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Supplementary Information for:

Gatorbulin-1, a Distinct Cyclodepsipeptide Chemotype, Targets a Seventh Tubulin Pharmacological Site

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METHODS – CHEMISTRY

General Experimental Procedures

Natural products analysis. Optical rotation was measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a SpectraMax M5 (Molecular Devices). IR spectrum was obtained on a Bruker Vector 22 spectrometer. ^{1}H NMR and 2D NMR data for GB1 (**1a**) and GB2 (**1b**) were initially recorded on a Bruker Avance II 600 MHz spectrometer, operating at 600 MHz (^{1}H) and 150 (^{13}C), using DMF-d_7 as solvent (referenced to δ_H 8.02, δ_C 162.9 for residual solvent signals). The instrument was equipped with a 1-mm triple resonance high-temperature superconducting cryogenic probe for **1b** analysis[1]. HMQC and HSQC experiments for GB1 (**1a**) and GB2 (**1b**) were optimized for $^{1}J_{CH}$ = 145 Hz, and ^{1}H–^{13}C HMBC experiments for both were optimized for $^{n}J_{CH}$ = 7 Hz. ^{1}H–^{15}N HMBC NMR data for **1a** was obtained on a Varian Unity Inova 500 spectrometer. HRMS data were obtained using an Agilent LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector. LRMS data were obtained using a 3200 Q TRAP LC/MS/MS (hybrid triple quadrupole linear ion trap mass spectrometer, Applied Biosystems) with an electrospray ionization (ESI) interface operated in positive mode. UV spectra were measured on a SpectraMax M5 (Molecular Devices).

Synthesis. All commercial reagents were used without further purification unless otherwise noted. Solvents were purified by PS-MD-5 solvent purification system (Innovative Technology Inc., now Inert Corp.). All reactions were performed in heat-gun dried flasks (400 °C under reduced pressure) under an inert atmosphere of anhydrous Ar unless otherwise noted. Thin layer chromatography was performed on EMD silica gel 60 Å F_{254} glass plates sand preparative thin layer chromatography was performed on Whatman silica gel 60 Å F_{254} glass plates (layer thick 1000 µm). Reversed-phase thin layer chromatography was performed on MilliporeSigma 60 RP-18 F254 s glass plate. Flash column chromatography was performed with Fisher 170-400 mesh silica gel. NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer or a Bruker Avance Neo-600 spectrometer with a broadband Prodigy cryogenic probe. Chemical shifts for ^{1}H and ^{13}C NMR spectra are reported in parts per million relative to the signal residual signal ($CDCl_3$: 7.26 ppm/77.16 ppm; D_2O 4.79 ppm; DMF-d_7 8.02 ppm/163.15 ppm). Optical rotation was measured on a Perkin-Elmer 341 polarimeter (Na D line) using a microcell of 1 dm path length. HRMS was conducted using a Thermo Fisher Q Exactive Focus mass spectrometer equipped with UltiMate™ 3000 RSLCnano System and electrospray probe on Universal Ion Max API source.

The abbreviations s, d, dd, ddd, dddd, t, q, p, br, and m stand for the NMR multiplicity singlet, doublet, doublet of doublets, doublet of doublet of doublets, doublet of doublet of doublet of doublets, triplet, quartet, pentet, broad and multiplet, respectively.

Isolation and Structure Determination

Extraction and Isolation. Samples of *Lyngbya* cf. *confervoides* were collected off the coast of Broward County (26º01.1414ʹN, 80º05.9973ʹW, 26º15.134ʹN, 80º03.908ʹW) at a depth of 7-15 meters in July 2004 and August of 2005. A voucher specimen is retained at the Smithsonian Marine Station. The freeze-dried organism was extracted with EtOAc-MeOH (1:1) to afford the lipophilic extract, VP56L (3.4 g). VP56L was applied to Diaion HP-20 polymeric resin and subsequently fractionated with water and increasing concentration of acetone. The fraction eluting with 50% aqueous acetone (80 mg) was applied to a C_{18} Alltech SPE cartridge and elution initiated with H_2O followed by aqueous MeOH was then purified by semipreparative reversed-phase HPLC (YMC-Pack ODS-AQ, 250 ×10 mm, 2.0 mL/min; UV detection at 220 and 240 nm) using a MeOH-H_2O linear gradient (20-100% over 70 min and then 100% MeOH for 10 min) to furnish compound **1a**, t_R 31.0 min (1.0 mg).

The same organism was recollected in 2006 during a cyanobacterial bloom, which was freeze-dried and extracted with EtOAc-MeOH (1:1) to afford a crude extract. The extract was suspended in water and defatted with hexanes and further partitioned between *n*-BuOH and H_2O. The combined n-BuOH extract (6.3 g) was applied on a Diaion HP-20 resin and subsequently fractionated with water and increasing concentrations of MeOH, and then with MeCN. The combined fractions eluting between 25-50% aqueous MeOH (350 mg) were subjected to reversed-phase preparative HPLC (Phenomenex Luna-C18 10 µ, 100 × 21.2 mm, 10 mL/min, UV detection at 220 and 240 nm) using a MeOH-H_2O linear gradient (10-55 in 20 min and 55-100% MeOH for 10 min) to afford compound **1a** (t_R 16.2 min, 2.0 mg). A fraction with minor peaks eluting between t_R 13.4-15.8 min was also collected and subjected to repeated semipreparative reversed-phase HPLC (YMC-Pack ODS-AQ, 250 ×10 mm, 2.0 mL/min; UV detection at 220 and 240 nm) using a MeOH-H_2O linear gradient (25-75% in 20 min, 75-100% in 10 min) to give compound **1b** (t_R 18 min, ~0.2 mg).

Gatorbulin-1 (1a). Colorless, amorphous solid; $[\alpha]^{20}_D$ –84.0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 209.8 (4.07) nm; IR (film) ν_{max} 3500, 1742, 1672, 1522, 1447, 1267, 1078 cm^{-1}; ^1H NMR, ^{13}C NMR, COSY, and HMBC data, see Table 1; HRESI/APCIMS *m*/*z* $[M + H]^+$ 484.2043 (calcd for $C_{20}H_{29}N_5O_9$, 484.2044).

Gatorbulin-2 (1b). Colorless, amorphous solid; $[\alpha]^{20}_D$ –62.0 (*c* 0.008, MeOH); UV (MeOH) λ_{max} (log ε) 210.0 (4.07) nm; ^1H NMR, ^{13}C NMR, COSY, HMBC, and NOESY data, see Table S1; HRESI/APCIMS *m*/*z* $[M + H]^+$ 468.2080 (calcd for $C_{20}H_{19}N_5O_8$, 468.2094).

Determination of absolute configuration of gatorbulin-1 (1a) by chiral HPLC and advanced Marfey's analysis

Chiral HPLC analysis. A sample of compound **1a** (0.5 mg) was dissolved in 6 N HCl (0.5 mL) and heated at 110 °C for 24 h. The hydrolyzate was concentrated to dryness, re-suspended in H_2O (100 µL), filtered and subjected to chiral HPLC analysis (Phenomenex Chirex 3126 *N*,S-dioctyl-(D)-penicillamine, 250 mm × 4.60 mm, 5 µm; solvent, 2 mM $CuSO_4$ or 2 mM $CuSO_4$–MeCN (95:5); flow rate, 1.0 mL/min; UV detection 254 nm). The absolute configurations of the amino

acid units in **1a** were established as L-Lac (38.5), (2*S*,4*S*)-4-Me-Pro (66.0) (solvent 2 mM $CuSO_4$); L-Lac (18.4), (*2S,4S*)-4-Me-Pro (19.8) (solvent 95:5) by comparison of the retention times t_R (min) with those of standard amino acids. The retention times t_R (min) for the other standard amino acid isomers were D-Lac (51.0) (solvent 2 mM $CuSO_4$); D-Lac (22.7), (2*S*,4*R*)-4-Me-Pro (17.5), (2*R*,4*S*)-4-Me-Pro (38.0), (2*R*,4*R*)-4-Me-Pro (40.0) (solvent 95:5).

Advanced Marfey's analysis. A portion of the hydrolysis product from **1a** was treated with 1 M $NaHCO_3$ (10 µL) and a 1% solution of either L- or DL-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide) in acetone and heated at 80 °C for 3 min. The solutions were cooled, neutralized with 2 N HCl (5 µL), dried and dissolved in H_2O–MeCN (1:1) and subjected to reversed-phase HPLC (Alltech Altima phenyl 5u, 250 × 4.6 mm; flow rate, 1 mL/min; PDA detection from 200–500 nm) using a linear gradient of MeOH in 0.1% aqueous HCOOH (20–40% for 20 min, 40–100% for 40 min). The retention times (t_R, min) of the L-FDLA derivatized amino acids in the hydrolyzate of **1a** matched with those of L-*erythro*-*N*-Me-β-OH-Asp ((2*S*,3*R*)-3-OH-*N*-Me-Asp) (19.58), and (2*S*,4*S*)-4-Me-Pro (24.0) and not that of L/D-*threo*-*N*-Me-β-OH-Asp (17.50, 18.28), D-*erythro*-NMe-β-OH-Asp (19.40).

Reduction of 1a to 1b. 1 mg of compound **1a** was dissolved in 2 mL of THF under argon. Then 1 mL of 4.5 M aqueous ammonium acetate followed by 0.5 mL of a 10% $TiCl_3$ solution in 20-30 wt.% HCl was added to the solution. The mixture was stirred at room temperature for 3 h. The product was extracted with THF (3 × 3 mL) and the organic layer was subsequently washed with saturated $NaHCO_3$ (10 mL) and brine (10 mL) solutions. The reduction product (**1b**) was purified by reversed-phase HPLC (Phenomenex Luna, ODS 250 × 10 mm, 5 micron, 2.0 mL/min; PDA detection) using a MeOH–H_2O linear gradient (40–100% MeOH for 30 min). The peak eluted at 11.7 min was identified as **1b** by low resolution ESI-MS *m/z* [M+H]+ 468.4. Equal amounts of starting material (**1a**) was also (12.1 min) detected by HPLC.

Chiral HPLC analysis of reduction product 1b. The reduction product thus obtained was hydrolyzed with 6 N HCl for 16 h at 116 °C. A portion of hydrolyzate was analyzed by ESI–LC–MS–MS in the positive mode, using multiple reaction monitoring (MRM). HPLC conditions: Astec Supelco Chirobiotic TAG column, 250 mm × 4.60 mm, 5 µm; solvent, 10 mM Ammonium acetate–MeOH, (60:40, pH ~ 5.3); flow rate 0.5 mL/min. The optimized MS instrument conditions were as follows: source temperature, 750 °C; curtain gas, 50 psi; nebulizer gas (GS1), 65 psi; turbo gas (GS2), 65 psi; collision energy (CE), 17.4 V. The following precursor and product ion transitions were used for multiple reaction monitoring: L and D-alanine, 90→44, and compared with Ala standards. L-Ala (9.3 min) was detected in the reduction product hydrolyzate but not D-Ala (18.1 min), verifying the configuration of the N-OH-Ala unit as L in **1a**.

Synthesis of *N*-Me-β-hydroxyaspartic acids (Scheme S1)

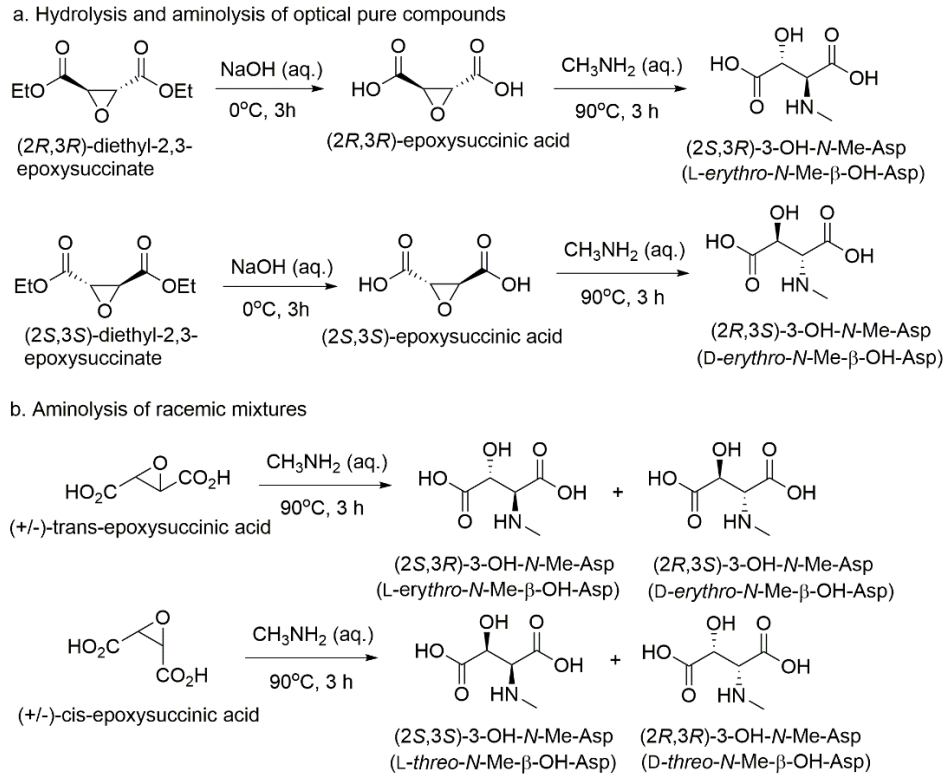
Enantiometrically pure L- and D-isomers of *N*-Me-β-hydroxyaspartic acids were obtained by saponification of corresponding diethyl-2,3-epoxysuccinate (**Scheme S1a**) as described below. A solution of aqueous NaOH (40 mg, 2 eq.) was added to (2*R*,3*R*)-diethyl-2,3-epoxysuccinate (94 mg, 1 eq.) in an ice bath. The resulting solution was stirred for 2 h at 0°C, then for 30 min at room temperature, after which the solution was neutralized and dried to yield (2*R*,3*R*)-2,3-epoxysuccinic acid. ^{1}H NMR (400 MHz, D_2O): δ 3.19 (s, 2H); ^{13}C NMR (100 MHz, D_2O): δ 175.3, 53.9.

100 mg (0.5 mmol) of (2*R*,3*R*)-2,3-epoxysuccinic acid was heated under reflux with concentrated methylamine (41% aq., 2 mL) for 3 h to afford L-*erythro*-*N*-Me-β-OH-Asp ((2*S*,3*R*)-3-OH-*N*-Me-Asp) (0.47 mmol, 94% overall yield). $[\alpha]^{20}_{D}$ +26.0 (*c* 0.24, H_2O); ^{1}H NMR (400 MHz, D_2O): δ 4.36 (d, *J* = 2.4, 1H), 3.84 (d, *J* = 2.4 Hz, 1H), 2.78 (s, 3H) ppm; ^{13}C NMR (100 MHz, D_2O): δ 170.2, 170.4, 69.9, 66.3, 32.3 ppm.

Similarly (2*S*,3*S*)-diethyl-2,3-epoxysuccinate (55 mg, 0.3 mmol) was treated as described above to yield D-*erythro*-*N*-Me-β-OH-Asp ((2*R*,3*S*)-3-OH-N-Me-Asp) (0.39 mmol, 100% overall yield). $[\alpha]^{20}_{D}$ – 15.0 (*c* 0.20, H_2O); ^{1}H NMR (400 MHz, D_2O/1,4 Dioxane): δ 4.61 (d, 1H, *J* = 2.8), 4.06 (d, 1H, *J* = 2.8 Hz), 2.82 (s, 3H) ppm. ^{13}C NMR (100 MHz, D_2O): δ 175.3, 170.1, 69.2, 65.7, 32.5 ppm.

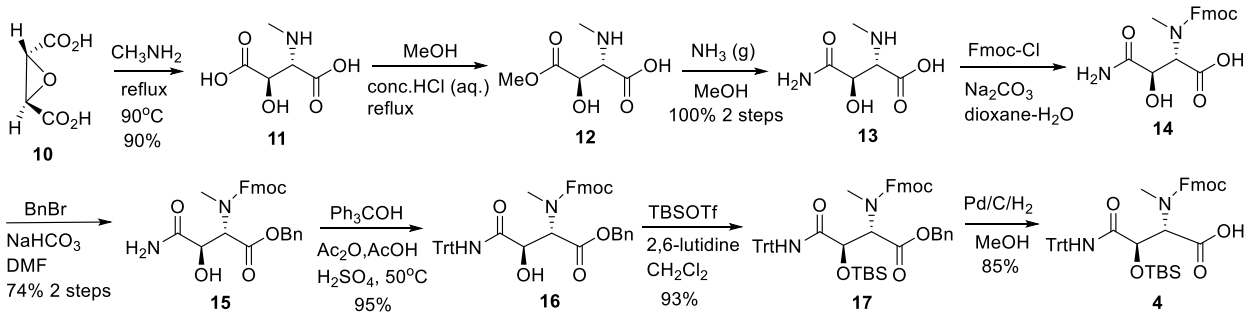
N-Me-DL-(*erythro*/*threo*)-β-hydroxyaspartic acid has been prepared by conversion of corresponding (+/-)-(trans/cis)-epoxysuccinic acid with concentrated solutions of aqueous methylamine to yield L/D-*threo*-*N*-Me-β-OH-Asp and L/D-*erythro*-*N*-Me-β-OH-Asp, respectively (**Scheme S1b**).

Similarly, L/D-*threo*-*N*-Me-β-OH-Asp was obtained from (+/-)-cis-epoxysuccinic acid as colorless amorphous powder (88 % yield). ^{1}H NMR (400 MHz, D_2O/1,4 Dioxane): δ 4.51 (d, 1H, *J* = 2.2), 3.93 (d, 1H, *J* = 2.2 Hz), 2.70 (s, 3H) ppm. L/D-*erythro*-*N*-Me-β-OH-Asp was obtained as colorless amorphous powder (92 % yield). ^{1}H NMR (400 MHz, D_2O/1,4 Dioxane): δ 4.62 (d, *J* = 3.0, 1H), 4.07 (d, *J* = 3.0 Hz, 1H), 2.80 (s, 3H) ppm. ^{13}C NMR (100 MHz, D_2O): δ 174.7, 169.8, 68.8, 65.3, 32.4 ppm.



Scheme S1. Synthesis of *N*-Me-β-hydroxyaspartic acids.

Total synthesis of gatorbulin-1 (1a)



Scheme S2. Synthesis of building block **4**.

(2*S*,3*R*)-3-Hydroxy-*N*-methylaspartic acid[2] (**11**). Methylamine water solution (41%) (100 mL) was added to solid of (2*R*,3*R*)-epoxysuccinic acid (**10**) (6.0 g, 45.43 mmol) at 0 °C. The mixture was stirred for 4.5 h under refluxing (75-80 °C), then cooled down to room temperature and concentrated under reduced pressure. Water (3 × 15 mL) was added to the concentrated residue and evaporated again for three times to remove unreacted methylamine. The crude product was purified by column of Dowex (H^+) resin, eluted by deionized water and then 4 M aqueous ammonia to provide product **11** (6.7 g, 90%). ^{1}H NMR (400 MHz, D_2O): δ 4.64 (d, J = 2.8 Hz, 1H), 4.10 (d, J = 3.2 Hz, 1H), 2.78 (s, 3H) ppm. HRMS (ESI) *m/z* calcd for $C_5H_9NO_5$ $(M+H)^+$ 164.0559, found 164.0550.

(2*S*,3*R*)-3-Hydroxy-*N*-methylaspartic acid β-methyl ester (12). Concentrated chloric acid (1.7 mL, 12 M) was added to the solution of compound **11** (1.649 g) in MeOH (50 mL) and the reaction mixture was refluxed for 3 h. The resulting mixture was concentrated under reduced pressure. MeOH (3 × 20 mL) was added to the concentrated residue and evaporated again for three times to remove excess chloric acid. The crude product was dried by oil pump to provide product **12** (1.73 g, 100%) as white foam. ^{1}H NMR (400 MHz, D_2O): δ 4.87 (d, J = 2.4 Hz, 1H), 4.44 (d, J = 2.8 Hz, 1H), 3.80 (s, 3H), 2.83 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.9, 168.0, 67.4, 63.0, 53.2, 31.6 ppm. HRMS (ESI) *m/z* calcd for $C_6H_{11}NO_5$ $(M+H)^+$ 178.0715, found 178.0712.

(2*S*,3*R*)-3-hydroxy-N_α-methyl-asparagine (13). Compound **12** (1.7 g, 10.49 mmol) was dissolved in anhydrous MeOH (40 mL). The resulting solution was saturated (bubbled) with ammonia (gas) for 5–7 min each day (total 3 days). After stirring for three days, the reaction solution was evaporated to dryness in vacuo. The resulting solid was washed with cold methanol, then diethyl ether and to give the compound **13** as a white solid (1.68 g, 100%). $[\alpha]^{20}_D$: +27 (*c* 0.09, H_2O). ^{1}H NMR (600 MHz, D_2O, mixtures of rotamers): δ 4.61 (br m, 1H), 4.00 (br m, 1H), 2.82 (s, 1H) ppm. ^{13}C NMR (150 MHz, D_2O, mixtures of rotamers): δ 175.4, 169.2, 68.7, 65.1, 32.0 ppm. HRMS (ESI) *m/z* calcd for $C_5H_{10}N_2O_4$ $(M+H)^+$ 163.0719, found 163.0710.

(2*S*,3*R*)-3-Hydroxy-N_α-methyl-N_α-Fmoc-asparagine (14). 9-Fluorenylmethyl chloroformate (Fmoc-Cl) (2.945g, 11.386 mmol) and Na_2CO_3 (2.413 g, 22.767 mmol) were dissolved in the solution of the mixture of 1,4-dioxane and water (45 mL-45 mL). Compound **13** (1.23 g, 7.589 mmol) was added to the above solution at 0 °C ant stirred 10 min at this temperature. After the reaction mixture was moved to room temperature and stirred at this temperature overnight, it was diluted with water (80 mL) and concentrated under reduced pressure to move most of 1,4-dioxane. The concentrated mixture was extracted with diethyl ether (20 mL × 4). The water layer was

acidified with 2M HCl (aq.) to pH 2 and extracted with EtOAc (150 mL × 3). The combined organic phase was dried with anhydrous $MgSO_4$ and evaporated in vacuo to give product **14** (2.393 g, 82%). $[\alpha]^{20}_D$: +1.5 (*c* 0.32, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixtures of rotamers): δ 7.68 (d, *J* = 7.7 Hz, 2H), 7.50 (t, *J* = 8.0 Hz, 2H), 7.36-7.30 (br m, 3H), 7.26-7.21 (br m, 3H), 4.86 (s, 1H), 4.49 (s, 1H), 4.36-4.25 (br m, 2H), 4.18-4.12 (br m, 1H), 3.01 (s, 3H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 176.2, 171.1, 158.4, 143.8, 143.5, 141.3, 141.3, 72.9, 68.5, 64.6, 47.0, 36.1 ppm. HRMS (ESI) *m/z* calcd for $C_{20}H_{20}N_2O_6$ $(M+H)^+$ 385.1400, found 385.1385. Usually, compound **14** was used in the next step without further purification and characterization.

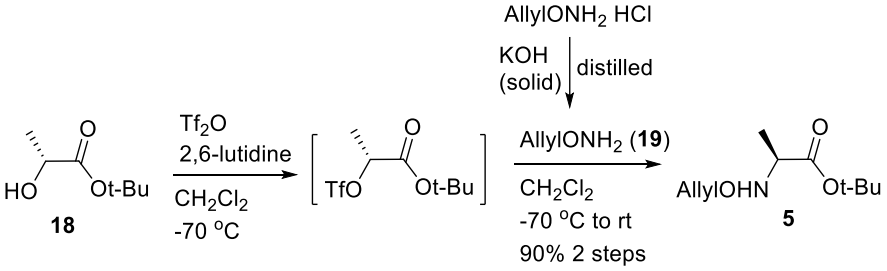
(2*S*,3*R*)-3-Hydroxy-N_α-methyl-N_α-Fmoc-asparagine benzyl ester (15). BnBr (2.94 mL, 24.735 mmol) was added to the solution of compound **14** (2.351 g, 6.121 mmol) and $NaHCO_3$ (1.55 g, 18.45 mmol) in anhydrous DMF (45 mL) at 0 °C and was stirred at this temperature for 1 h, room temperature for 20 h. The resulting mixture was quenched with water (100 mL) and was extracted with EtOAc (150 mL × 4). The combined organic phase was washed with water (100 mL × 4), dried with anhydrous $MgSO_4$, evaporated in vacuo and purified by flash chromatography column on silica gel (eluted by 40-80% ethyl acetate in hexane) to give product **15** (2.14 g, 74%). $[\alpha]^{20}_D$: +2.8 (*c* 0.12, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixtures of rotamers): δ 7.78 (d, *J* = 7.6 Hz, 2H), 7.58 (d, *J* = 7.5 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.33-7.27 (br m, 7H), 6.83 (s, 1H), 5.96-5.86 (br m, 2H), 5.23-5.15 (m, 2H), 4.76 (br s, 1H), 4.50-4.45 (m, 2H), 4.39-4.35 (m, 1H), 4.24 (t, *J* = 7.0 Hz, 1H), 3.08 (s, 3H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.7, 167.7, 158.6, 143.9, 143.5, 141.4, 141.3, 135.2, 128.5, 128.4, 128.2, 127.9, 127.9, 127.2, 125.1, 125.0, 120.1, 73.7, 68.5, 67.5, 66.4, 47.1, 37.5 ppm. HRMS (ESI) *m/z* calcd for $C_{27}H_{26}N_2O_6$ $(M+H)^+$ 475.1869, found 475.1854.

(2*S*,3*R*)-3-Hydroxy-N_α-methyl-N_α-Fmoc-N_γ-trityl-asparagine benzyl ester (16). Compound **15** (2.443 g, 5.153 mmol) and trityl alcohol (13.414 g, 51.528 mmol) was dissolved in AcOH (17.5 mL). The solution was heated to 50 °C and was treated successively with concentrated sulfuric acid (168 μL, 3.092 mmol) and acetic anhydride (1.22 mL, 12.882 mmol). The reaction mixture was cooled to room temperature after stirred at 50 °C for 2.5 h, then it was diluted with EtOAc (100 mL) and quenched with saturated aqueous $NaHCO_3$ solution (70 mL). Excessive $NaHCO_3$ powder was added slowly to the quenched solution to that there was no bubble (CO_2) produced any more. EtOAc layer was separated and the aqueous layer was further extracted with EtOAc, and the combined organic layers were dried ($MgSO_4$), filtered, concentrated in vacuo and purified by flash chromatography column (SiO_2, 33% EtOAc in hexane) to provide **16** as a white solid (3.5 g, 95%). $[\alpha]^{20}_D$: +28.4 (*c* 0.0.48, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (s, 1H), 7.79 (d, *J* = 7.6 Hz, 2H), 7.59 (dd, *J* = 7.6, 3.2 Hz, 2H), 7.43 (dd, *J* = 7.6, 7.6 Hz, 2H), 7.35-7.16 (m, 22H), 6.03 (s, 1H), 5.42 (d, *J* = 12.4 Hz, 1H), 5.11 (d, *J* = 12.4 Hz, 1H), 4.72 (br s, 1H), 4.50-4.46 (m, 2H), 4.41-4.36 (m, 1H), 4.26 (dd, *J* = 7.2, 7.2 Hz, 1H), 3.03 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.1, 167.7, 158.8, 144.8, 144.0, 143.5, 141.5, 141.4, 135.5, 128.8, 128.6, 128.5, 128.4, 128.0, 128.0, 127.2, 127.1, 125.1, 125.1, 120.1, 75.1, 70.2, 68.5, 67.4, 67.2, 47.1, 38.1 ppm. HRMS (ESI) *m/z* calcd for $C_{46}H_{40}N_2O_6$ $(M+Na)^+$ 739.2748, found 739.2761.

(2*S*,3*R*)-3-OTBS-N_α-methyl-N_α-Fmoc-N_γ-trityl-asparagine benzyl ester (17). To the solution of **16** (1.48 g, 2.066 mmol) in anhydrous CH_2Cl_2 (20 mL) were added 2,6-lutidine (2.4 mL, 20.661 mmol) and *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBSOTf) (2.37 mL, 10.33 mmol) at 0 °C under argon. After stirring at the same temperature for 20 min, the reaction mixture was moved to room temperature and stirred for another 1 h, then it was quenched with MeOH (10 mL)

and saturated aq. NH_4Cl (30 mL), and extracted with EtOAc (50 mL × 3). The combined organic layer was washed with 0.5 M HCl (20 mL × 3), saturate aq. $NaHCO_3$ (20 mL × 2) and brine (20 mL), dried with anhydrous $MgSO_4$ and evaporated in vacuo. The resulting crude mixture was purified by flash chromatography column (SiO_2, eluted by 10-12.5% EtOAc in hexane) to give product **17** (1.71 g, 93%) as a white solid. $[\alpha]^{20}_D$ +11.6 (*c* 0.22, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major and minor (7/3)): δ 7.82 (s, 1H), 7.77 (d, *J* = 7.6 Hz, 1.7H), 7.73-7.70 (m, 1H), 7.57-7.53 (m, 2H), 7.49 (d, *J* = 7.6 Hz, 0.3H), 7.40 (dd, *J* = 7.6, 7.6 Hz, 2H), 7.36-7.11 (m, 21H), 5.30 (d, *J* = 6.8 Hz, 0.7H), 5.19-5.07 (m, 2.3H) 4.73 (d, *J* = 6.8 Hz, 0.7H), 4.63 (d, *J* = 6.0 Hz, 0.3H), 4.58-4.52 (m, 0.3H), 4.37 (dd, *J* = 10.0, 7.2 Hz, 0.7H), 4.29 (dd, *J* = 7.2, 7.2 Hz, 0.7H), 4.23 (dd, *J* = 7.2, 7.2 Hz, 0.7H), 4.11-4.05 (m, 0.6 H), 2.83 (s, 3H), 0.81 (s, 9H), 0.15 (s, 2.1H), 0.06 (s, 2.1H), -0.01 (s, 0.9H), -0.02 (s, 0.9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers, major and minor (7/3)): δ 169.0, 168.5, 168.1, 157.0, 155.8, 144.4, 144.3, 144.0, 143.9, 141.4, 135.7, 135.5, 128.8, 128.7, 128.6, 128.5, 128.4, 128.3, 128.1, 128.0, 127.8, 127.7, 127.3, 127.2, 127.2, 127.1, 125.3, 125.0, 120.1, 120.0, 73.2, 72.3, 70.6, 70.5, 68.2, 68.0, 67.3, 67.1, 61.9, 61.2, 34.8, 34.6, 32.2, 31.9, 31.7, 25.7, 25.7, 25.4, 22.8, 17.9, 17.9, 14.3, -4.6, -4.7, -5.2, -5.3 ppm. HRMS (ESI) *m/z* calcd for $C_{52}H_{54}N_2O_6Si$ $(M+H)^+$ 831.3829, found 831.3802.

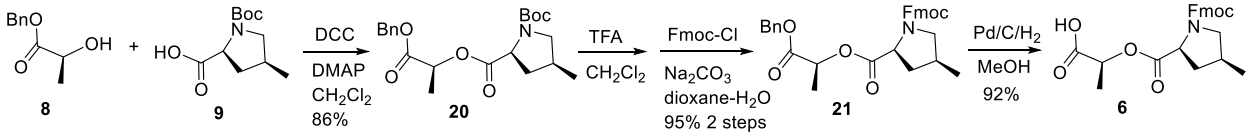
(2*S*,3*R*)-3-OTBS-N_α-methyl-N_α-Fmoc-N_γ-trityl-asparagine (4). MeOH (50 mL) was added cautiously to the mixture of compound **17** (1.6 g, 1.927 mmol) and Pd/C (10% wt) (160 mg). The suspension was degassed with argon (balloon) and hydrogen (balloon) successively, then it was stirred under hydrogen gas (balloon) at room temperature for 30 min. The catalyst was removed by filtration through Celite and the filtrate cake was washed by MeOH. The combined filtrate was concentrated in vacuo and purified by flash chromatography column (SiO_2, eluted by 2-7% MeOH in CH_2Cl_2) to provide product **4** as a white solid (1.21 g, 85%). $[\alpha]^{20}_D$ +32.4 (c 0.18, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major and minor (3/1)): δ 9.03 (br s, 1H), 7.93 (br s, 0.75H), 7.78 (d, *J* = 7.2 Hz, 1.75H), 7.72 (dd, *J* = 7.6, 7.6 Hz, 0.5H), 7.59 (dd, *J* = 6.4, 6.4 Hz, 1.75H), 7.53 (d, *J* = 7.6 Hz, 0.25H), 7.43-7.38 (m, 1.75H), 7.36-7.20 (m, 17.25H), 5.17 (d, *J* = 6.4 Hz, 0.75H), 4.99 (d, *J* = 6.0 Hz, 0.25H), 4.84 (d, *J* = 6.4 Hz, 0.75H), 4.71-4.66 (m, 0.5H), 4.42-4.32 (m, 1.5H), 4.26 (dd, *J* = 7.2, 7.2 Hz, 1H), 4.18-4.10 (m, 0.5H), 2.75 (s, 0.75H), 2.74 (s, 2.25H), 0.87 (s, 6.75H), 0.85 (s, 2.25H), 0.20 (s, 2.25H), 0.16 (s, 2.25H), 0.04 (s, 0.75H), 0.00 (s, 0.75H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers, major and minor (3/1)): δ 177.2, 172.5, 172.0, 169.3, 169.0, 157.3, 155.9, 144.1, 144.1, 144.0, 143.9, 141.4, 141.3, 128.8, 128.1, 127.8, 127.3, 127.2, 127.1, 125.3, 125.2, 120.0, 72.7, 72.0, 70.5, 68.3, 67.9, 61.8, 61.5, 47.2, 36.2, 34.8, 34.6, 32.5, 32.1, 29.2, 27.0, 25.8, 25.7, 25.4, 20.8, 18.9, 17.9, 14.2, 11.6, -4.7, -5.2 ppm. HRMS (ESI) *m/z* calcd for $C_{45}H_{48}N_2O_6Si$ $(M+H)^+$ 741.3360, found 741.3341.



Scheme S3. Synthesis of building block **5**.[3,4]

Allyloxamine (19)[3,4]. Alloxyamine.HCl (6.0 g) was mixed with KOH pellets (15.0 g) in a distillation flask with distillation assembly. The mixture was subjected to distillation by heating at 1 atm under the atmosphere of nitrogen. The product was collected at 86-90 °C as colorless liguid (3.5 g, 88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.93-5.83 (m, 1H), 5.37 (br s, 2H), 5.29-5.19 (m, 2H), 1.06 (d, J = 6.5 Hz, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 134.0, 118.5, 76.8 ppm.

(*S*)-*N*-Allyloxyalanine t-butyl ester (5)[3,4]. Triflic anhydride (4.2 mL, 24.981 mmol) was added dropwise to the solution of *tert*-butyl (*R*)-lactate (3.044 g, 20.817 mmol) in anhydrous CH_2Cl_2 (90 mL) at -70 °C. The mixture was stirred 5 min, then 2,6-lutidine (3.044 mL, 26.022 mmol) was added dropwise at the same temperature. After the reaction mixture was stirred at -70 °C for 1.5 h, alloxyamine (**19**) (3.35 mL, 41.635 mmol) was added dropwise at the same temperature. When the resulting mixture was stirred at -70 °C for another 15 min, it was warmed to room temperature and stirred overnight (about 19 h). The reaction was diluted with CH_2Cl_2 (40 mL) and quenched with water (40 mL). The organic layer was washed successively with water (50 mL × 2), 2% citric acid (50 mL × 2), 5% $NaHCO_3$ (50 mL × 2), dried with anhydrous $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by flash chromatography column (SiO_2, eluted by 10% EtOAc in hexanes) to provide product **5** (3.77 g, 90%). $[\alpha]^{20}_D$: -8.3 (*c* 0.12, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.90 (dddd, J = 17.3, 10.4, 5.6, 5.6 Hz, 1H), 5.20 (m, 2H), 5.86-5.76 (m, 1H), 5.70 (br, 1H), 4.17 (d, J = 5.6 Hz, 2H), 3.59 (q, J = 6.8 Hz, 1H), 1.46 (s, 9H), 1.17 (d, J = 7.2 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.7, 134.6, 117.5, 81.4, 75.1, 59.7, 28.1, 15.0 ppm. HRMS (ESI) *m/z* calcd for $C_{10}H_{19}NO_3$ $(M+H)^+$ 202.1443, found 202.1449.

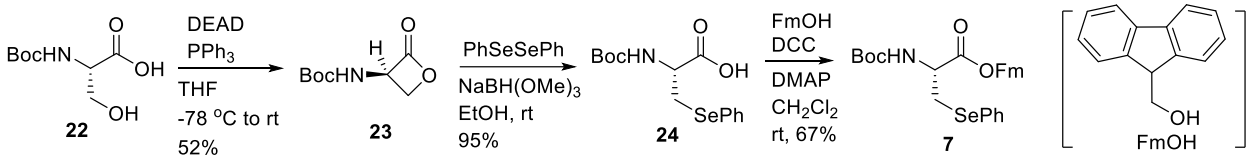


Scheme S4. Synthesis of building block **6**.

***N*-Boc-4-MePro-Lac-OBn** (**20**). To the suspension of 4-MePro-OH (**9)** (3.247 g, 14.160 mmol) in toluene (5.0 mL) was added *N, N*-diisopropylethylamine (DIEA) (3.69 mL, 21.24 mmol), *2,4,6*-trichlorobenzoyl chloride (3.32 mL, 21.24 mmol) at room temperature under argon, and stirred at the same temperature for 40 min. Then the benzyl lactate (**8**) (2.5 mL, 15.575 mmol) and DMAP (2.941 g, 24.07 mmol) were added to the above mixture at 0 °C. The reaction was stirred at 0 °C for 20 min and at room temperature for another 4 h, then it was quenched with water (50 mL) and extracted with diethyl ether (50 mL × 4). The combined organic layer was washed with saturated NH_4Cl (50 mL × 2), saturated $NaHCO_3$ (50 mL × 2), brine (50 mL), dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by silica gel flash chromatography column (eluted by 10% ethyl acetate in hexane) to give ester **20** (4.738 g, 86%). $[\alpha]^{20}_D$: -86 (*c* 0.43, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major and minor (1/1)): δ 7.39-7.26 (m, 5H), 5.25-5.09 (m, 3H), 4.65 (d, J = 10.8 Hz, 1H), 4.31 (t, J = 8.4 Hz, 0.5H), 4.22 (t, J = 8.4 Hz, 0.5H), 4.22 (t, J = 8.4 Hz, 0.5H), 3.73 (dd, J = 10.0, 6.8 Hz, 0.5H), 3.65 (dd, J = 10.4, 7.2 Hz, 0.5H), 3.65 (dd, J = 10.4, 7.2 Hz, 0.5H), 2.95 (dd+dd, J = 10.0, 10.0 Hz, 1H), 2.41-2.32 (m, 1H), 2.26-2.13 (m, 1H), 1.58-1.49 (m, 4H), 1.45 (s, 4.5H), 1.39 (s, 4.5H), 1.00 (d, J = 6.4 Hz, 1.5H), 0.98 (d, J = 6.0 Hz, 1.5H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers, major and minor (1/1)): δ 172.8, 172.4, 170.7, 170.3, 154.3, 153.5, 135.3, 135.2, 128.6, 128.6, 128.5, 128.5, 128.4, 128.3, ,128.3, 80.0, 79.9, 68.8, 68.7, 67.2, 67.0, 59.1, 58.7, 53.9, 53.3, 38.7, 37.7, 33.3, 32.6, 28.5, 28.2, 17.0, 16.9, 16.9, 16.8 ppm. HRMS (ESI) *m/z* calcd for $C_{21}H_{29}NO_6$ $(M+Na)^+$ 414.1893, found 414.1878.

***N*-Fmoc-4-MePro-Lac-OBn** (**21**). Trifluoroacetic acid (TFA) (30 mL) was added to the solution of compound **20** (4.738 g, 12.108 mmol) in CH_2Cl_2 (30 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 20 min, then moved room temperature and stirred for 1.5 h. Toluene (30 mL) was added in and the solvent was evaporated. Another toluene (5 mL × 3) was added to the concentrated residue and evaporated again for three times to move excess TFA. The residue was dried under vacuum and used in the next step without purification. The dried residue was dissolved in 1,4-dioxane (30 mL) and water (30 mL). $NaHCO_3$ (2.543 g, 30.271 mmol), Fmoc-Cl (3.759 g, 14.53 mmol) were added the above solution successively at 0 °C. After the reaction mixture was stirred at 0 °C for 20 min, at room temperature for 4.5 h, it was diluted water (30 mL) and extracted with EtOAc (50 mL × 3). The combined organic layer was washed water (30 mL × 3), dried with anhydrous $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by flash chromatography column (SiO_2, eluted by 10-25% EtOAc in hexane) to provide product **21** (5.874 g, 95%). $[\alpha]^{20}_D$: -81 (*c* 0.21, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$, mixture of rotamers, major and minor (5.5/4.5)): δ 7.77 (dd, *J* = 7.0, 7.0 Hz, 2H), 7.64 (d, *J* = 7.5 Hz, 0.55H), 7.61 (dd, *J* = 6.5, 6.5 Hz, 1H), 7.56 (d, *J* = 7.5 Hz, 0.45H), 7.42-7.30 (m, 9H), 5.27 (q, *J* = 7.0 Hz, 0.55H), 5.23-5.07 (m, 2.45H), 4.51 (dd, *J* = 10.5, 6.0 Hz, 0.45H), 4.45 (dd, *J* = 10.0, 6.5 Hz, 0.55H), 4.41 (t, *J* = 8.5 Hz, 0.55H), 4.35 (t, *J* = 8.0 Hz, 0.45H), 4.33-4.26 (m, 1.55H), 4.16 (dd, *J* = 6.5, 6.5 Hz, 0.45H), 3.85 (dd, *J* = 10.5, 7.0 Hz, 0.45H), 3.80 (dd, *J* = 10.0, 7.0 Hz, 0.55H), 3.13 (dd, *J* = 10.0, 10.0 Hz, 0.55H), 3.05 (dd, *J* = 10.0, 10.0 Hz, 0.45H), 2.46 (dq, *J* = 7.5, 7.5 Hz, 1H), 2.34-2.26 (m, 0.55H), 2.26-2.18 (m, 0.45H), 1.69-1.62 (m, 1H), 1.56 (d, *J* = 7.0 Hz, 1.65H), 1.47 (d, *J* = 7.0 Hz, 1.35H), 1.07 (d, *J* = 6.5 Hz, 1.65H), 1.03 (d, *J* = 6.5 Hz, 1.35H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$, mixture of rotamers, major and minor (5.5/4.5)): δ 172.2, 172.1, 170.6, 170.3, 154.8, 154.3, 144.5, 144.2, 144.0, 143.7, 141.4, 141.4, 141.3, 135.3, 135.2, 128.7, 128.7, 128.6, 128.5, 128.3, 128.3, 127.8, 127.7, 127.7, 127.1, 127.1, 127.0, 125.3, 125.2, 125.2, 125.0, 120.0, 120.0, 120.0, 69.0, 69.0, 67.6, 67.4, 67.2, 67.1, 59.3, 58.8, 54.2, 53.7, 47.4, 47.3, 38.8, 37.6, 33.5, 32.6, 17.1, 17.0, 16.9, 16.9 ppm. HRMS (ESI) *m/z* calcd for $C_{31}H_{31}NO_6$ $(M+Na)^+$ 536.2049, found 536.2029.

