed to stir for 24 h. The solvent was removed, and the residue co-evaporated with acetone (2 x 5 mL). MeOH (2 mL) was added, Et_2O (5 mL) was added to complete precipitation, and the solvent was evaporated. Lastly, co-evaporation with 1:1 Et_2O -acetone (10 mL), and the foam was suspended in EtOH (4 mL), Et_2O (10 mL) was added to complete precipitation, followed by evaporation of the solvent. The resulting foam was triturated with 1:1 CH_2Cl_2 - Et_2O (2 x 4 mL), and the resulting solid was washed with n-pentane (2 x 5 mL) to afford 7 (0.608 g, 85%). $R_f= 0.55$ (8:8:3:1 n-BuOH- H_2O -AcOH-acetone); mp = 160 - 162 °C; 1H NMR (400 MHz, D_2O) δ 5.01 (m, 1H), 4.30 (dd, $J = 5.9$ Hz, 1H), 3.37 (m, 4H), 3.10 (m, 2H), 1.87 – 1.56 (m, 8H); ^{13}C NMR (101 MHz, D_2O)

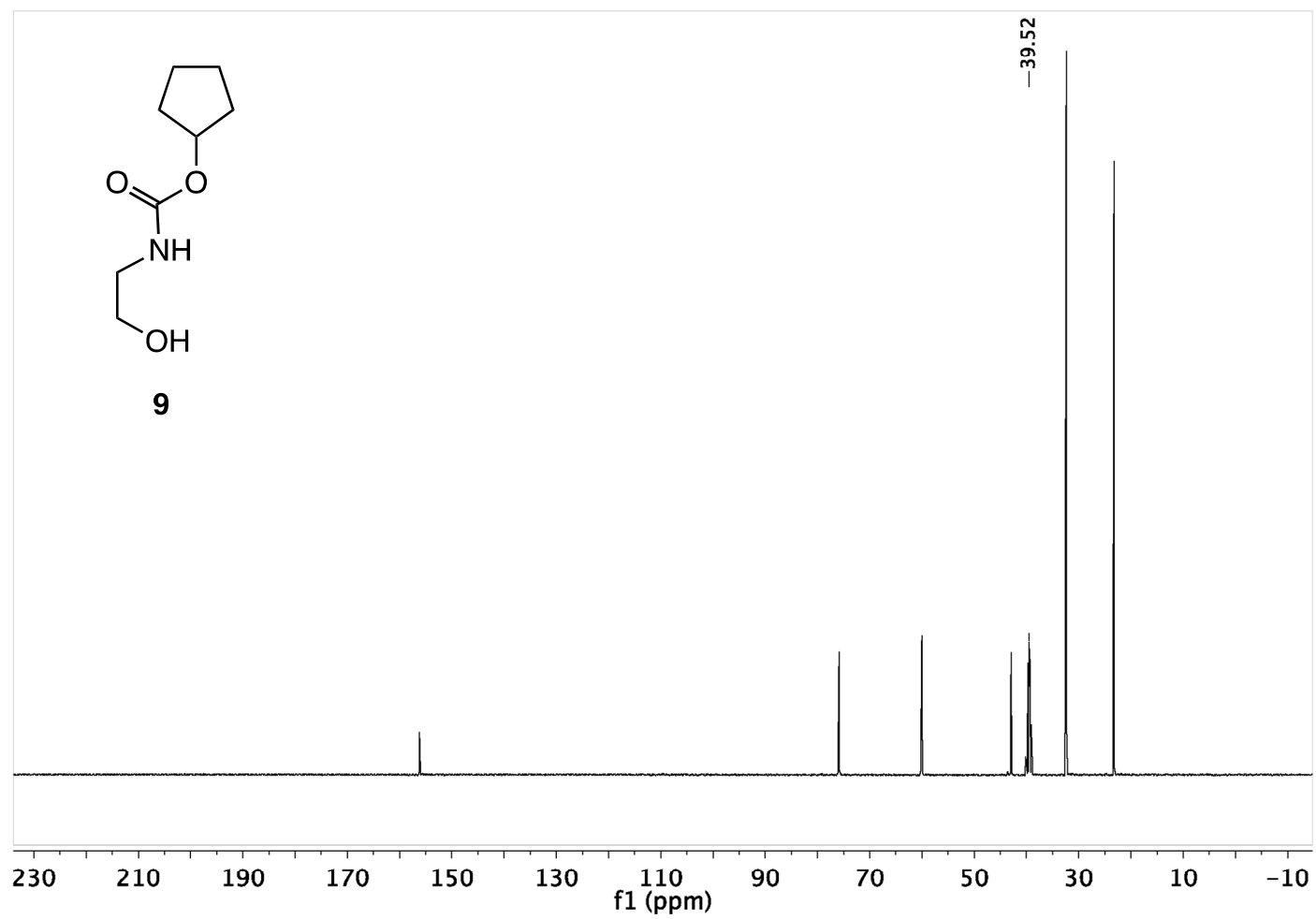
δ 198.6, 171.2, 158.7, 79.0, 49.8, 42.3, 39.3, 32.2, 32.2, 29.0, 23.0; HRMS (ESI) $[M+H]^+_{calc} = 305.1171$, $[M+H]^+_{obs} = 305.1174$.

9. NMR spectra

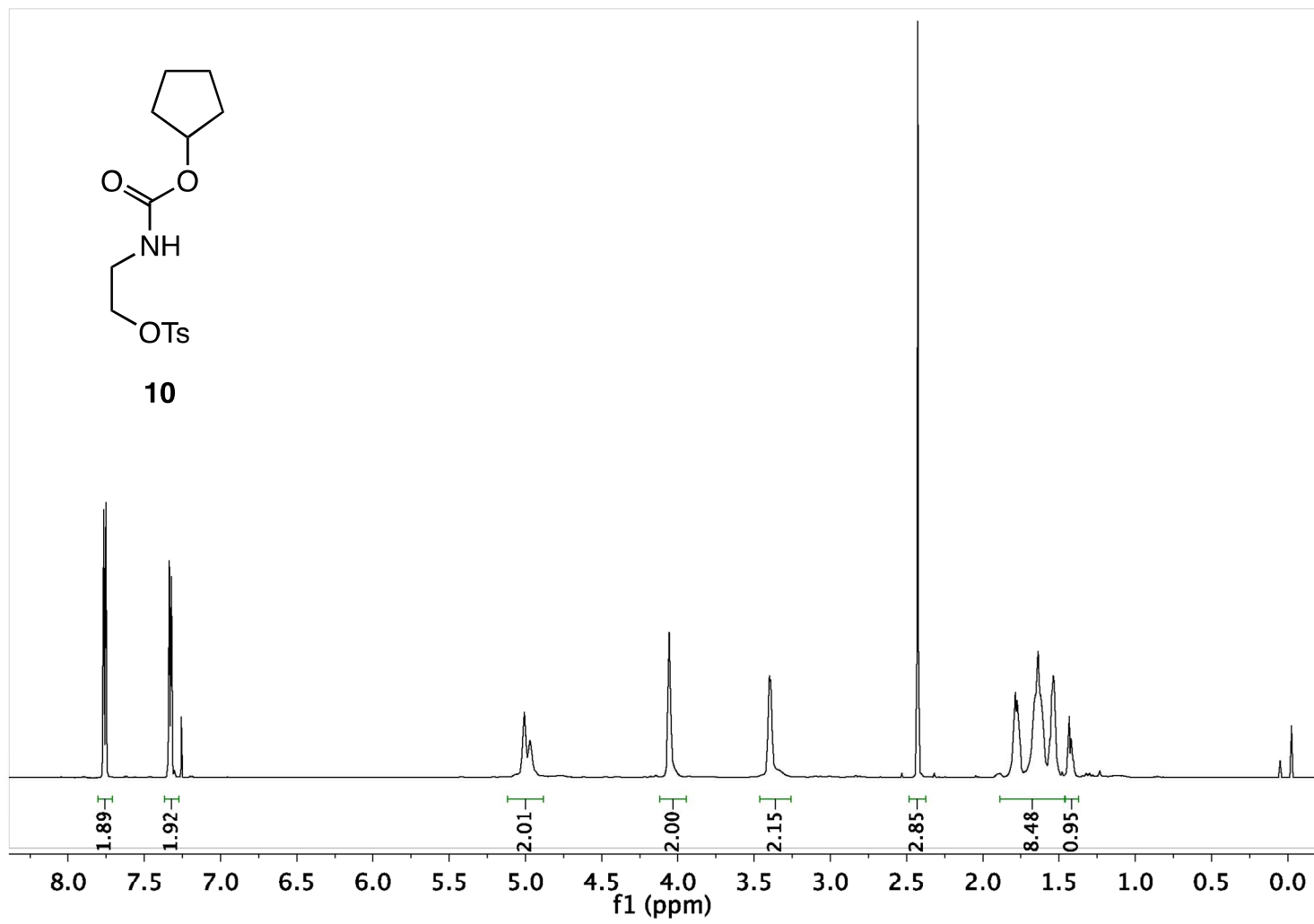
300 MHz ^1H NMR, CDCl_3 , 25 $^\circ\text{C}$