***N*-Boc-4-MePro-Lac-OH (6).** MeOH (250 mL) was added cautiously to the mixture of compound **21** (6.6 g, 12.86 mmol) and Pd/C (10% wt) (660 mg). The suspending mixture was degassed with argon (balloon) and hydrogen (balloon) successively, then it was stirred under hydrogen gas (balloon) at room temperature for 30 min. The catalyst was removed by filtration through Celite and the filtrate cake was washed with MeOH. The combined filtrate was concentrated in vacuo and purified by flash chromatography column (SiO_2, eluted by 10-15% MeOH in CH_2Cl_2) to provide product **6** as a white solid (5.0 g, 92%). $[\alpha]^{20}_D$: -82.5 (*c* 0.11, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major and minor (5.5/4.5)): δ 8.73 (br, 1H), 7.77-7.74 (m, 2H), 7.62-7.58 (m, 1.55H), 7.55 (d, *J* = 7.6 Hz, 0.45H), 7.39 (d, *J* = 7.6,7.6 Hz, 2H), 7.33-7.28 (m, 2H), 5.20-5.13 (m, 0.55H), 5.00 (q, *J* = 7.2 Hz, 0.45H), 4.52-4.39 (m, 1.55H), 4.36-4.24 (m, 2H), 4.15 (dd, *J* = 6.8, 6.8 Hz, 0.45H), 3.83 (dd, *J* = 10.8, 7.6 Hz, 0.45H), 3.78-3.72 (m, 1H), 3.12-3.01 (m, 1H), 2.54-2.45 (m, 1H), 2.35-2.19 (m, 1H), 1.78-1.68 (m, 1H), 1.51 (d, *J* = 7.2 Hz, 1.65H), 1.44 (d, *J* = 7.2 Hz, 1.35H), 1.08 (d, *J* = 6.4 Hz, 1.65H), 1.04 (d, *J* = 6.4 Hz, 1.35H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers, major and minor (5.5/4.5)): δ 175.2, 175.0, 172.2, 172.0, 155.1, 154.5, 144.3, 144.1, 143.8, 143.6, 141.3, 141.3, 141.2, 127.8, 127.8, 127.7, 127.1, 127.1, 127.0, 125.2, 125.2, 125.2, 125.0, 120.0, 120.0, 120.0, 119.9, 69.4, 69.1, 67.8, 67.6, 59.4, 58.8, 54.2, 53.7, 53.5, 47.3, 47.2, 38.6, 37.6, 33.3, 33.3, 32.6, 17.3, 17.1, 17.0, 16.9 ppm. HRMS (ESI) *m/z* calcd for $C_{24}H_{25}NO_6$ $(M+Na)^+$ 446.1580, found 446.1563.

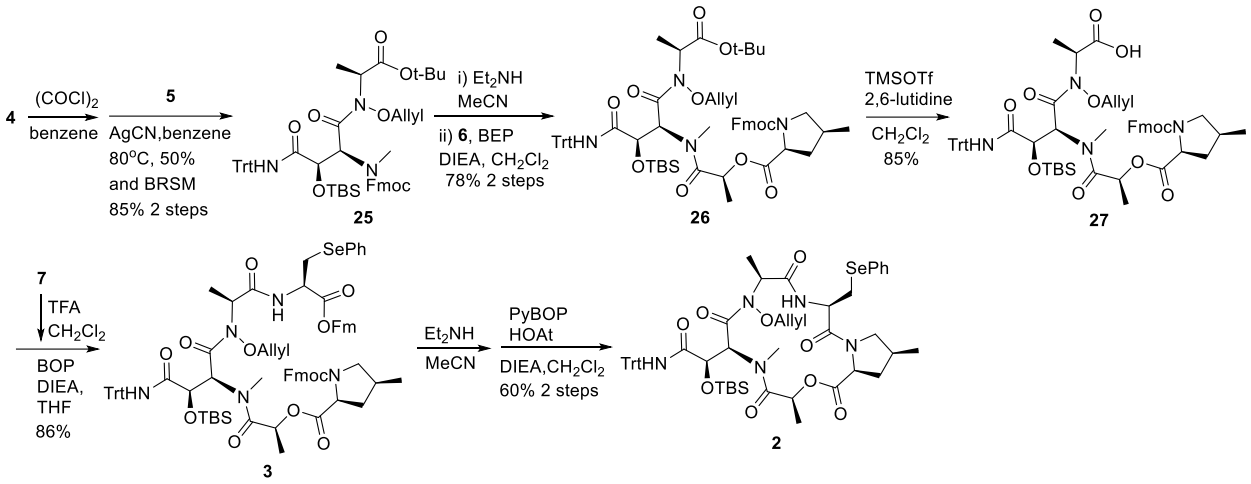


Scheme S5. Synthesis of building block **7**.

***N*-Boc-L-serine *β*-lactone (23)**[5,6]. Diethyl azodicarboxylate (DEAD) (14.0 mL, 30.73 mmol) was added to the solution of triphenylphosphine (Ph_3P) (8.06 g, 30.73 mmol) in THF (250 mL) at -78 °C. The resulting solution was stirred at -78 °C for 15 min, then warmed to room temperature and stirred for another 15 min. Then it was cooled to -78 °C. A solution of *N*-Boc-Ser-OH (6.31 g, 30.73 mmol) in THF (30 mL) was added dropwise to the above solution at -78 °C. The reaction mixture was stirred at -78 °C for 30 minutes, room temperature for another 2 h, then concentrated in vacuo. The residue was triturated in EtOAc/hexane (200 mL, 1:1) and filtered. The filtrate was concentrated in vacuo and purified by flash column chromatography (SiO_2, EtOAc/hexane, 1:3) to provide *β*-lactone **23** (3.01 g, 52%) as white solid, which was confirmed by MS (ESI) and used in next step directly without characterization by NMR. MS (ESI) *m/z* calcd for $C_8H_{13}NO_4$ $(M+Na)^+$ 210.08, found 210.1; $C_8H_{13}NO_4$ $(M-H)^-$ 186.08, found 186.0.

(*S*)-*N*-Boc-L-(*Se*)-phenylselenocysteine (24)[5,6]. Sodium trimethoxyborohydride ($NaBH(OMe)_3$) (2.47 g, 19.316 mmol) was added to the solution of diphenyl diselenide ($(PhSe)_2$) (3.014 g, 9.659 mmol) in absolute ethanol (150 mL) at room temperature under argon. The resulting mixture was stirred for 30 min at room temperature, then *β*-lactone (**23**) (2.583 g, 13.797 mmol) was added. The reaction mixture was stirred for 3 h at room temperature under argon and concentrated in vacuo. Saturated aqueous $NaHCO_3$ (100 mL) was added to the residue and stirred 20 min at room temperature. The above mixture was washed with Et_2O (30 mL × 3). The water layer was acidified with 3 M aqueous HCl to pH 2 and extracted with EtOAc (100 mL × 3). The combined organic layers were washed with brine (50 mL × 3), dried with anhydrous $MgSO_4$ and concentrated to give a yellowish oil, which was added hexanes (20 mL) and kept -20 °C freezer overnight. The precipitate was filtrated to give product **24** (4.52 g, 95%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56-7.54 (m, 2H), 7.27-7.25 (m, 3H), 5.29 (d, J = 6.8 Hz, 1H), 4.65-4.61 (m, 1H), 3.39-3.29 (m, 2H), 1.41 (s, 9H) ppm. LRMS (ESI) *m/z* calcd for $C_{14}H_{19}NO_4{}^{78}Se$ $(M+Na)^+$ 368.05, found 368.0; $C_{14}H_{19}NO_4{}^{78}Se$ $(M-H)^-$ 344.05, found 344.1.

Fm (*S*)-*N*-Boc-L-(*Se*)-phenylselenocysteine ester (7)[7]. DMAP (150 mg, 1.228 mmol), DCC (2.767 g, 13.409 mmol) were added to a solution of **24** (4.205 g, 12.19 mmol) and 9-fluorenylmethanol (FmOH) (2.632 g, 13.415 mmol) in anhydrous CH_2Cl_2 (70 mL) at 0 °C under argon. After stirring at room temperature for 4 h, the reaction mixture was evaporated in vacuo. EtOAc (200 mL) was added to the residue and the suspension was filtered. The filtrate was evaporated, and purified by flash chromatography column on silica gel (eluted by 6-12% AcOEt in hexane) to provide **7** (4.791 g, 67%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78-7.75 (m, 2H), 7.55-7.51 (m, 4H), 7.44-7.39 (m, 2H), 7.35-7.29 (m, 2H), 7.25-7.19 (m, 3H), 5.38 (d, J = 8.4 Hz, 1H), 4.76-4.71 (m, 1H), 4.25 (dd, J = 10.4, 7.2 Hz, 1H), 4.14-4.04 (m, 2H), 3.29 (br d, J = 5.2 Hz, 2H), 1.45 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.7, 155.1, 143.5, 143.4, 141.4, 141.3, 133.8, 129.2, 128.9, 128.0, 127.6, 127.3, 127.2, 125.2, 125.1, 120.1, 120.1, 80.2, 67.3, 53.4, 46.6, 30.6, 28.4 ppm. HRMS (ESI) *m/z* calcd for $C_{28}H_{29}NO_4Se$ $(M+H)^+$ 524.1340, found 524.1319.



Scheme S6. Fusing of all fragments and macrocyclization.

((2*S*,3*R*)-3-OTBS-N_α-methyl-N_α-Fmoc-N_γ-trityl)-Asn-(*N*-OAllyl)-Ala-O*t*-Bu (25). $(COCl)_2$ (2.0 mL, 22.89 mol) was added to the solution of acid **4** (1.107 g, 1.496 mmol) in benzene (22 ml) under nitrogen. After the reaction mixture was stirred at room temperature for 1 h, it was concentrated *in vacuo*. The residue was added dry benzene (5 mL× 3) and evaporated again for another three times to remove traces of $(COCl)_2$, and dried under high vacuum. The residue was dissolved in dry benzene (24 mL) and the solution of compound **5** (352 mg, 1.75 mmol) in dry benzene (2 mL) was added under N_2. The reaction flask was equipped with a reflux condenser and covered with Al foil. AgCN (260 mg, 1.942 mmol) was added in a single portion. The reaction mixture was stirred at room temperature for 10 min and then at 80 °C to for 20 min (pre-heated oil bath). The reaction mixture was cooled to room temperature and diluted with CH_2Cl_2 (40 mL) and filtered through Celite. The filtrate was then concentrated *in vacuo* and purified by flash chromatography column (SiO_2, eluted by 7-15% EtOAc in hexanes) to afford **25** (690 mg, 50%, 85% BRSM) as a white solid. $[\alpha]^{20}_D$: +20.3 (*c* 0.1, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major and minor (3/1)): δ 7.88 (br s, 0.25H), 7.78 (d, *J* = 7.6 Hz, 2H), 7.75 (br s, 0.75H), 7.72 (d, *J* = 7.6 Hz, 0.75H), 7.64-7.60 (m, 2H), 7.49 (d, *J* = 6.8 Hz, 0.25H), 7.41 (dd, *J* = 7.6, 7.6 Hz, 2H), 7.36-7.19 (m, 16H), 6.12-5.92 (m, 0.75H), 5.61 (d, *J* = 8.4 Hz, 0.75H), 5.47 (d, *J* = 6.0 Hz, 0.25H), 5.38-5.19 (m, 2H), 4.73-4.41 (m, 5H), 4.30-4.20 (m, 2H), 3.06 (s, 2.25H), 2.96 (s, 0.75H), 1.53-1.45 (m, 12H), 0.84 (s, 2.25H), 0.81 (s, 6.75H), 0.09 (s, 2.25H), 0.03 (s, 0.75H), -0.01 (s, 0.75H), -0.07 (s, 2.25H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers, major and minor (3/1)): δ 169.5, 169.4, 168.7, 456.4, 155.7, 144.8, 144.6, 144.5, 144.2, 144.1, 143.4, 141.5, 141.3, 141.3, 141.1, 131.7, 131.0, 129.0, 128.9, 128.1, 128.0, 127.9, 127.8, 127.7, 127.2, 127.1, 127.1, 127.0, 125.5, 125.4, 120.6, 120.2, 120.1, 120.0, 119.1, 82.0, 81.9, 78.0, 77.4, 77.3, 73.4, 71.9, 70.8, 70.3, 68.2, 60.1, 58.8, 58.2, 58.0, 47.2, 46.9, 34.8, 34.6, 32.0, 31.9, 29.8, 29.8, 28.0, 26.0, 25.9, 22.8, 22.8, 17.9, 17.8, 14.8, 14.5, 14.2, -4.7, -4.8, -4.9 ppm. HRMS (ESI) *m/z* calcd for $C_{55}H_{65}N_3O_8Si$ $(M+H)^+$ 924.4619, found 924.4594.

***(N*-Fmoc-4-Me)-Pro-Lac-((2*S*,3*R*)-3-OTBS-N_α-methyl-N_α-Fmoc-N_γ-trityl)-Asn-(*N*-OAllyl)-Ala-O*t*-Bu (26).** Dipeptide **25** (283.0 mg, 0.306 mmol) was treated with diethylamine (Et_2NH) (4.0 mL) in MeCN (8.0 mL) at room temperature for 1 h. The reaction mixture was concentrated in vacuo and the residue was co-evaporated with toluene (5 mL × 3) for three times to remove traces of Et_2NH, and dried with high vacuum for 1 h. DIEA (160 μL, 0.919 mmol) was added to

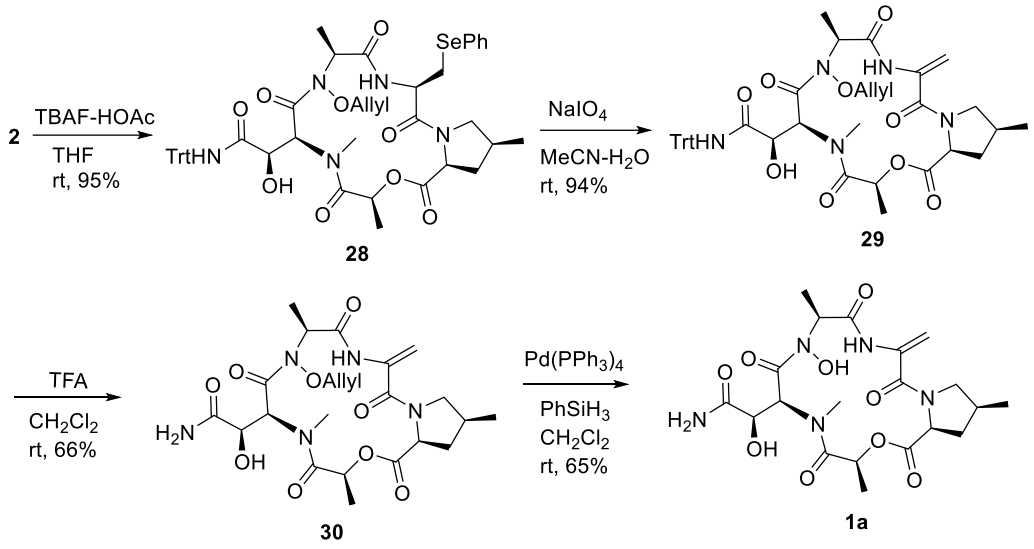
the solution of the above residue and BEP (125.9 mg, 0.46 mmol) in dry CH_2Cl_2 (18 mL) at 0 °C. The reaction mixture was stirred at room temperature overnight under argon, then concentrated in vacuo and purified by flash chromatography column (SiO_2, eluted by EtOAc/hexane (1:3, v/v) to afford product **26** (359 mg, 78%). $[\alpha]^{20}_D$: -42.0 (*c* 0.2, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major and minor (6/4)): δ 7.82-7.76 (m, 3H), 7.68 (d, *J* = 6.8 Hz, 0.4H), 7.63-7.58 (m, 1.6H), 7.41-7.38 (m, 2.4H), 7.35-7.10 (m, 16.6H), 6.00 (br, 1H), 5.81 (br m, 0.6H), 5.72 (br d, *J* = 5.6 Hz, 0.6H), 5.65 (br m, 0.4H), 4.62 (br m, 1H), 4.58-4.48 (m, 2.4H), 4.44-4.20 (m, 4H), 3.79 (br m, 0.4H), 3.72 (br m, 0.6H), 3.10-2.95 (m, 4H), 2.16 (br m, 2H), 1.77-1.69 (m, 0.4H), 1.66-1.57 (m, 0.6H), 1.50-1.45 (m, 13.8H), 1.34 (d, *J* = 6.4 Hz, 1.2H), 1.00-0.92 (m, 3H), 0.92-0.83 (m, 2.4H), 0.79 (s, 3.6H), 0.78 (s, 5.4H), 0.07 (s, 1.8H), -0.12 (s, 1.8H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers, major and minor (6/4)): δ 172.1, 172.0, 169.6, 169.5, 169.3, 169.0, 168.9, 154.8, 154.4, 144.6, 144.5, 144.3, 144.0, 143.7, 141.1, 141.3, 141.3, 131.8, 131.7, 129.0, 128.0, 127.8, 127.7, 127.7, 127.2, 127.1, 127.1, 127.0, 125.5, 125.3, 125.2, 125.1, 121.0, 120.0, 82.0, 78.1, 77.4, 72.1, 72.1, 70.4, 67.8, 67.5, 67.0, 59.8, 59.3, 58.7, 58.6, 57.1, 54.3, 53.8, 47.4, 47.3, 38.7, 37.6, 34.6, 34.8, 33.5, 32.9, 32.6, 29.8, 28.1, 26.0, 17.9, 17.1, 17.0, 15.0, -4.7, -4.9 ppm. HRMS (ESI) *m/z* calcd for $C_{64}H_{78}N_4O_{11}Si$ $(M+Na)^+$ 1129.5334, found 1129.5320.

***(N*-Fmoc-4-Me)-Pro-Lac-((2*S*,3*R*)-3-OTBS-N_α-methyl-N_α-Fmoc-N_γ-trityl)-Asn-(*N*-OAllyl)-Ala-OH (27).** To the solution of **26** (355 mg, 0.321 mmol) in anhydrous CH_2Cl_2 (20 mL) were added 2,6-lutidine (1.86 mL, 16.04 mmol) and trimethylsilyl trifluoromethanesulfonate (TMSOTf) (1.74 mL, 9.62 mmol) at 0 °C under argon. After stirred at the same temperature for 20 min, the reaction mixture was moved to room temperature and stirred for 2.5 h, then it was quenched with saturated aqueous $NaHCO_3$ (30 mL) and extracted with EtOAc (50 mL × 3). The combined organic layer was washed with 5% $KHSO_4$ (30 mL × 5), water (30 mL × 2) and brine (30 mL), dried with anhydrous $MgSO_4$ and evaporated in vacuo. The resulting crude mixture was purified by flash chromatography column (SiO_2, eluted by 5% MeOH in CH_2Cl_2) to provide acid **27** (285 mg, 85%) as a white solid. $[\alpha]^{20}_D$: -55.0 (*c* 0.09, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major and minor (5.5/4.5)): δ 8.15 (s, 0.45H), 8.11 (s, 0.55H), 7.76 (dd, *J* = 6.4, 6.4 Hz, 2H), 7.63-7.55 (m, 2H), 7.41-7.36 (m, 2H), 7.32-7.25 (m, 11H), 7.16-7.14 (m, 6H), 6.03-5.91 (m, 1H), 5.87 (dd, *J* = 7.6 Hz, 1H), 5.57 (br d, J = 6.0 Hz, 0.55H), 5.41-5.27 (m, 2.45H), 4.90-4.80 (m, 2H), 4.60-4.50 (m, 2H), 4.48-4.41 (m, 1H), 4.38-4.26 (m, 2.55H), 4.17 (dd, *J* = 6.4 Hz, 0.45H), 3.85-3.75 (m, 1H), 3.09-3.03 (m, 0.55H), 2.98 (br s, 3.45 H), 2.50-2.38 (br m, 1H), 2.31-2.17 (br m, 1H), 1.70-1.56 (m, 1H), 1.50-1.45 (m, 4.65H), 1.37 (d, J = 6.4 Hz, 1.35H), 1.05 (d, *J* = 6.4 Hz, 1.65H), 1.01 (d, *J* = 6.4 Hz, 1..35H), 0.80 (s, 4.95H), 0.79 (s, 4.05H), 0.09 (s, 1.65H), 0.07 (s, 1.35H), 0.07 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of rotamers, major and minor (5.5/4.5)): δ 171.9, 171.8, 171.5, 171.3, 170.8, 170.9, 170.8, 169.6, 169.6, 154.8, 154.4, 144.4, 144.3, 143.9, 143.7, 141.4, 141.3, 141.3, 131.0, 130.9, 128.7, 128.2, 127.8, 127.8, 127.7, 127.5, 127.2, 127.1, 127.0, 125.4, 125.3, 125.2, 125.0, 121.7, 120.0, 120.0, 79.4, 77.3, 71.2, 70.3, 70.1, 67.6, 67.5, 59.3, 58.8, 58.1, 57.2, 54.2, 53.8, 47.4, 47.3, 38.7, 37.5, 33.4, 33.0, 32.5, 31.7, 25.9, 22.8, 17.9, 17.2, 16.6, 16.4, 14.2, -4.7, -5.1 ppm. HRMS (ESI) *m/z* calcd for $C_{60}H_{70}N_4O_{11}Si$ $(M+Na)^+$ 1073.4708, found 1073.4691.

***(N*-Fmoc-4-Me)-Pro-Lac-((2*S*,3*R*)-3-OTBS-N_α-methyl-N_α-Fmoc-N_γ-trityl)-Asn-(*N*-OAllyl)-Ala- ((*S*)-*N*-Boc-*L*-(*Se*)-phenylseleno)-Cys-OFm (3)**. Compound **7** (244.7 mg, 0.468 mmol) was treated with TFA (2.5 mL) in CH_2Cl_2 (5 mL) at room temperature for 30 min. The reaction mixture was concentrated in vacuo, and the residue was co-evaporated with toluene (5 mL × 3) for three times to remove traces of TFA. The residue was dried under vacuum and used in next step without

purification. Compound **27** (277 mg, 0.264 mmol), BOP (256.4 mg, 0.58 mmol), DIEA (230 μL, 1.321 mmol) were added successively to the solution of the above residue from **7** in dry THF (20 mL) at room temperature. After the reaction mixture was stirred at the same temperature for 3 h, it was evaporated in vacuo and purified by flash chromatography column (SiO_2, eluted by 15-20% EtOAc in hexane) to provide product **3** (330 mg, 86%). $[\alpha]^{20}_D$: -54.4 (*c* 0.09, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of rotamers, major and minor (5.5/4.5)): δ 8.21 (s, 0.45H), 8.19 (s, 0.55H), 8.09 (d, *J* = 7.6 Hz, 1H), 7.79-7.76 (m, 4H), 7.65-7.57 (m, 2H), 7.53 (d, *J* = 7.6 Hz, 1H), 7.48-7.12 (m, 28H), 6.07-5.90 (m, 2H), 5.59 (q, *J* = 5.6 Hz, 0.55H), 5.50-5.38 (m, 1.45H), 5.30 (dd, *J* = 10.0, 1H), 5.25-5.19 (br m, 1H), 4.98 (d, *J* = 7.6 Hz, 1H), 4.78-4.71 (m, 1H), 4.66-4.60 (m, 1H), 4.50-4.26 (m, 6H), 4.18 (dd, *J* = 6.8, 6.8 Hz, 0.55H), 4.09 (t, *J* = 8.8 Hz, 1H), 4.02 (dd, *J* = 6.8, 6.8 Hz, 1H), 3.89-3.79 (m, 1H), 3.12-3.00 (m, 4.45H), 2.89-2.84 (br m, 1H), 2.58-2.47 (m, 1H), 2.37-2.27 (m, 1H), 2.25-2.14 (m, 1.55H), 1.74-1.62 (m, 1.45H), 1.50 (br d, *J* = 6.8 Hz, 4.65H), 1.40 (d, *J* = 6.8 Hz, 1.35H), 1.09 (d, *J* = 6.4 Hz, 1.65H), 1.05 (d, *J* = 6.4 Hz, 1.35H), 0.82 (s, 4.05H), 0.81 (s, 4.95H), 0.15-0.12 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.0, 171.9, 171.8, 171.8, 170.5, 170.1, 169.3, 168.8, 168.7, 154.8, 154.4, 144.4, 144.3, 144.1, 144.0, 143.8, 143.3, 141.4, 141.4, 141.4, 141.4, 141.2, 133.7, 133.7, 131.1, 131.0, 129.3, 129.0, 128.8, 128.2, 127.9, 127.8, 127.8, 127.8, 127.8, 127.7, 127.7, 127.5, 127.5, 127.3, 127.2, 127.1, 127.1, 127.0, 125.7, 125.3, 125.3, 125.3, 125.2, 125.0, 120.7, 120.1, 120.0, 120.0, 79.0, 79.0, 77.4, 71.0, 70.9, 70.4, 70.3, 67.6, 67.6, 67.5, 67.2, 59.3, 58.7, 57.0, 57.0, 55.4, 54.2, 53.8, 52.9, 47.5, 47.3, 46.7, 38.7, 37.6, 33.5, 33.3, 33.3, 32.6, 31.7, 29.8, 28.5, 25.9, 22.8, 17.9, 17.3, 17.2, 16.6, 15.0, 14.2, -4.7, -5.0 ppm. HRMS (ESI) *m/z* calcd for $C_{83}H_{89}N_5O_{12}SeSi$ $(M+Na)^+$ 1478.5340, found 1478.5337.

Macrocycle 2. Linear compound **3** (222.5 mg, 0.153 mmol) was treated with diethylamine (Et_2NH) (10 mL) in MeCN (20 mL) under nitrogen atmosphere at room temperature for 2.5 h. The reaction mixture was concentrated in vacuo and the residue was co-evaporated with the mixture of toluene/CH_2Cl_2/DIEA (10:10:1, 5 mL × 3) for three times and CH_2Cl_2 (5 mL × 2) two times to remove traces of Et_2NH, and dried with high vacuum for 0.5 h. The reaction mixture was purified by a preparative column (Alltech, 1 gram, silica). The column was eluted sequentially by CH_2Cl_2, EtOAc/hexane (1:1) and CH_2Cl_2/MeOH (4:1). The fraction eluted by CH_2Cl_2/MeOH (4:1) was collected, evaporated, dried and used in next step directly. DIEA (239.2 μL, 1.376 mmol) was added to the solution of the above residue, PyBOP (238.6 mg, 0.459 mmol) and HOAt (64.5 mg, 0.474 mmol) in dry CH_2Cl_2 (250 mL) at room temperature. The reaction mixture was stirred at the same temperature for 24 h under argon, then concentrated in vacuo and purified by flash chromatography column (SiO_2, eluted by EtOAc/hexane (1:3 to 1:1, v/v) to afford product **2** (95.2 mg, 60%). $[\alpha]^{20}_D$: -42.3 (*c* 0.11, MeOH). ^{1}H NMR of **2** (400 MHz, $CDCl_3$): δ 7.75 (s, 1H), 7.46-7.43 (m, 2H), 7.29-7.20 (m,12H), 7.15-7.12 (m, 6H), 6.82 (d, *J* = 9.2 Hz, 1H), 6.01 (dddd, *J* = 17.0, 10.7, 6.4, 6.0 Hz, 1H), 5.52-5.46 (m, 2H), 5.40-5.30 (m, 2H), 4.89 (ddt, *J* = 11.2, 6.0, 1.2 Hz, 1H), 4.79 (td, *J* = 9.6, 4.8 Hz, 1H), 4.69 (d, *J* = 9.2 Hz, 1H), 4.66 (q, *J* = 6.8 Hz, 1H), 4.56 (dd, *J* = 8.8, 5.2 Hz, 1H), 4.19 (ddt, *J* = 10.8, 6.4, 1.6 Hz, 1H), 3.57 (dd, *J* = 12.0, 7.2 Hz, 1H), 3.15 (dd, *J* = 12.8, 10.4, 1H), 3.06 (dd, *J* = 12.8, 5.2 Hz, 1H), 2.92 (s, 3H), 2.78 (dd, *J* = 12.0, 6.4 Hz, 1H), 2.47-2.40 (m, 1H), 2.15-2.07 (m, 1H), 1.69-1.63 (m, 1H), 1.60 (d, *J* = 6.8 Hz, 3H), 1.11 (d, *J* = 7.2 Hz, 3H), 0.90 (d, *J* = 7.2 Hz, 3H), 0.82 (s, 9H), 0.21 (s, 3H), -0.05 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.9, 169.7, 169.4, 168.5, 167.8, 165.4, 144.3, 131.3, 131.1, 129.6, 129.1, 128.0, 127.2, 127.2, 120.1, 78.7, 77.4, 74.8, 70.5, 69.7, 59.8, 59.2, 56.5, 53.7, 49.9, 39.2, 32.3, 30.9, 29.8, 28.4, 26.0, 18.6, 18.0, 16.6, 16.1, -3.6, -5.0 ppm. HRMS (ESI) *m/z* calcd for $C_{54}H_{67}N_5O_9SeSi$ $(M+Na)^+$ 1060.3771, found 1060.3753.



Scheme S7. Fully unmasking and completion of final target gatorbulin-1 (**1a**).

Macrocycle 28. The pre-mixed buffer of TBAF (1.35 mL, 0.5 M in THF, 0.675 mmol) and AcOH (1.62 mL, 0.5 M in THF, 0.81 mmol) was added to the solution of macrocycle **2** (70 mg, 0.0675 mmol) in dry THF (14 mL). After the reaction mixture was stirred at room temperature under argon for 5 h, it was diluted with EtOAc and quenched with saturate $NaHCO_3$ (10 mL) extracted with EtOAc (15 mL × 3). The combined organic layers were dried over anhydrous $MgSO_4$ and purified by preparative TLC plate (SiO_2, developed by acetone/hexanes 1:2, Rf = 0.4) to provide product **28** (59.2 mg, 95%). $[\alpha]^{20}_D$: -91.5 (*c* 0.09, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (s, 1H), 7.47-7.44 (m, 2H), 7.30-7.18 (m,18H), 6.76 (d, *J* = 9.2 Hz, 2H), 6.09-5.99 (m, 1H), 5.49 (q, *J* = 6.8 Hz, 1H), 5.43-5.33 (m, 2H), 4.91 (dd, *J* = 10.8, 5.6 Hz, 1H), 4.85-4.79 (m, 1H), 4.53 (dd, *J* = 8.8, 6.0 Hz, 1H), 4.46 (br s, 2H), 4.30 (dd+dd, *J* = 11.2, 6.8 Hz, 2H), 3.64 (dd, *J* = 11.6, 7.2 Hz, 1H), 3.18 (dd, *J* = 12.4, 10.0 Hz, 1H), 3.02 (dd, *J* = 12.4, 5.6 Hz, 1H), 3.03 (s, 3H), 2.88-2.83 (m, 1H), 2.51-2.44 (m, 1H), 2.17-2.09 (m, 1H), 1.65 (ddd, *J* = 13.2, 6.4, 6.4 Hz, 1H), 1.57 (d, *J* = 6.8 Hz, 3H), 1.26 (d, *J* = 6.8 Hz, 3H), 0.96 (d, *J* = 6.8 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.6, 169.5, 169.5, 169.3, 169.0, 166.8, 144.5, 131.6, 131.2, 129.5, 129.2, 128.8, 128.0, 127.3, 127.1, 120.8, 79.2, 77.4, 71.7, 70.1, 60.0, 59.5, 53.7, 49.7, 39.3, 31.1, 28.9, 18.3, 16.7, 16.5 ppm. HRMS (ESI) *m/z* calcd for $C_{48}H_{53}N_5O_9Se$ $(M+Na)^+$ 946.2906, found 946.2878.

Macrocycle 29. $NaIO_4$ (67.8 mg, 0.314 mmol) was added to the solution of compound **28** (73.2 mg, 0.0793 mmol) in the mixture of MeCN-H_2O (15 mL–11.2 mL) at the room temperature. After the reaction mixture was stirred at the same temperature under argon for 2 h, CH_2Cl_2 (25 mL) and aqueous saturate $NaHCO_3$ (15 mL) were added and the resulting mixture was extracted with CH_2Cl_2 (20 mL × 4). The combined organic layers were dried with anhydrous $MgSO_4$, evaporated in vacuo and purified by preparative TLC plate (SiO_2, developed by acetone/hexanes 2:3, Rf = 0.5) to afford product **29** (57.2 mg, 94%). $[\alpha]^{20}_D$: -48.4 (*c* 0.07, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of three conformers, major, medium and minor (0.65:0.25:0.1)): δ 8.38 (s, 0.1H), 8.30 (s, 0.1H), 8.28 (s, 0.65H), 8.24 (s, 0.25H), 8.19 (s, 0.65H), 7.99 (s, 0.25H), 7.28-7.20 (m, 15H), 6.48 (s, 0.65H), 6.25 (s, 0.25H), 6.23 (s, 0.1H), 6.14-6.04 (m, 0.25H), 5.95-5.86 (m, 0.65H), 5.86-5.77 (m, 0.1H), 5.63 (q, *J* = 6.8 Hz, 0.75H), 5.48-5.27 (m, 3H), 5.13 (s, 0.65H), 5.05 (br m, 0.25H), 5.01 (s, 0.25H), 4.91 (s, 0.1H), 4.72 (dd, *J* = 8.4, 6.0 Hz, 0.35H), 4.69 (d, *J* = 9.2 Hz, 1H),

4.56 (dd, *J* = 9.6, 7.2 Hz, 0.65H), 4.48 (dd, *J* = 10.8, 6.8 Hz, 0.65H), 4.42-4.39 (m, 2.65H), 4.34-4.31 (m, 1H), 4.30-4.24 (m, 0.35H), 4.18 (q, *J* = 6.8 Hz, 1H), 4.07 (dd, *J* = 10.4, 6.8 Hz, 0.9H), 4.02 (dd, *J* = 11.6, 7.2 Hz, 0.1H), 3.75 (dd, *J* = 11.6, 7.2 Hz, 0.35H), 3.40 (dd, *J* = 10.0, 8.4 Hz, 0.65H), 3.24 (t, *J* = 11.2, 0.25H), 3.14 (t, *J* = 11.2 Hz, 0.1H), 3.05 (s, 1.95H), 3.03 (s, 0.3H), 2.97 (s, 0.75H), 2.63-2.42 (m, 2.25H), 2.37-2.27 (m, 0.65H), 2.00 (d, *J* = 7.2 Hz, 0.3H), 1.81 (d, *J* = 7.6 Hz, 1.95H), 1.62-1.55 (m, 1.75H), 1.48 (d, *J* = 7.2 Hz, 0.3H), 1.40 (d, *J* = 6.8 Hz, 1.95H), 1.33 (d, *J* = 6.4 Hz, 0.75H), 1.15-1.11 (m, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of three conformers, major, medium and minor): δ 172.3, 171.4, 171.0, 170.4, 170.2, 170.0, 169.6, 169.4, 169.0, 168.7, 167.9, 166.5, 166.0, 165.8, 144.5, 144.5, 144.5, 144.4, 135.8, 134.3, 134.3, 131.5, 131.3, 130.6, 130.5, 129.5, 128.7, 128.7, 128.4, 128.2, 128.0, 127.8, 127.1, 121.7, 120.6, 120.3, 104.8, 104.2, 103.1, 78.8, 77.4, 76.3, 74.7, 73.5, 73.4, 70.1, 70.0, 67.9, 67.6, 63.9, 63.6, 63.4, 62.0, 61.7, 60.7, 56.1, 54.7, 53.4, 40.0, 37.4, 36.9, 36.0, 35.9, 34.0, 31.7, 29.8, 17.7, 17.6, 17.3, 17.0, 16.9, 16.7, 16.0, 14.6, 14.2 ppm. HRMS (ESI) *m/z* calcd for $C_{42}H_{47}N_5O_9$ $(M+Na)^+$ 788.3271, found 788.3244.

Macrocycle 30. TFA (2.0 mL) was added to the solution of **29** (26.3 mg, 0.0344 mmol) in dry CH_2Cl_2 (10 mL) at 0 ^{o}C. The reaction was stirred room temperature for 1 h, then it was diluted with toluene (10 mL). The solvent was evaporated in vacuo and the residue was purified by preparative TLC plate (SiO_2, developed by MeOH/CH_2Cl_2 5:95, Rf = 0.2) to afford product **30** (11.8 mg, 66%). $[\alpha]^{20}_D$: -54.5 (c 0.08, MeOH). ^{1}H NMR (400 MHz, $CDCl_3$, mixture of conformers, major/minor (2:1)): δ 8.24 (s, 0.67H), 7.95 (s, 0.33H), 6.49 (s, 0.67H), 6.27 (s, 0.33H), 6.06-5.96 (m, 1H), 5.80 (br m, 1H), 5.67 (q, *J* = 6.8 Hz, 0.67H), 5.29 (d, *J* = 10.0 Hz, 0.33H), 5.14 (s, 0.67H), 5.02 (br s, 1H), 4.96-4.92 (m, 0.33H), 4.60-4.36 (m, 5H), 4.20-4.07 (m, 2H), 3.74 (dd, *J* = 12.0, 7.2 Hz, 0.33H), 3.35 (dd, *J* = 10.4, 7.6 Hz, 0.67H), 3.23 (t, *J* = 11.6, 0.33H), 3.14-3.12 (m, 1.32H), 3.09-3.06 (m, 2.68H), 2.55-2.43 (m, 2H), 2.38-2.27 (m, 0.67H), 1.67-1.64 (m, 3.33H), 1.47-1.43 (m, 3H), 1.14-1.11 (m, 3H), 7-5.03 (m, 2H), 4.65 (d, *J* = 10.8 Hz, 1H), 4.45 (d, *J* = 10.8 Hz, 1H), 3.86-3.80 (m, 1H), 3.80 (s, 3H), 3.08 (dd, *J* = 7.2, 3.6 Hz, 1H), 2.31-2.20 (m, 2H), 1.87 (br m, 1H), 1.66 (ddd, *J* = 13.2, 9.2, 2.8 Hz, 1H),1.46-1.34 (m, 2H), 1.06 (d, *J* = 6.8 Hz, 3H),0.91 (s, 9H), 0.86 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, mixture of conformers, major/minor (2:1)): δ 173.8, 173.6, 170.7, 170.5, 170.0, 169.4, 168.6, 168.4, 166.9, 166.3, 165.3, 134.3, 134.2, 131.2, 130.6, 122.4, 120.3, 104.8, 104.1, 78.2, 77.4, 70.7, 70.4, 63.4, 62.0, 61.8, 60.9, 56.2, 54.7, 40.0, 36.1, 35.3, 33.9, 32.0, 31.8, 29.8, 29.8, 17.9, 17.3, 17.1, 16.7, 15.8, 14.3 ppm. HRMS (ESI) *m/z* calcd for $C_{23}H_{33}N_5O_9$ $(M+H)^+$ 524.2357, found 524.2341.

Gatorbulin-1 (1a). $PhSiH_3$ (10 μL, 0.081 mmol) and $Pd(PPh_3)_4$ (2.43 mg, 0.0021 mmol, in degassed CH_2Cl_2 (0.5 mL) were added to the solution of **30** (11.0 mg, 0.021 mmol) in degassed dry CH_2Cl_2 (4.0 mL) sequentially at room temperature. The reaction flask was protected by aluminum foil and the reaction mixture was stirred at the room temperature for 1.5 h under argon. Then the reaction was concentrated under reduced pressure and the residue was purified by preparative reverse TLC plate (C18, developed by MeOH/H_2O (1:1), Rf = 0.5). The product band was scraped and washed down by MeOH/CH_2Cl_2 (3:7). The washed down product fraction was concentrated to dryness under reduced pressure and rinsed with hexanes. The residue was purified again by reverse preparative cartridge (C18, 100 mg, Alltech, eluted by MeOH). The product fractions were collected, concentrated and dried to provide product **1a** (6.6 mg, 65%) as off-white solid. The ^{1}H NMR and ^{13}C NMR were identical to natural product **1a** (mixture of two conformers (1:1)). $[\alpha]^{20}_D$: –119.2 (*c* 0.17, MeOH) (natural **1a**, $[\alpha]^{20}_{D:}$ – 84.0 (*c* 0.10, MeOH)).

(Integrated two conformers separately) **Conformer 1**: ^{1}H NMR (600 MHz, DMF-d_7), δ 11.38 (br s, 1H), 8.28 (s, 1H), 7.28 (s, 1H), 7.09 (s, 1H), 6.46 (s, 1H), 6.07 (br, 1H), 5.51 (br d, *J* = 9.6 Hz, 1H), 5.47 (q, *J* = 7.2 Hz, 1H), 5.23 (s, 1H), 4.51 (br d, 1H), 4.41 (t, *J* = 7.8 Hz, 1H), 4.31 (m, 1H), 3.25 (dd, *J* = 10.2, 7.8 Hz, 1H), 3.09 (s, 3H), 2.58 (m, 1H), 2.53 (m, 1H), 1.58 (m, 1H), 1.53 (d, *J* = 6.6 Hz, 3H), 1.42 (d, *J* = 7.2 Hz, 3H), 1.12 (d, *J* = 6.6 Hz, 3H) ppm; ^{13}C NMR (150 MHz, DMF-*d7*), δ 175.1, 170.8, 170.5, 169.9, 165.9, 136.2, 101.9, 72.9, 68.8, 64.6, 62.6, 57.9, 56.9, 37.0, 34.7, 34.0, 18.0, 17.6, 15.6, 13.8 ppm. **Conformer 2:** ^{1}H NMR (400 MHz, d_7-DMF): δ 10.61 (br s, 1H), 7.91 (s, 1H), 7.41 (s, 1H), 7.21 (s, 1H), 6.22 (s, 1H), 5.90 (br, 1H), 5.38 (q, *J* = 7.2 Hz, 1H), 5.22 (s, 1H), 5.07 (br, 1H), 4.93 (dd, *J* = 9.6, 7.2 Hz, 1H), 4.71 (br q, *J* = 7.2 Hz, 1H), 4.64 (br m, 1H), 4.29 (q, J = 6.6 Hz, 1H), 3.71 (dd, J = 11.4, 7.2 Hz, 1H), 3.13 (s, 3H), 3.11 (dd, *J* = 11.4, 11.4 Hz, 1H), 2.38 (m, 1H), 1.66 (m, 1H), 1.52 (d, *J* = 6.0 Hz, 3H), 1.40 (d, *J* = 6.6 Hz, 3H), 1.10 (d, J = 6.6 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.9, 170.4, 170.2, 169.7, 165.4, 166.8, 136.2, 72.2, 69.7, 63.8, 60.0, 55.8, 55.6, 40.8, 34.5, 32.4, 17.3, 16.9 ppm. HRMS (ESI) *m/z* calcd for $C_{20}H_{29}N_5O_9$ $(M+H)^+$ 484.2044, found 484.2028.

(Integrated two conformers together): ^{1}H NMR (600 MHz, DMF-d_7, mixture of conformers (1:1)): δ 11.38 (br s, 0.5H), 10.61 (br s, 0.5H), 8.28 (s, 0.5H), 7.91 (s, 0.5H), 7.41 (s, 0.5H), 7.28 (s, 0.5H), 7.21 (s, 0.5H),7.09 (s, 0.5H), 6.46 (s, 0.5H), 6.22 (s, 0.5H), 6.07 (br, 0.5H), 5.90 (br, 0.5H), 5.51 (br d, *J* = 9.6 Hz, 0.5H), 5.47 (q, *J* = 7.2 Hz, 0.5H), 5.38 (q, *J* = 7.2 Hz, 0.5H), 5.23 (s, 0.5H), 5.22 (s, 0.5H), 5.07 (br, 0.5H), 4.93 (dd, *J* = 9.6, 7.2 Hz, 0.5H), 4.71 (br q, *J* = 7.2 Hz, 0.5H), 4.64 (br m, 0.5H), 4.51 (br d, 0.5H), 4.41 (t, *J* = 7.8 Hz, 0.5H), 4.33-4.28 (m, 1H), 3.71 (dd, *J* = 11.4, 7.2 Hz, 0.5H), 3.25 (dd, *J* = 10.2, 7.8 Hz, 0.5H), 3.15-3.11 (m, 2H), 3.09 (s, 1.5H), 2.60-2.50 (m, 1.5H), 2.41-2.34 (m, 0.5H), 1.69-1.63 (m, 1H), 1.60-1.56 (m, 1H), 1.53 (d, *J* = 6.6 Hz, 1.5H), 1.52 (d, *J* = 6.0 Hz, 1.5H), 1.42 (d, *J* = 7.2 Hz, 1.5H), 1.40 (d, *J* = 6.6 Hz, 1.5H), 1.12 (d, *J* = 6.6 Hz, 1.5H), 1.10 (d, J = 6.6 Hz, 1.5H) ppm; ^{13}C NMR (150 MHz, DMF-d_7, mixture of conformers (1:1)): δ 175.1, 174.9, 170.8, 170.5, 170.4, 170.2, 169.9, 169.7, 169.4, 166.8, 165.9, 165.4, 136.2, 136.2, 101.9, 72.9, 72.2, 69.7, 68.8, 64.6, 63.8, 62.6, 60.0, 57.9, 56.9, 55.8, 55.6, 40.8, 37.0, 34.7, 34.5, 34.0, 32.4, 18.0, 17.6, 17.3, 16.9, 15.6, 13.8 ppm. HRMS (ESI) *m/z* calcd for $C_{20}H_{29}N_5O_9$ $(M+H)^+$ 484.2044, found 484.2028.