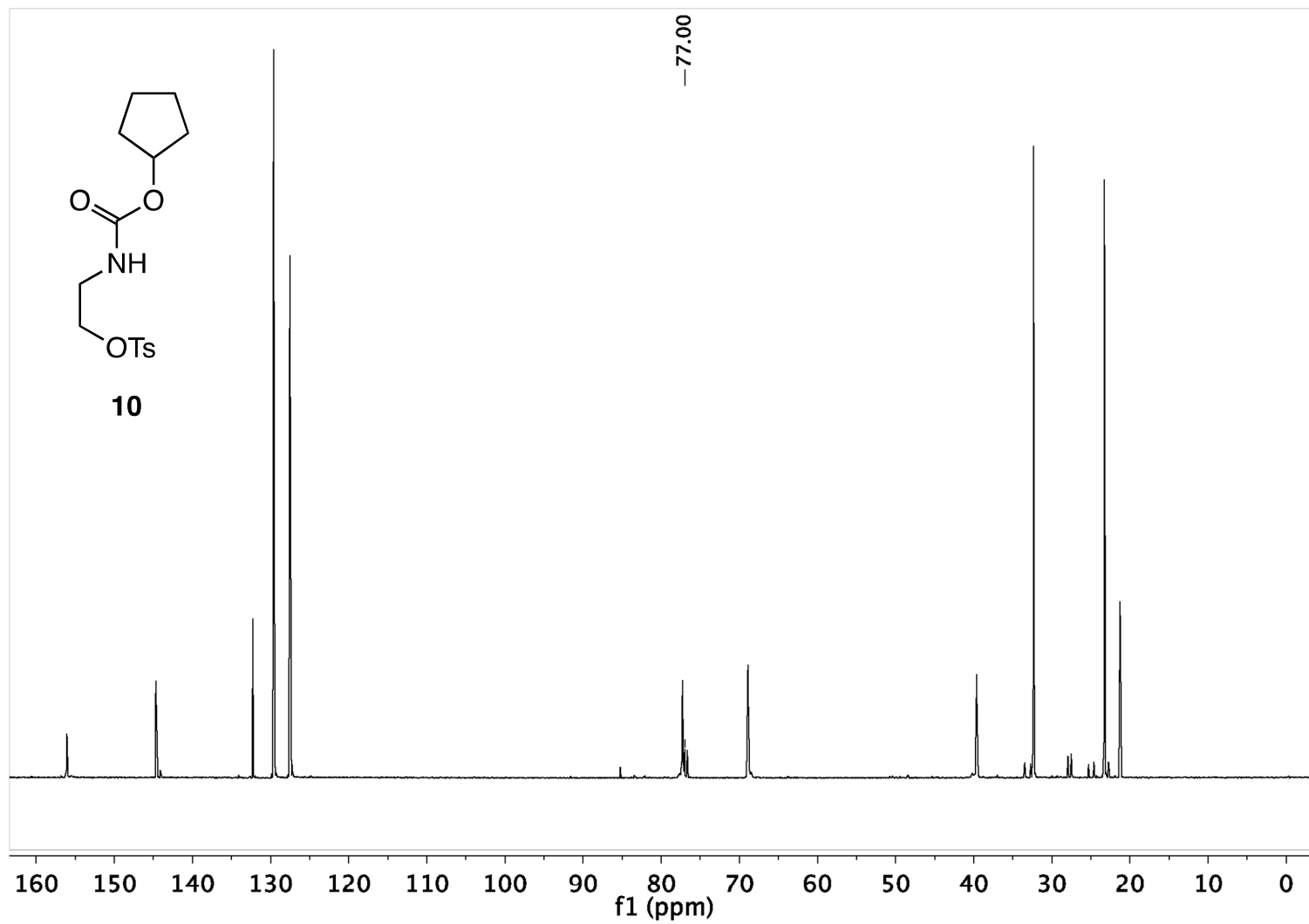
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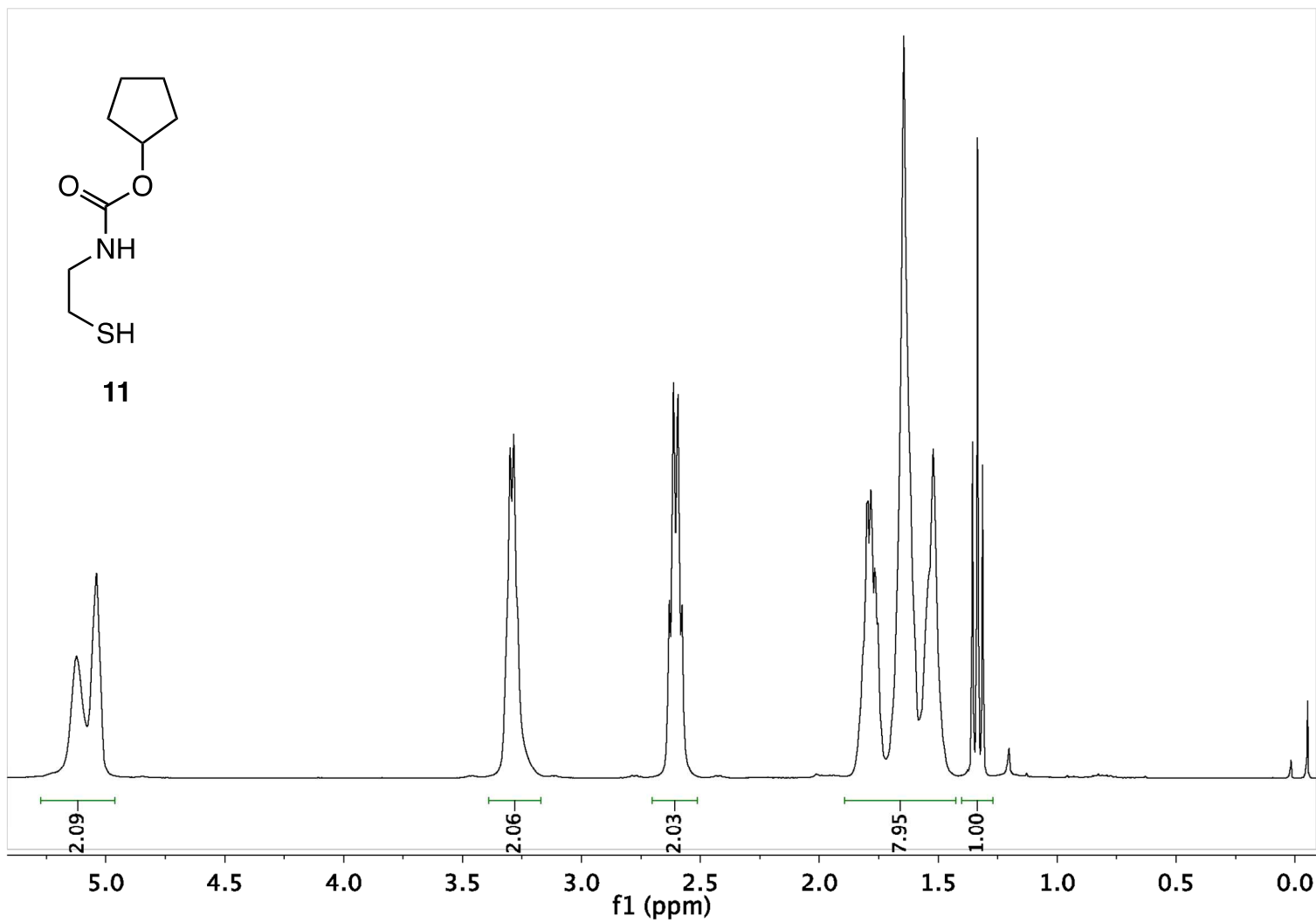
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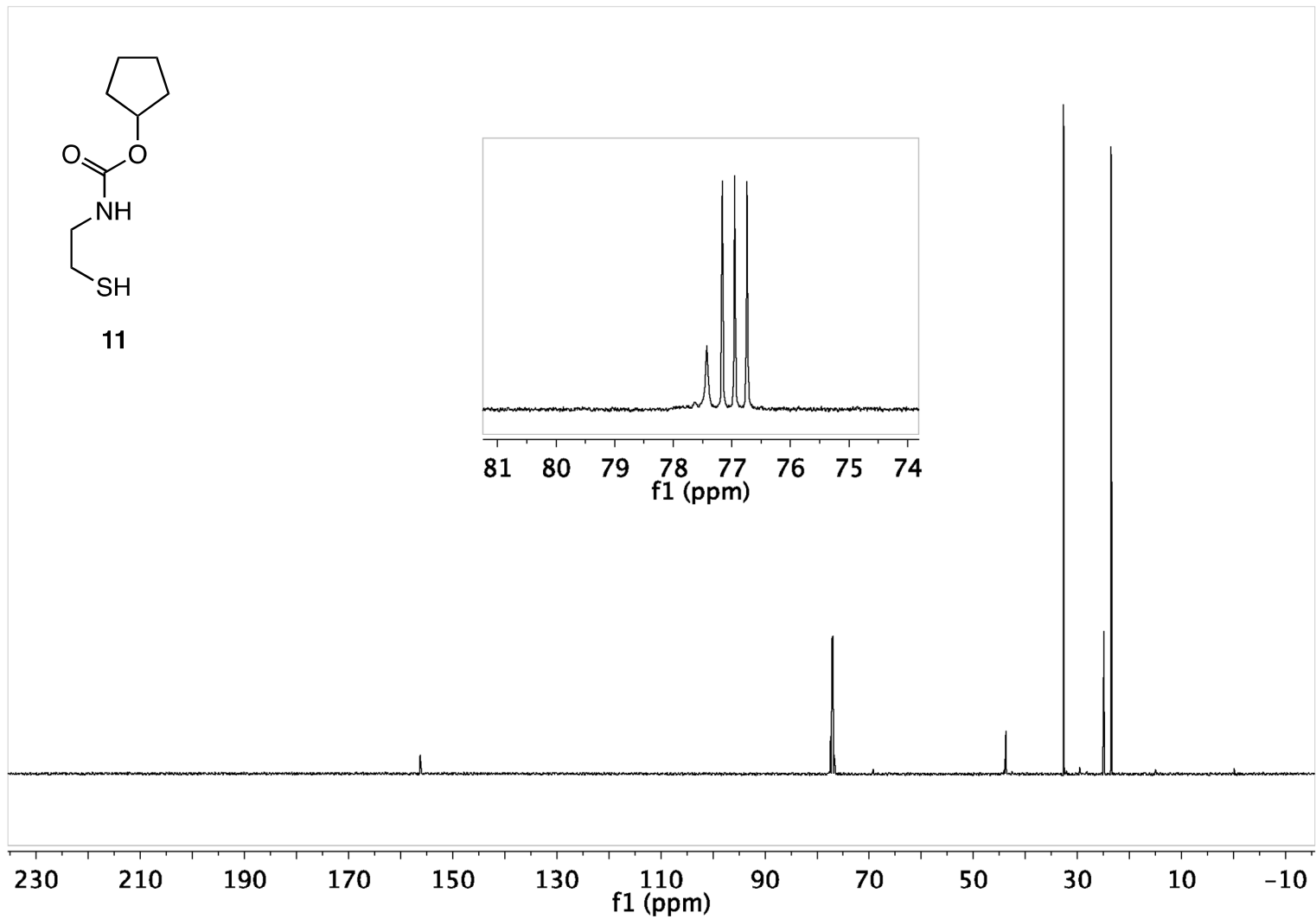
101 MHz ^1H NMR, CDCl_3 , 25 $^\circ\text{C}$