Qualitative test for Fe(III) chelating ability of GB1 (1a)

We applied a solution of GB1 (**1a**) in CH_2Cl_2 to reverse TLC (C18) (MeOH/H_2O 1:1, v/v), then dipped the TLC into $FeCl_3$ solution in ethanol (5% wt). The compound quickly caused an orange spot (positive reaction).

METHODS – BIOLOGY

Cell Lines

Isogenic HCT116 cell lines were generated and authenticated as described[8], and normal colon CCD841-CoN cells obtained from ATTC. HeLa, A-10, HCC1806, HCC1937, MDA-MB-453, and MDA-MB-231 were purchased from the American Type Culture Collection (ATCC) and authenticated by Genetica. The BT-549 cell line was obtained from the Lombardi Cancer Center at George Washington University and was authenticated in April 2014 via STR-based profiling (Promega). HeLa βIII-tubulin and SK-OV-3-MDR1-M6/6 cell lines were characterized as previously described[9]. Those experiments were performed on cells within 4 months of liquid nitrogen cryo-recovery. Cells were grown at 37°C in a humidified incubator with 5% CO_2. The types of media sued for all these cells are indicated for the corresponding assays. Human umbilical vein endothelial cells (HUVEC) were maintained in endothelial cell growth medium-2 (EGM™-2, Lonza, Switzerland) supplemented with EGM™-2 Endothelial SingleQuots™ bullet kit according to supplier's instructions.

Proteins and Reagents

Combretastatin A-4 was obtained from Sigma Aldrich, brought up in DMSO and stored at -20 °C. Calf brain tubulin was purified as described[10]. MTC[11] was a kind gift from Prof. Wei-Shuo Fang (Institute of Materia Medica, Beijing). Eribulin-A488[12], was a gift of Michel Steinmetz (PSI, Vilingen) synthesized by Eisai chemist Daniel Custar. FcMaytansine[13] was a kind gift from Prof. Karl-Heinz Altmann (ETH, Zürich). The compounds were diluted in 99.8% DMSO-d_6 (Merck, Darmstadt, Germany) to a final concentration of 10 mM and stored at –80 °C.

MTT Cell Viability Assay

Parental HCT116, HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}HIF\text{-}2\alpha\text{-}/\text{-}}$, HCT116$^{HIF\text{-}1\alpha\text{-}/\text{-}}$, HCT116$^{HIF\text{-}2\alpha\text{-}/\text{-}}$, HCT116$^{WT\ KRAS}$ along with normal colon cell line CCD841-CoN were cultured in Dulbecco's modified Eagle medium (DMEM, Life Technologies, USA) supplemented with 10% Fetal Bovine Serum (FBS, Sigma, USA) and maintained in 5% CO_2 at 37 °C. HCT116 cells (8,000 cells/well) and normal cells (3,000 cells/well) were seeded in 96-well plates, allowed to attach overnight and then treated with different concentrations of GB1 (**1a**) or solvent control (0.5% DMSO). Cell viability was measured 48 h following treatment with MTT dye using manufacturer's protocol (Promega). IC_{50} was determined by non-linear regression analysis using GraphPad Prism 8. Data are represented as average ± SD (n = 3).

HCT116 Cell Cycle Analysis

HCT116 cells were seeded in 6-well plates (400,000 cells/well, DMEM/10% FBS and allowed to attach overnight prior to treatment with GB1. Media was replaced by fresh DMEM/10% FBS after 24 h of incubation and treated with compound and solvent control (0.25% DMSO) for 24 h. The medium in each well was collected separately, and the cells washed with 500 µL PBS and collected into the corresponding tubes. Cells were detached using trypsin (Invitrogen) and collected into the corresponding tubes and centrifuged at 400*g* for 10 min at 4 °C. The supernatant was discarded and the cell pellets were resuspended in 500 µL ice-cold PBS, centrifuged at 400*g* for 10 min at 4

°C and supernatant was discarded. The cell pellets were resuspended in 300 μL ice-cold PBS and 700 μL ice-cold EtOH was added slowly to each cell suspension with gentle pipetting. Cells were incubated at –20 °C overnight and centrifuged at 400*g* for 10 min at 4 °C next day. The EtOH/PBS was discarded and cells resuspended in 300 μL PBS containing 1 mM EDTA and 100 μg/mL RNase A (Invitrogen). The cells were incubated at 37 °C for 30 min, with shaking at 800 rpm, followed by addition of 1 μL propidium iodide (1 mg/mL, Invitrogen). Fluorescence from propidium iodide–DNA complexes were quantified using FACScan (BD Biosciences LSRFortessa) and data were analyzed using ModFit LT (Verity Software House).

RNA Isolation, Reverse Transcription and Quantitative Polymerase Chain Reaction

HCT116 cells were seeded in 6-well plates at a density of 4×10^5 per well and incubated overnight for cells to attach. Cells were treated with 3.2 μM GB1 or 0.25 % DMSO (vehicle). RNA was isolated 16 h later using the RNeasy mini kit (QIAGEN, Valencia, CA). Total RNA was quantified using NanoDrop 2000. cDNA synthesis was carried out using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and oligo (dT) (Invitrogen) from 2 μg of total RNA. The qPCR after reverse transcription (RT-qPCR) was performed on a 25 μL reaction solution containing a 0.3 μL aliquot of cDNA, 12.5 μL of TaqMan gene expression master mix, 1.25 μL of probes, and 11 μL RNase-free water. qPCR was carried out on an ABI 7300 sequence detection system using the thermocycler program: 2 min at 50 °C, 10 min at 95 °C, and 15 s at 95 °C (40 cycles) and 1 min at 60 °C. Experiments were performed in triplicate. Probes for target gene: *VEGFA* (Hs00900055_m1) and for endogenous control: *β-actin* (Hs99999903_m1). Statistical analysis for comparison between treatment and vehicle group was done using a student *t* test ($^*p < 0.05$).

Sulforhodamine B Assay

The sulforhodamine B (SRB) assay was used to evaluate the antiproliferative and cytotoxic potency of compounds in cell lines (HCC1806, HCC1937, MDA-MB-453, MDA-MB-231, HeLa and SK-OV-3, HeLa, SK-OV-3, BT-549, SK-OV-3/MDR-1-6/6 and WTβIII) as previously described.[14,15] The following cell culture media were used: RPMI with 5% FBS (HCC1806, BT-549), RPMI with 10% FBS (HCC1937), IMEM with 5% FBS (MDA-MB-453, MDA-MB-231), BME with 10% FBS (HeLa, A10, SK-OV-3, M6/6, HeLa BIII). Briefly, cells were plated at a density of 4,000–6,000 cells per well in 96-well plates and allowed to adhere overnight. The cells were subsequently treated with indicated concentrations of GB1, CA-4 or vehicle (0.5% DMSO) for 48 h after which cells were fixed, processed, and data acquired using a SpectraMax plate reader (Molecular Devices) with the exception of the SK-OV-3-MDR1-M6/6 line that was treated for 96 h to allow for the same number of population doublings. A separate T0 plate was processed at the time of compound addition as an indication of the cellular density when compounds were added and plotted as a dashed line at y = 0. Antiproliferative effects elicited by compounds as compared to vehicle treated controls were calculated using the equation [(FinalValue-T0)/(Vehicle-T0)] and depicted as positive numbers on the graphs. The concentration of compounds that caused a 50% decrease in proliferation as compared to vehicle controls (GI_{50}) was determined by non-linear regression analysis using GraphPad Prism 6. Percent cytotoxicity was calculated using the equation [100-(FinalValue/T0)*100] and depicted on the graph as negative values. Data are representative of the average of three independent experiments ± SEM.

Indirect Immunofluorescence

A-10 cells, grown in BME with 10% FBS, were plated on glass coverslips in 24 well plates and allowed to adhere overnight. Cells were then treated for 18 h with either vehicle (0.1% DMSO), GB1, or CA-4 at indicated concentrations and fixed using cold methanol. Coverslips were blocked with 10% bovine calf serum (Sigma-Aldrich) and incubated in primary antibody (T-4026, Sigma-Aldrich) at 1:1000 for 2 h at 37°C and a FITC-conjugated secondary antibody (F-3008, Sigma Aldrich) at 1:250 for 30 min at 37°C before staining with DAPI (Sigma-Aldrich) for 15 minutes. Images were acquired using a Nikon Eclipse 80i fluorescence microscope and analyzed using NIS Elements. All images show non-deconvolved stacks and are representative of three independent experiments.

HeLa Cell Cycle Distribution

HeLa cells, grown in BME with 10% FBS, were seeded in a 6 well plate and allowed to adhere overnight. Cells were then treated for 18 h with vehicle (0.1% DMSO), GB1, or CA-4 at indicated concentrations. Cells were harvested on ice by collecting the media after scraping up the adherant cells by centrifuging at 500*g* for 5 min at 4 ºC. Cells were resuspended in Krishan's reagent (50 µg/mL propidium iodide, 1 mg/mL sodium citrate, 3 µL/mL IGEPAL CA-630 and 20 µg/mL ribonuclease A) and DNA content as a function of propidium iodide incorporation analyzed using a Muse Cell Analyzer (Millipore). Analysis was performed based on three independent experiments.

Caspase Activity in HeLa Cells

HeLa cells were seeded in DMEM media (supplemented with 10% FBS) at a density of 4000/well in 96-well, white clear bottom plates and incubated for 24 h. Cells were then treated with varying concentrations of GB1, solvent control (0.5% DMSO) and CA-4 (100 nM) and allowed to incubate. The medium was used for determination of background luminescence. Caspase-Glo 3/7 reagent (Promega) was prepared according to the manufacturer's instruction and equal volume (100 µL) of reagent mix was added to each well after incubation with compounds and solvent control for 14 h, 24 h, and 36 h. The plate was gently shaken and incubated at room temperature for 30 minutes to ensure complete cell lysis. Luminescence was measured using Envision (Perkin Elmer, Waltham, MA, USA) multilabel plate reader and the relative caspase 3/7 activity of GB1 and CA-4 reported relative to DMSO control. The assay was carried out in triplicate.

Tubulin Polymerization

The effects of GB1 and CA-4 on tubulin polymerization were evaluated using purified porcine brain tubulin obtained from Cytoskeleton. Briefly, 20 µM of purified tubulin was mixed with cold GPEM buffer (1 mM GTP, 10% glycerol, 80 mM PIPES pH 6.9, 2 mM $MgCl_2$, and 0.5 mM EGTA) containing either vehicle (DMSO; 1%), GB1, or CA-4 (10 µM) in a total volume of 100 µL. Tubulin polymerization was monitored every minute for 60 minutes by light scattering at 340 nm after samples were warmed to 37 °C in a SpectraMax plate reader. Analysis was performed based on three independent experiments.

Measuring of the Binding Constants

Competition of up to 20 µM GB1 with Eribulin-A488 was assayed as described[12]. Competition of up to 20 µM GB1 with FcMaytansine was assayed as described[13]. The apparent binding constant of GB1 for the colchicine site of tubulin was measured by competition with 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC)[11] a well characterized reversible colchicine-binding site ligand[16]. Briefly, a solution of 10 µM tubulin plus 10 µM MTC was incubated for 30 minutes with growing amounts of GB1 up to 50 µM (vehicle: DMSO; 1%). To discard any fluorescence or inner filter effect that could interfere with the assay results, the spectra of GB1 dissolved in 10 mM sodium phosphate, 0.1 mM GTP was determined in a Thermo Scientific Evolution 201 spectrophotometer. The fluorescence spectrum from 360 to 550 nm (5 nm slits) of the solution was measured with excitation at 350 nM (5 nm slits). The data from triplicate experiments were analyzed with Equigra V5 as described[17].

MTS Cell Viability Assay

HUVEC cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) assay. HUVECs (2×10^4) were seeded in 96-well plates and allowed to settle overnight before being subjected to either DMSO (0.1%) or GB1 treatments in EGM-2 for 24 h. Then, 10 µL of CellTiter 96® AQueous One Solution reagent (Promega, USA) was added to each well of a 96-well plate. Cell viability was determined 3 h post additional incubation with the reagent by measuring absorbance readings at 490 nm using the Synergy H1 multi-mode reader (Biotek, USA). Data are represented as mean ± SEM (n = 3).

Tube Formation Assay

A volume of 50 µL of Growth Factor Reduced Matrigel Basement Membrane Matrix (Corning, USA) was added in each well of a 96-well plate and incubated at 37 °C for 30 minutes. 2×10^4 HUVECs were resuspended in 100 µL of EGM-2 with the presence of either DMSO (0.1%) or GB1 and seeded onto the polymerized Matrigel. After a 9-hour incubation, the vasculature was visualized using Eclipse Ti-E Inverted Research Microscope (Nikon, USA) and the complexity of the vascular network was analyzed by Image J (National Institutes of Health, USA). Triplicates were performed for each treatment.

Trypan Blue Exclusion Assay

Human breast cancer cell line MDA-MB-231 was maintained in DMEM supplemented with 10% FBS (Gibco). MDA-MB-231 cells (2×10^5) were first seeded and starved overnight in serum-free DMEM followed by 24-h pre-treatment with DMSO (0.1%) or GB1 at various doses (10 µM, 1 µM or 0.1 µM) in DMEM containing 1% FBS. Thereafter, cells were washed once in PBS, trypsinized and pelleted by centrifugation. The resulting cell pellet was resuspended in 500 µL of serum free DMEM which were diluted 1:3 in 0.04% Trypan Blue solution (Sigma, USA). 10 µL of the final mixture was transferred to a hemocytometer (Celeromics Technology, France) the number of viable cells was counted at 10x magnification using Leica DM IL LED Inverted Microscope (Leica Microsystems, Germany). Triplicates were performed for each treatment.

Migration and Invasion Assay

Transwell migration and invasion assays were carried out in 24-well (6.5mm in diameter, 8 μm pore size) Transwell Permeable Support inserts (Corning Costar, Sigma, USA) precoated with rat tail collagen (Corning, USA) or with Matrigel (Corning, USA) (300 μg/mL in 0.01 M Tris (pH 8.0) and 0.7% NaCl coating buffer) for migration and invasion assays respectively.

Human breast cancer cell line MDA-MB-231 was maintained in DMEM supplemented with 10% FBS. MDA-MB-231 cells (2×10^5) were first seeded and starved overnight in serum-free DMEM followed by 24 h pre-treatment with DMSO (0.1%) or GB1 at various doses (10 μM, 1 μM or 0.1 μM) in DMEM containing 1% FBS. Following which, 5×10^4 DMSO or GB1 pre-treated cells were resuspended in serum-free DMEM and were seeded onto the upper chamber of the transwell. DMEM supplemented with 10% FBS was added in the lower chamber as a chemoattractant. 5 h post-incubation, cells that had migrated through the membrane were fixed and stained with DAPI dye. Cells in six random optical fields (10x magnification) were then visualized using Eclipse Ti-E Inverted Research Microscope (Nikon, Japan) to determine the total number of migrated/invaded cells. Triplicates were performed for each treatment.

Crystallography

Synthetic DNA encoding for the DARPin D1 was gently provided by Michel O. Steinmetz. The DARPin protein was purified as described[18]. Calf brain tubulin was purified as described[19] and lyophilized for storage. Tubulin was resuspended in 15 mM MES, pH 6.8, 0.5 mM EGTA, 0.1 mM GTP, equilibrated through a G-25 column and centrifugated at 50 K rpm / 10 min at 4 ºC to remove aggregates. The T1D complex was formed by mixing tubulin and DARPin in a 1:1.5 ratio in a buffer containing 0.5 mM $MgCl_2$ and a final GTP concentration that was 5 times tubulin concentration. The mixture was incubated 15 minutes on ice and subsequently gel filtrated in a Superdex 200 column in buffer 15 mM Pipes, pH 6.8, 0.2 mM EGTA, 0.3 mM $MgCl_2$, 0.2 mM GTP. T1D complex was concentrated to 20 mg/mL and flash freeze in liquid nitrogen. T1D complex was crystallized by hanging drop vapor diffusion at 20 ºC in 0.1 M Bis-Tris Methane pH 5.5, 0.20 Ammonium Sulfate, 18-22% PEG 3350, mixing 1 μL of the complex with 1 μL of the precipitant solution. Suitable T1D crystals were exchanged into reservoir solutions containing 2 mM GB1 and soaked for 15 to 30 minutes. Soaked crystals were cryoprotected into a cryo-solution containing 20% glycerol and flash cooled in liquid nitrogen. T1D-GB1 data were collected at beamline XALOC at ALBA synchrotron (Spain). Images were indexed and processed using XDS[20], and scaled using AIMLESS[21]. Molecular replacement was performed with PHASER[22] using the previously determined structure (PDB 4drx) as a search model. Structure was completed with cycles of manual building in COOT[23] and refined in PHENIX[24]. Figures were prepared in PyMol (Schrödinger). Data collection and refinement statistics are given in **Table S2**. The atomic coordinates have been deposited in the Protein Data Bank www.rcsg.org (PDB 7ALR).

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Table S1. NMR Data for Gatorbulin-2 (**1b**) in DMF-d_7 (600 MHz)

		conformer 1					conformer 2	
unit	C/H no.	δ_H (J in Hz)	δ_C, mult[a]	COSY	HMBC	NOESY	δ_H (J in Hz)	δ_C, mult[a]
DhAla	1		166.5, qC					165.6, qC
(A)	2		136.7, qC					136.6, qC
	3a	6.15, s	102.1, CH_2	H-3b	1, 2	H-3b, NH, H-2 (Pro)	6.22, s	101.7, CH_2
	3b	5.03, s		H-3a	1, 2	H-3a, NH, H-2 (Pro), for conf. 1 only: H-2 (MePro), for conf. 2 only: H-5a (MePro)	5.11, s	
	NH	8.79, s			1, 1 (Ala)	H-3a, H-3b, H-2 (Ala)	8.60, s	
4-Me-Pro	1		170.8, qC					170.5, qC
(B)	2	4.78, dd (7.8, 7.8)	61.4, CH	H-3a, H-3b	1, 5	H-3a, H-3b, H-4, H-5a, for conformer 1 only: H-3b (DhAla)	4.28, dd (7.8, 7.8)	61.2, CH
	3a (*proS*)	2.63, ddd (–12.2, 8.9, 7.8)	37.4, CH_2	H-2, H-3b, H-4	1, 2, 4, 5, 6	H-2, H-3b, H-4, H_3-6	2.53, ddd (–11.5, 8.4, 7.8,)	35.7, CH_2
	3b (*proR*)	1.71, ddd (–12.2, 7.8, 4.5)		H-2, H-3a, H-4	1, 2, 4, 5, 6	H-2, H-3a, H-4, H-5b, H_3-6	1.54, ddd (–11.5, 7.8, 4)	
	4	2.34, ddqdd (10.8, 8.9, 7.8, 6, 4.5)	31.1, CH	H-3a, H-3b, H-5a, H-5b, H_3-6	2, 3, 5, 6	H-2, H-3a, H-3b, H_3-6	2.51, ddqdd (9.6, 8.4, 7.5, 6, 4)	33.5, CH
	5a (*proR*)	3.89, dd (–10.4, 7.8)	53.6, CH_2	H-4, H-5b	2, 3, 4, 6	H-2, H-4, H-5b, H_3-6, for conf. 2 only: H-3 (DhAla)	4.23, dd (–9.2, 7.5)	55.6, CH_2
	5b (*proS*)	3.04. dd (–10.4, 10.8)		H-4, H-5a	2, 3, 4, 6, for conf. 2 only: 1 (DhAla)	H-3b, H-4, H-5a, H_3-6	3.24, dd (–9.2, 9.6)	
	6	1.12, d (6)	16.7, CH_3	H-4	3, 4, 5	H-3a, H-3b, H-4, H-5a, H-5b	1.10, d (6)	16.7, CH_3
Lac	1		171.4, qC					170.6, qC
(C)	2	5.42, q (6.7)	67.2, CH	H_3-3	1, 3, 1 (MePro)	H_3-3, NMe (Asn)	5.62, q (6.5)	66.8, CH
	3	1.39, d (6.7)	15.3, CH_3	H-2	1, 2	H-2, NMe (Asn)	1.38, d (6.5)	16.5, CH_3
N(α)-Me-*β*-OH-Asn	1		169.6, qC					169.5, qC
(D)	2	3.68, d (9.4)	68.5, CH	H-3,	1, 2, 4	H-3, 3-OH, H-4, NHa, H-4NHb, NMe, NH (Ala)	3.99, dd (10.3, 7.7)	66.9, CH
	3	4.88, br	67.8, CH	H-2, 3-OH		H-2, NMe, H_3-3 (Ala), NH (Ala)	4.77, br	68.3, CH
	3-OH	6.41, br		H-3		H-2	6.48, br	
	4		173.9, qC					not determined
	NMe	3.20, s	38.6, CH_3		2, 1 (Lac)	H-2, NH (Ala), H-2 (Lac), H_3-3 (Lac)	3.18, s	37.4, CH_3
	4-NHa	7.70, s		4-NHb	3	H-2	7.55, s	
	4-NHb	7.24, s		4-NHa	3	H-2	7.23, s	
Ala	1		172.8, qC					171.5, qC
(E)	2	4.17, qd (6.4, 4.5)	51.5, CH	H_3-3, NH	1, 3, 1 (Asn)	H_3-3, NH, NH (DhAla)	4.42, qd (7.2, 7.1)	49.6, CH
	3	1.41, d (6.4)	16.6, CH_3	H-2	2	H-2, NH, H-2 (Asn)	1.42, d (7.1)	15.2, CH_3
	NH	8.21, d (4.5)		H-2	2, 1 (Asn)	H-2, H_3-3	8.38, d (7.2)	

[a]Deduced from HSQC and HMBC.

Table S2. Data Collection and Refinement Statistics

	Native TD1-Gatorbulin-1 (PDB 7ALR)*
Data collection	
Space group	$P2_1$
Cell dimensions	
a, b, c (Å)	73.462, 91.292, 82.970
α, β, γ (º)	90.00, 97.437, 90.00
Resolution (Å)	49.21 – 1.93 (1.999 – 1.93)**
Total reflections	397791 (21749)
Unique reflections	81042 (4364)
Redundancy	4.9 (5.0)
Completeness (%)	99.4 (98.8)
Mn (I/sd)	13.1 (2.7)
CC_{half}	0.997 (0.931)
R_{merge}	0.064 (0.494)
R_{pin}	0.032 (0.244)
R_{meas}	0.072 (0.551)
Wilson B factor	25.1
Refinement	
Resolution (Å)	49.21 – 1.93
No. of reflections	80966
R_{work}/R_{free}	0.1677/0.2039
No. non-hydrogen atoms	8501
Protein	7994
Ligand	123
Water	384
Average B-factor	36.3
Protein	36.5
Ligand	28.4
Water	34.2
r.m.s deviation	
Bond lengths (Å)	0.011
Bond angles (º)	1.01
Ramachandran statistics (%) (allowed/favored/outliers)	98.29/1.71/0.00
Rotamer outliers (%)	0.00
Clashscore /MolProbity Overall score	2.50 / 1.03

* Data were collected from one crystal. ** Values in parenthesis are for highest-resolution Shell.