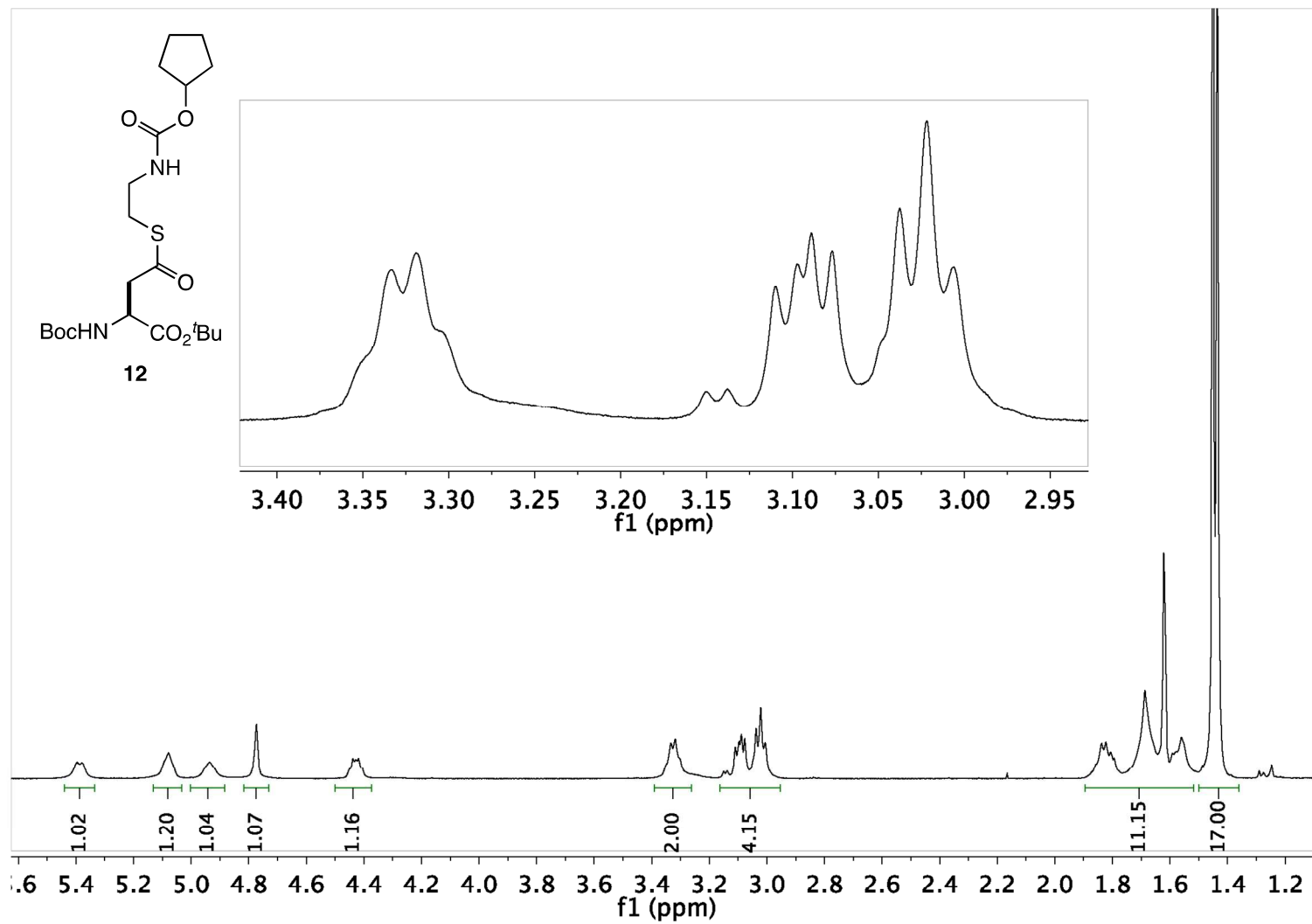
400 MHz ^1H NMR, CDCl_3 , 25 $^\circ\text{C}$



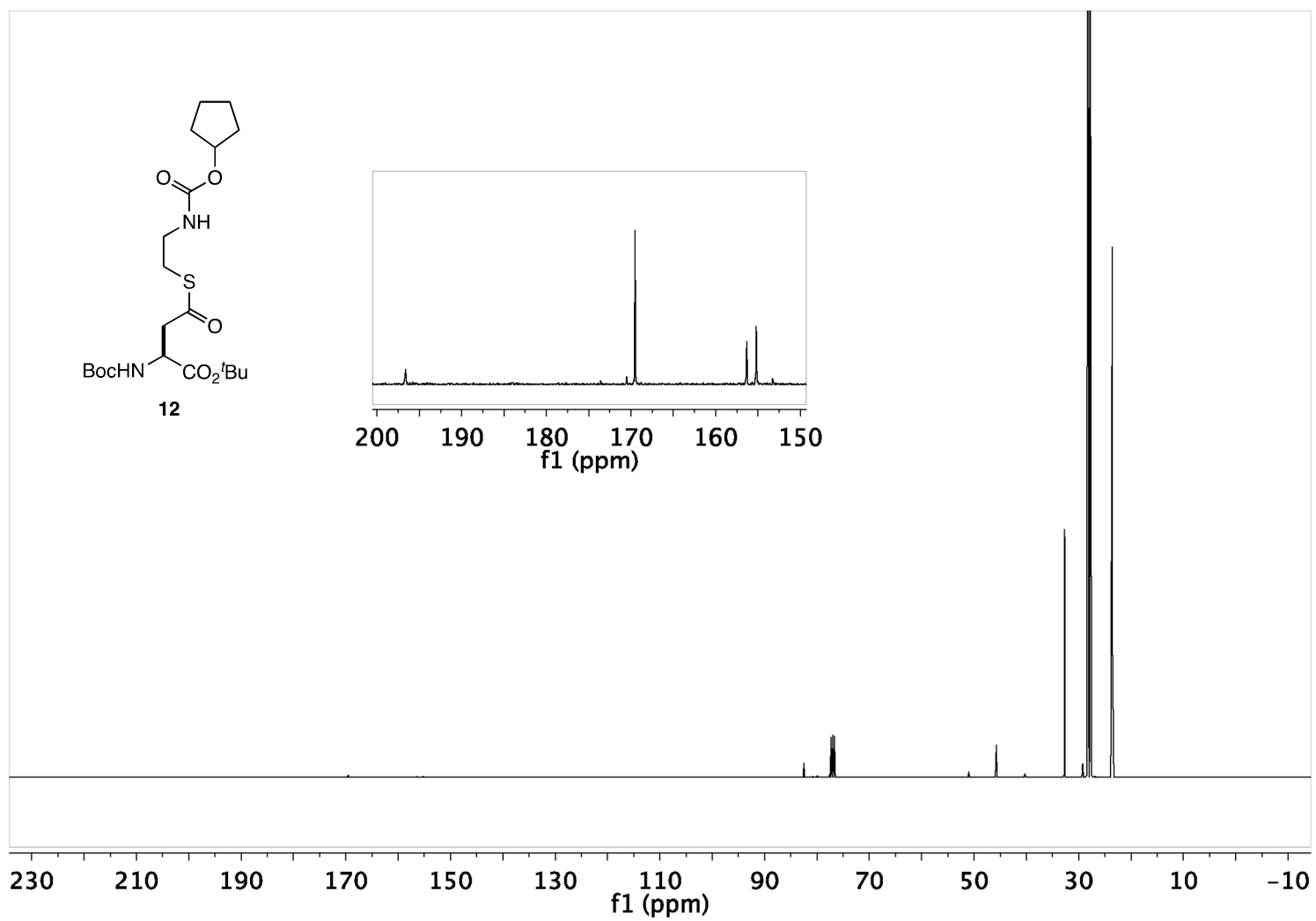
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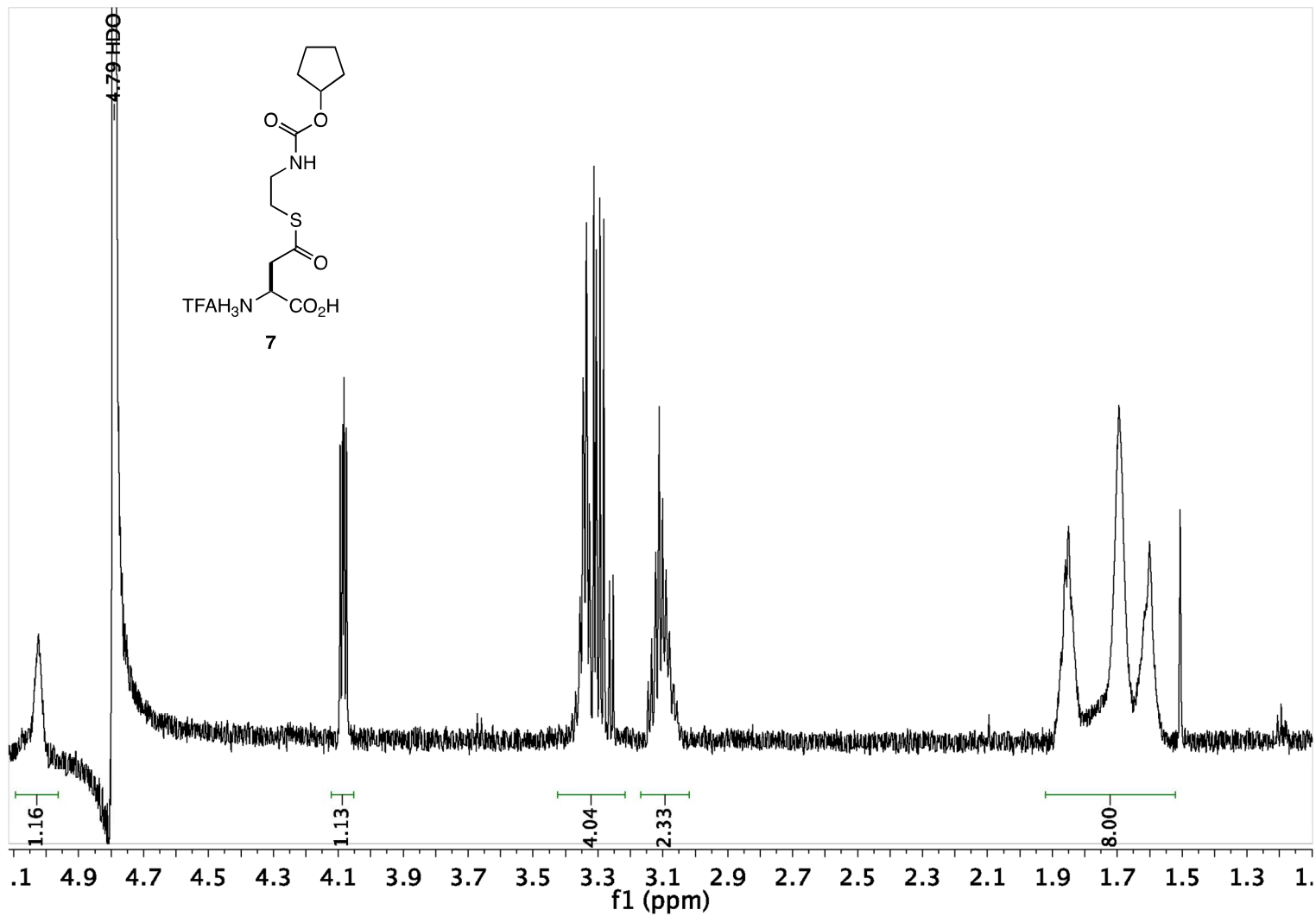
400 MHz ^1H NMR, CDCl_3 , 25 $^\circ\text{C}$