Figure S1. ^{1}H NMR spectrum of natural **1a** in DMF-d_7 (600 MHz) at 25 °C

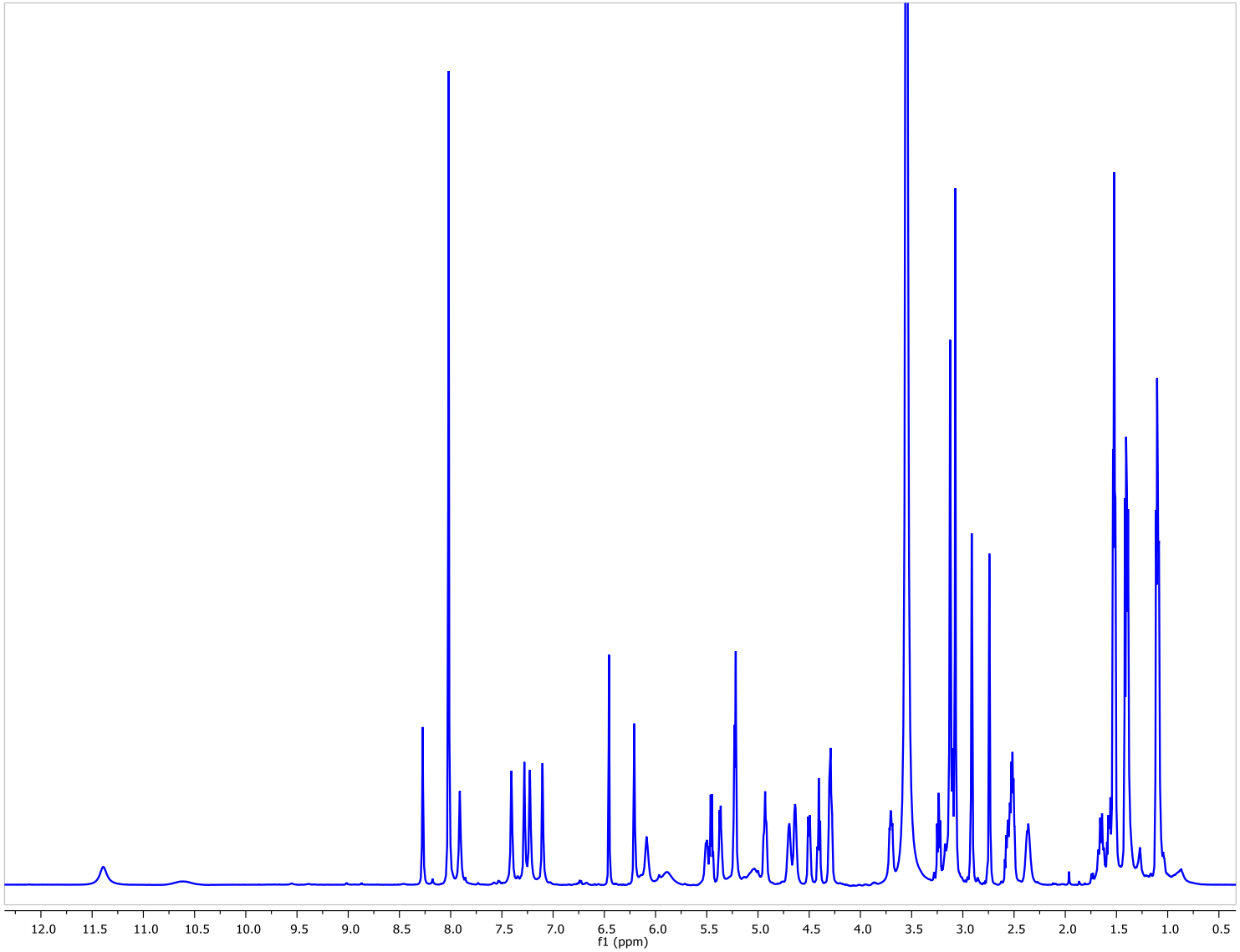


Figure S2. ^{13}C NMR spectrum of natural **1a** in DMF-d_7 (150 MHz) at 25 °C

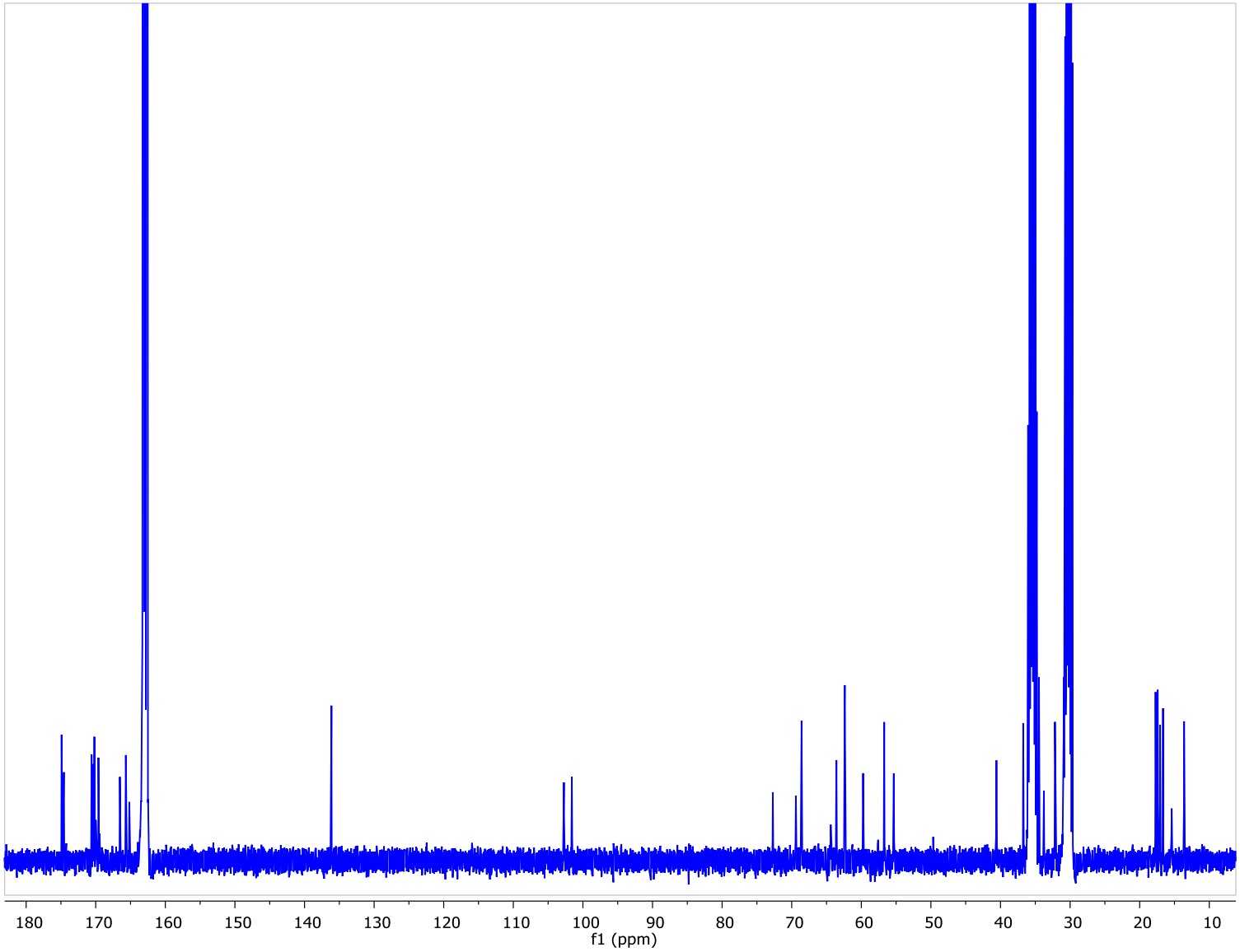


Figure S3. COSY spectrum of natural **1a** in DMF-d_7 (600 MHz) at 25 °C

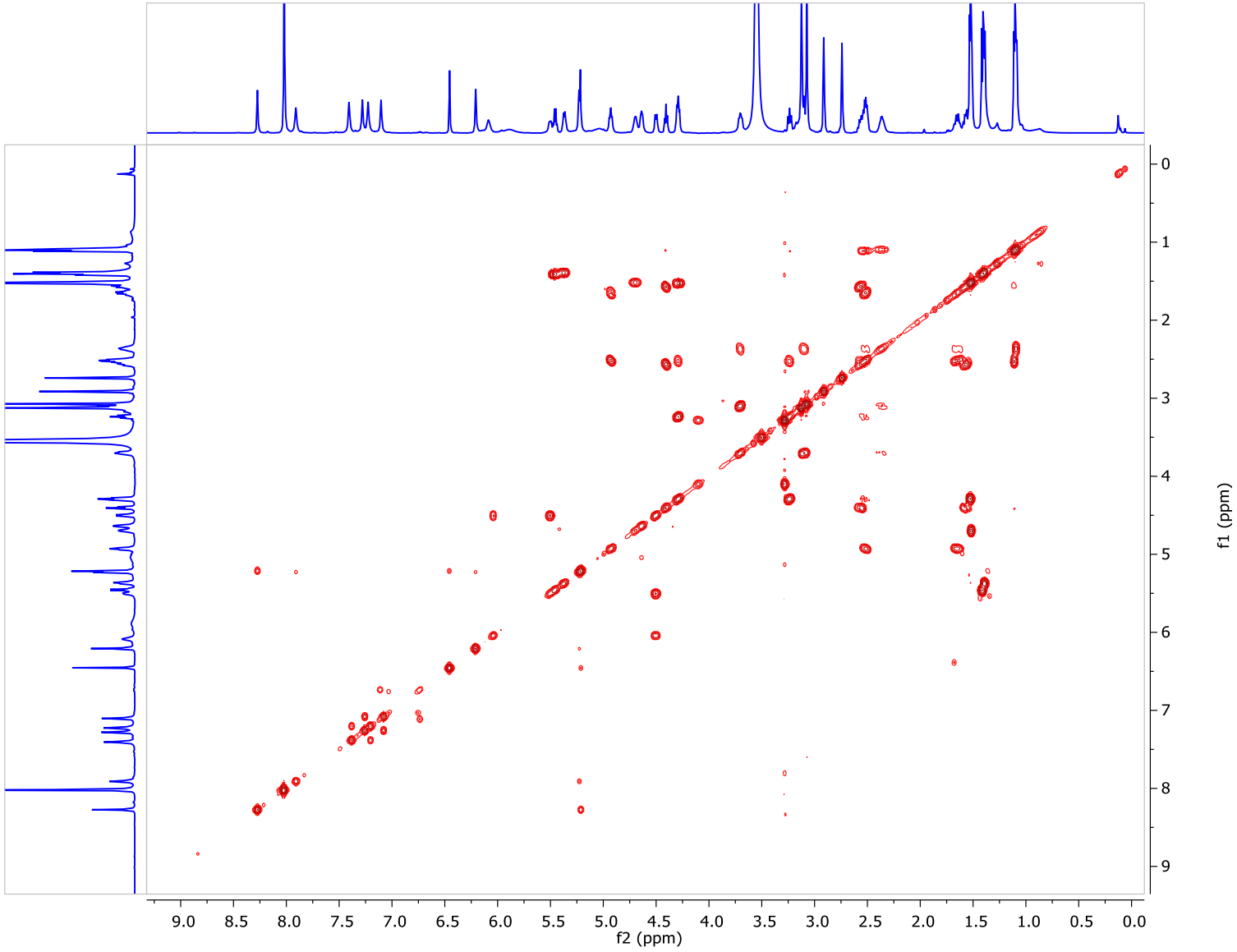


Figure S4. HMQC spectrum of natural **1a** in DMF-d_7 (600 MHz) at 25 °C

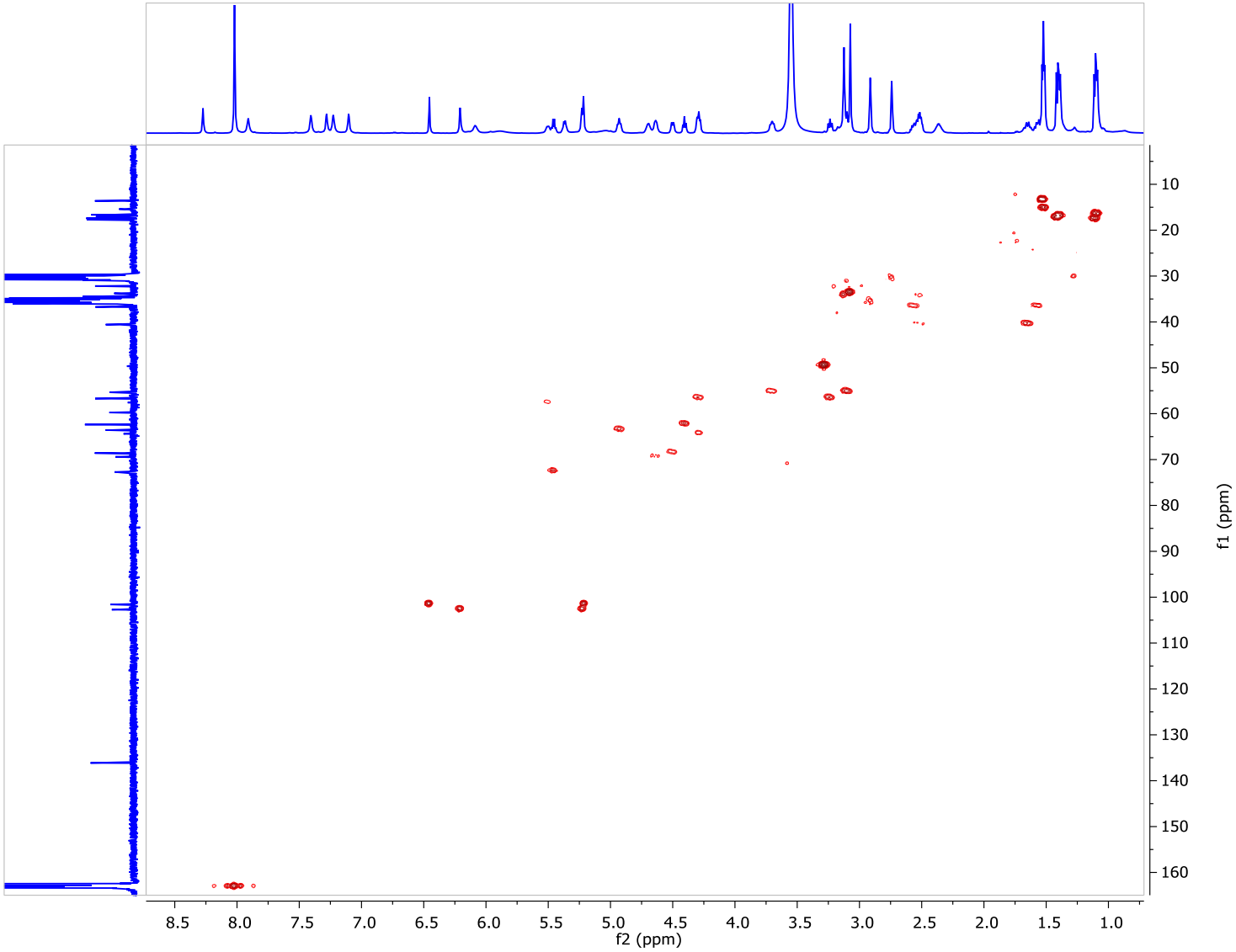


Figure S5. HMBC spectrum of natural **1a** in DMF-d_7 (600 MHz) at 25 °C

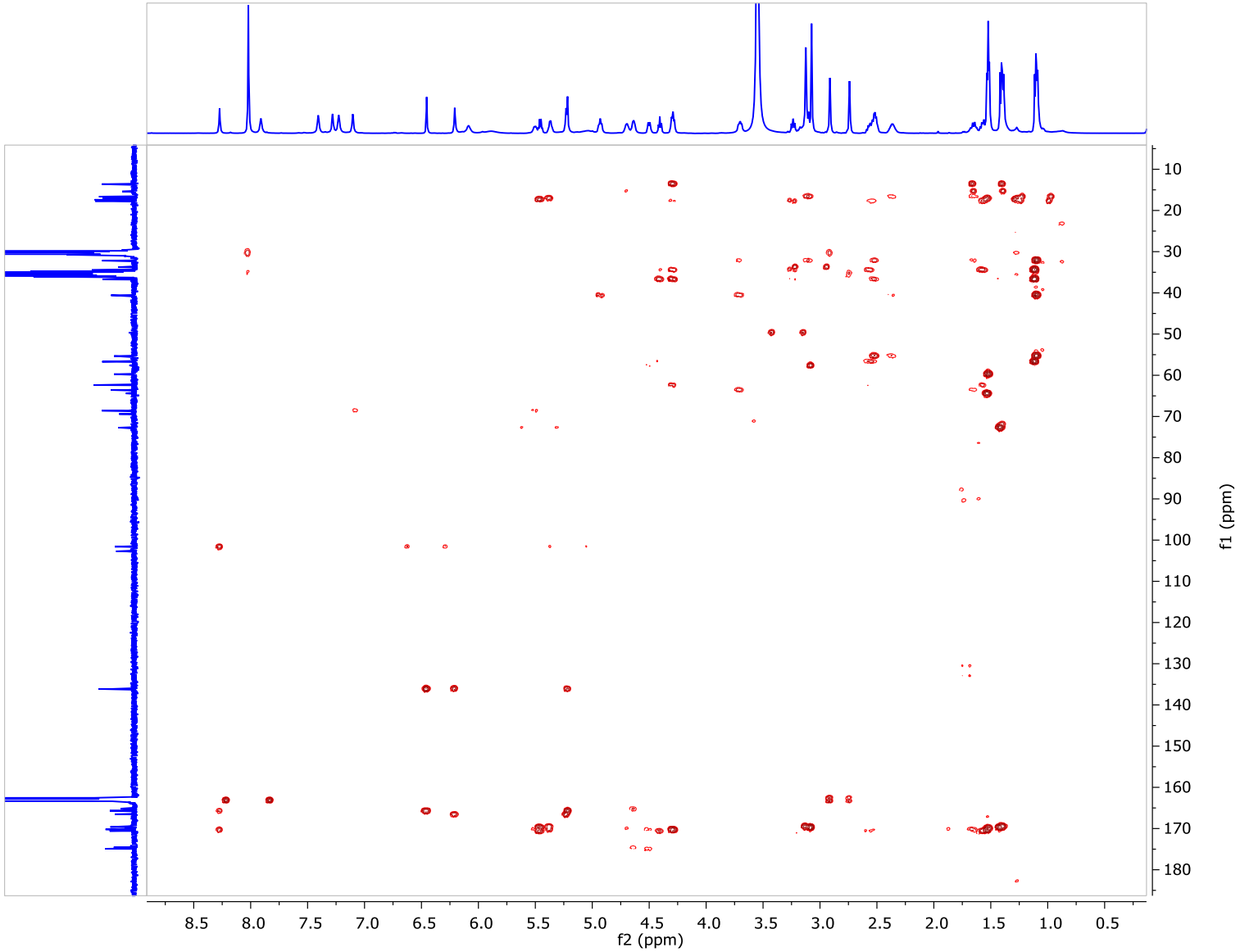


Figure S6. TOCSY spectrum of natural **1a** in DMF-d_7 (600 MHz) at 25 °C

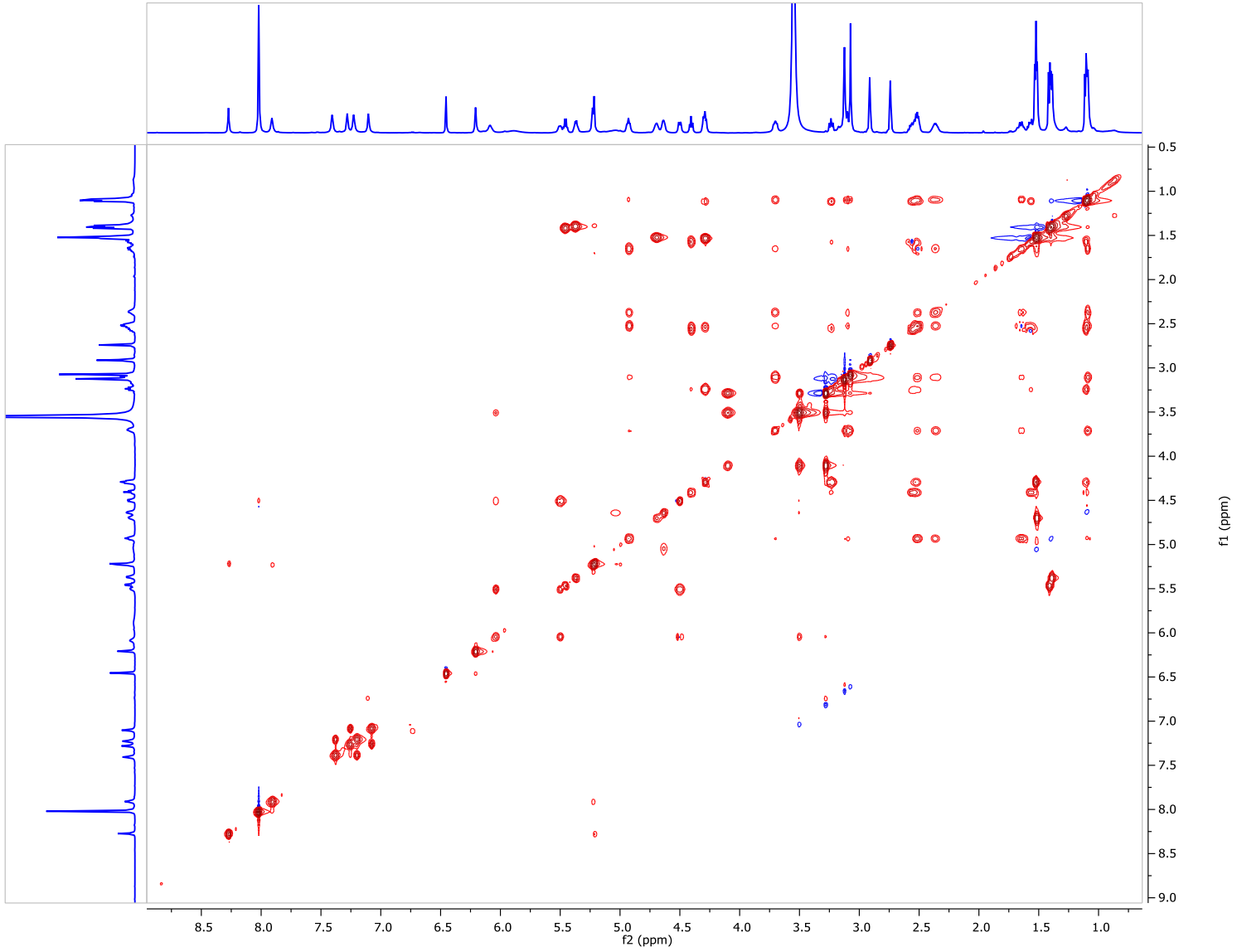


Figure S7. NOESY spectrum of natural **1a** in DMF-d_7 (600 MHz) at 25 °C

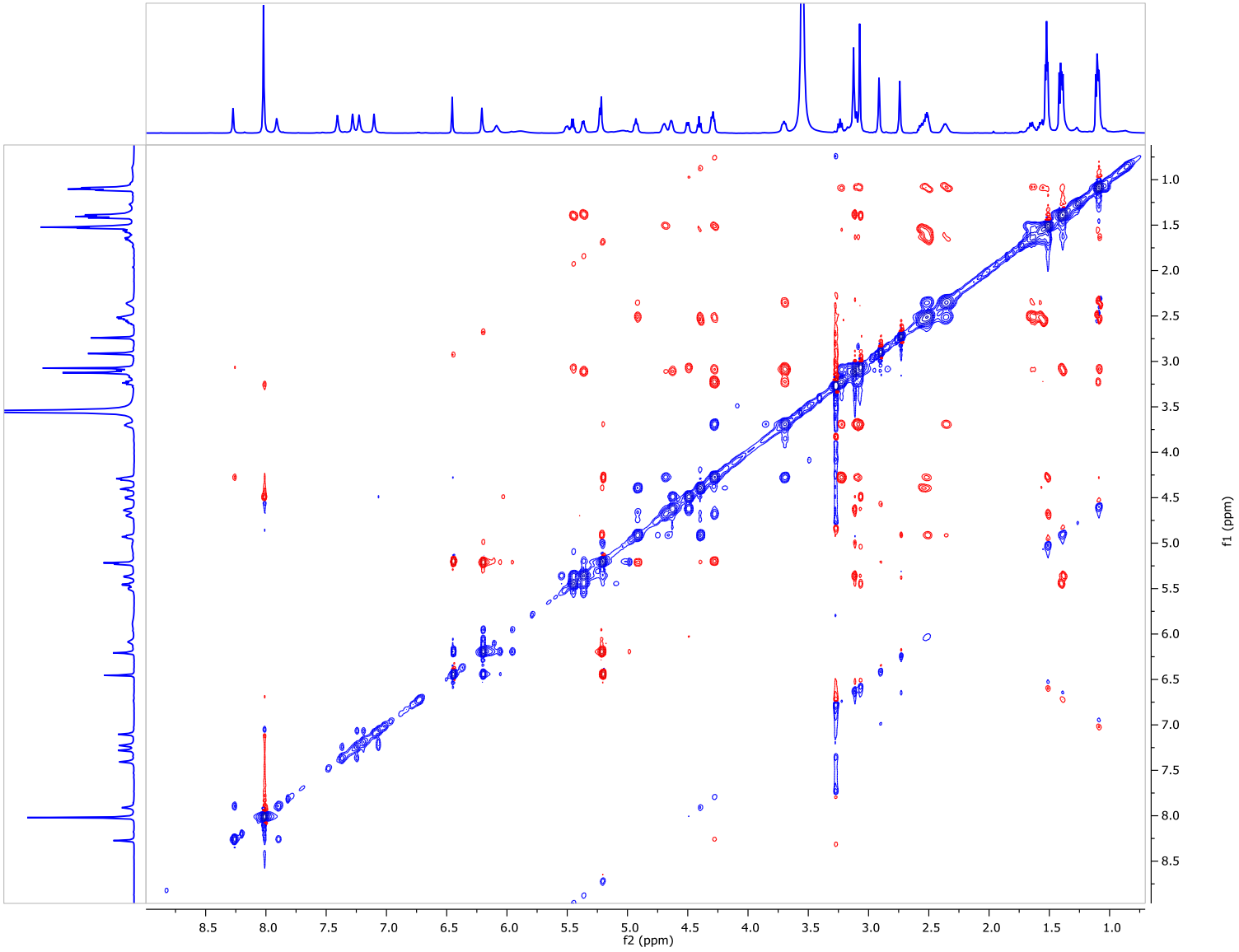


Figure S8. ^{1}H-^{15}N HSQC spectrum of natural **1a** in DMF-d_7 (500 MHz) at 25 °C

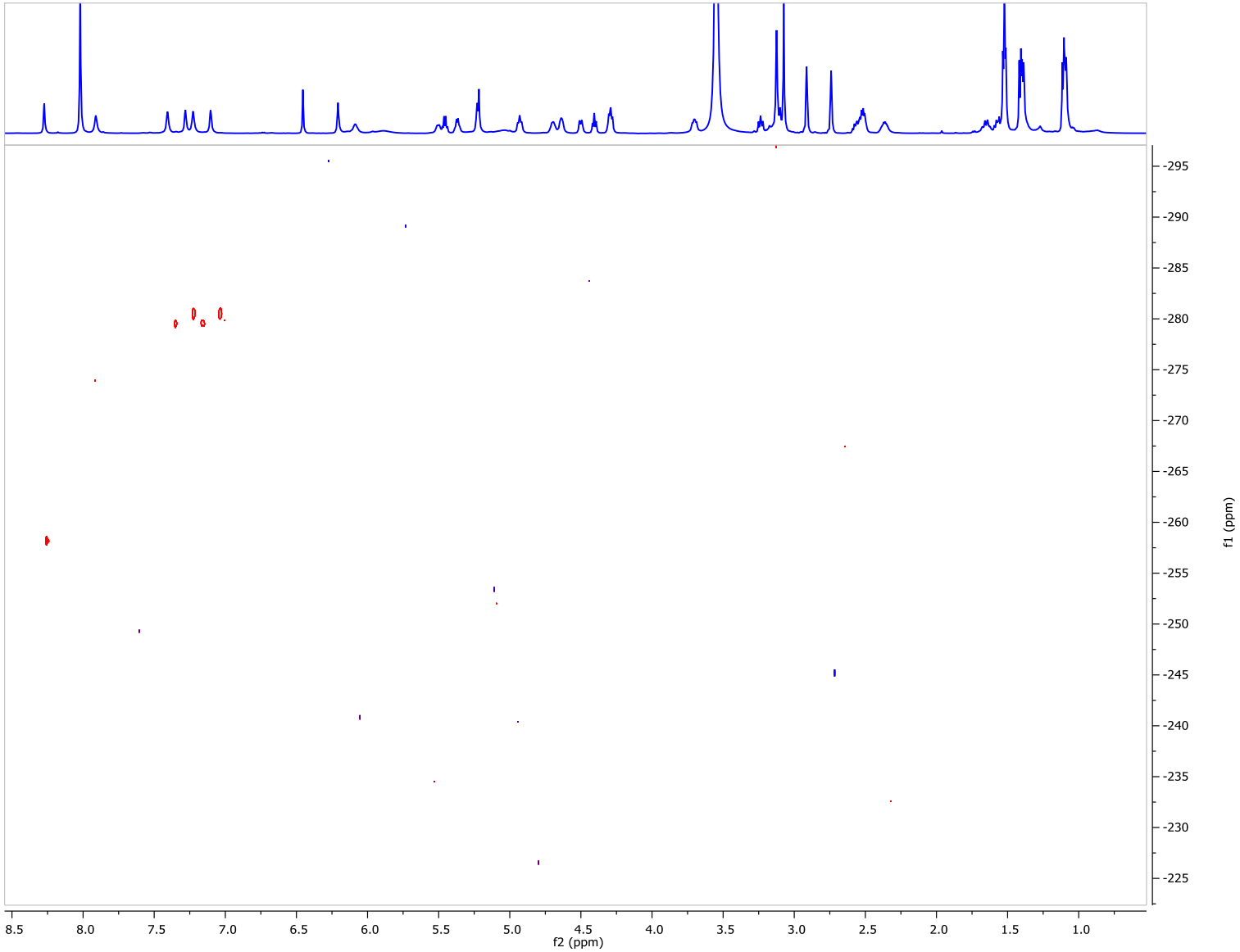


Figure S9. ^{1}H-^{15}N HMBC spectrum of natural **1a** in DMF-d_7 (500 MHz) at 25 °C