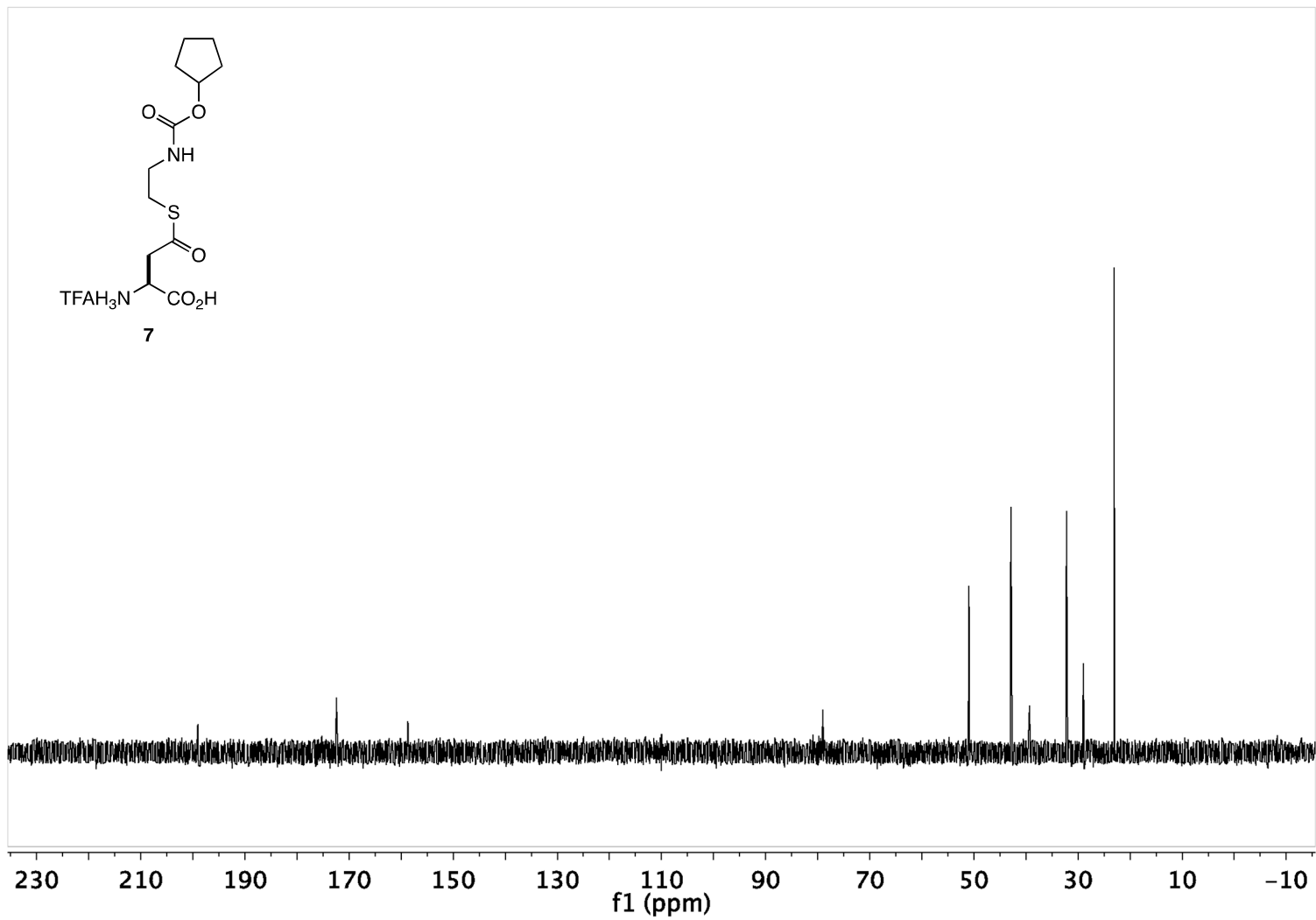
101 MHz ^{13}C NMR, CDCl_3 , 25 $^\circ\text{C}$



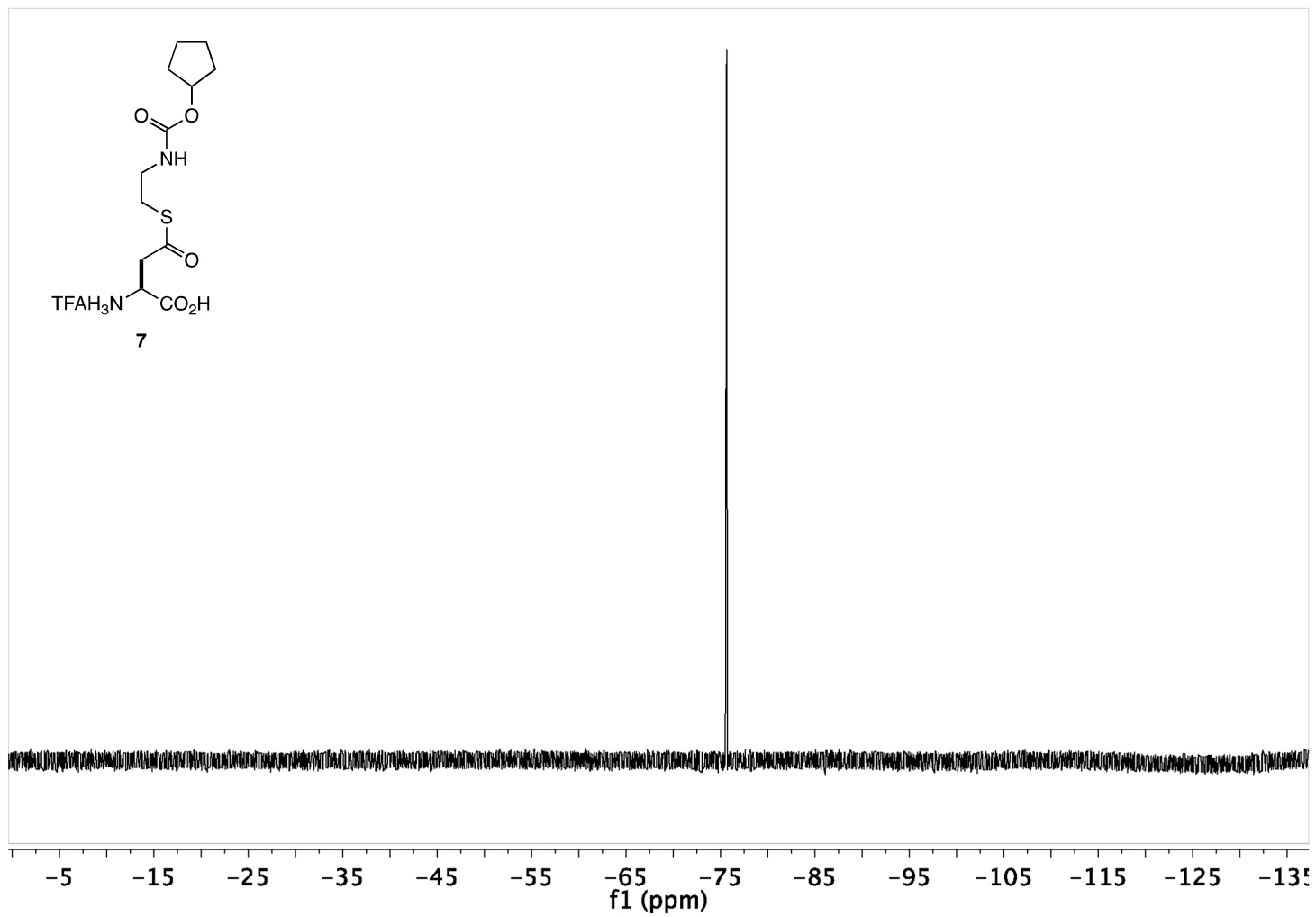
600 MHz ^1H NMR, D_2O , 25 $^\circ\text{C}$