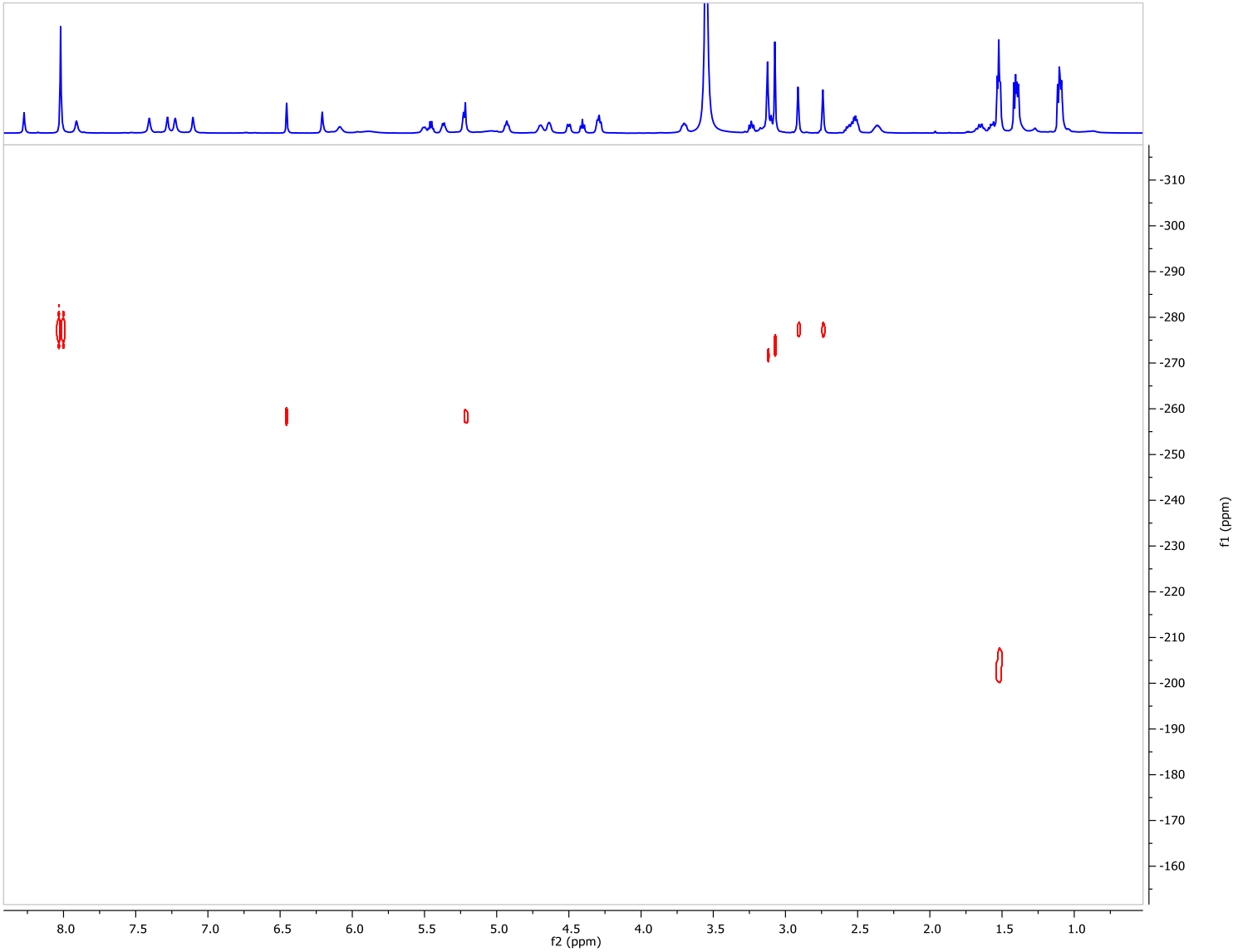


Figure S10. ^{1}H NMR spectrum of natural **1b** in DMF-d_7 (600 MHz) at 25 °C

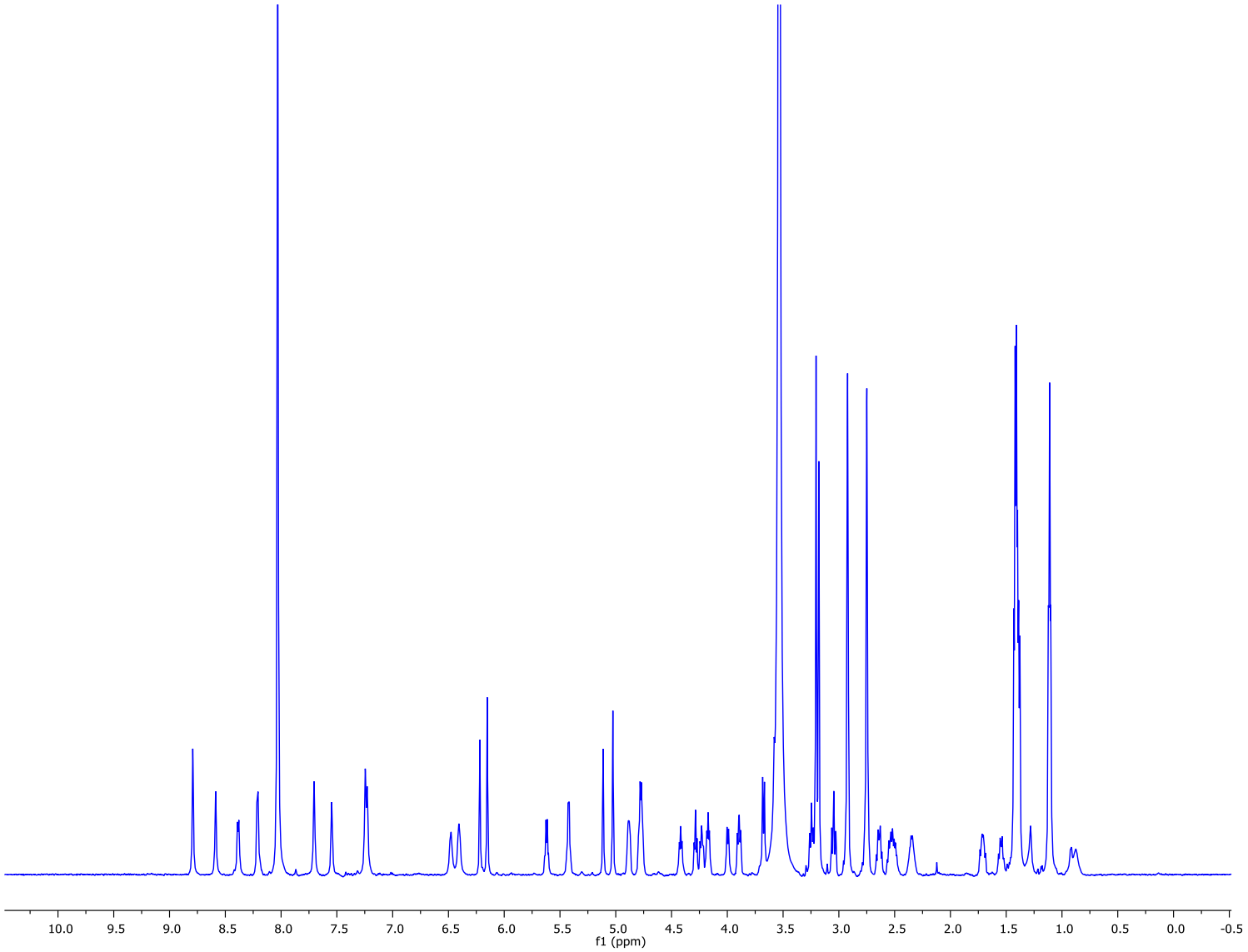


Figure S11. COSY spectrum of natural **1b** in DMF-d_7 (600 MHz) at 25 °C

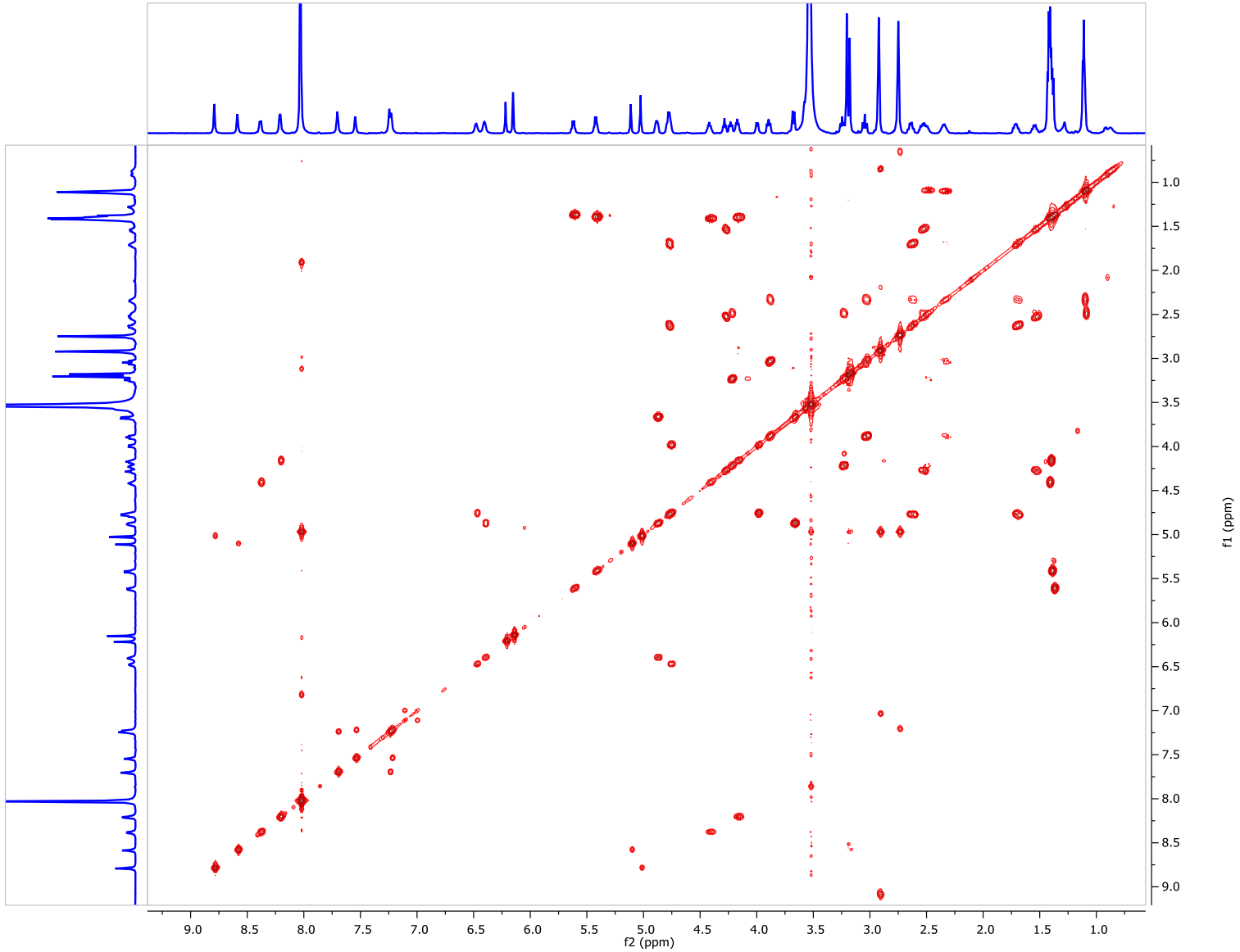


Figure S12. HSQC spectrum of natural **1b** in DMF-d_7 (600 MHz) at 25 °C

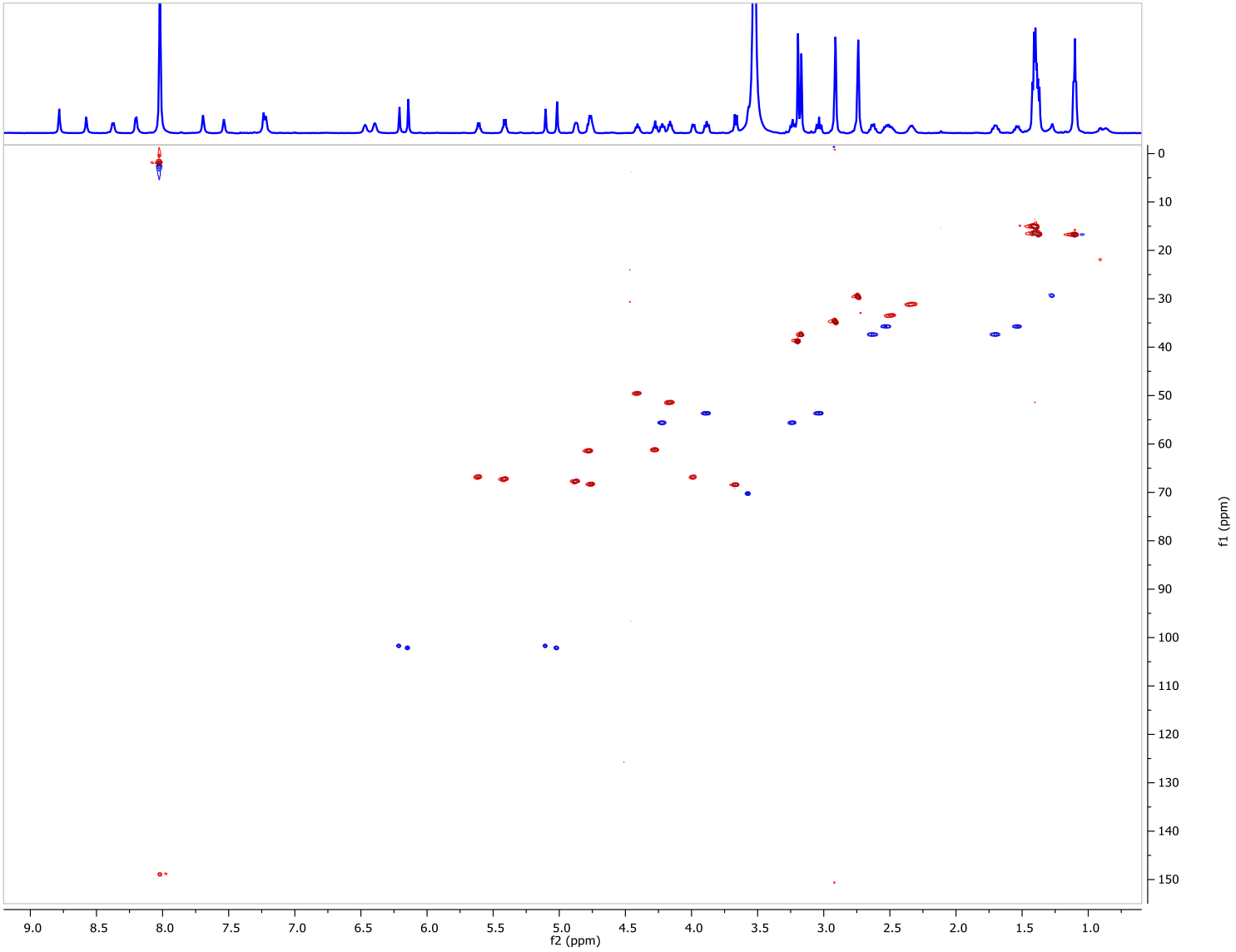


Figure S13. HMBC spectrum of natural **1b** in DMF-d_7 (600 MHz) at 25 °C

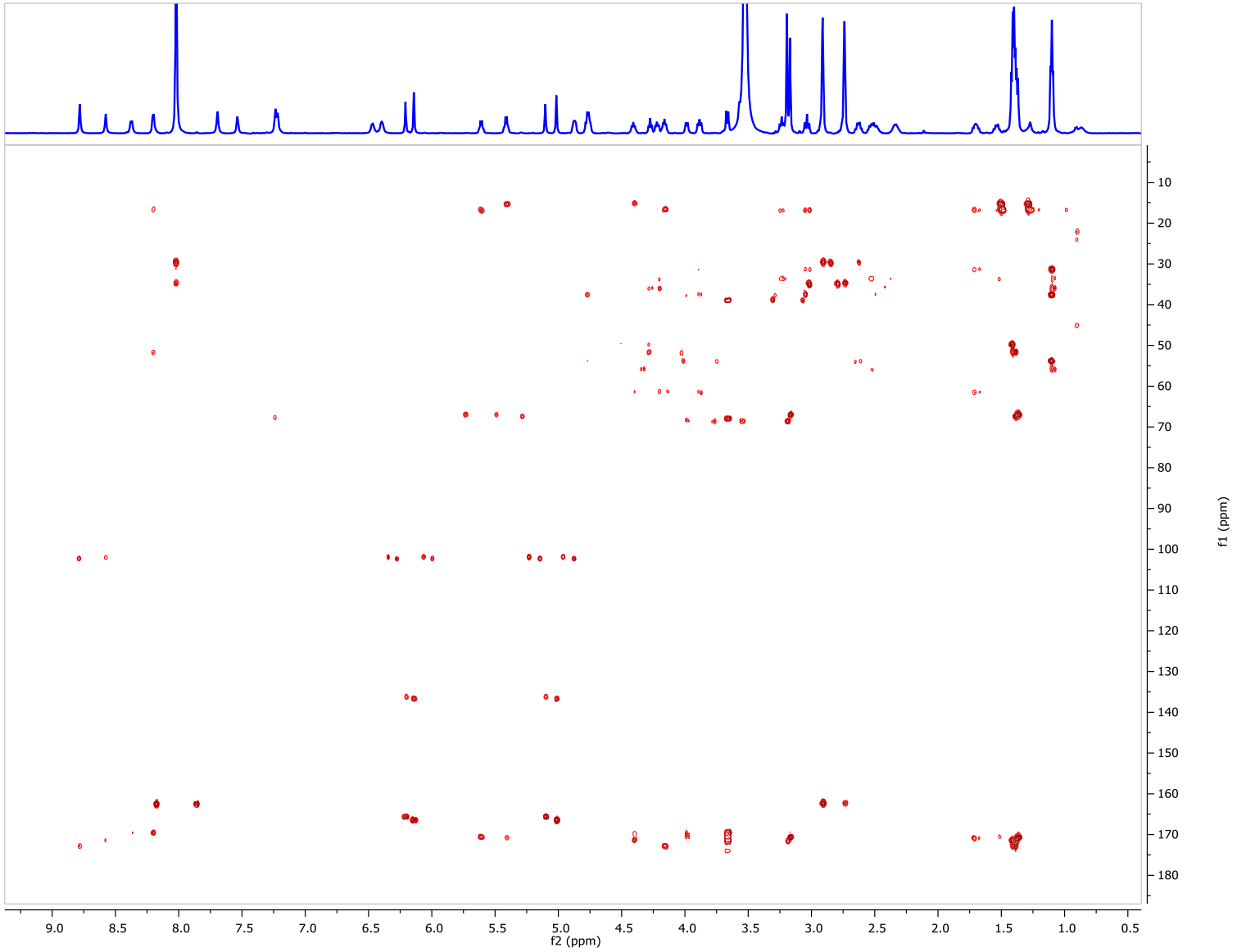


Figure S14. TOCSY spectrum of natural **1b** in DMF-d_7 (600 MHz) at 25 °C

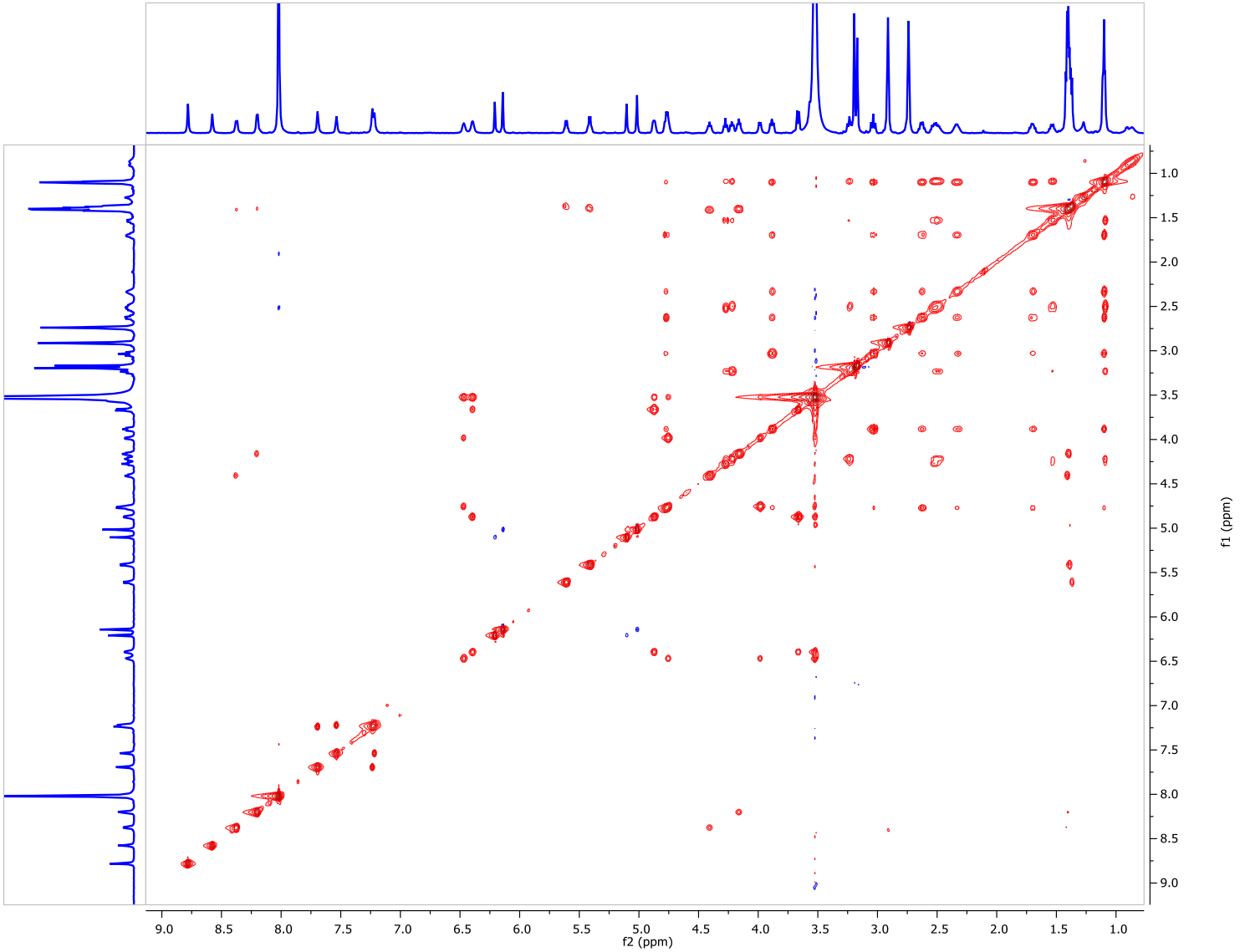


Figure S15. NOESY spectrum of natural **1b** in DMF-d_7 (600 MHz) at 25 °C

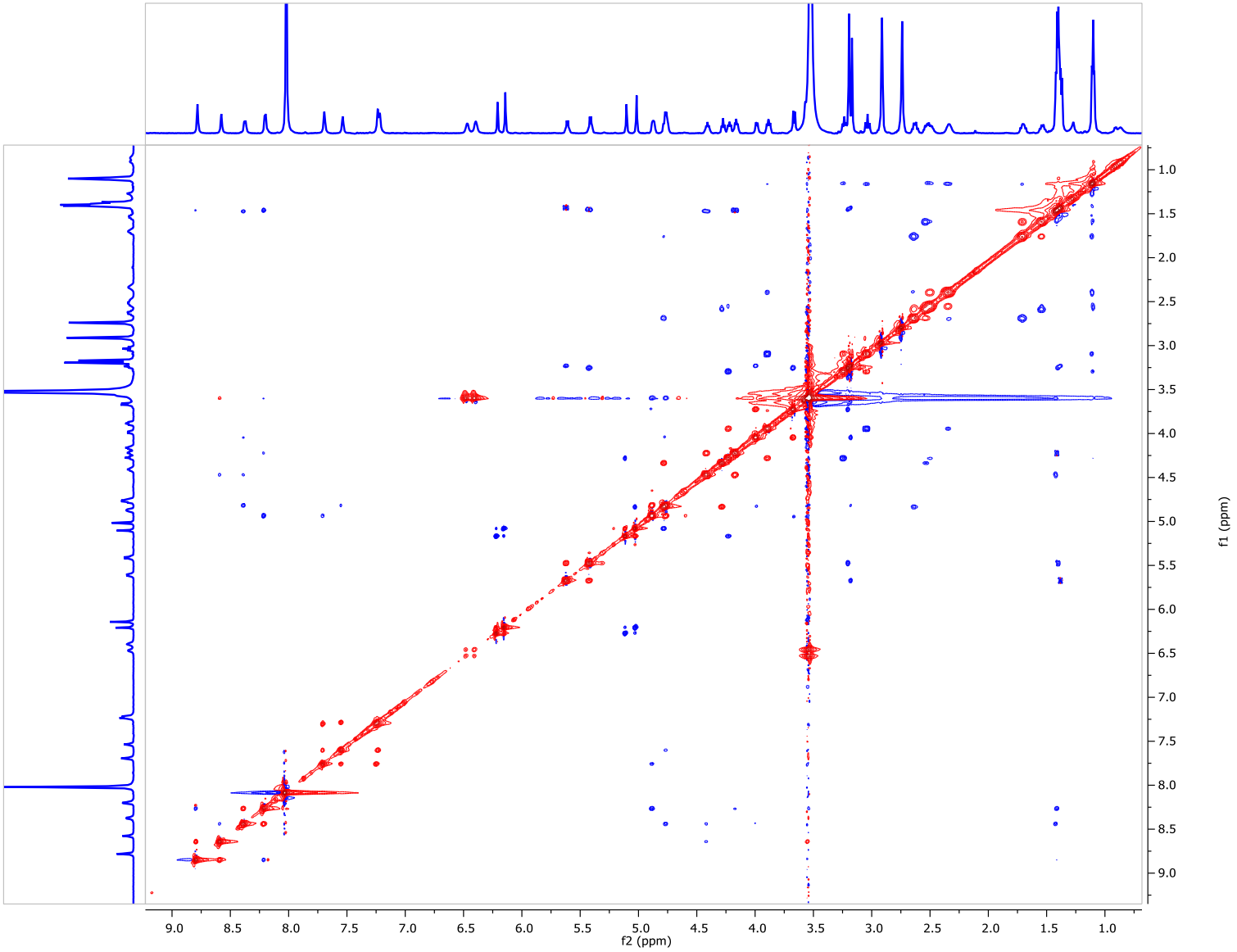


Figure S16. ^{1}H NMR Spectrum of **11** in D_2O (400 MHz) at 25 °C

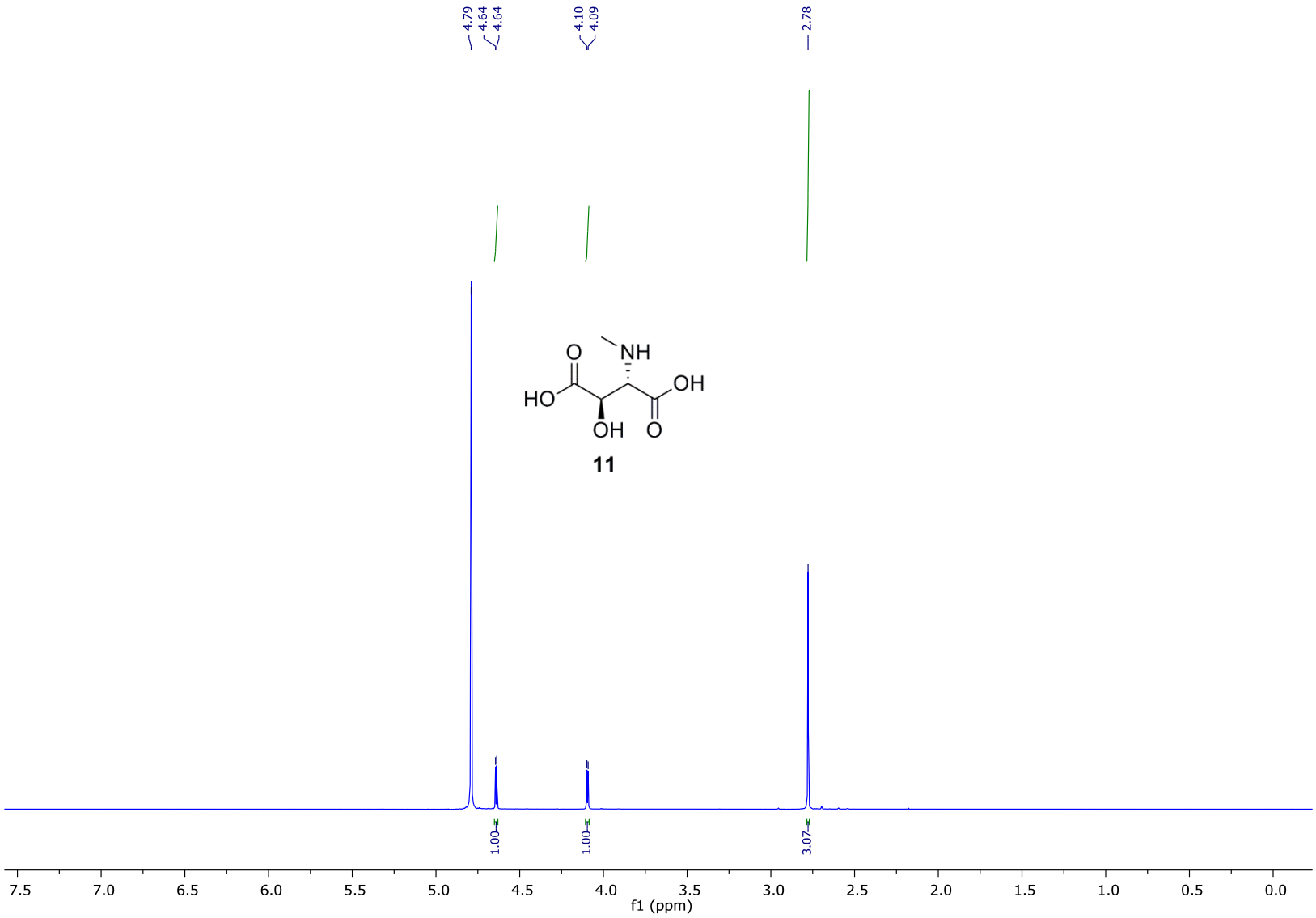


Figure S17. ^{1}H NMR Spectrum of **12** in D_2O (400 MHz) at 25 °C

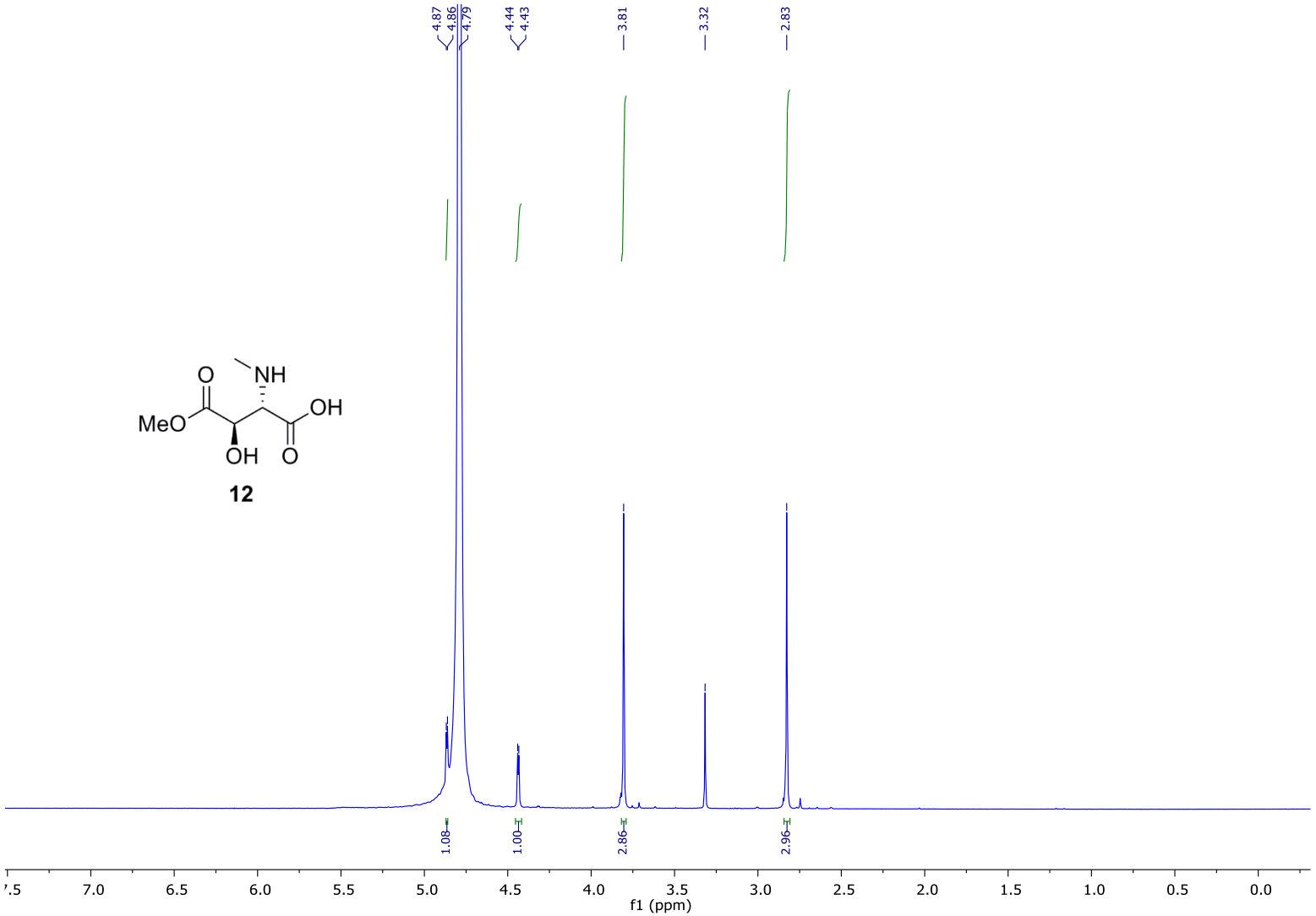


Figure S18. ^{13}C NMR Spectrum of **12** in D_2O (100 MHz) at 25 °C

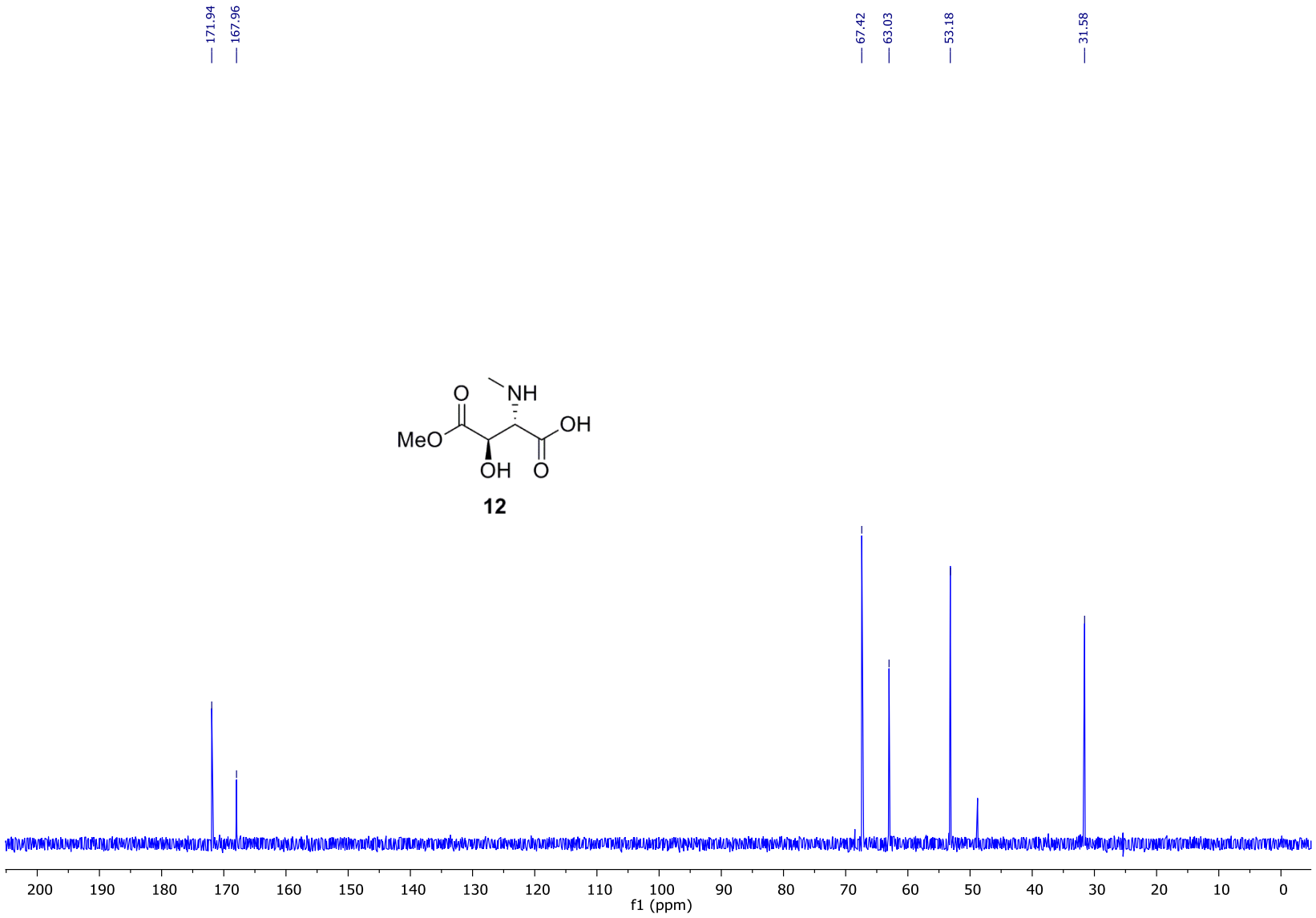


Figure S19. ^{1}H NMR Spectrum of **13** in D_2O (600 MHz) at 25 °C

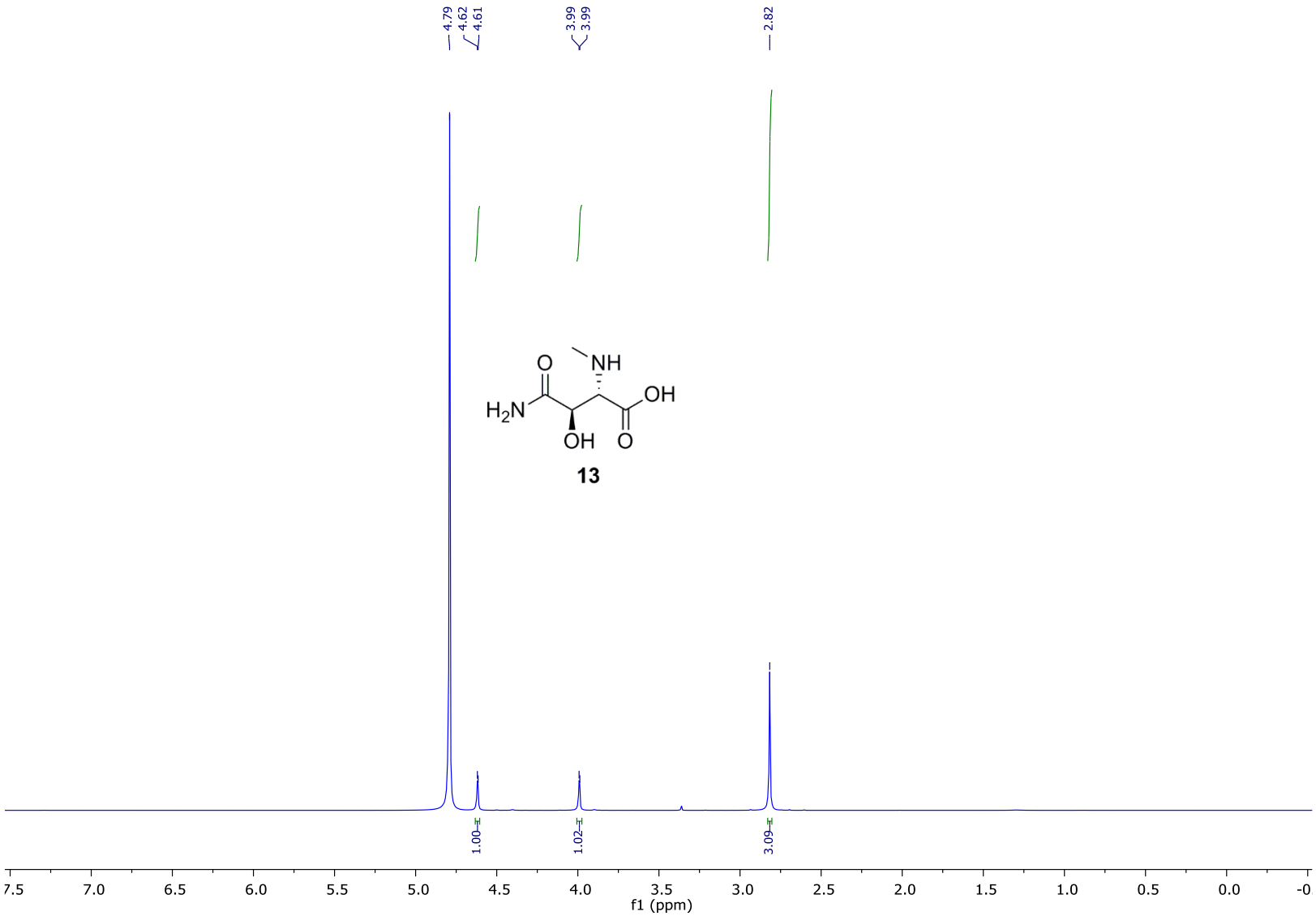


Figure S20. ^{13}C NMR Spectrum of **13** in D_2O (150 MHz) at 25 °C

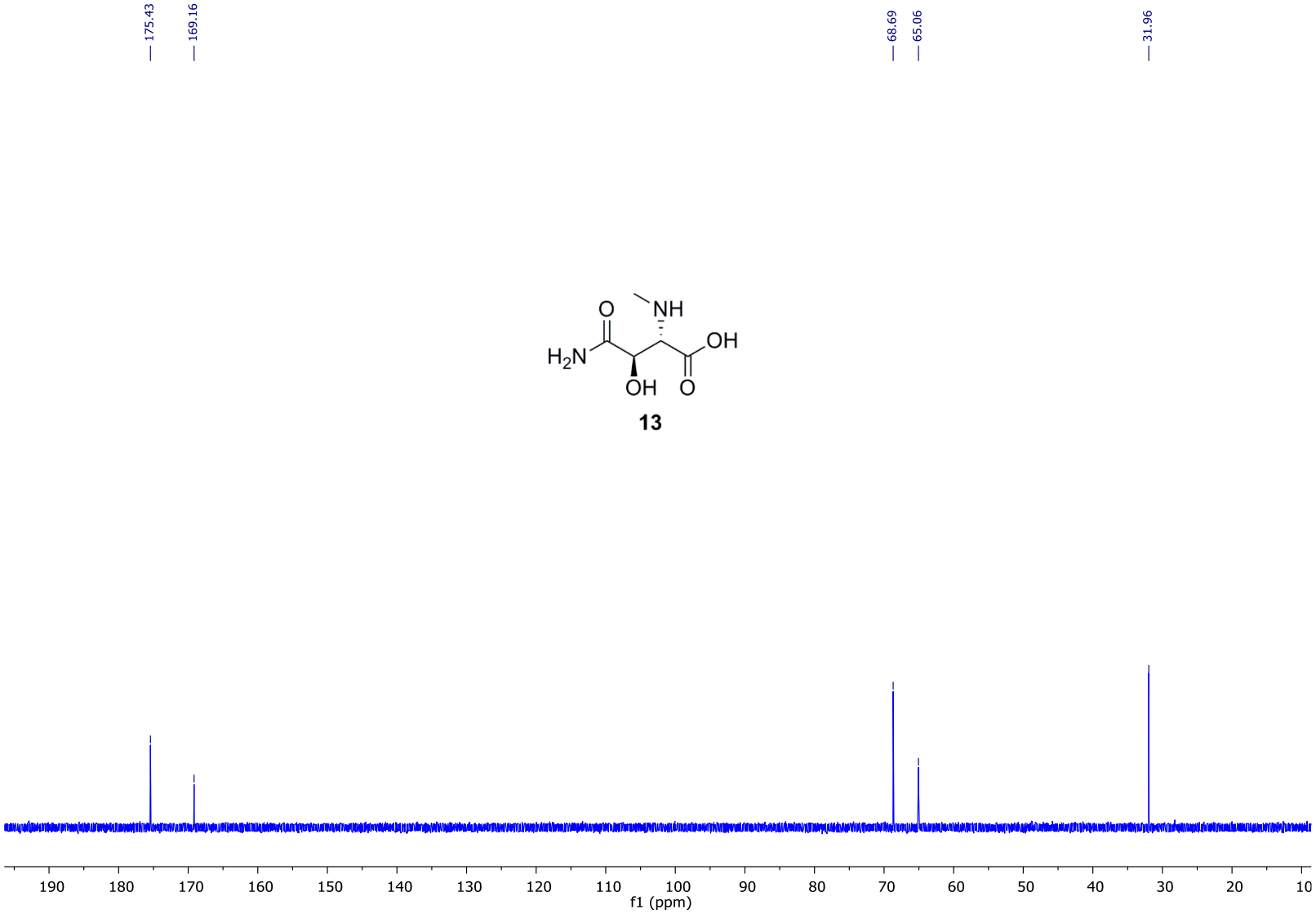


Figure S21. ^{1}H NMR Spectrum of **14** in $CDCl_3$ (400 MHz) at 25 °C

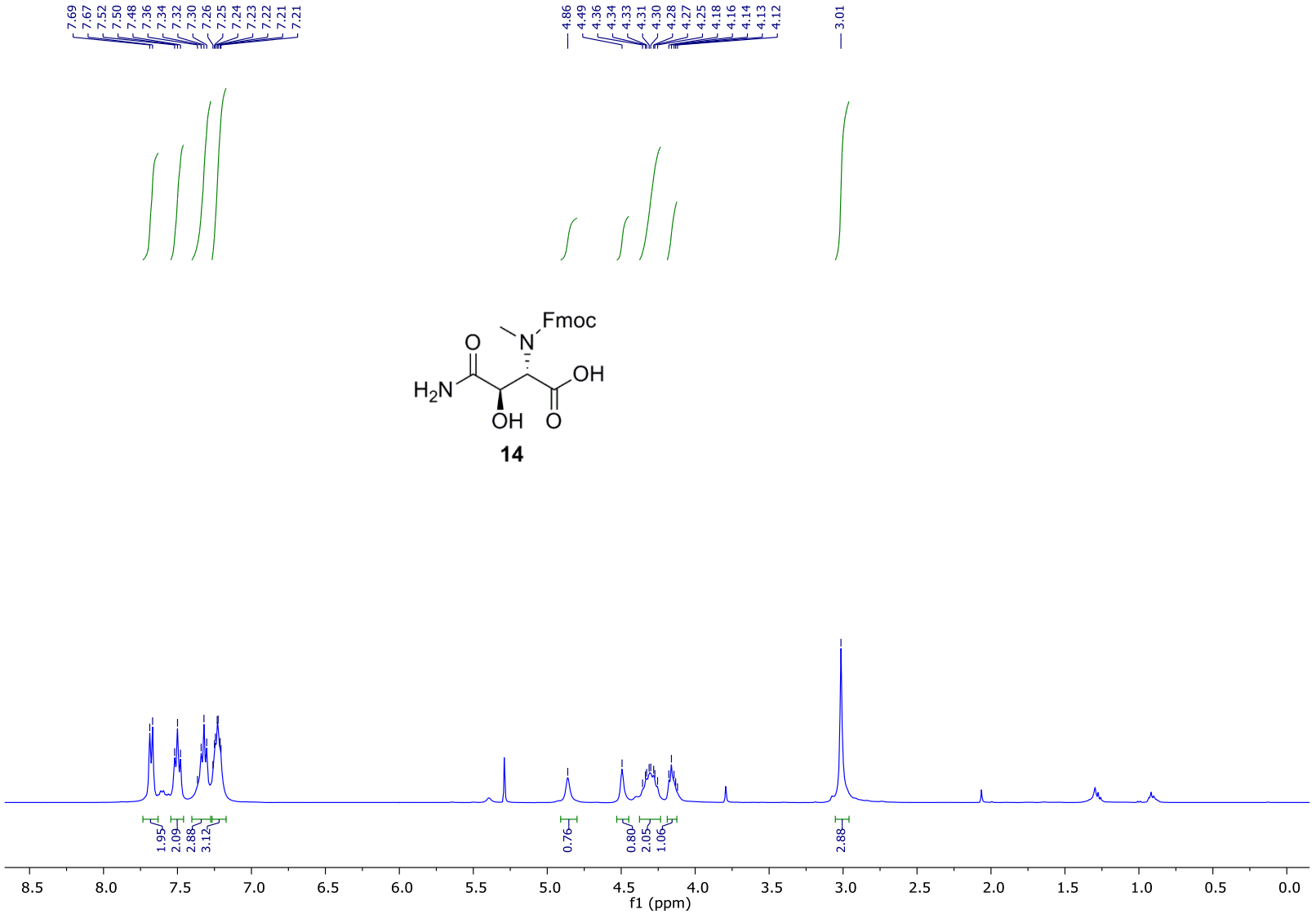


Figure S22. ^{13}C NMR Spectrum of **14** in $CDCl_3$ (100 MHz) at 25 °C

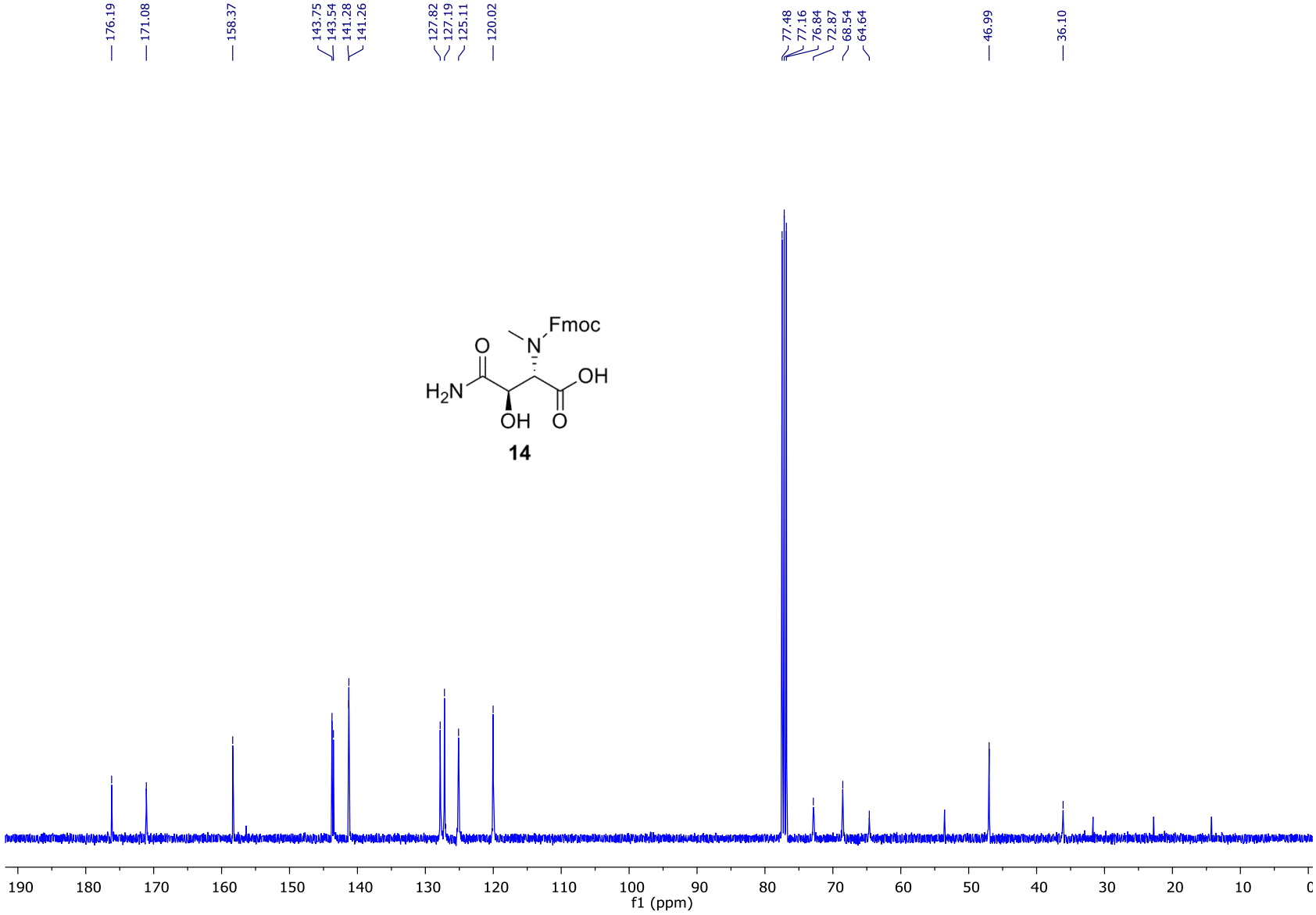


Figure S23. ^{1}H NMR Spectrum of **15** in $CDCl_3$ (400 MHz) at 25 °C