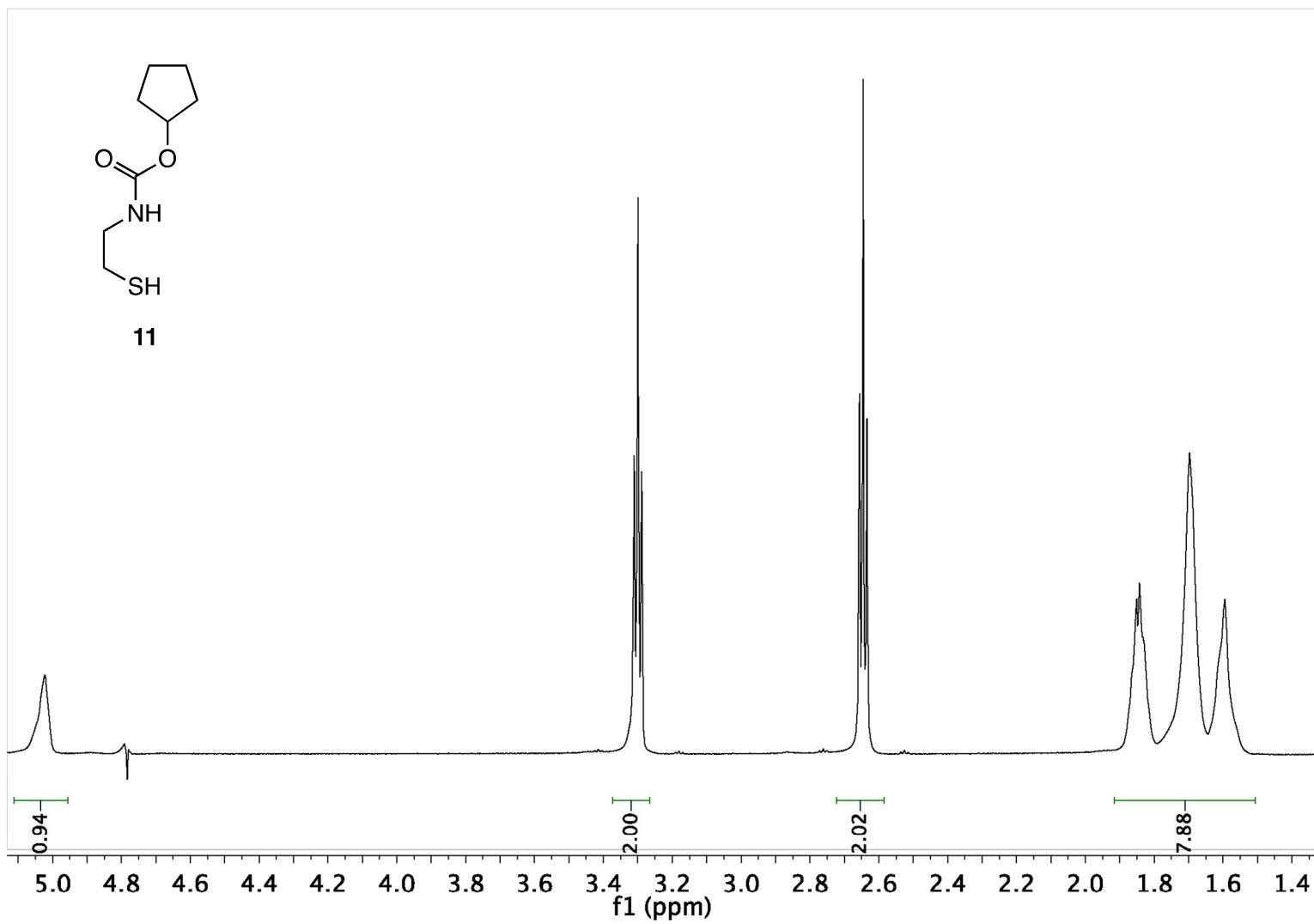
101 MHz ^{13}C NMR, D_2O , 25 °C



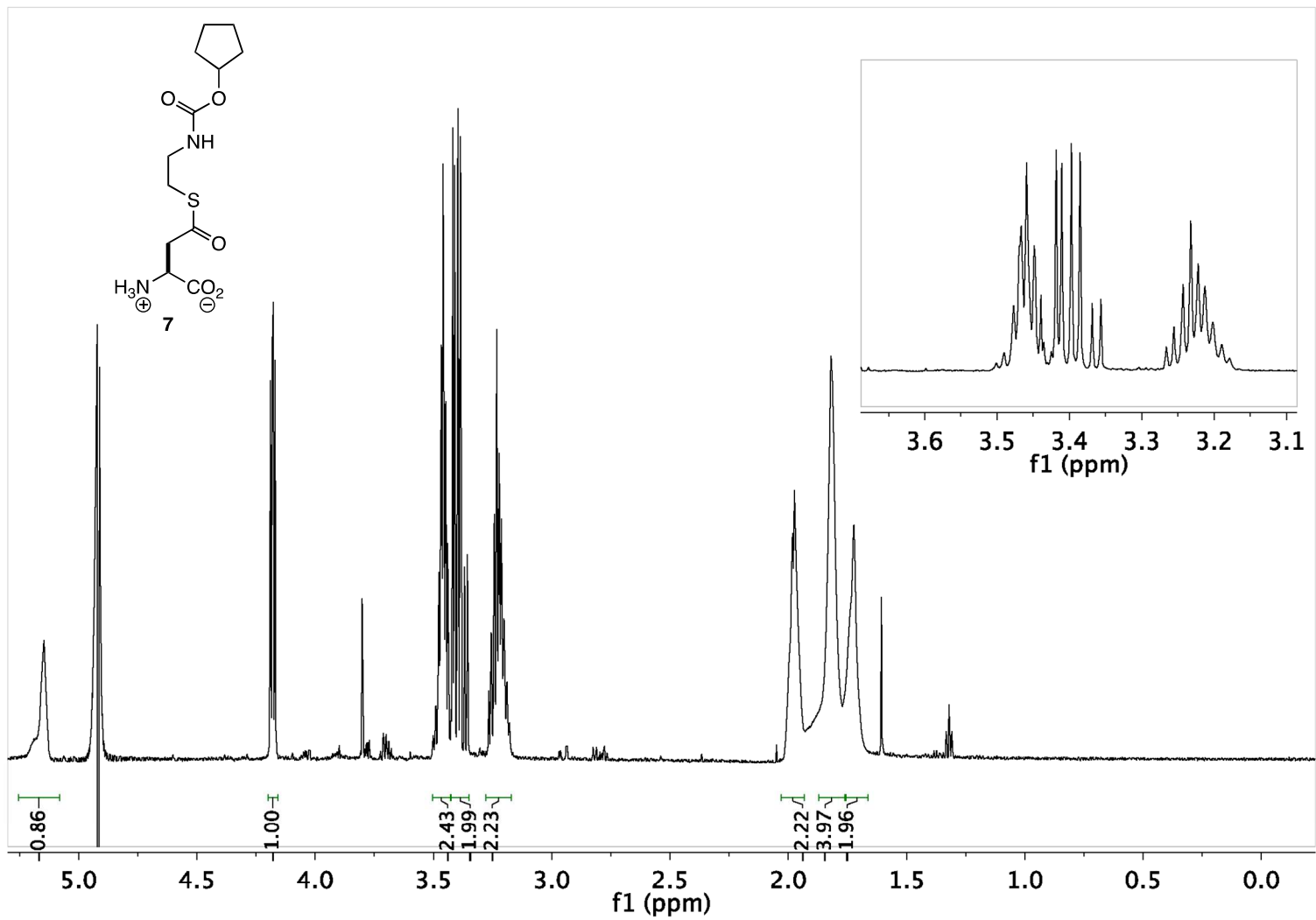
564 MHz ^{19}F NMR, D_2O , 25 $^\circ\text{C}$



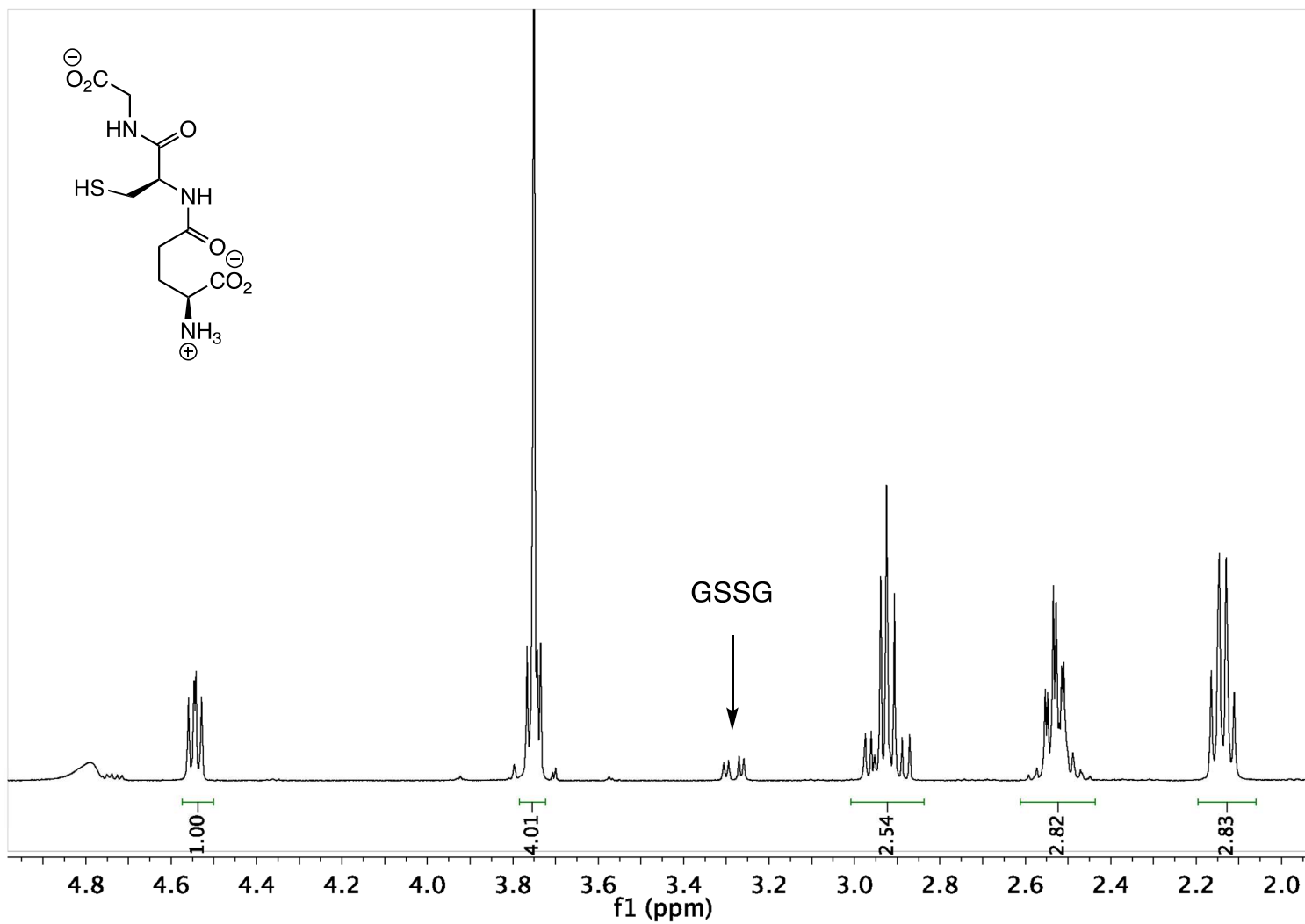
600 MHz ^1H NMR, D_2O , 25 $^\circ\text{C}$