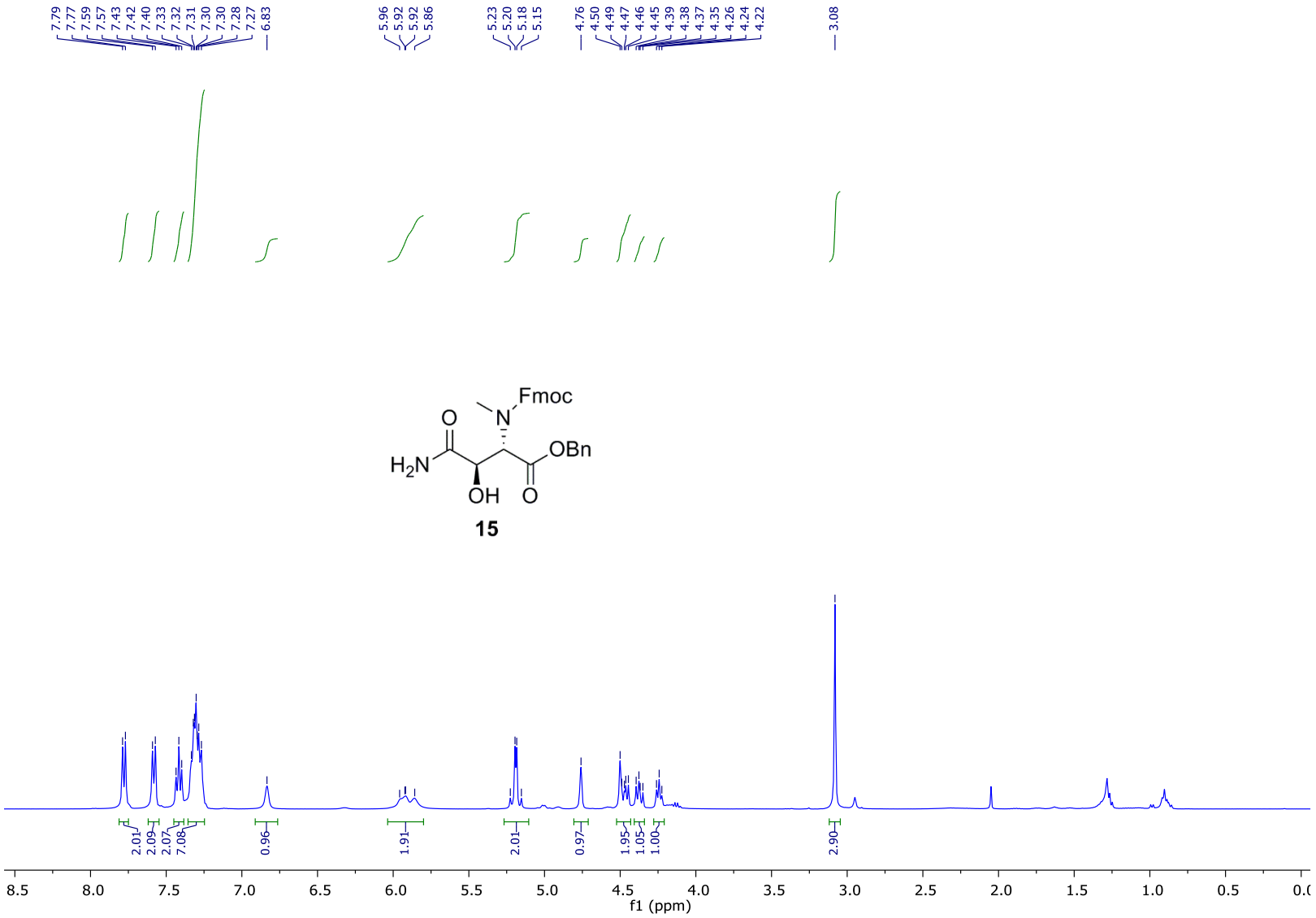


Figure S24. ^{13}C NMR Spectrum of **15** in $CDCl_3$ (100 MHz) at 25 °C

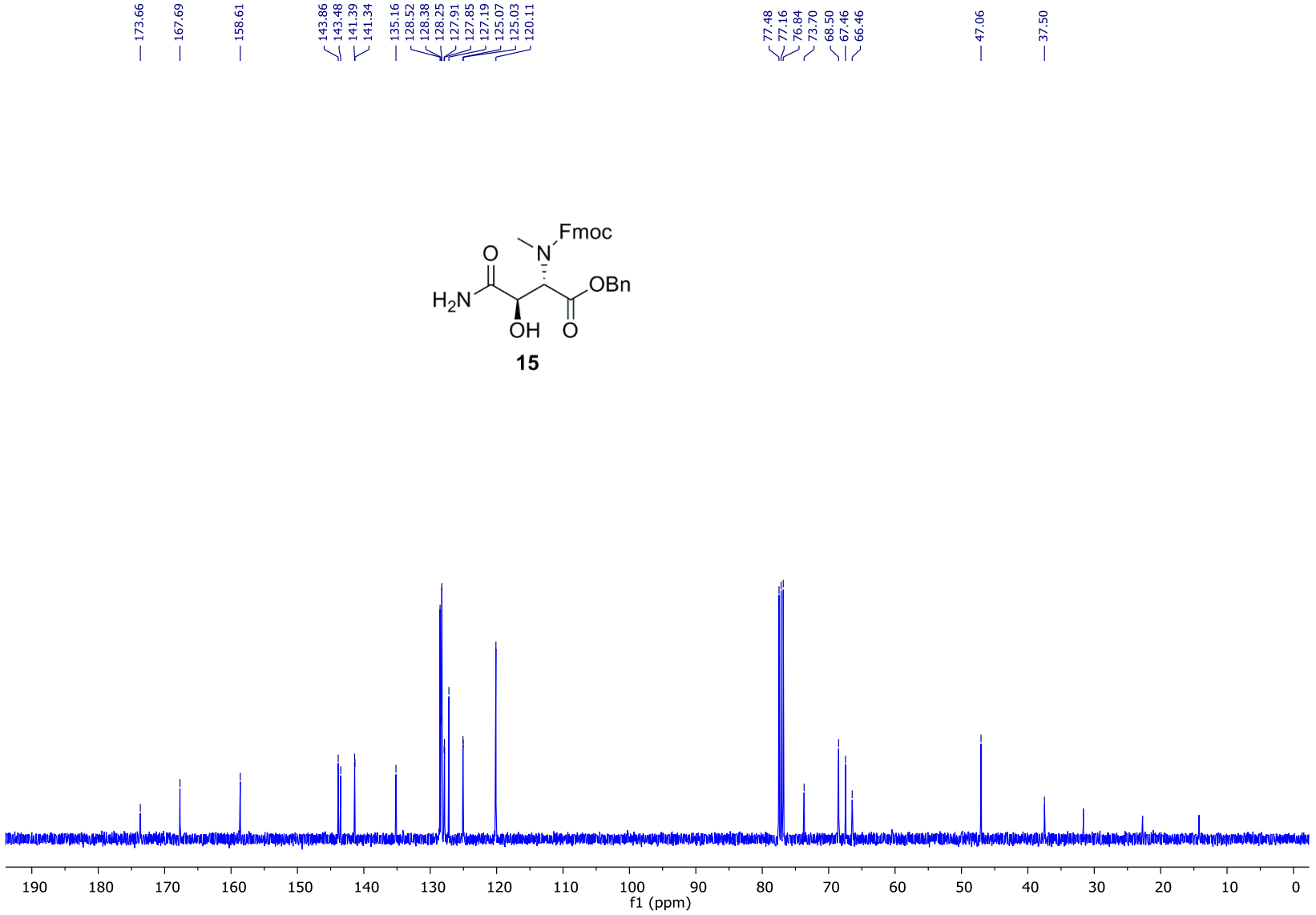


Figure S25. ^{1}H NMR Spectrum of **16** in $CDCl_3$ (400 MHz) at 25 °C

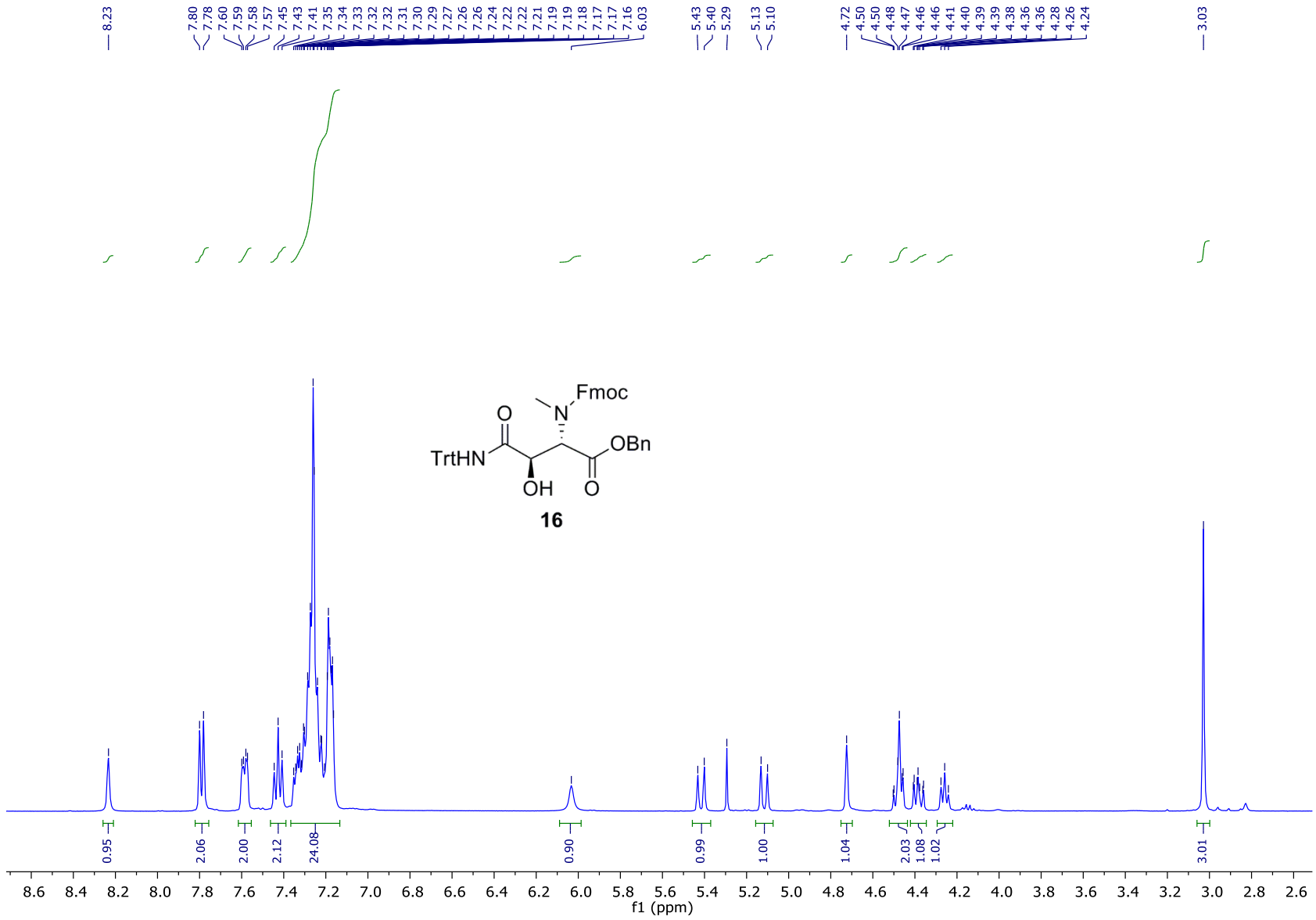


Figure S26. ^{13}C NMR Spectrum of **16** in $CDCl_3$ (100 MHz) at 25 °C

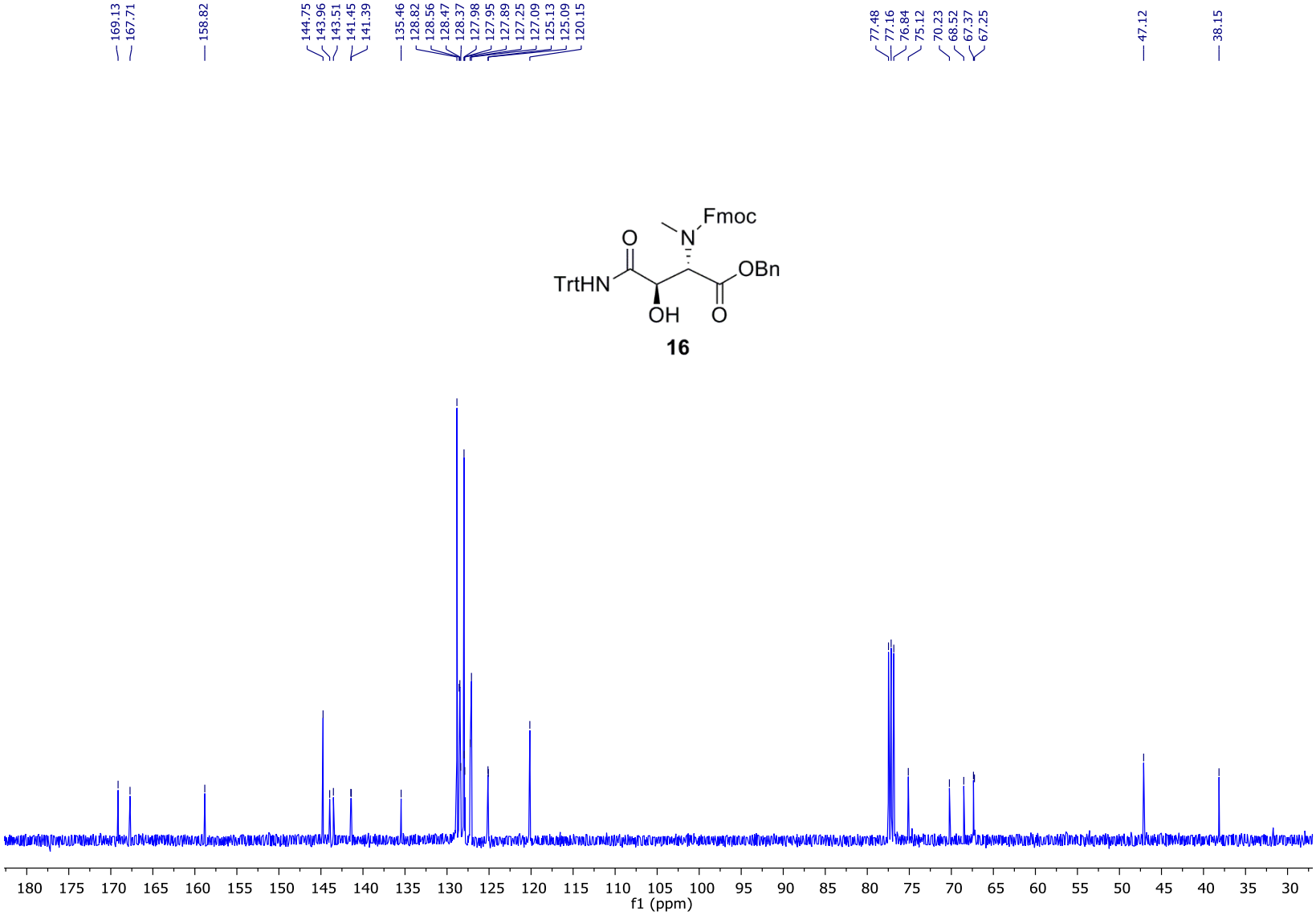


Figure S27. ^{1}H NMR Spectrum of **17** in $CDCl_3$ (400 MHz) at 25 °C

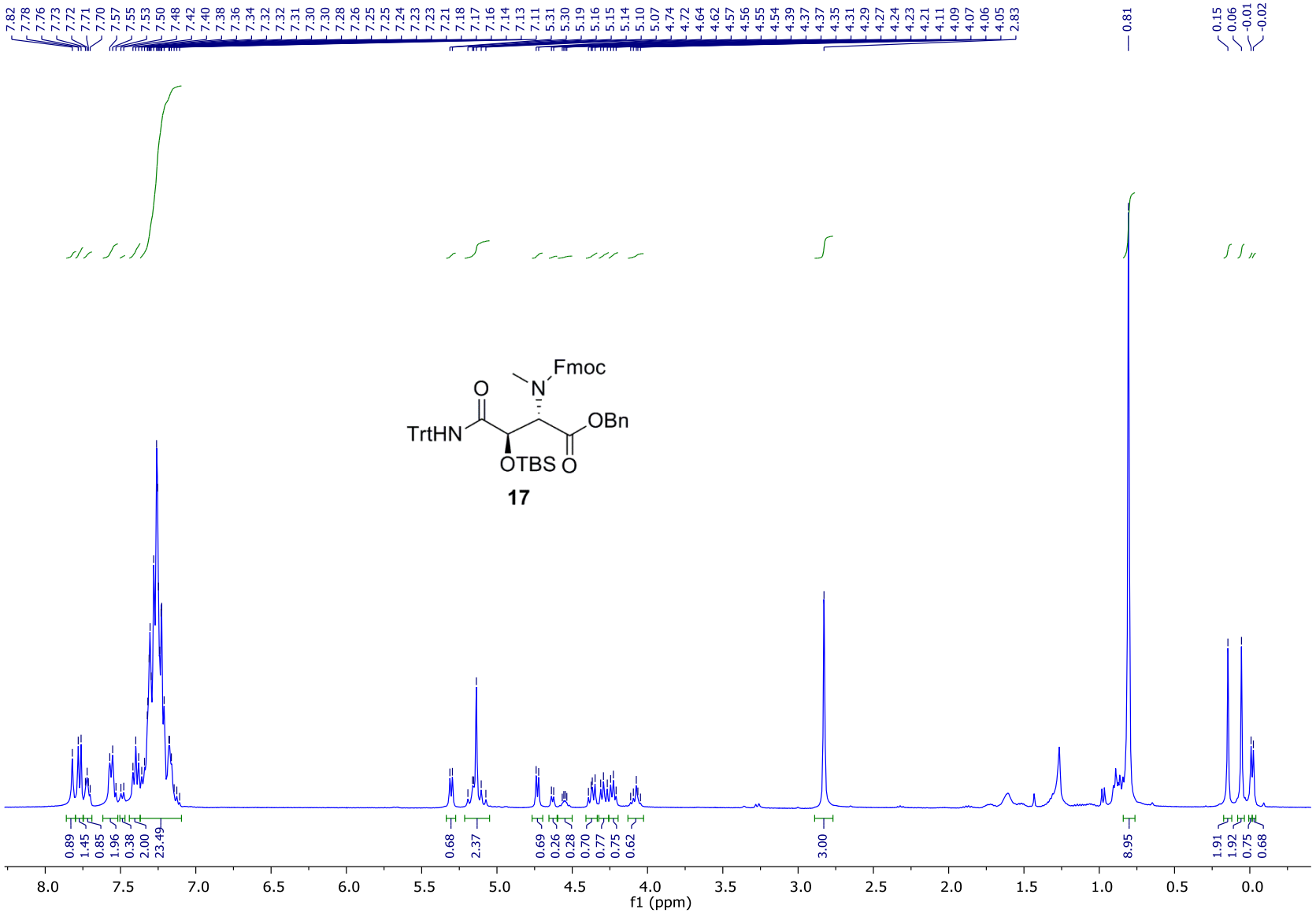


Figure S28. ^{13}C NMR Spectrum of **17** in $CDCl_3$ (100 MHz) at 25 °C

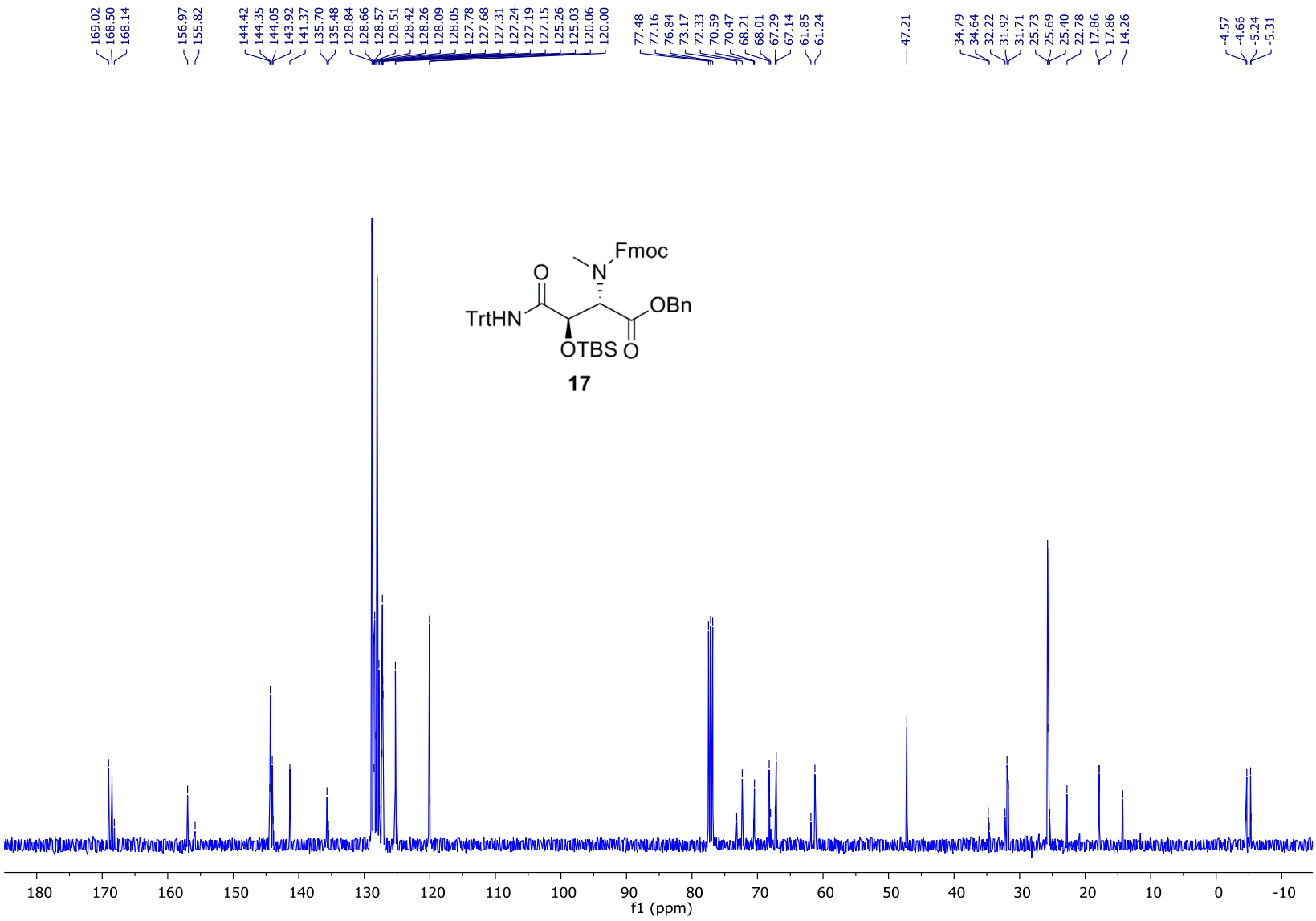


Figure S29. ^{1}H NMR Spectrum of **4** in $CDCl_3$ (400 MHz) at 25 °C

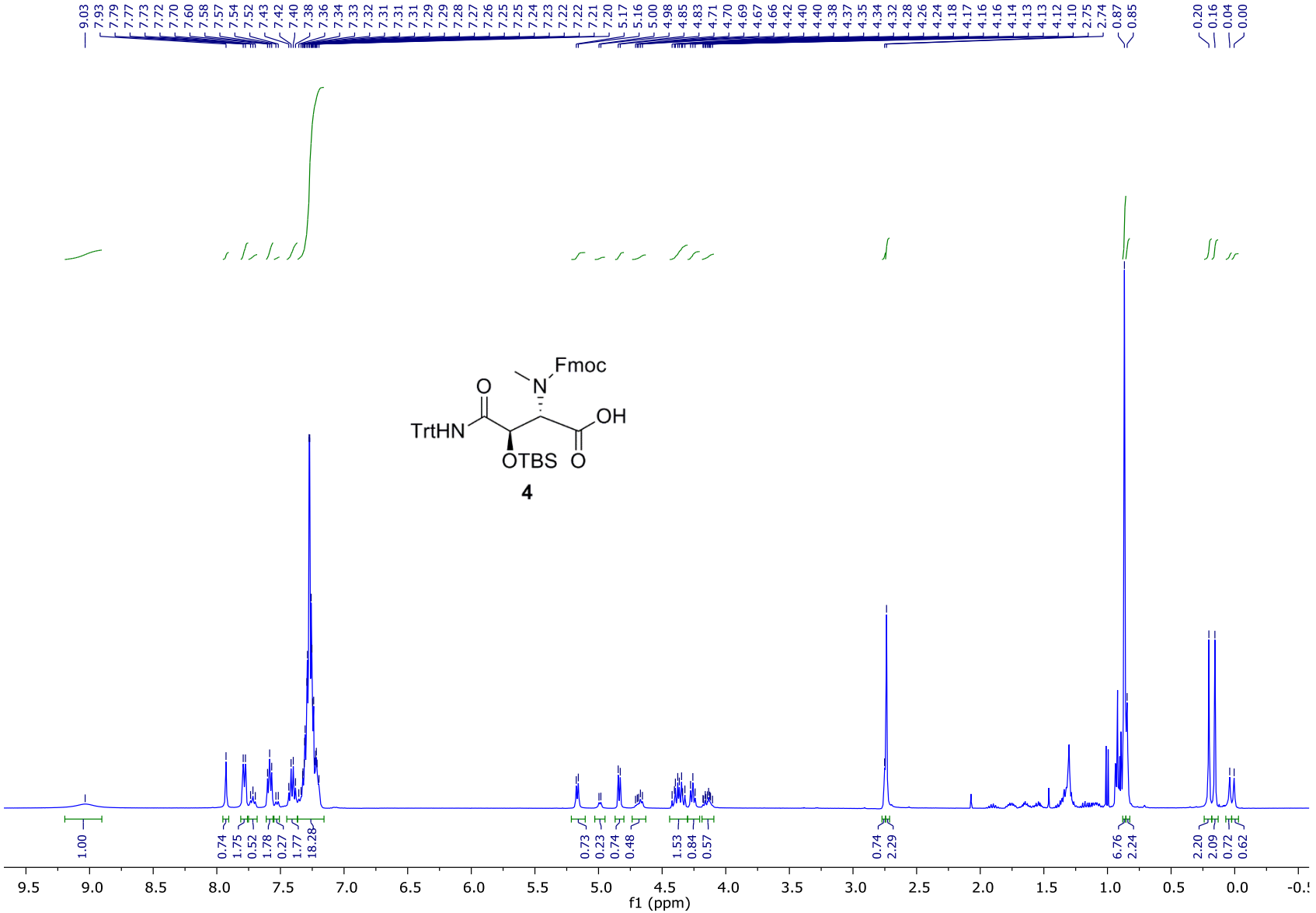


Figure S30. ^{13}C NMR Spectrum of **4** in $CDCl_3$ (100 MHz) at 25 °C

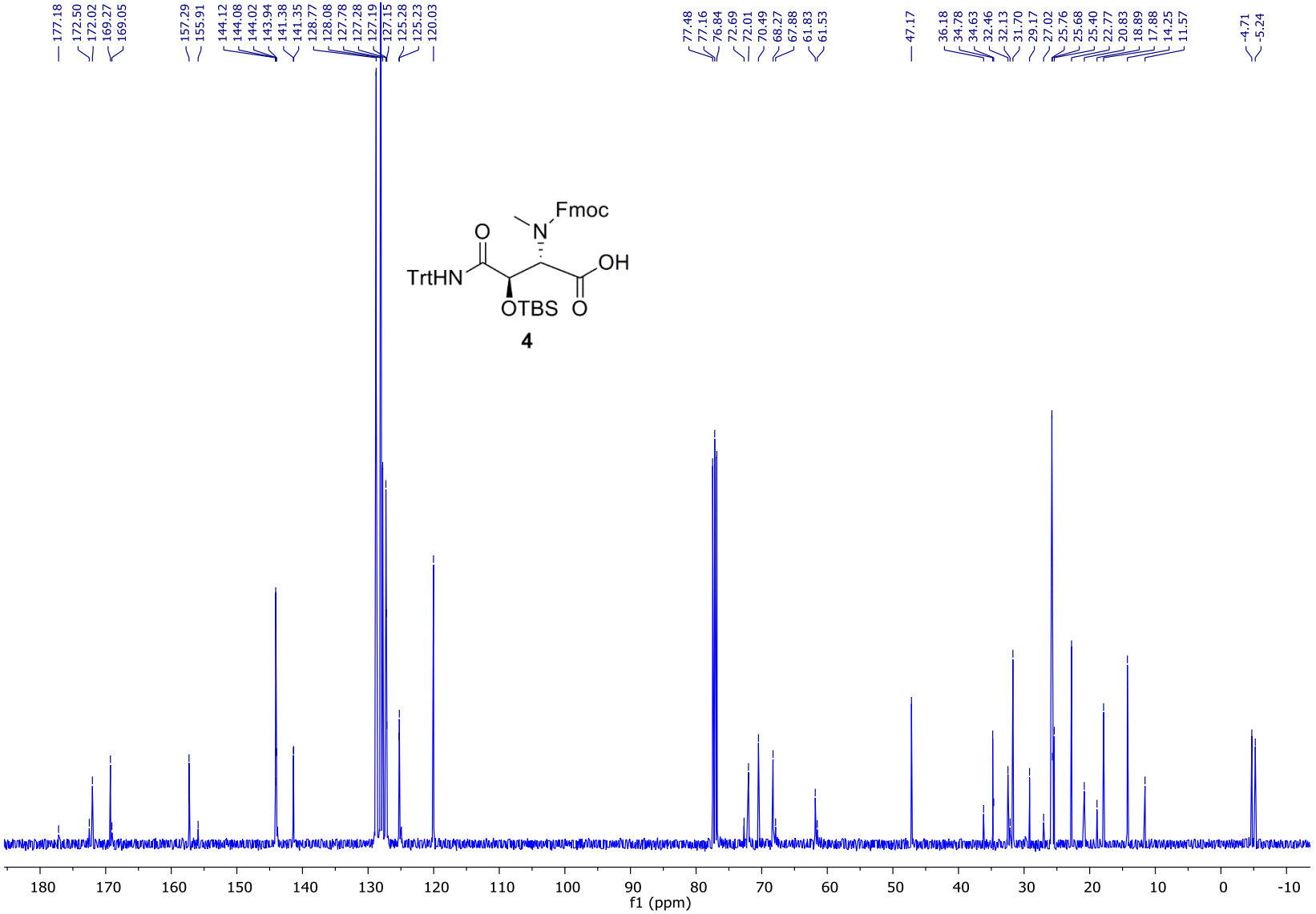


Figure S31. ^{1}H NMR Spectrum of **19** in $CDCl_3$ (400 MHz) at 25 °C

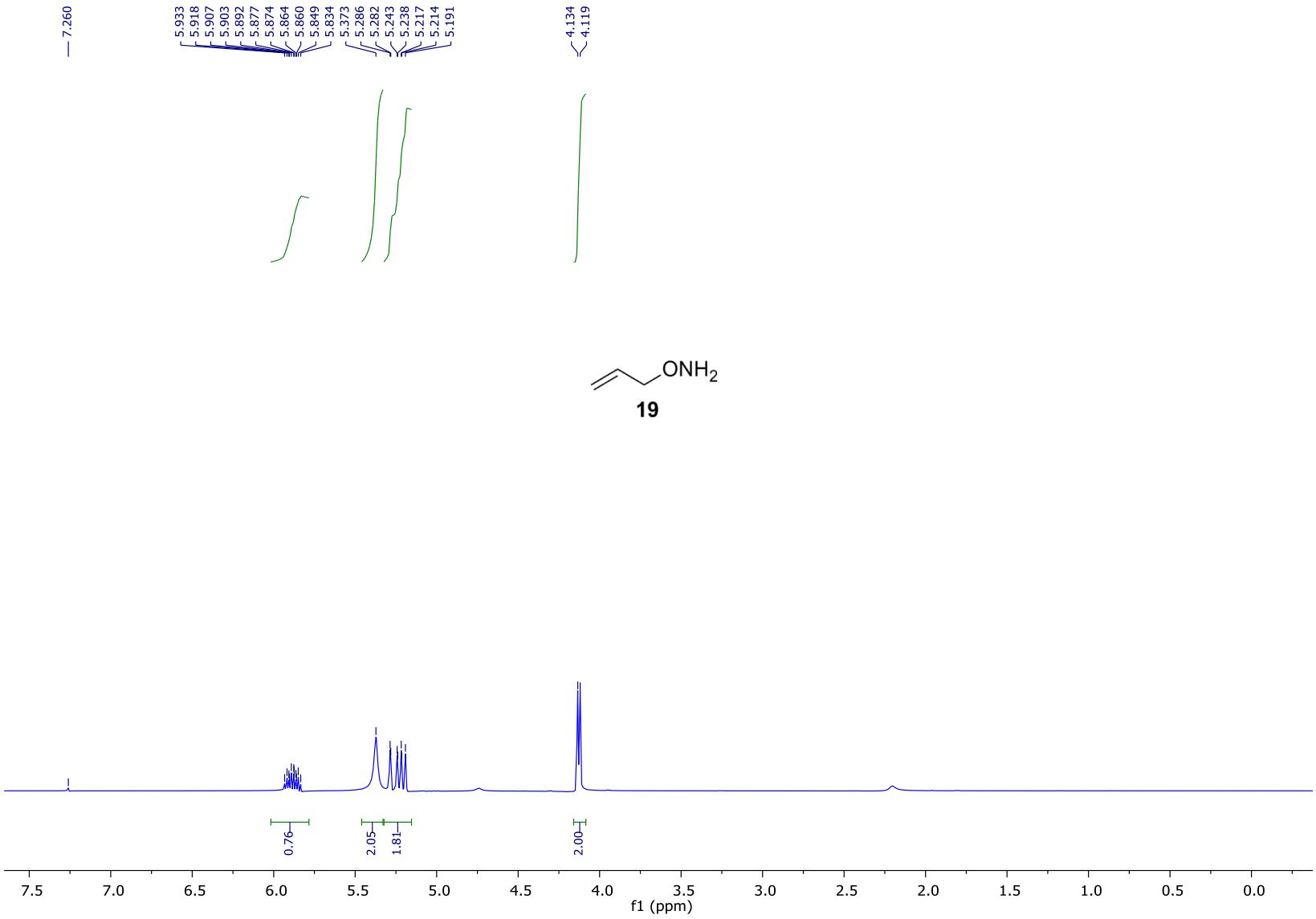


Figure S32. ^{13}C NMR Spectrum of **19** in $CDCl_3$ (100 MHz) at 25 °C

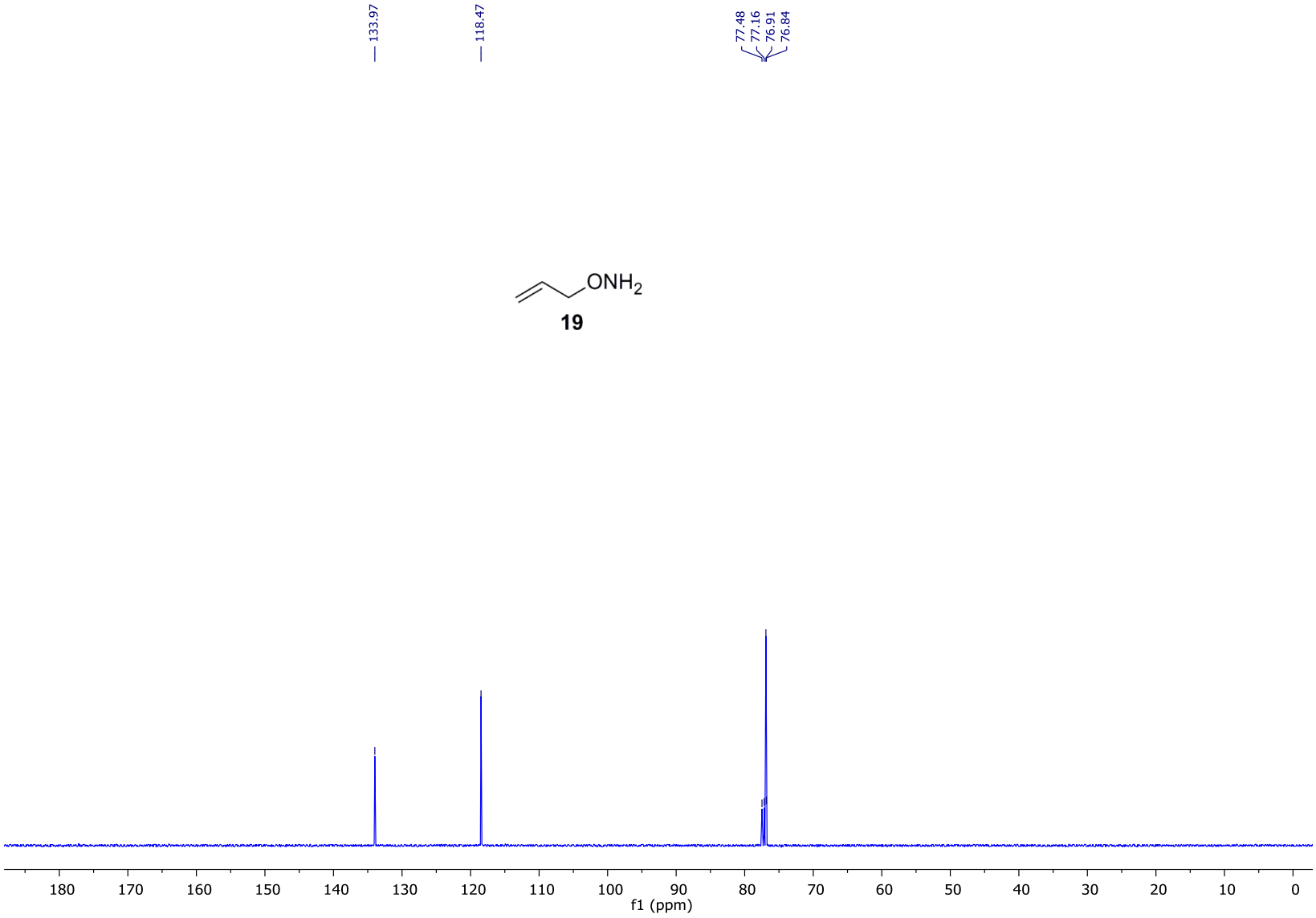


Figure S33. ^{1}H NMR Spectrum of **5** in $CDCl_3$ (400 MHz) at 25 °C

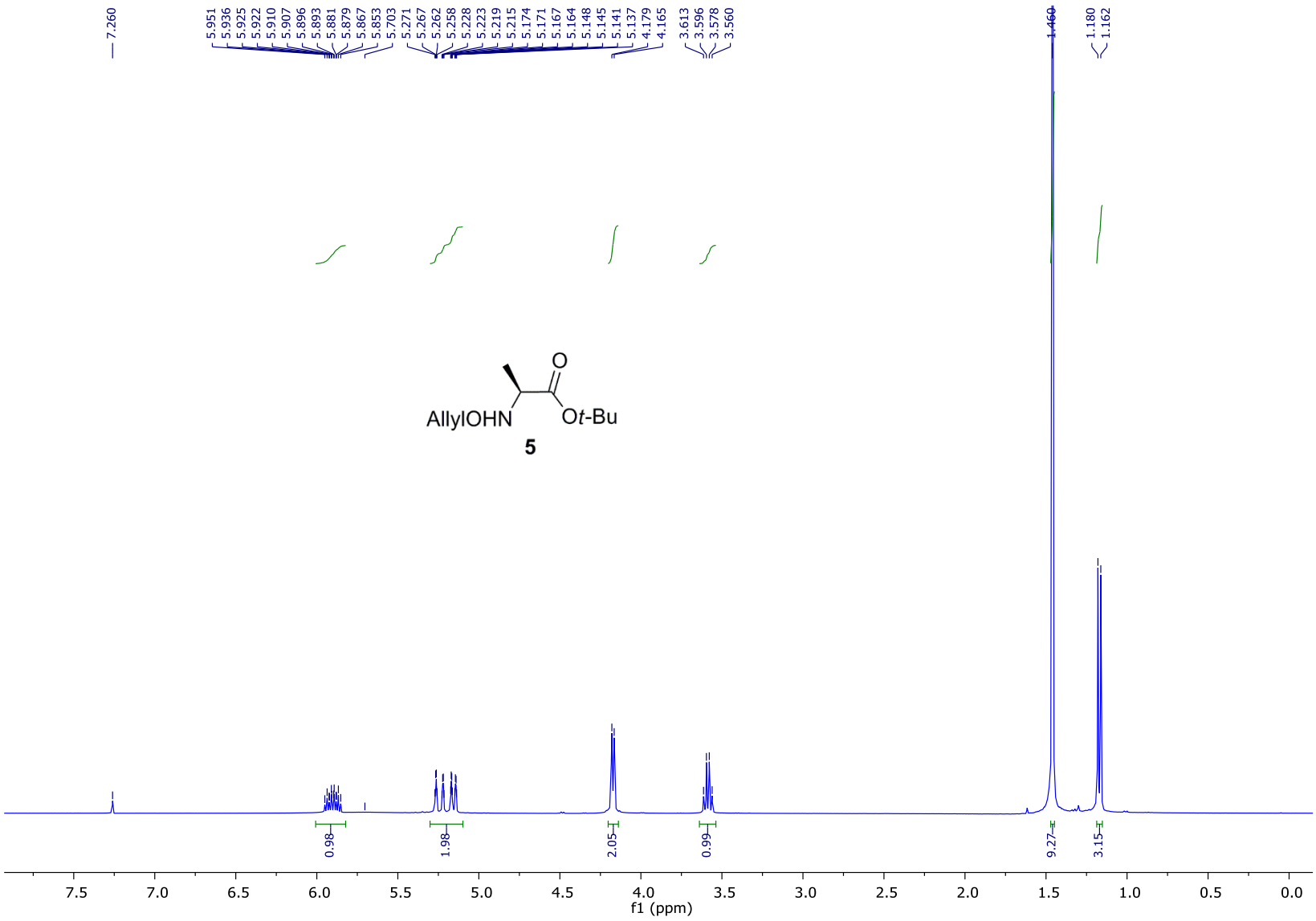


Figure S34. ^{13}C NMR Spectrum of **5** in $CDCl_3$ (100 MHz) at 25 °C

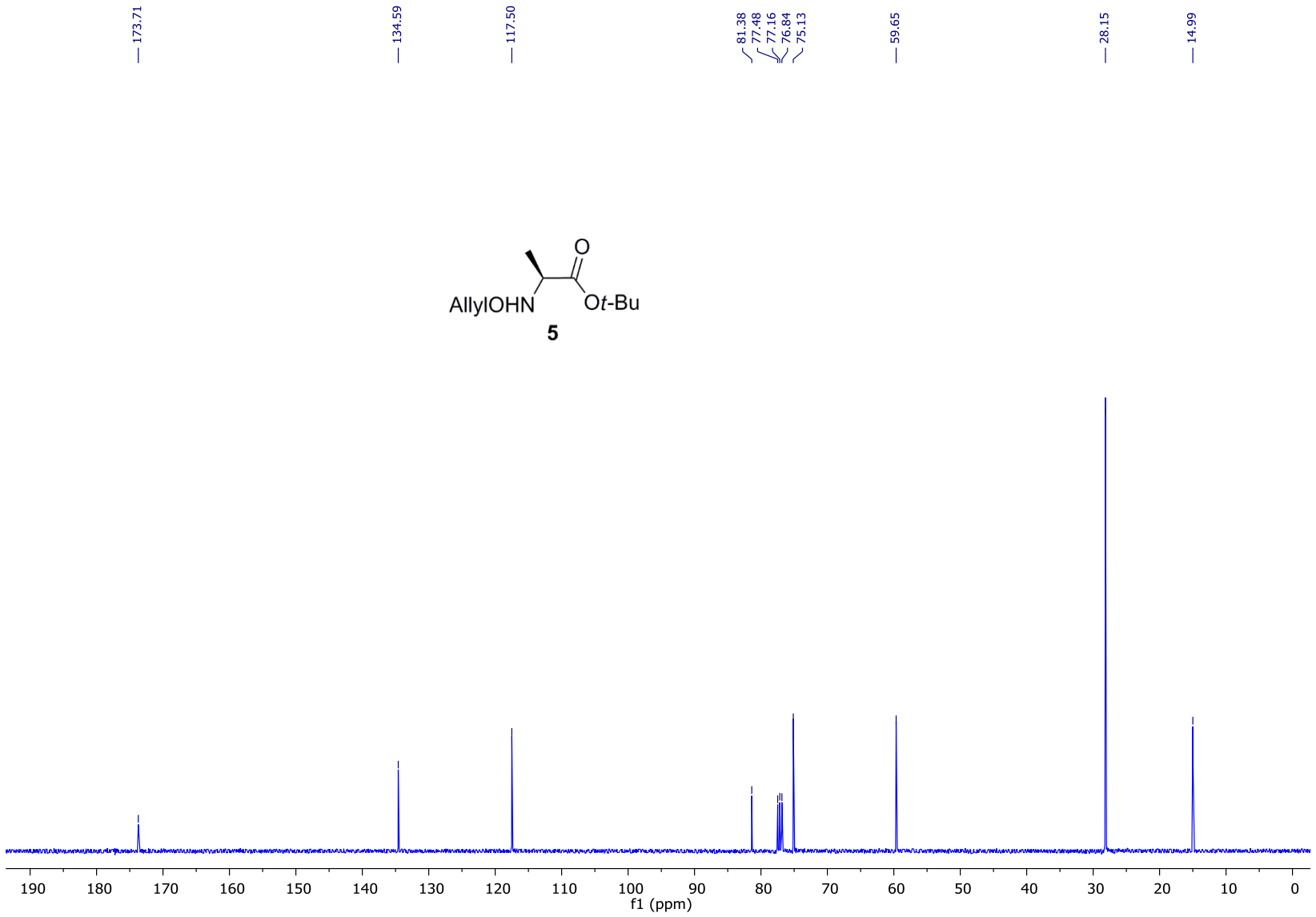


Figure S35. ^{1}H NMR Spectrum of **20** in $CDCl_3$ (400 MHz) at 25 °C

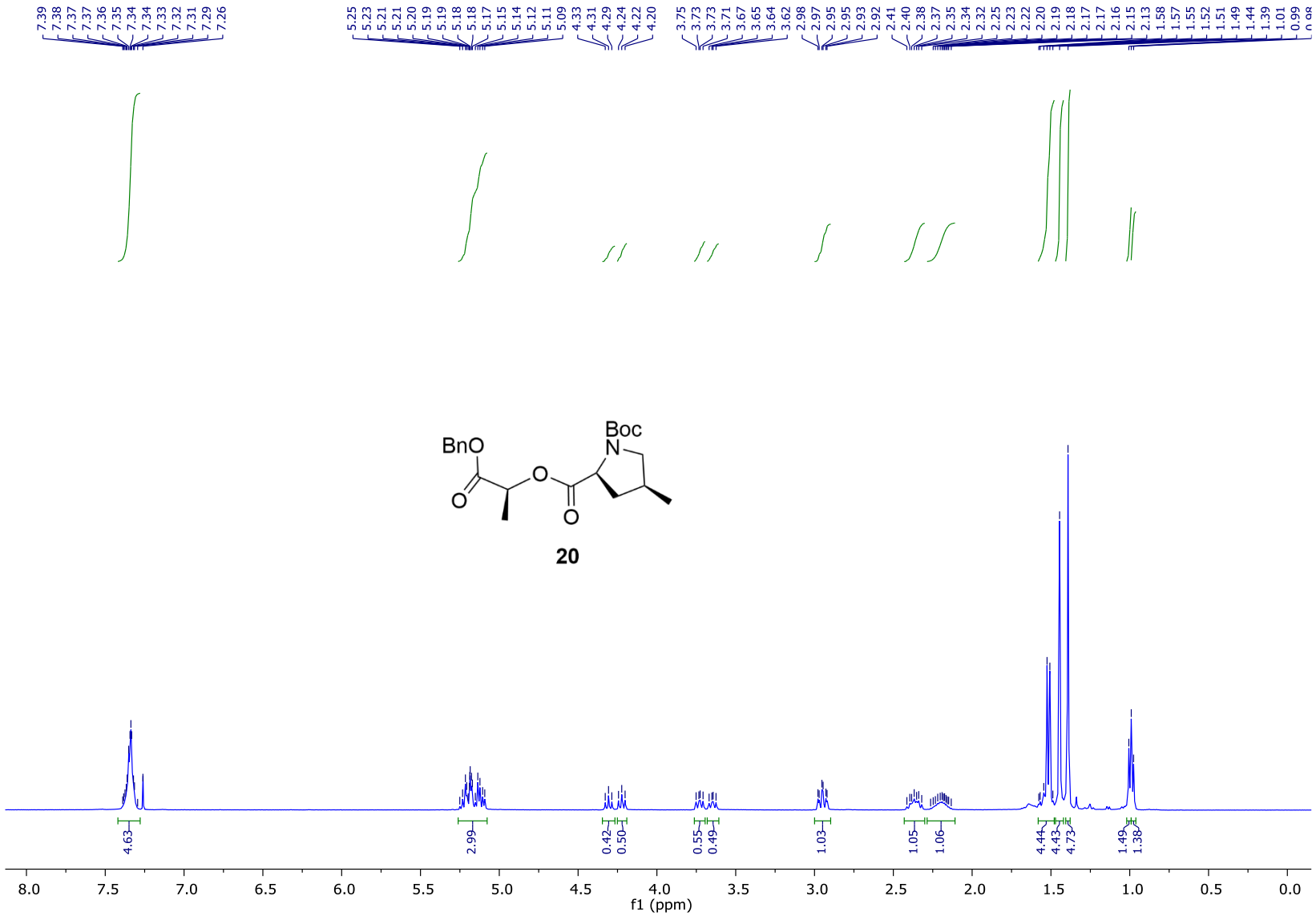


Figure S36. ^{13}C NMR Spectrum of **20** in $CDCl_3$ (100 MHz) at 25 °C

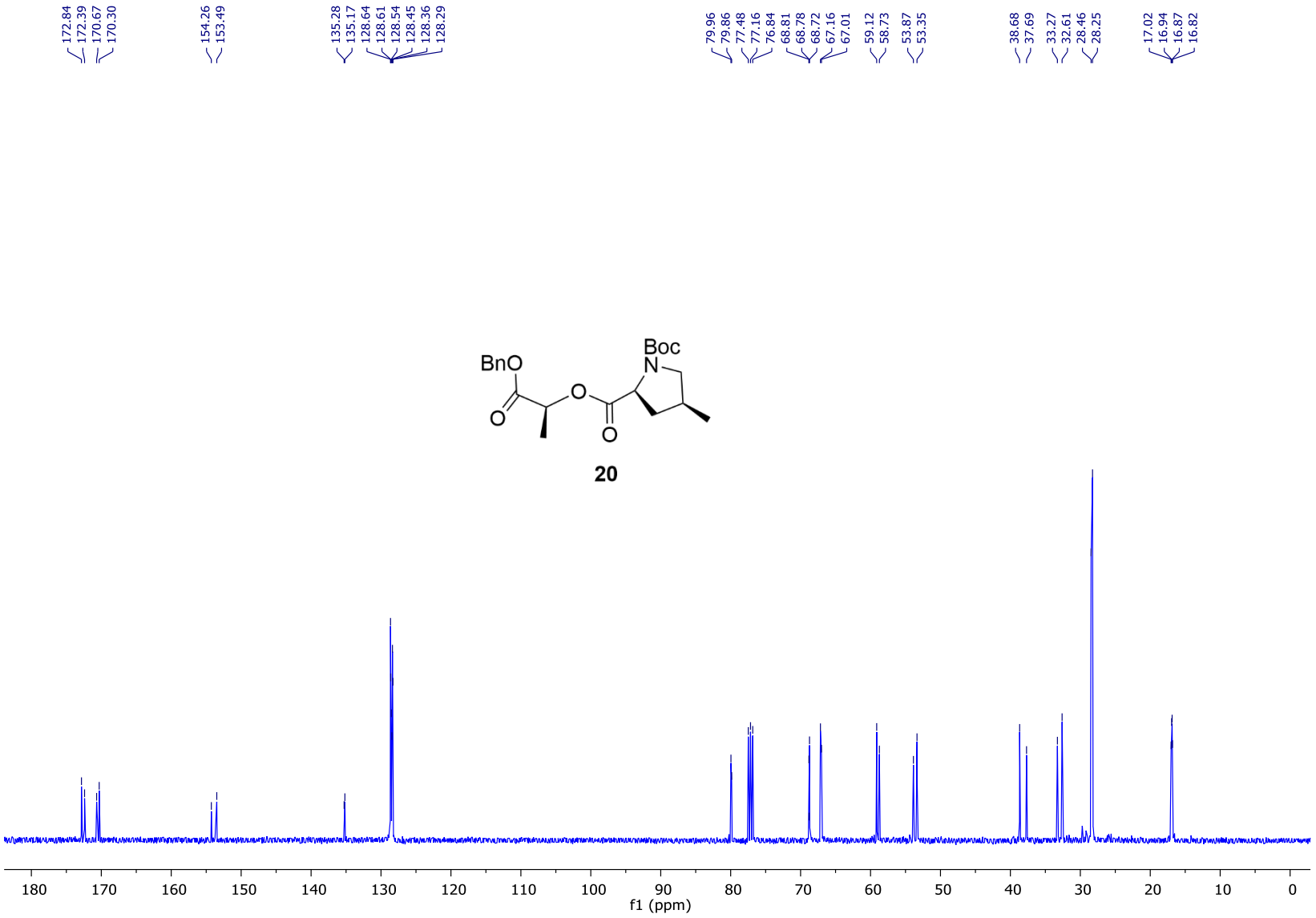


Figure S37. ^{1}H NMR Spectrum of **21** in $CDCl_3$ (500 MHz) at 25 °C

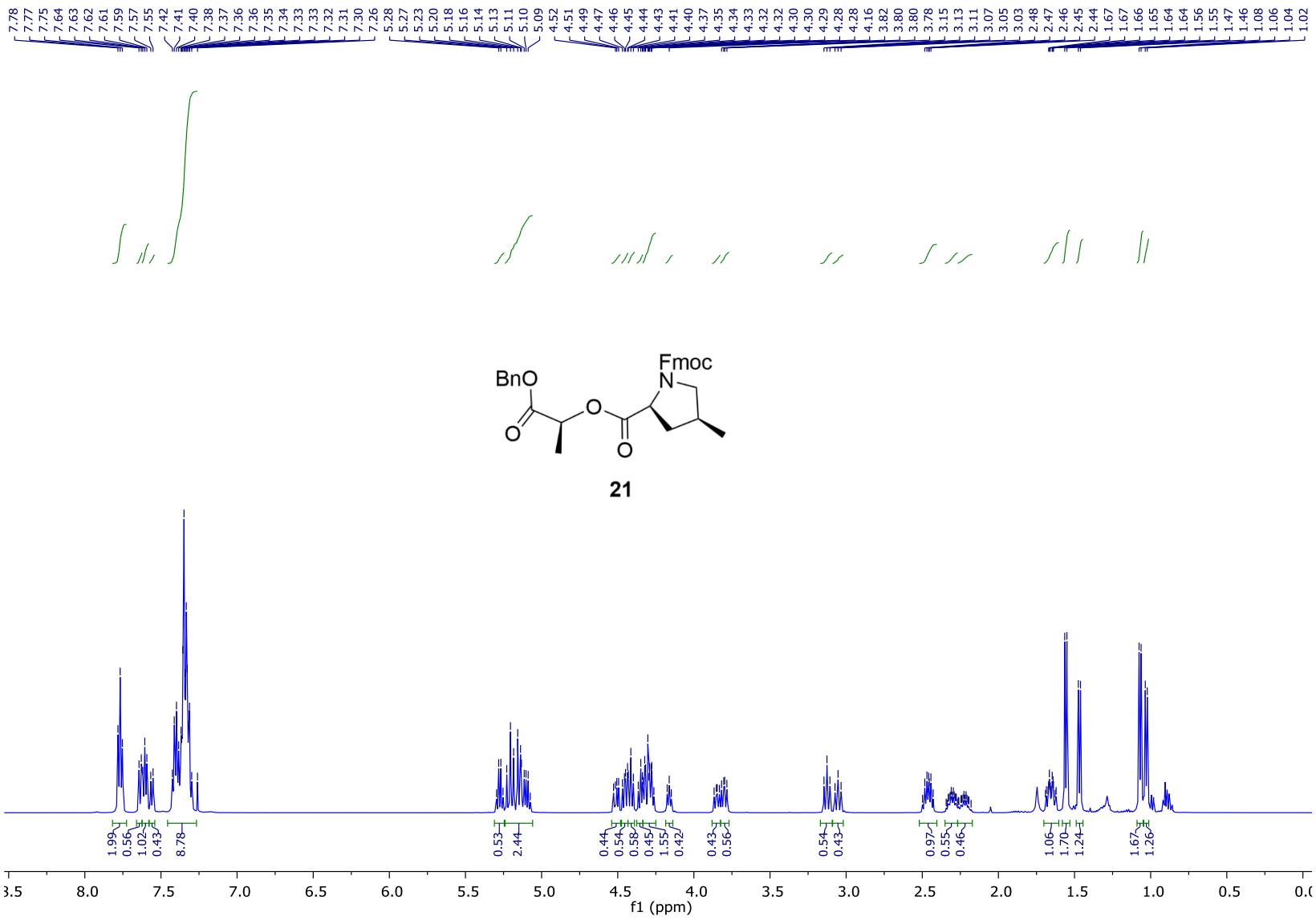


Figure S38. ^{13}C NMR Spectrum of **21** in $CDCl_3$ (125 MHz) at 25 °C

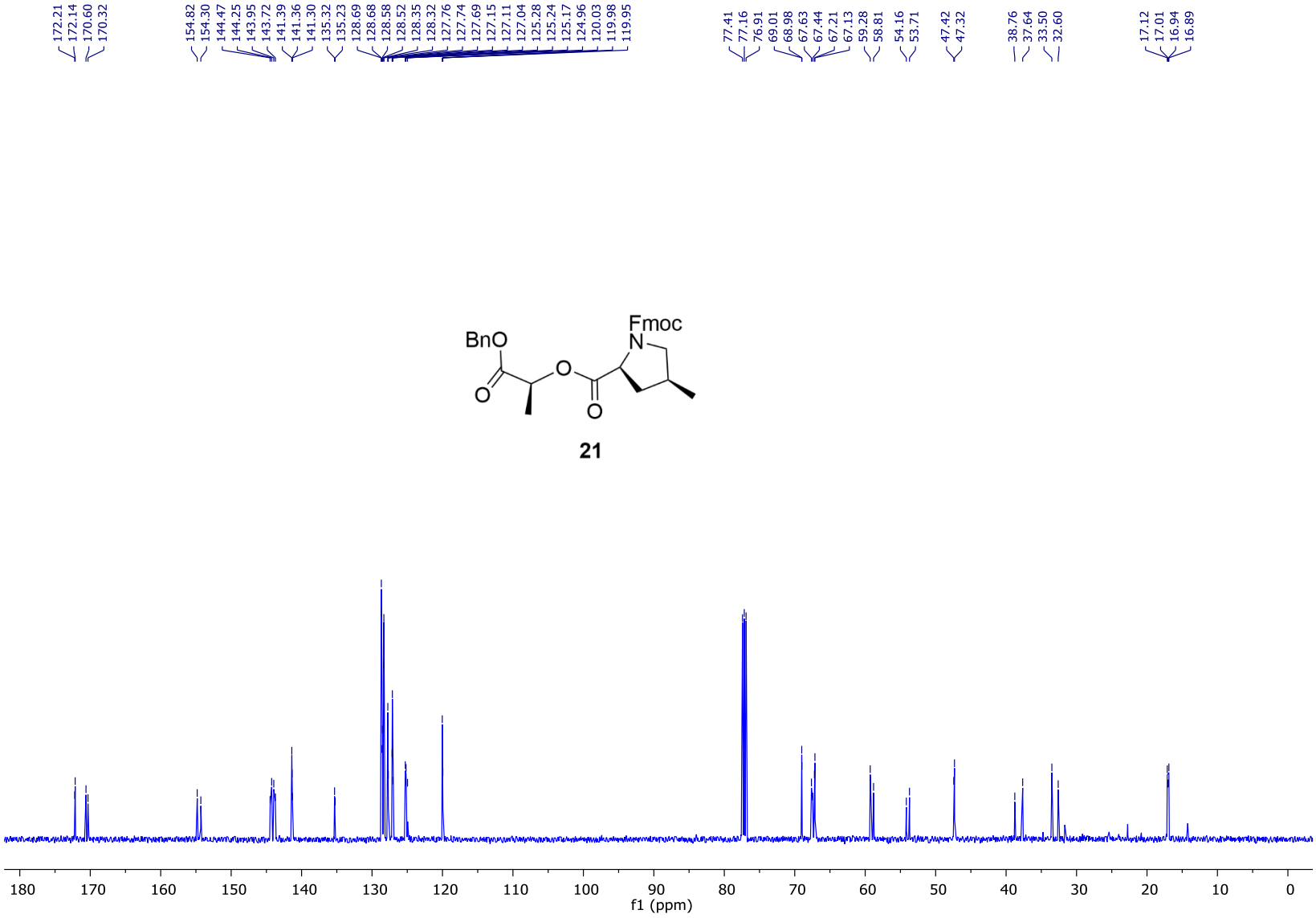


Figure S39. ^{1}H NMR Spectrum of **6** in $CDCl_3$ (400 MHz) at 25 °C

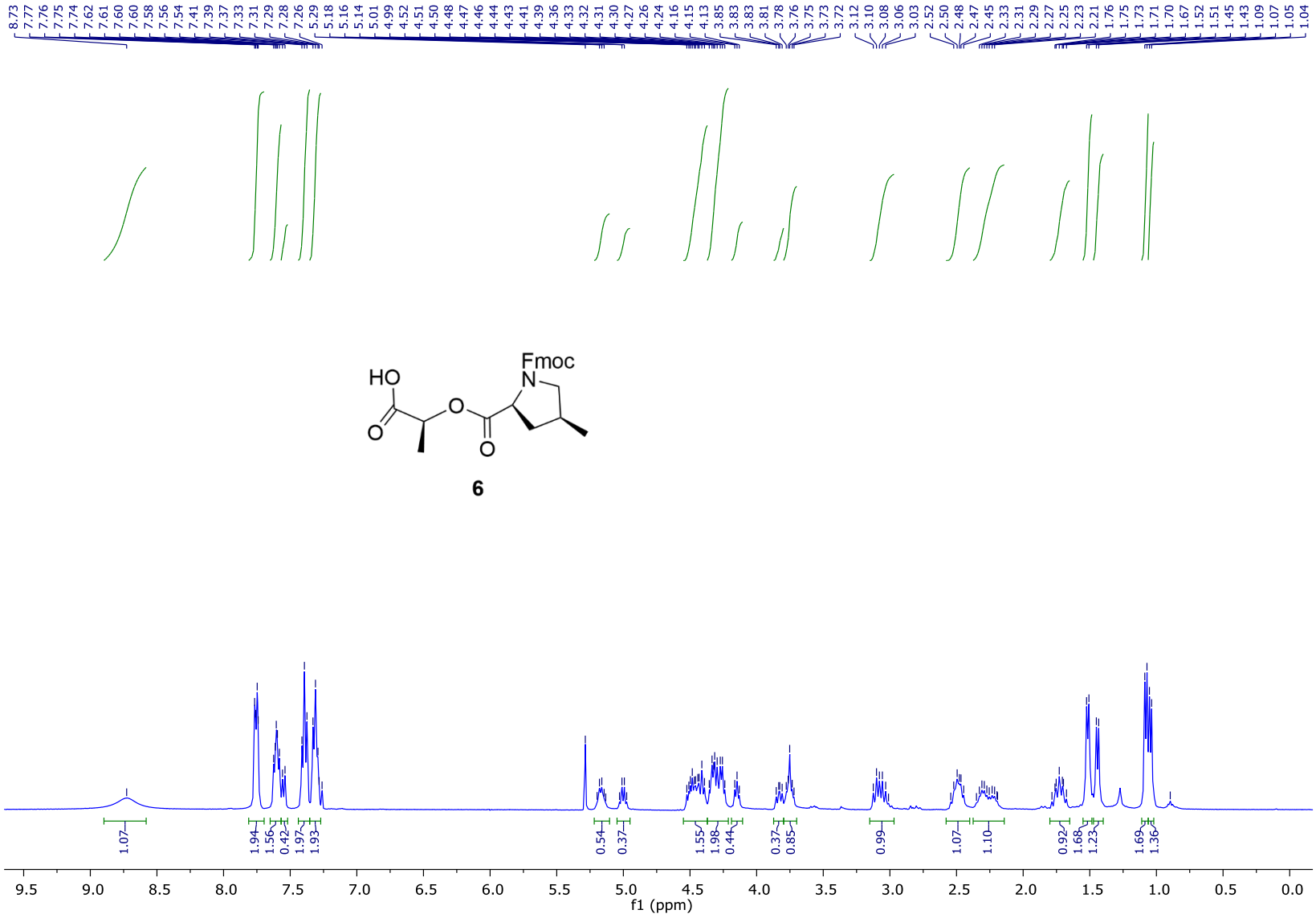


Figure S40. ^{13}C NMR Spectrum of **6** in $CDCl_3$ (100 MHz) at 25 °C

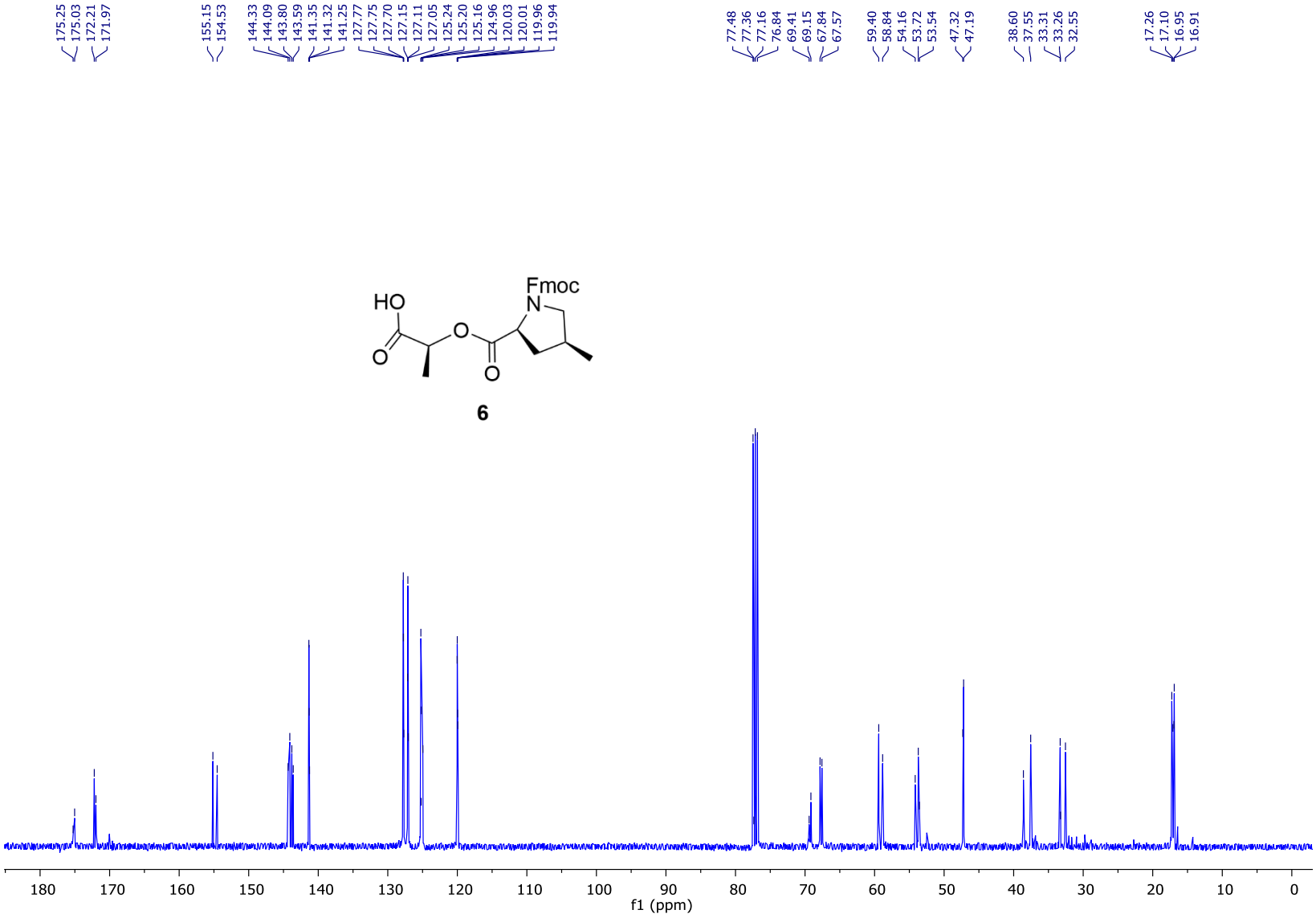


Figure S41. ^{1}H NMR Spectrum of **24** in $CDCl_3$ (400 MHz) at 25 °C

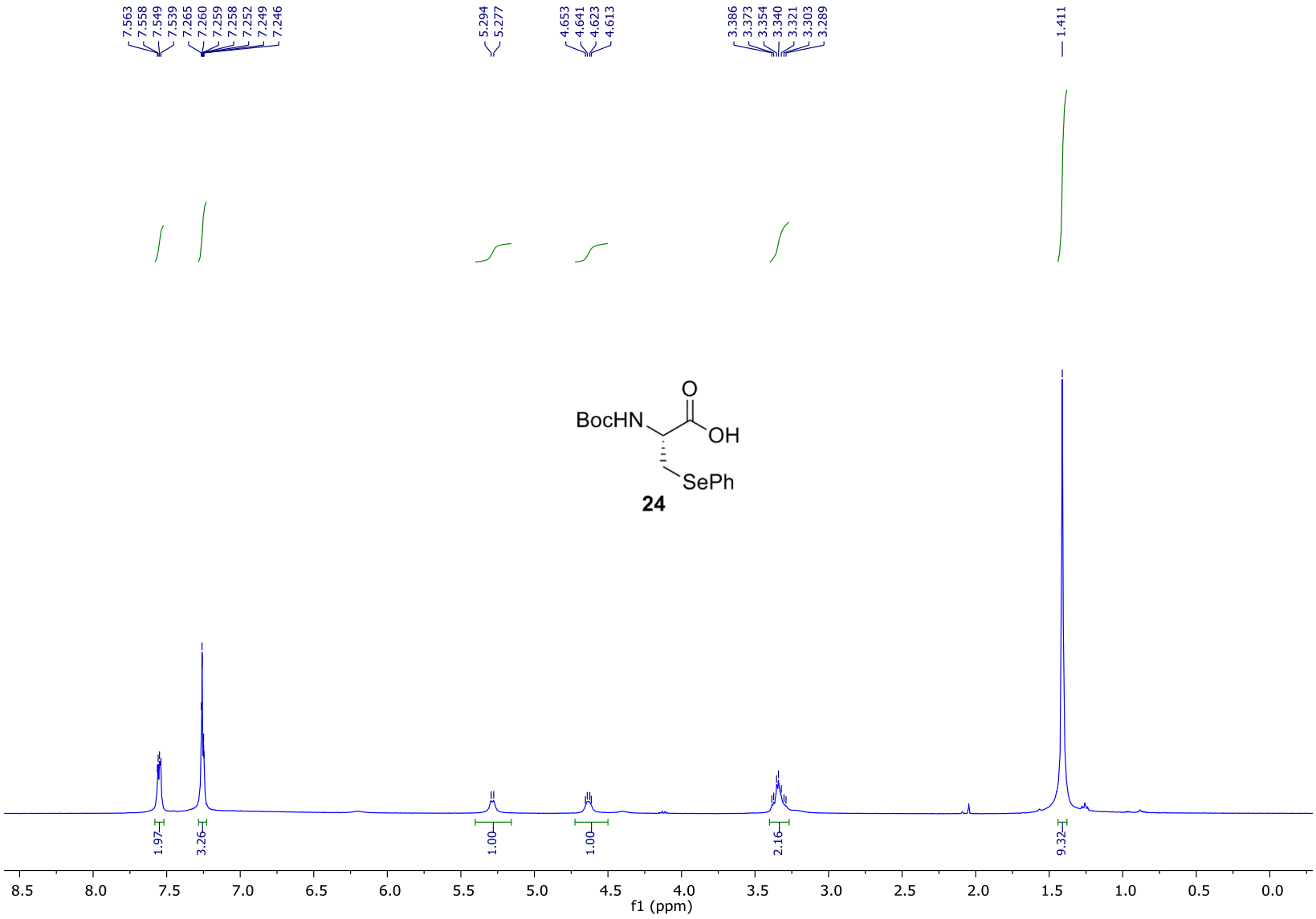


Figure S42. ^{1}H NMR Spectrum of **7** in $CDCl_3$ (400 MHz) at 25 °C

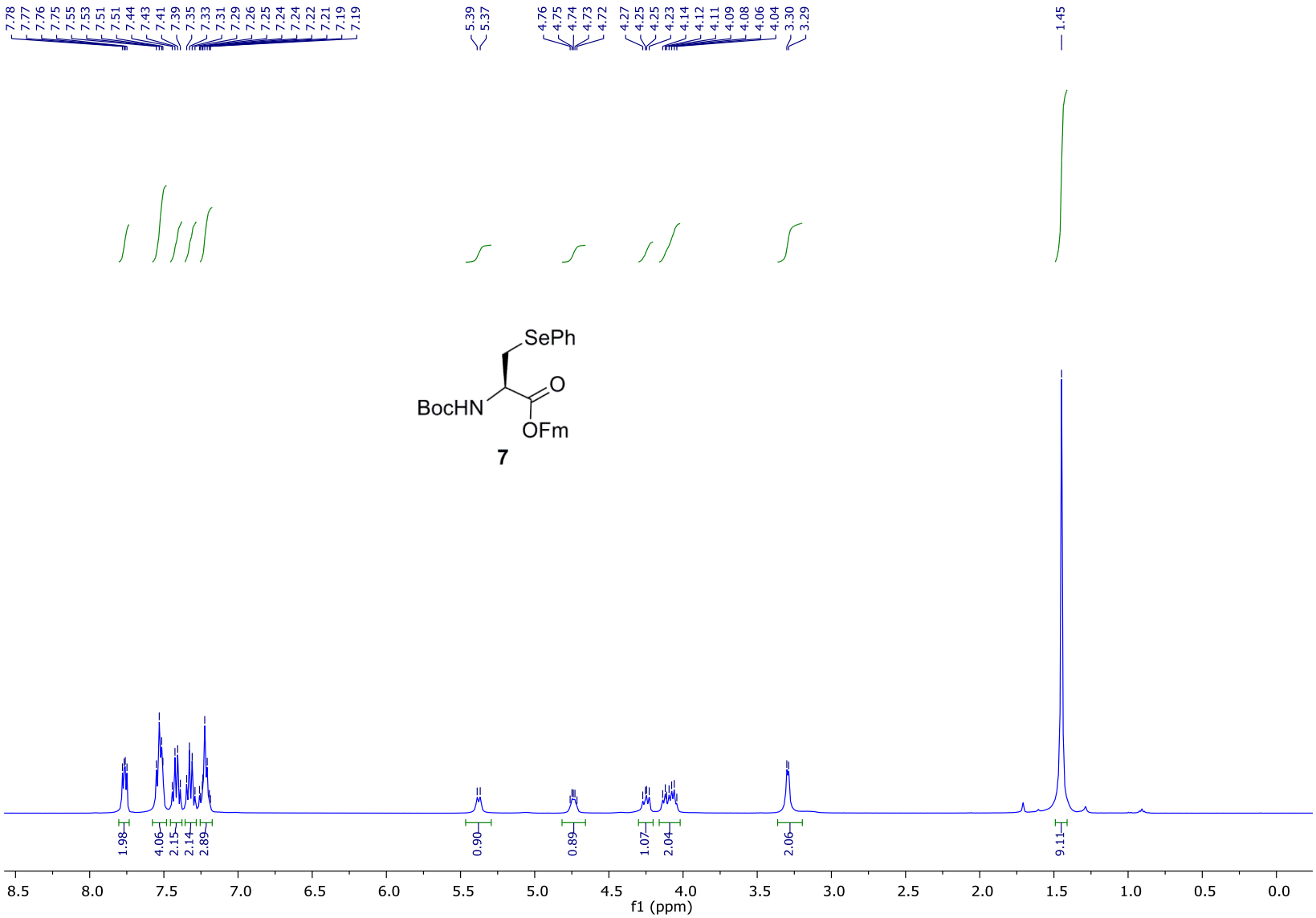


Figure S43. ^{13}C NMR Spectrum of **7** in $CDCl_3$ (100 MHz) at 25 °C

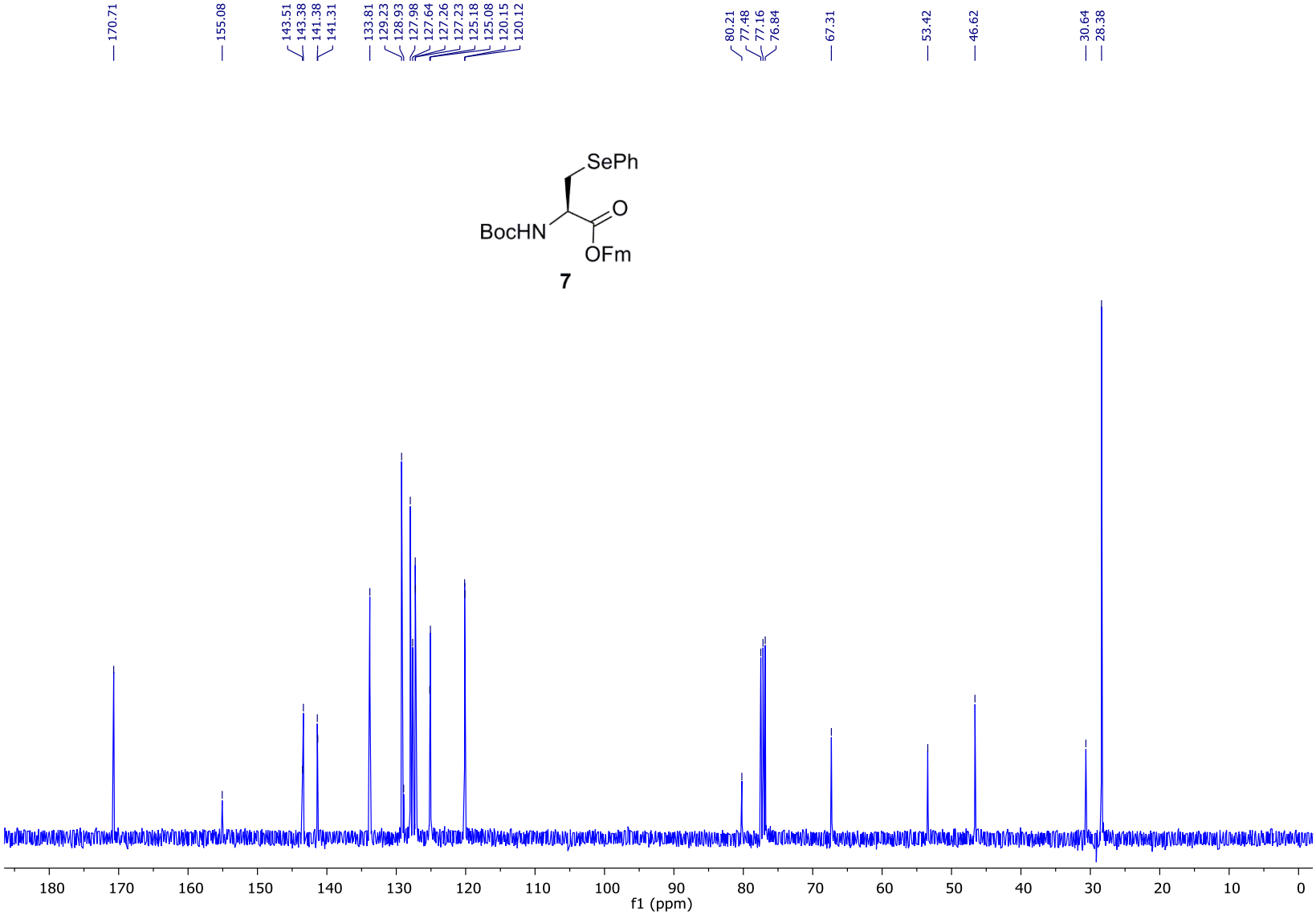


Figure S44. ^{1}H NMR Spectrum of **25** in $CDCl_3$ (400 MHz) at 25 °C

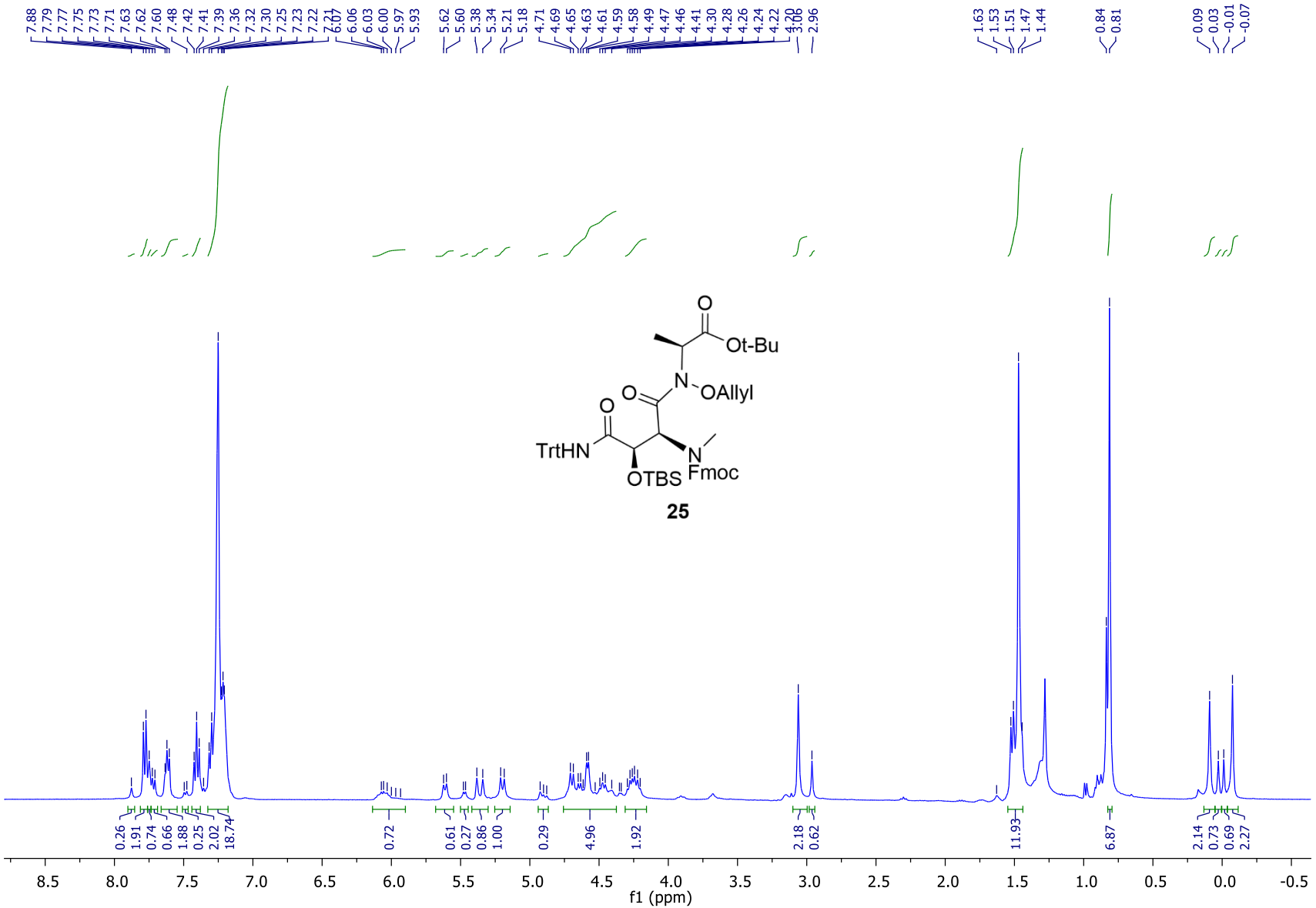


Figure S45. ^{13}C NMR Spectrum of **25** in $CDCl_3$ (100 MHz) at 25 °C

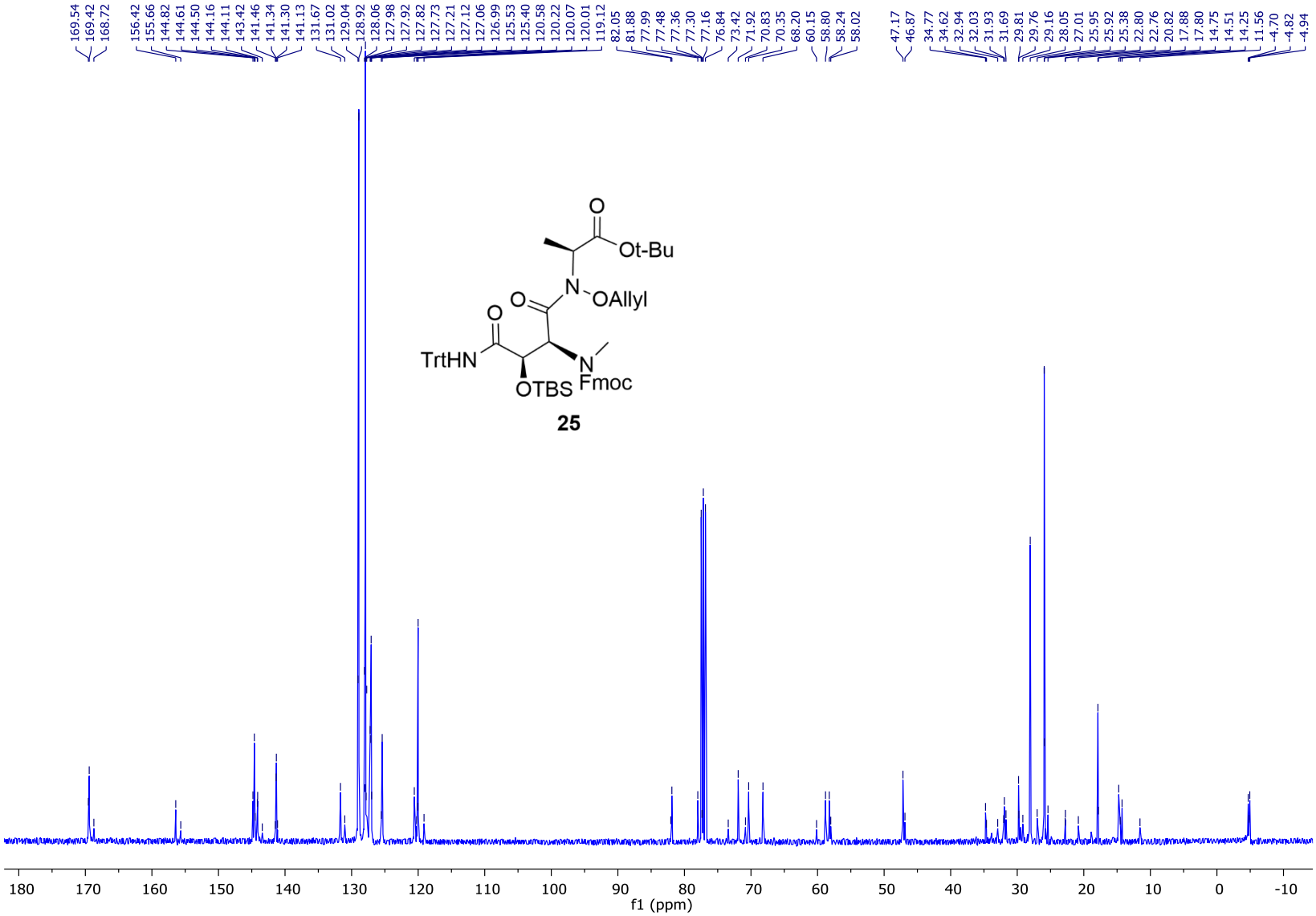


Figure S46. ^{1}H NMR Spectrum of **26** in $CDCl_3$ (400 MHz) at 25 °C

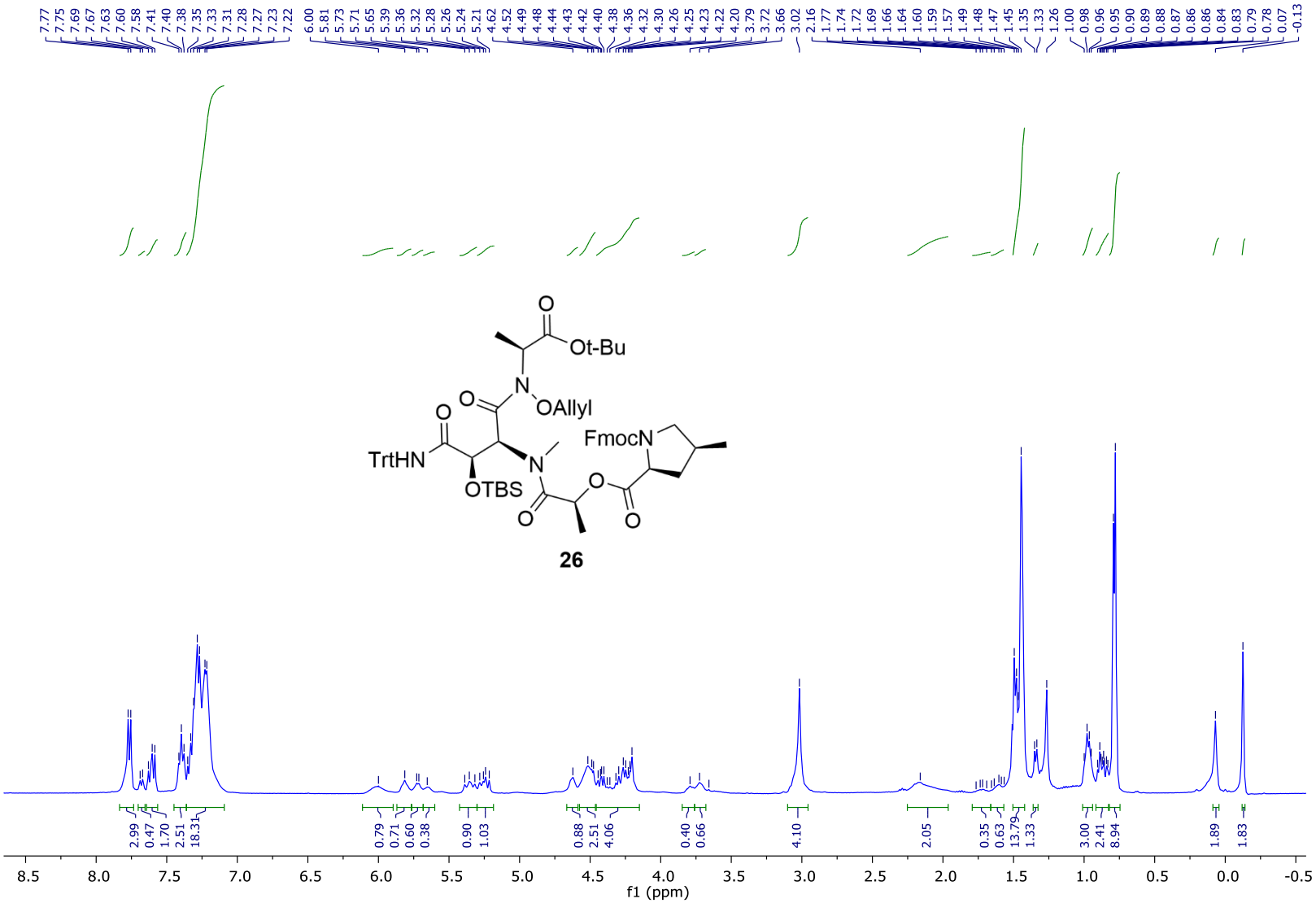


Figure S47. ^{13}C NMR Spectrum of **26** in $CDCl_3$ (100 MHz) at 25 °C

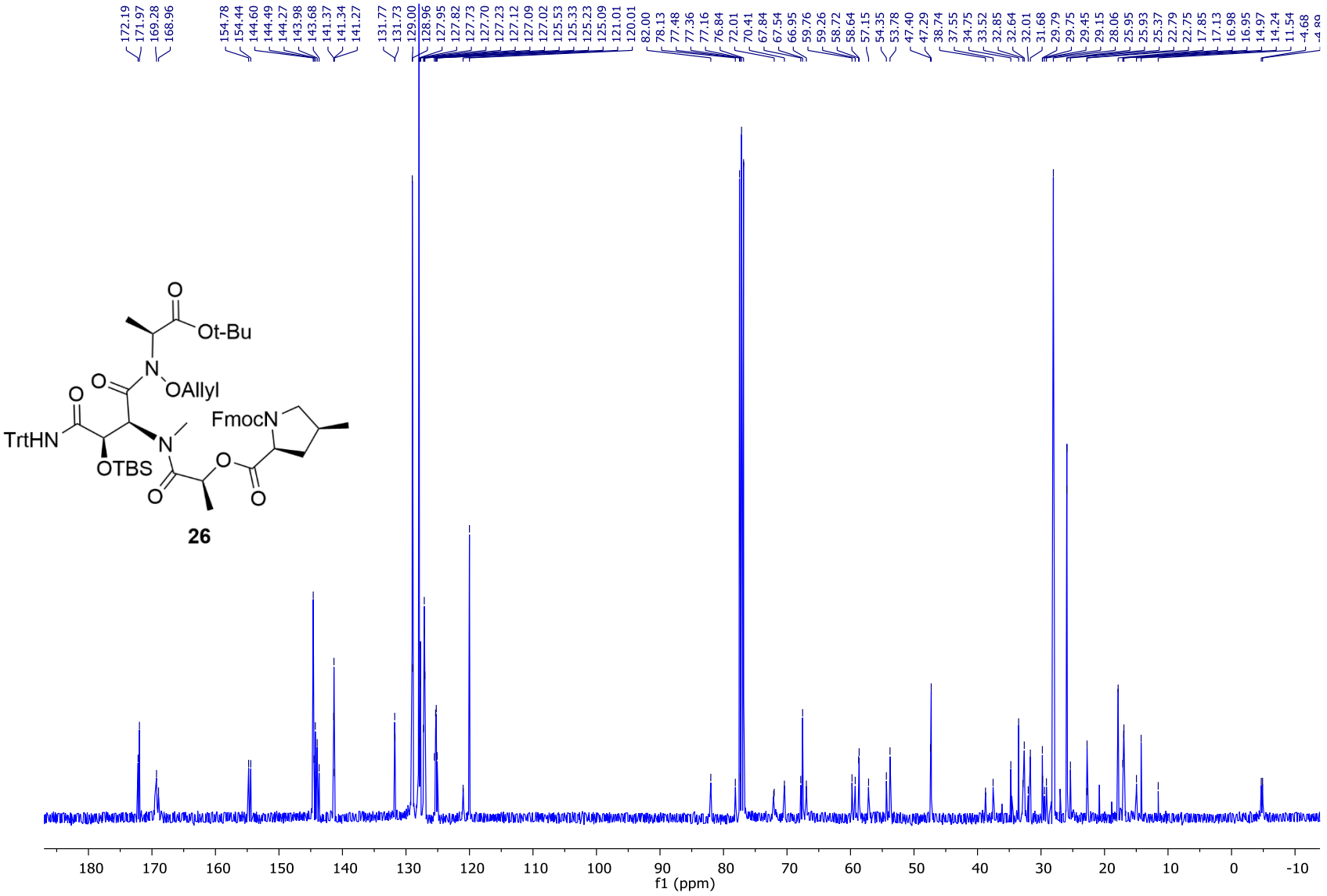


Figure S48. ^{1}H NMR Spectrum of **27** in $CDCl_3$ (400 MHz) at 25 °C

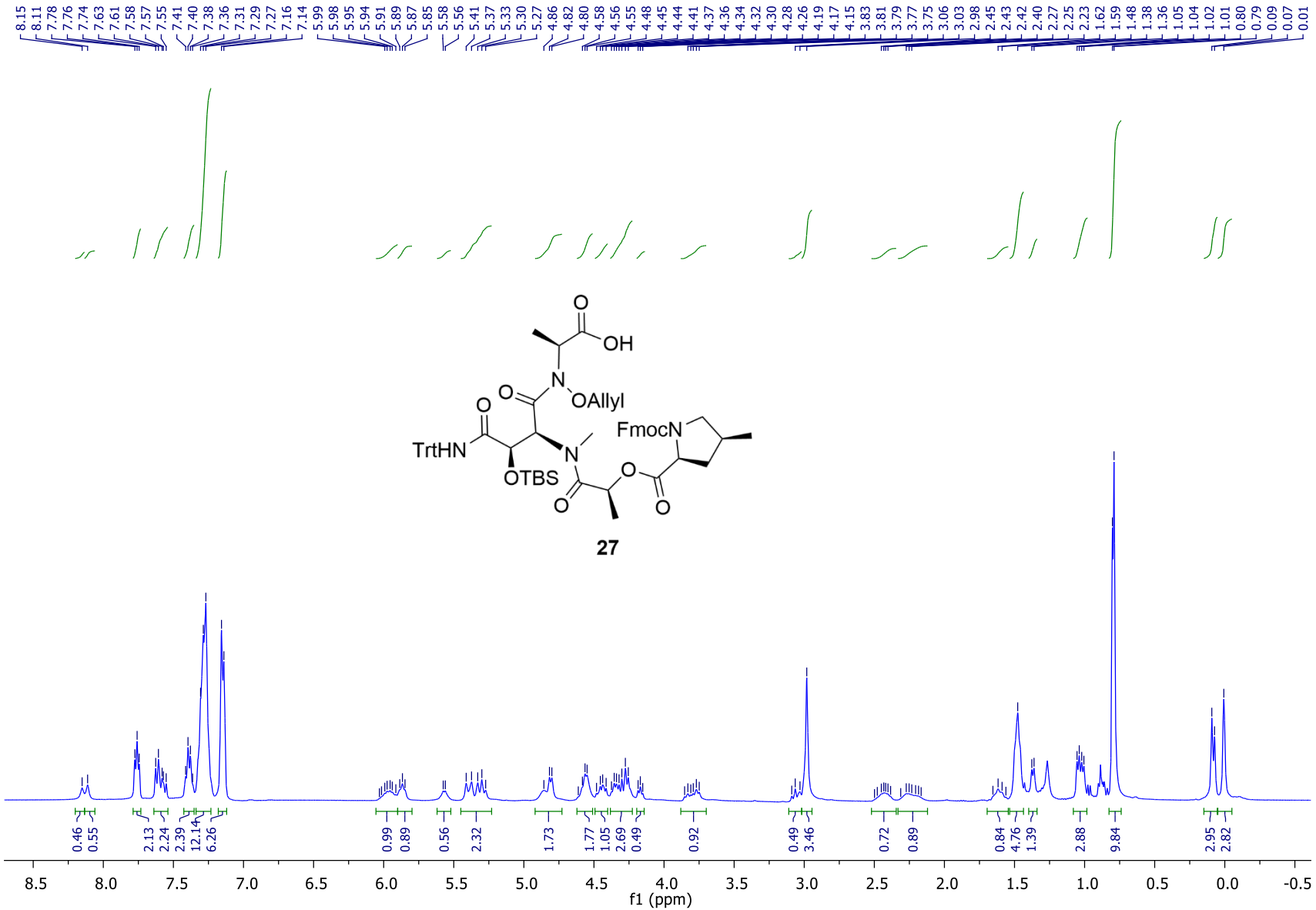


Figure S49. ^{13}C NMR Spectrum of **27** in $CDCl_3$ (100 MHz) at 25 °C

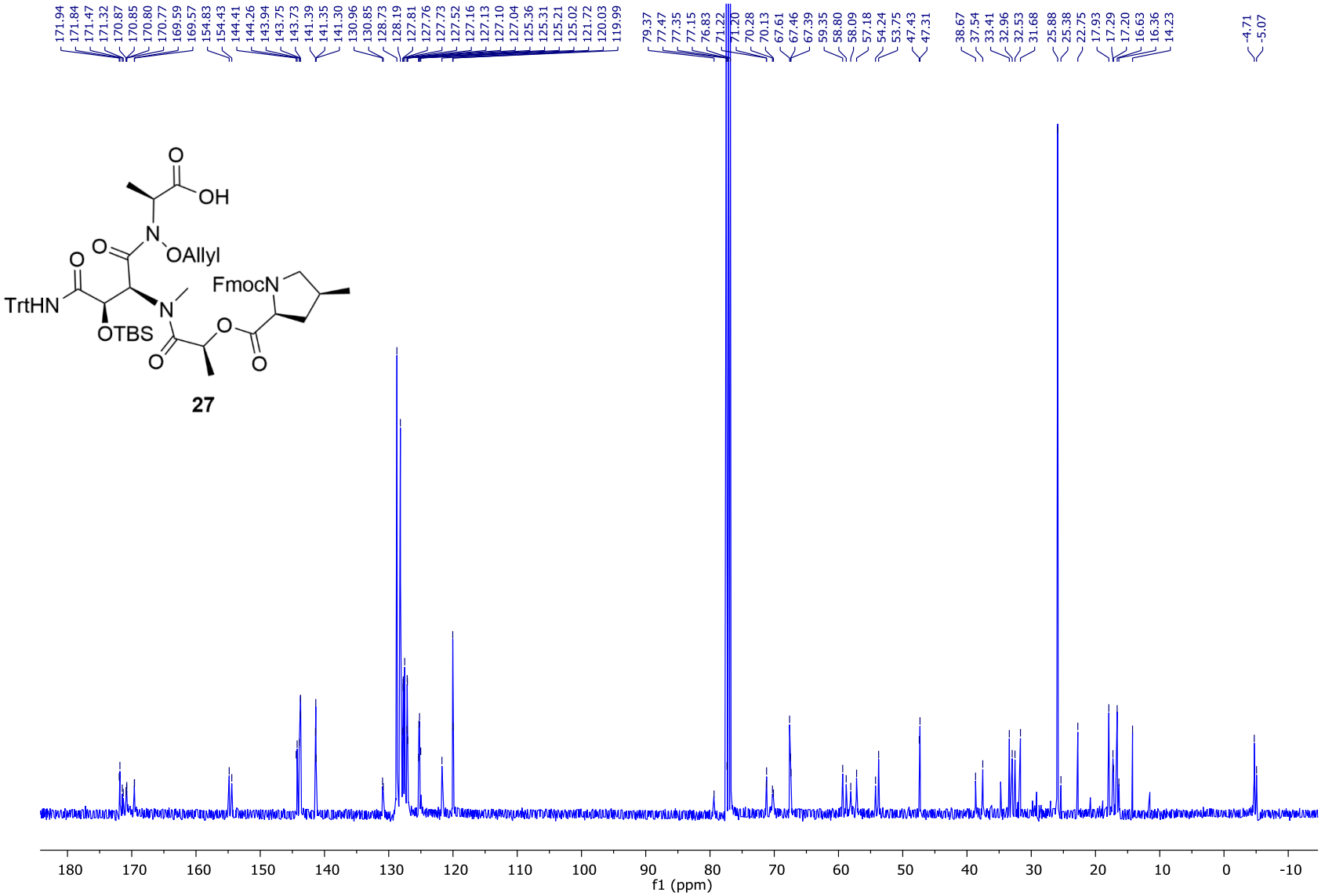


Figure S50. ^{1}H NMR Spectrum of **3** in $CDCl_3$ (400 MHz) at 25 °C

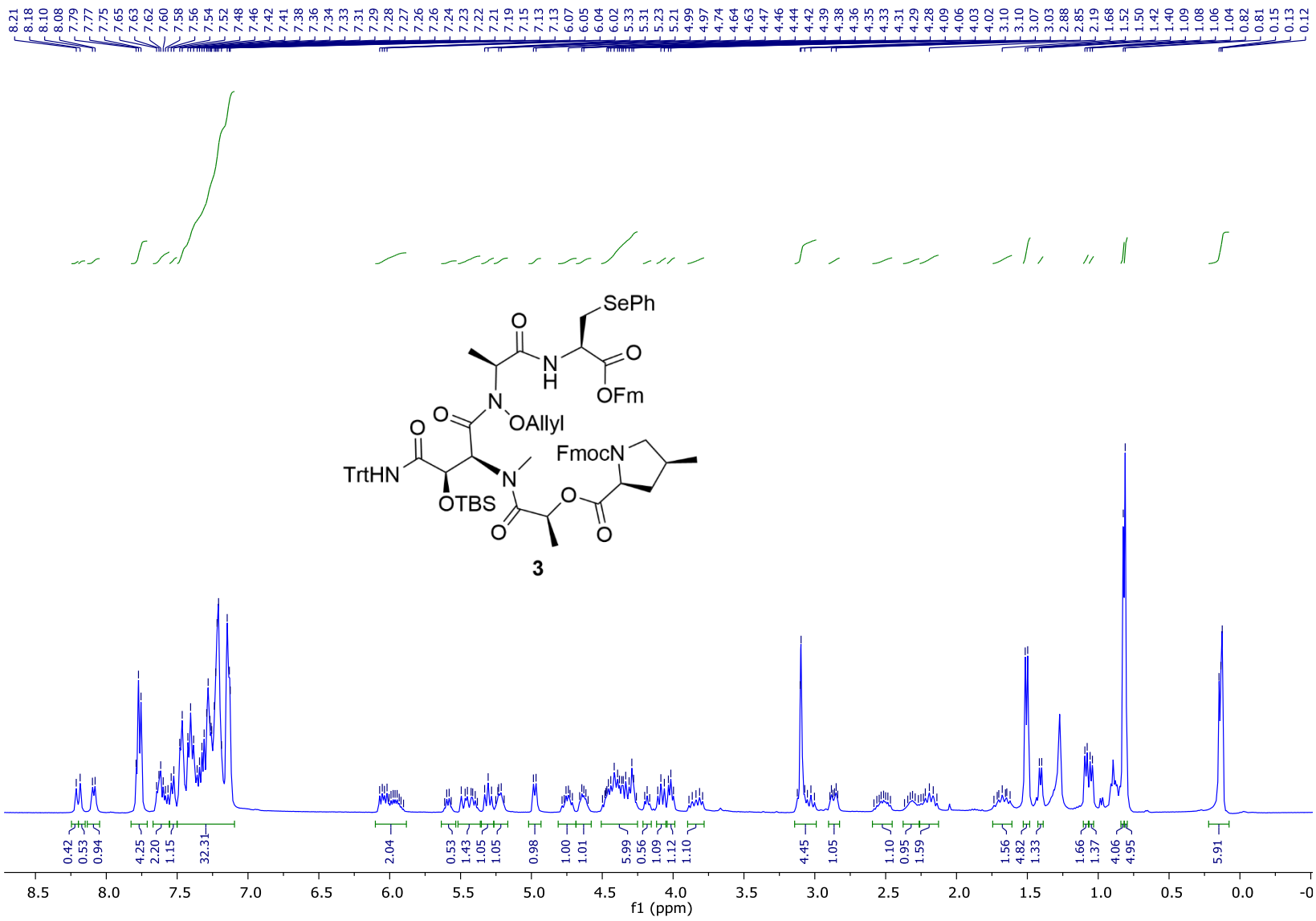