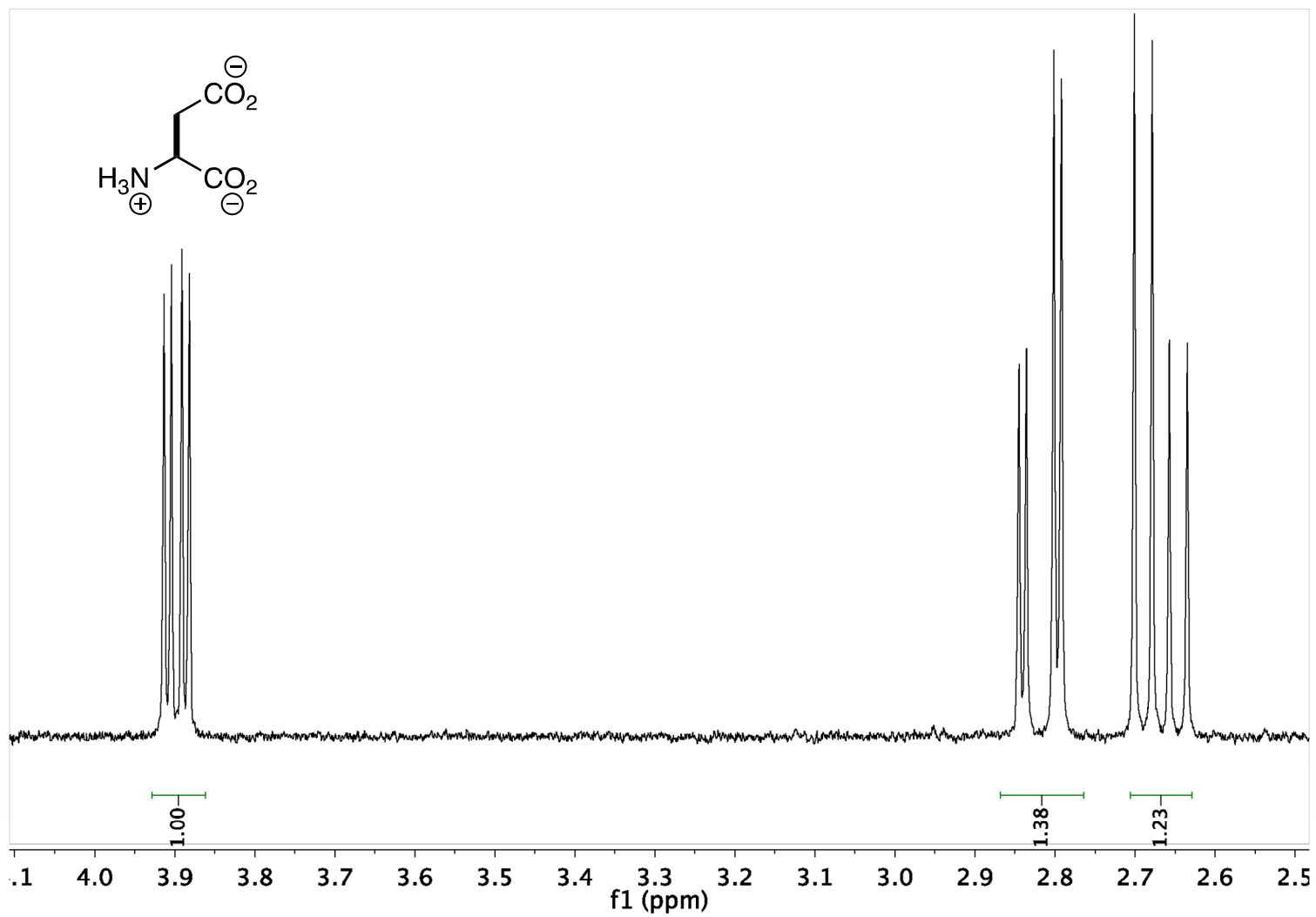
Zwitterionic 7 @ pD 7.2, 400 MHz ^1H NMR, D_2O , 25 $^\circ\text{C}$



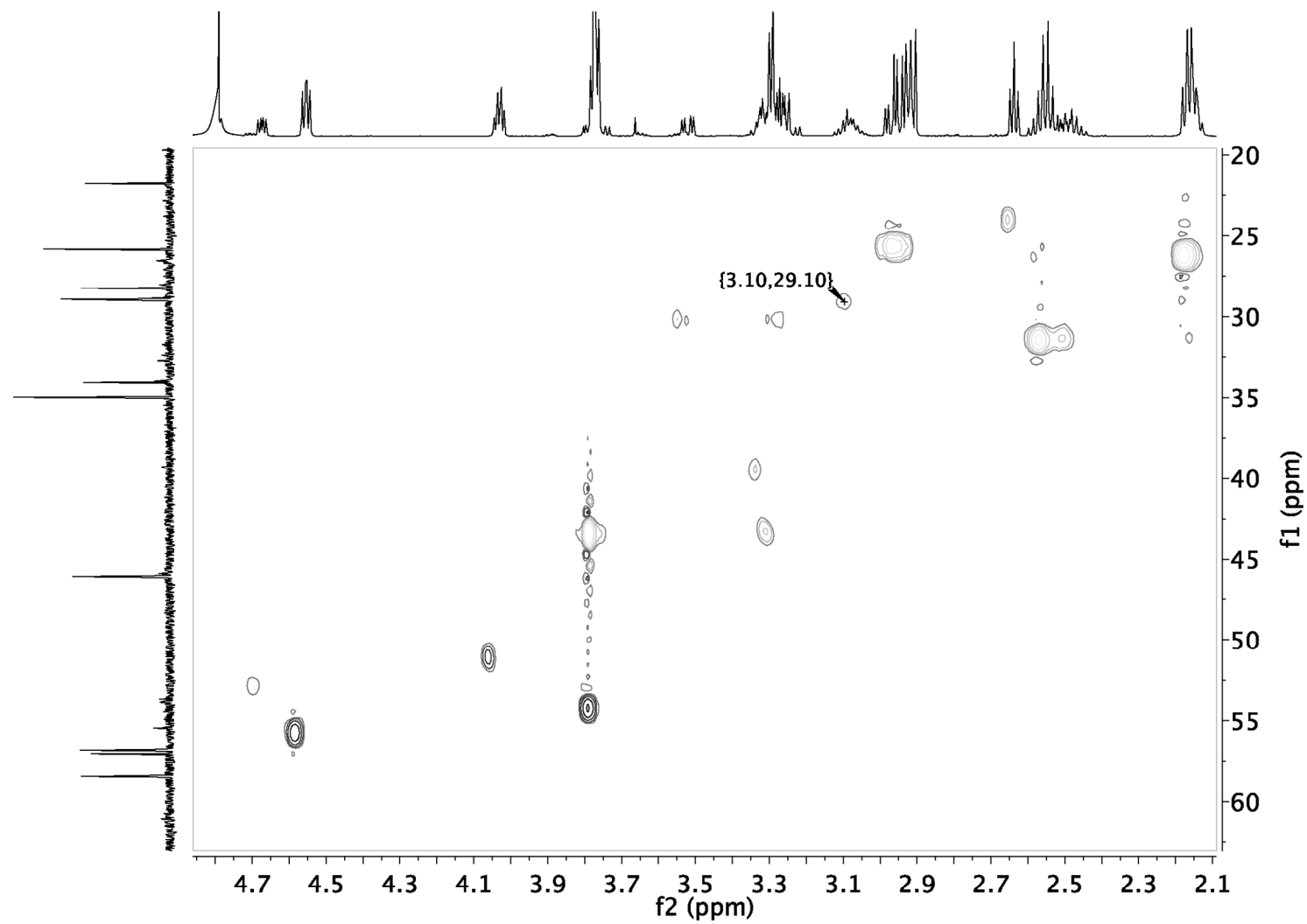
Glutathione @ pD 7.1, 400 MHz ^1H NMR, D_2O , 25 $^\circ\text{C}$



L-aspartate @ pD 7.1, 400 MHz ^1H NMR, D_2O , 25 $^\circ\text{C}$



Stability study of ThioD, 600 MHz HSQC, D₂O, 25 °C



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