Figure S51. ^{13}C NMR Spectrum of **3** in $CDCl_3$ (100 MHz) at 25 °C

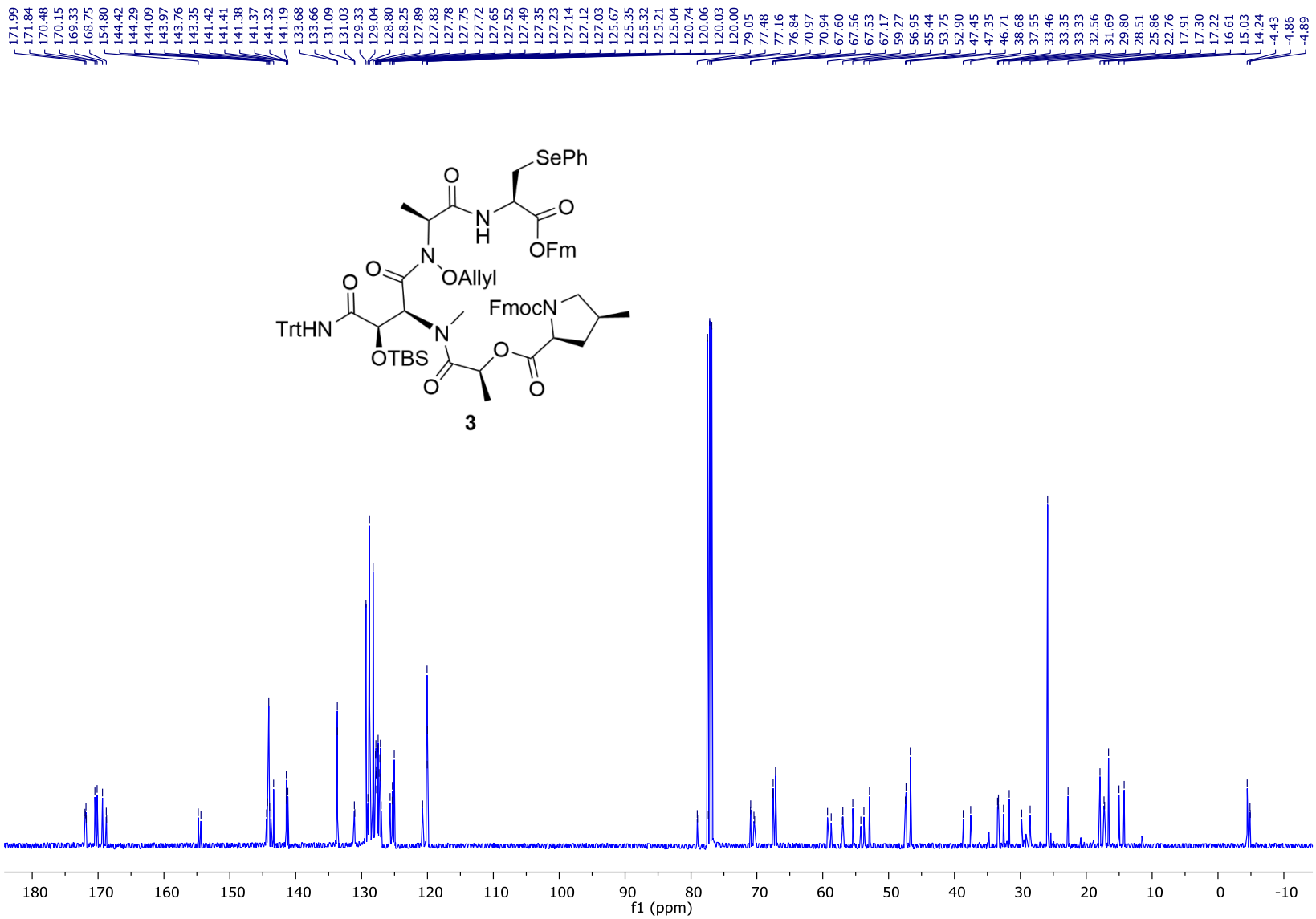


Figure S52. ^{1}H NMR Spectrum of **2** in $CDCl_3$ (400 MHz) at 25 °C

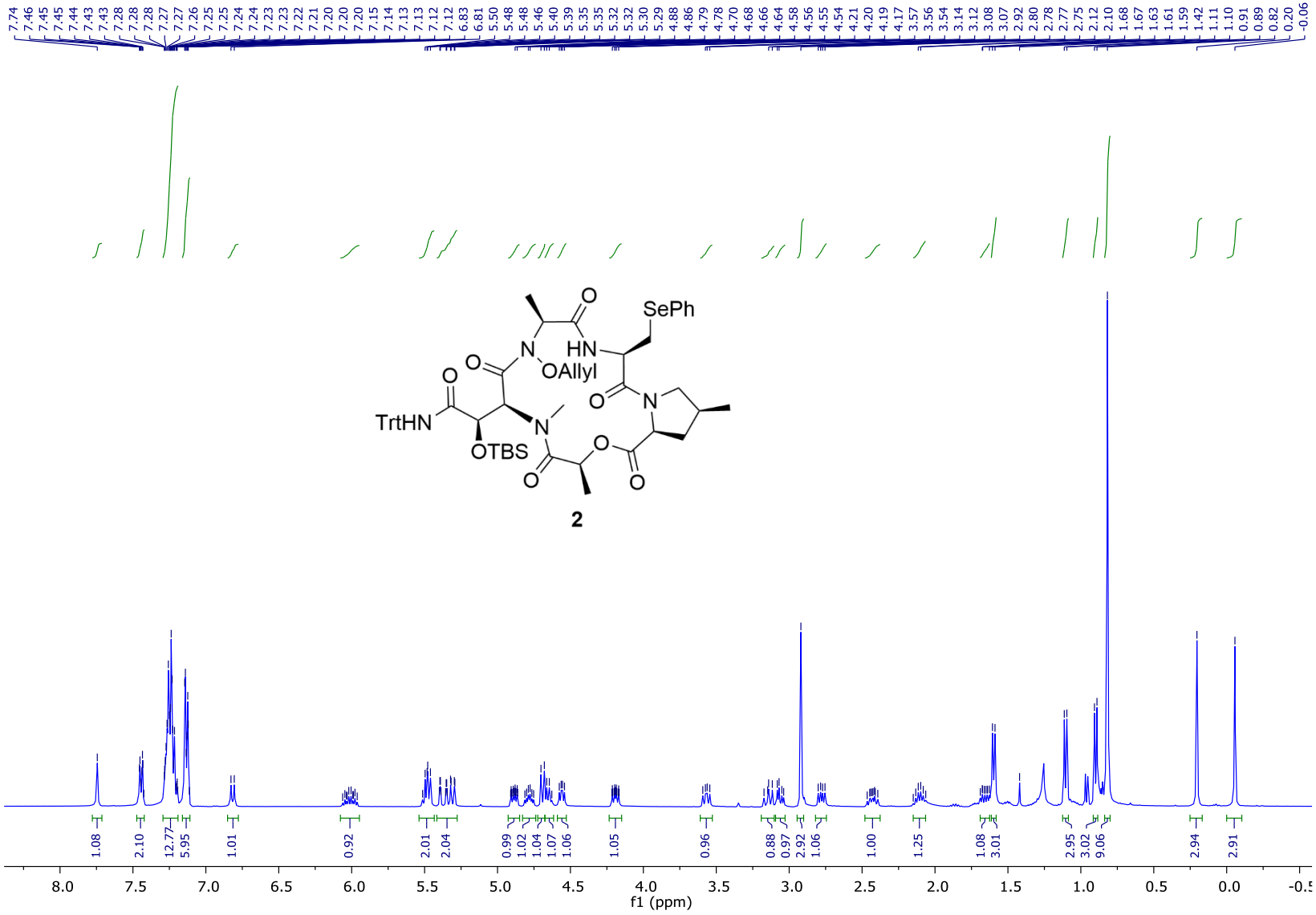


Figure S53. ^{13}C NMR Spectrum of **2** in $CDCl_3$ (100 MHz) at 25 °C

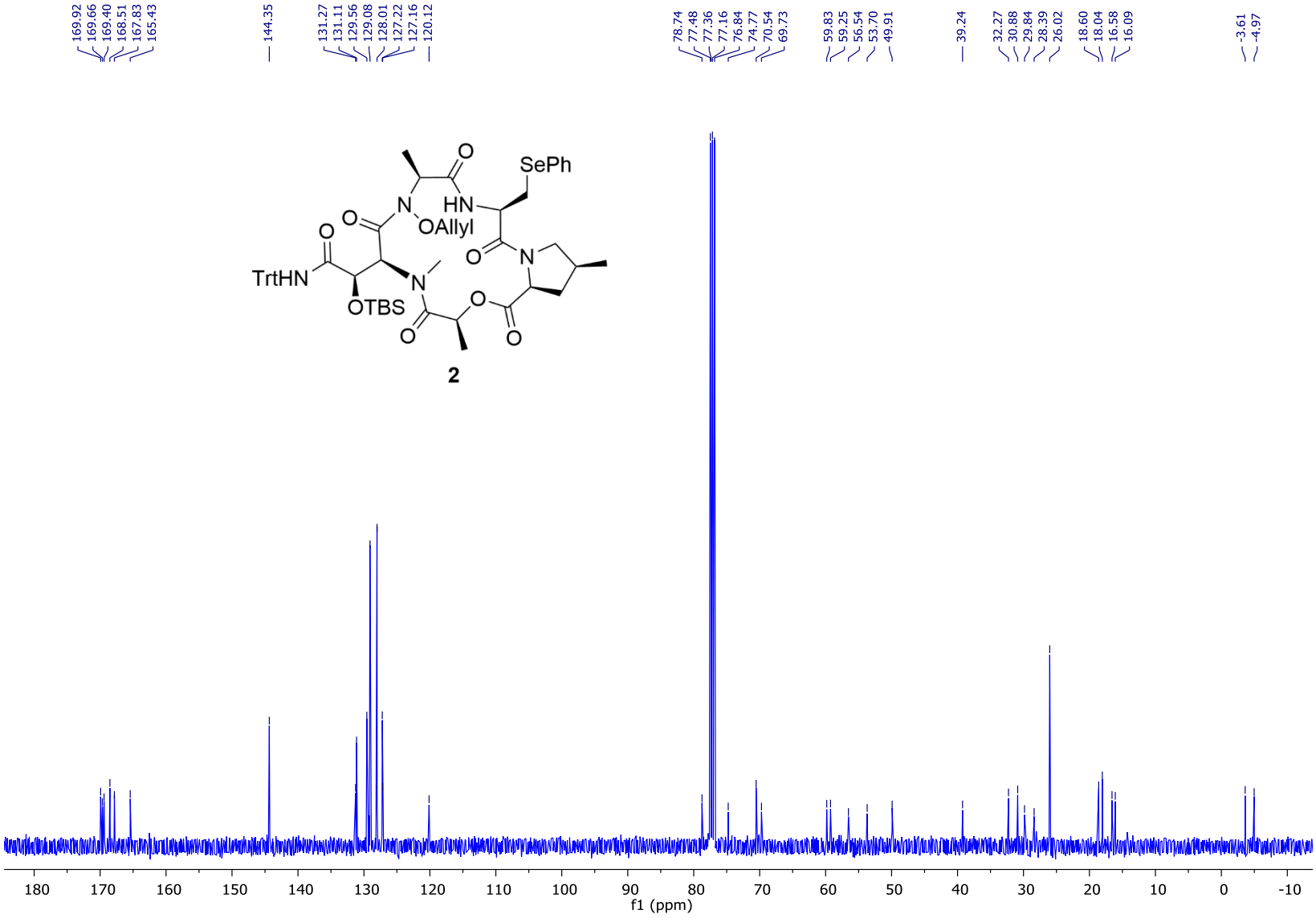


Figure S54. ^{1}H NMR Spectrum of **28** in $CDCl_3$ (400 MHz) at 25 °C

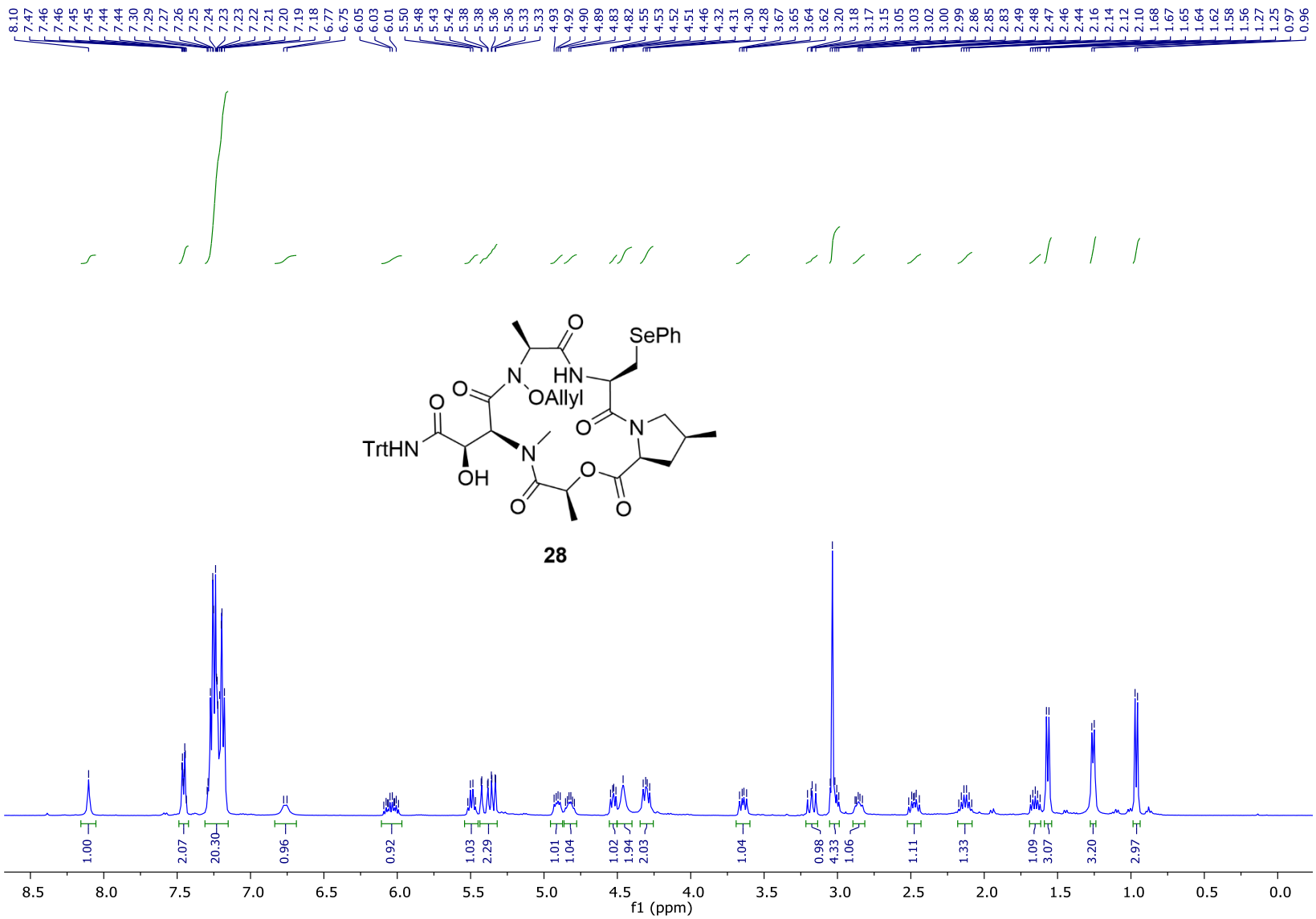
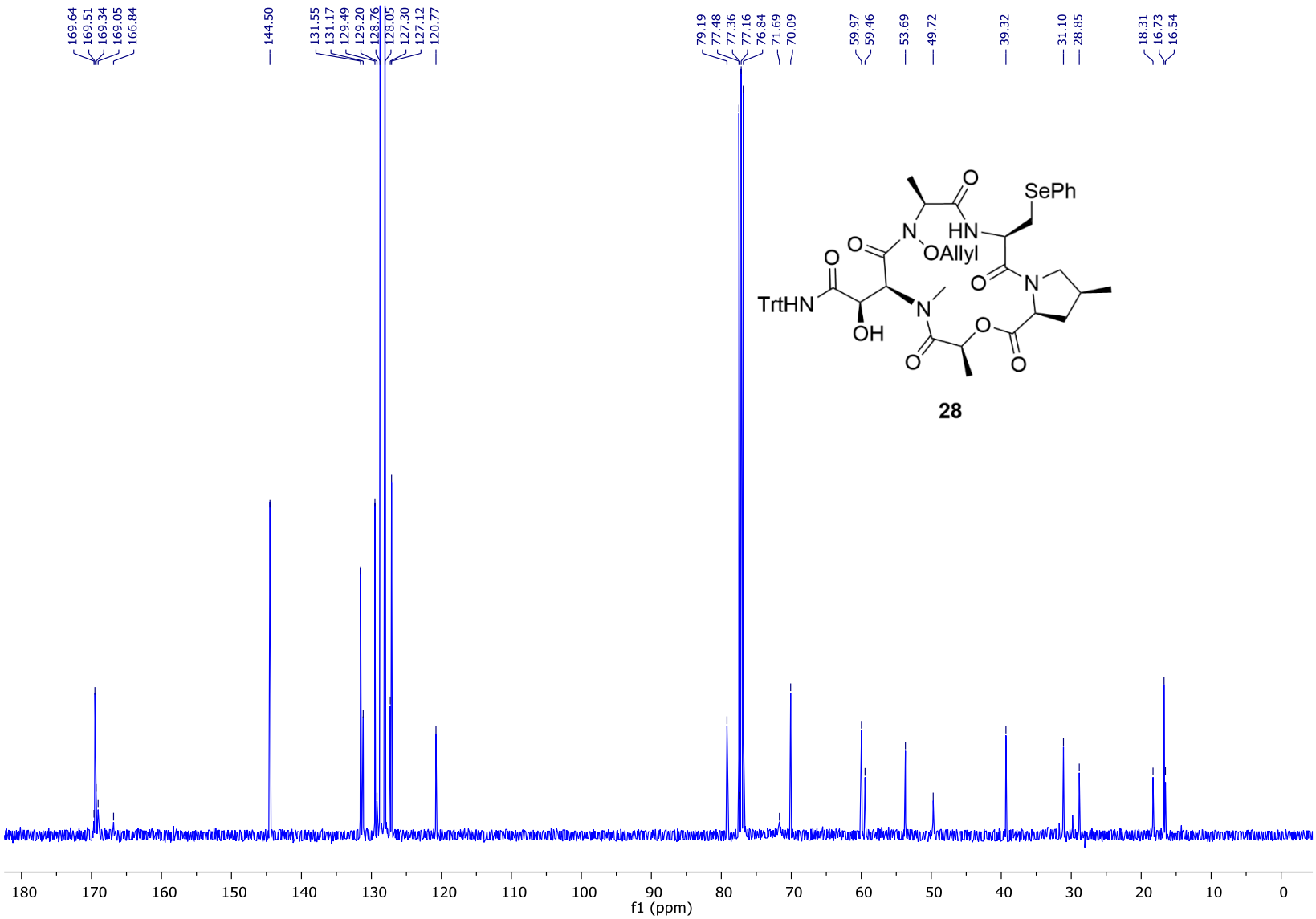
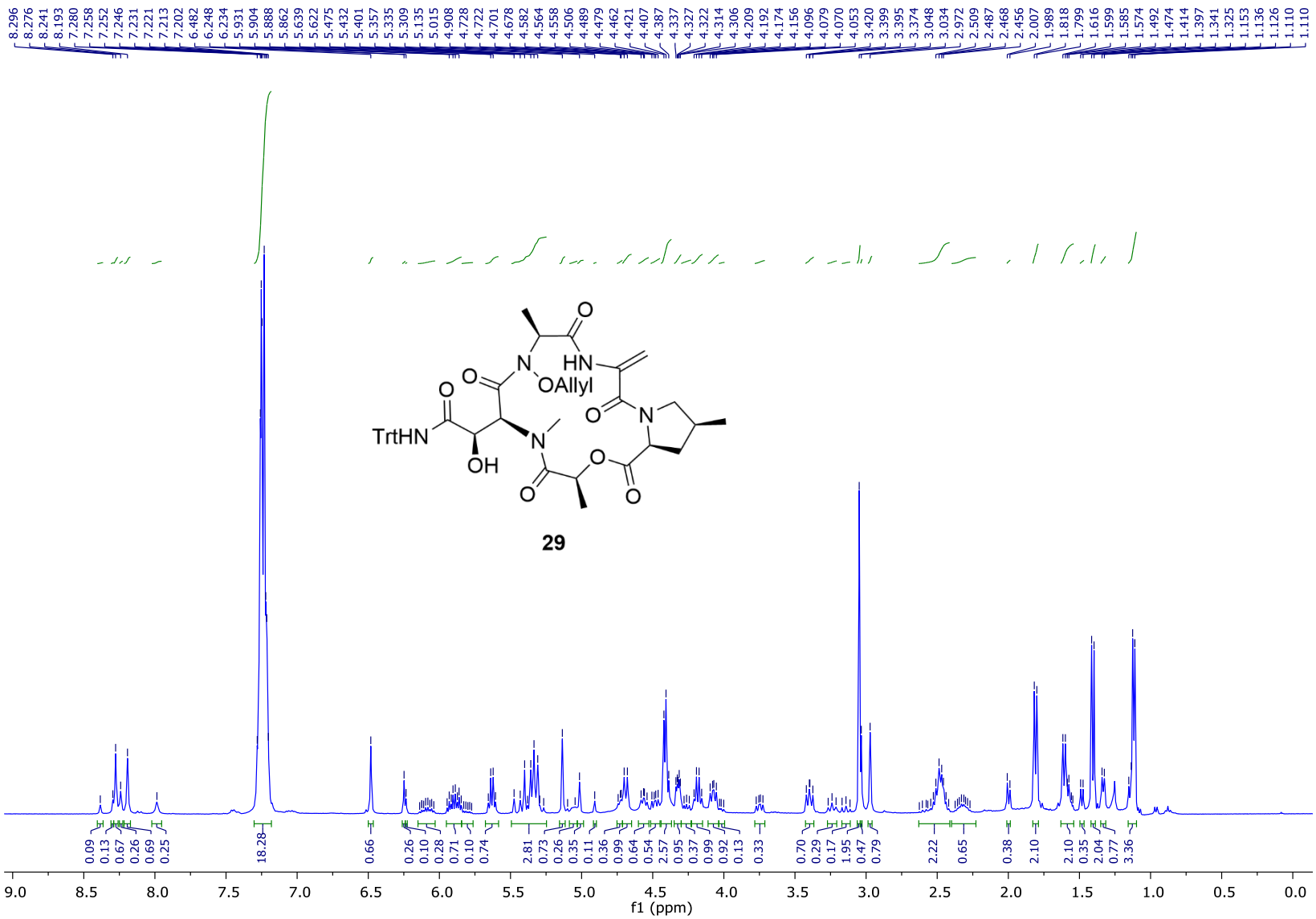


Figure S55. ^{13}C NMR Spectrum of **28** in $CDCl_3$ (100 MHz) at 25 °C



28

Figure S56. ^{1}H NMR Spectrum of **29** in $CDCl_3$ (400 MHz) at 25 °C



29

Figure S57. ^{13}C NMR Spectrum of **29** in $CDCl_3$ (100 MHz) at 25 °C

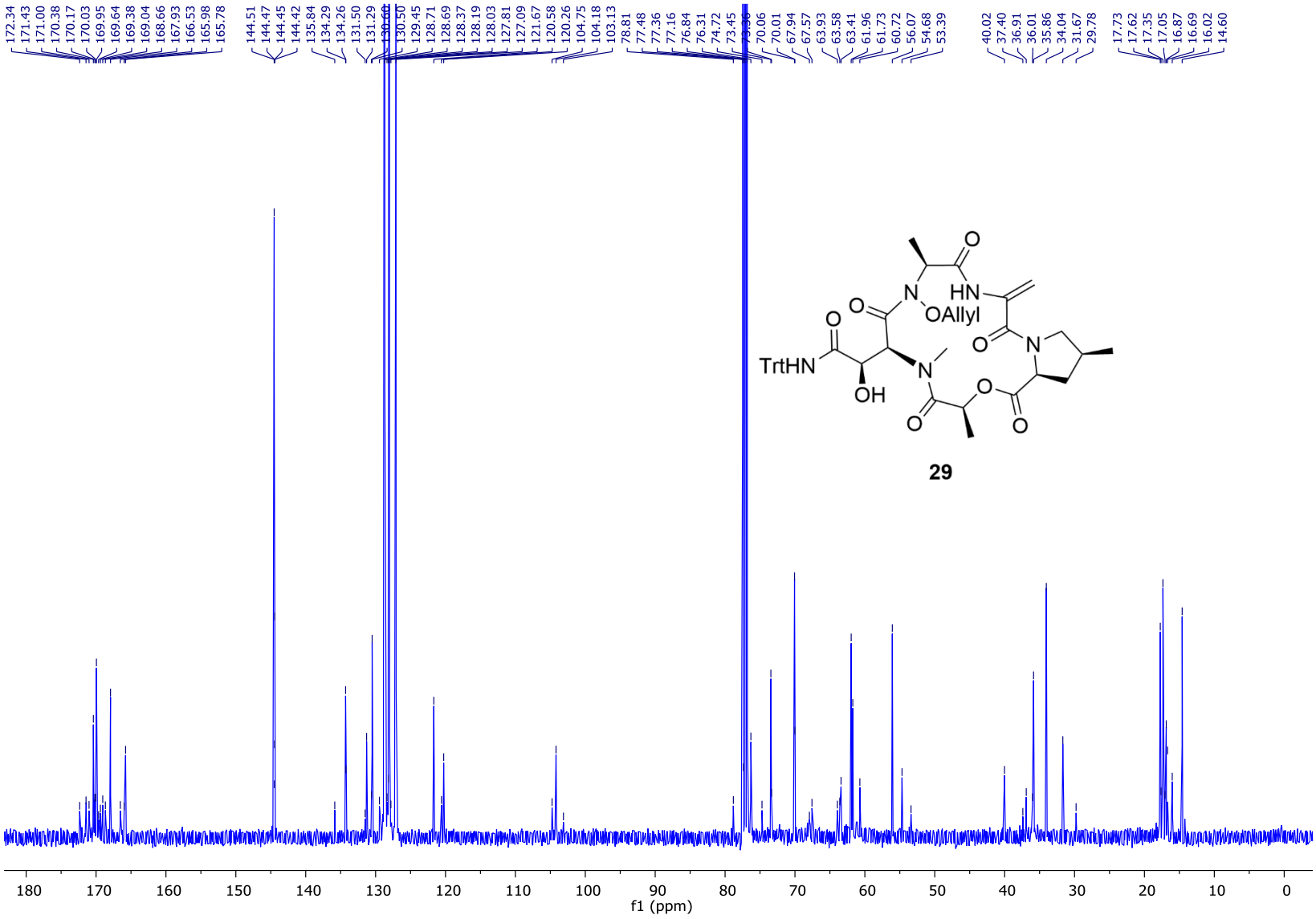
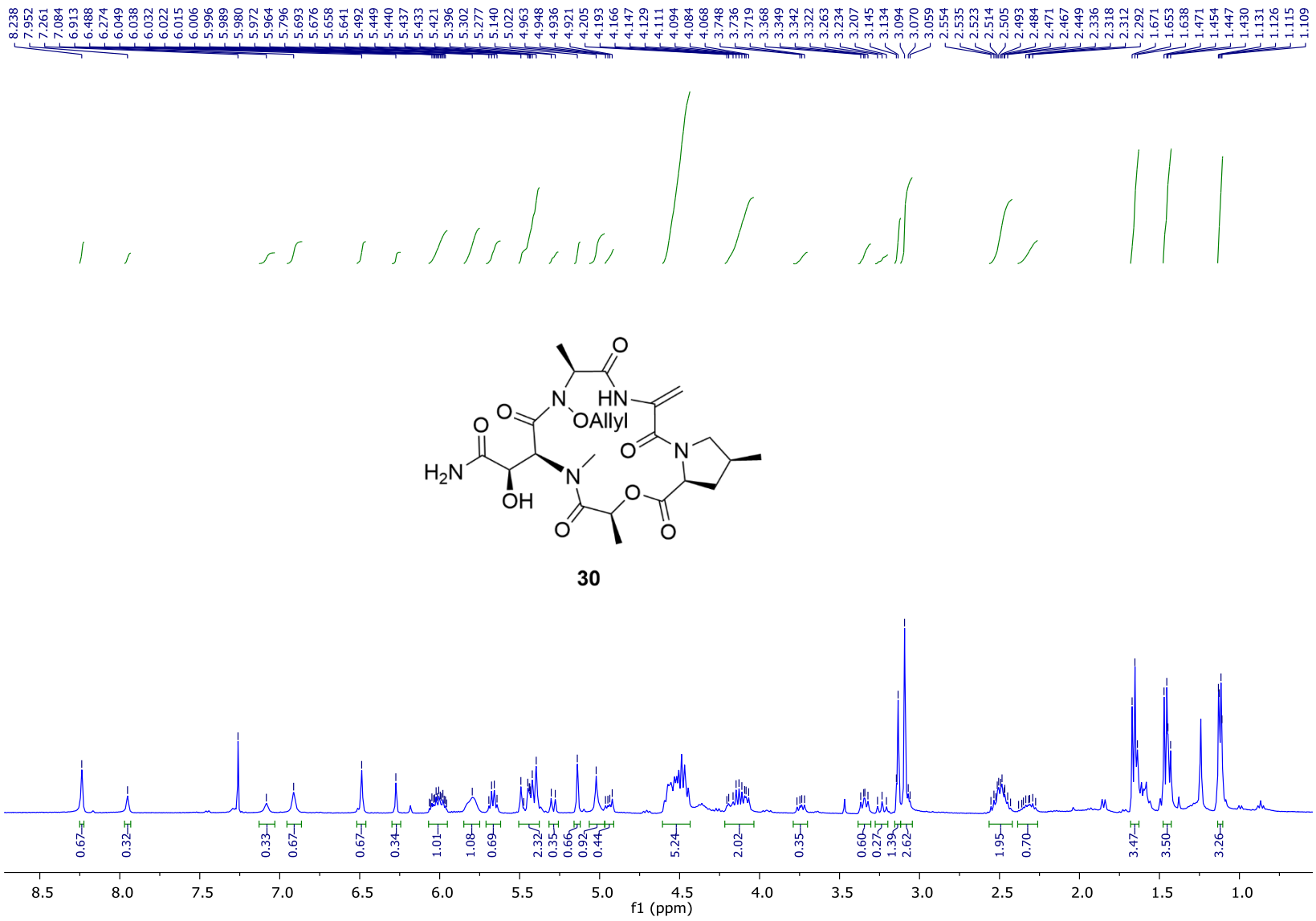


Figure S58. ^{1}H NMR Spectrum of **30** in $CDCl_3$ (400 MHz) at 25 °C



30

Figure S59. ^{13}C NMR Spectrum of **30** in $CDCl_3$ (100 MHz) at 25 °C

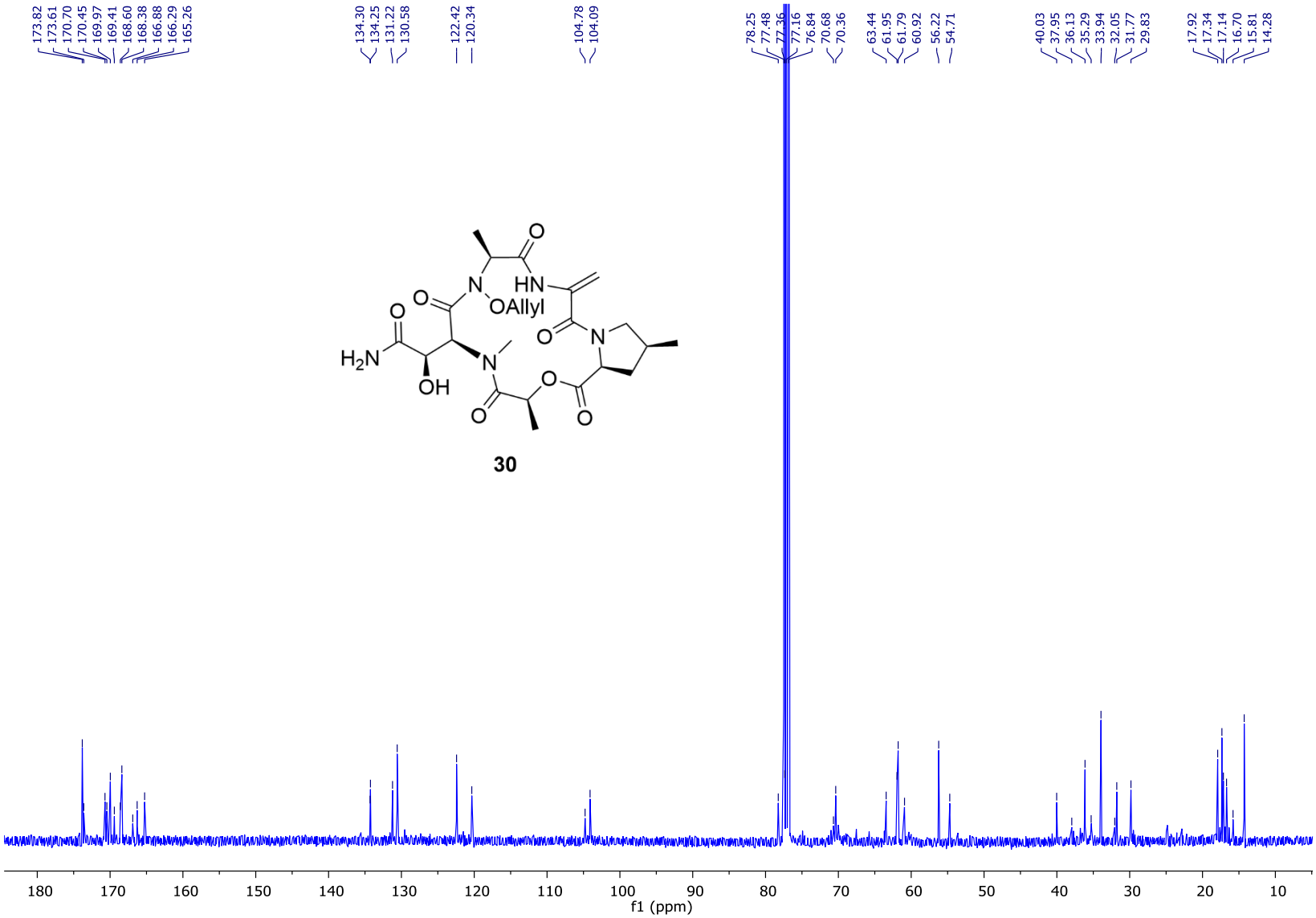
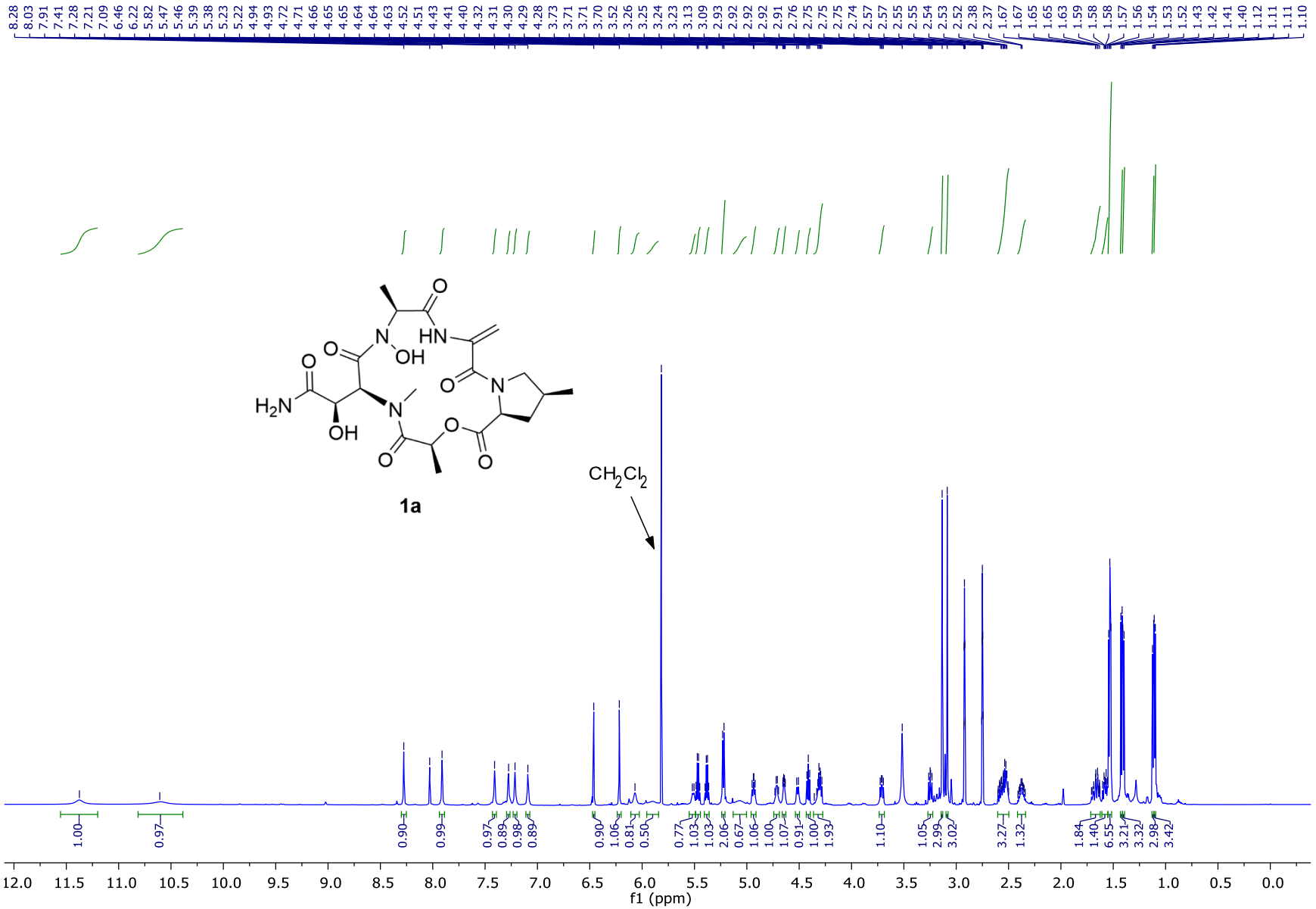
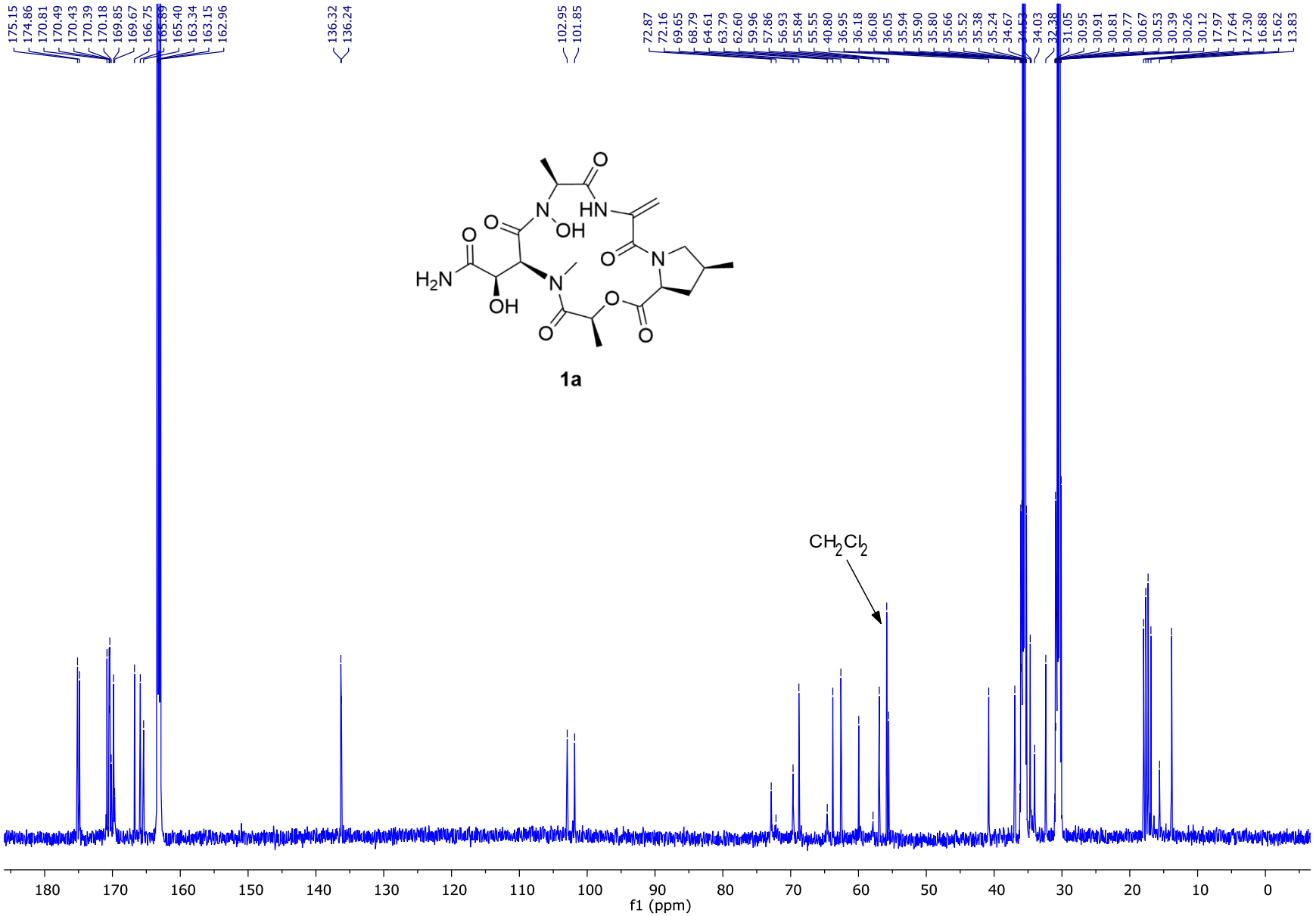


Figure S60. ^{1}H NMR spectrum of synthetic **1a** in DMF-d_7 (600 MHz) at 25 °C



1a

Figure S61. ^{13}C NMR spectrum of synthetic **1a** in DMF-d_7 (150 MHz) at 25 °C



1a

Figure S62. Comparison of ^{1}H NMR spectra of synthetic **1a** (maroon) and natural gatorbulin-1 (**1a**) (blue) in DMF-d_7 (600 MHz) at 25 °C

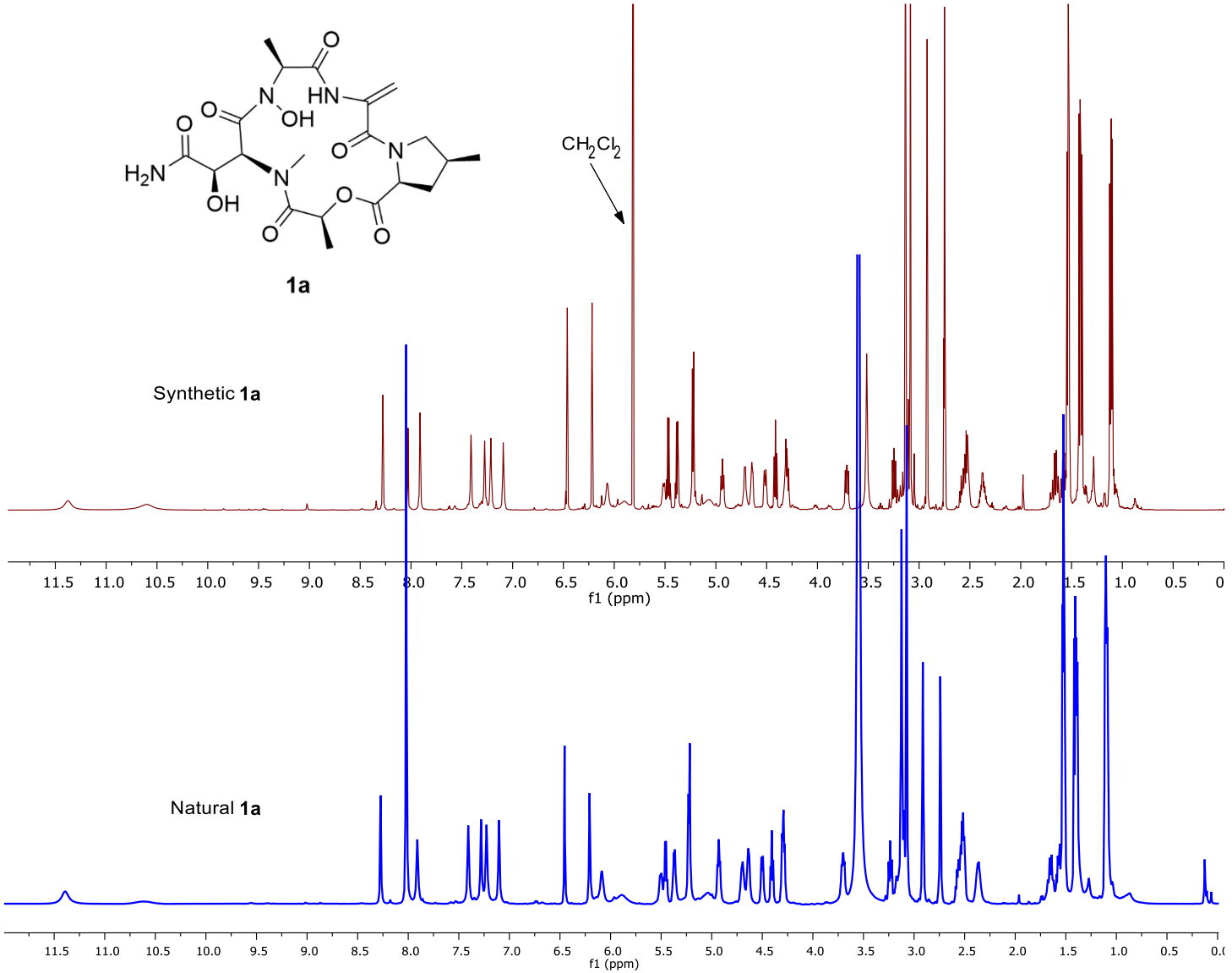


Figure S63. Comparison of ^{13C} NMR spectra of synthetic **1a** (maroon) and natural gatorbulin-1 (**1a**) (blue) in DMF-d_7 (150 MHz) at 25 °C.