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Supporting Information for:

Caged cyclopropenes for controlling bioorthogonal reactivity

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List of abbreviations

BME	β-Mercaptoethanol
Calcd	Calculated
CDI	1,1’-Carbonyldiimidazole
CTAB	Cetyltrimethylammonium bromide
DCM	Dichloromethane
DIPEA	*N,N*-diisopropylethylamine
DMF	Dimethylformamide
DOPE	1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine
ECL	Enhanced Chemiluminescence
ESI	Electrospray ionization
EtOAc	Ethyl acetate
EtOH	Ethanol
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate
HMPA	Hexamethylphosphoramide
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
LAH	Lithium aluminum hydride
LiHMDS	Lithium bis(trimethylsilyl)amide
MeCN	Acetonitrile
MeOH	Methanol
MHz	Megahertz
MOMCl	Methoxymethyl chloride
R_f	Retention factor
R_t	Retention time
TBDPS	tert-Butyldiphenylsilane
TCEP	Tris(2-carboxyethyl)phosphine
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV	Ultraviolet

General materials and methods. All chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification unless otherwise specified. Reactions were monitored by thin layer chromatography (TLC) on pre-coated glass TLC plates (Analtech UNIPLATE™ silica gel HLF w/ organic binder, 250 μm thickness, with UV254 indicator) or by LC/MS (Agilent LC-MSD, direct-injection mode, 1–10 μL, ESI). TLC plates were visualized by UV illumination or developed with either potassium permanganate stain ($KMnO_4$ stain: 1.5 g $KMnO_4$, 10 g K_2CO_3 and 1.25 mL of 10% NaOH dissolved in 200 mL H_2O), ceric ammonium molybdate stain (CAM stain: 12 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.5 g $Ce(NH_4)_2(NO_3)_6$ and 15 mL of concentrated H_2SO_4 dissolved in 235 mL H_2O), or ninhydrin stain (1.5 g ninhydrin dissolved in 100 mL of 1-butanol and 3 mL of conc. AcOH). Flash chromatography was carried out using Sorbtech, 60 Å, 40–63 μm or Millipore 60 Å, 35–70 μm silica gel according to the procedure described by Still[1]. HPLC was performed using a Shimadzu HPLC (FCV-200AL) equipped with an Agilent reversed phase Zorbax Sb-Aq C18 column (Preparatory column: 4.6 × 250 mm or Analytical column: 21.2 × 250 mm) fitted with an Agilent stand-alone prep guard column. HPLC purifications were carried using solvents spiked with 0.1% TFA unless specified otherwise. NMR spectra (^1H and ^{13}C) were obtained using a 300, 400, 500, or 700 MHz Bruker spectrometer and analyzed using Mestrenova 9.0. ^1H and ^{13}C chemical shifts (δ) were referenced to residual solvent peaks. Following residual solvent peaks were chosen: (for ^1H NMR) $CDCl_3$, 7.2600 ppm; CD_3OD, 3.3100 ppm; $(CD_3)_2SO$, 2.5000 ppm (for ^{13}C NMR) $CDCl_3$, 77.1600 ppm; CD_3OD, 49.0000 ppm; $(CD_3)_2SO$, 39.5200 ppm; D_2O, 4.7900 ppm. Further, following abbreviations were used to define ^1H NMR peaks: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Low-resolution electrospray ionization (ESI) and High-resolution electrospray ionization (ESI) mass spectra were obtained at the Stony Brook University Institute for Chemical Biology and Drug Discovery (ICB&DD) Mass Spectrometry Facility with an Agilent LC/MSD and LC-UV-TOF spectrometer respectively. Reaction kinetics were determined by measuring absorbance over time in a 96-well plate format with a BioTek Synergy2 microplate reader. Autoradiographic films (5×7 in, BioExcell) were developed using an ALPHATEK AX300SE developer.

Generation of 1,2,4,5-tetrazine-conjugated bovine serum albumin (BSA-tetrazine): BSA (10.0 mg) was dissolved in 1.930 mL of a pH 8.3–8.4 solution containing 20 parts PBS (5.0 mL, pH 7.4, ThermoFisher Scientific# 10010023) to 1 part $NaHCO_3$ (250 μL, 0.2 M, adjusted to pH 9 using 2M NaOH). To this solution was added Tetrazine-PEG_5-NHS ester (70 μL, 8.0 eq, 1 mg/100 μL in DMSO, CAS# 1682653-80-0). The resulting solution was allowed to stand at rt for 2 h and then overnight at 4 °C. Unreacted tetrazine was removed by dialysis using a 10,000 molecular weight cutoff dialysis cassette (Thermo Scientific product# 87730, 3.0 mL capacity) using 1X PBS (6×2.0 L, pH 7.4, 12–24 h for each dialysis run) at 4 °C. The final concentration of **BSA-PEG_5-Tetrazine** conjugate was adjusted to 5 mg/mL, and stored at 4 °C.

SDS-PAGE and western blotting: 10 μL reactions containing 5.0 μg of protein (BSA alone or BSA modified with PEG_5-Tet) and 500 nM cyclopropene (**10b** or **11**, either exposed to dark or 365 nm light for 4 hours) in 1X PBS (pH 7.4, ThermoFisher Scientific# 10010023) were incubated at rt for 12–14 h. 4X western loading buffer (3.33 μL, containing 5% (v/v) β-mercaptoethanol) was added to each reaction well and the mixture was incubated at rt for 1 h. The reactions were subjected to SDS-PAGE using 12% polyacrylamide gel (mini-PROTEAN® TGX™ precast gels BIORAD# 4561043, 6.67 μL each well, 2.5 μg BSA/BSA-PEG_5-Tet, 250 V, ~25 mins, 1X Tris/Glycine/SDS running buffer). The proteins were transferred to a nitrocellulose membrane (100 V, 1 h on ice, 1X western transfer buffer containing 10% MeOH (v/v)). The membrane was stained with Ponceau S solution (1.0 g Ponceau S in 1.0 L of 5% (v/v) acetic acid/ddH_2O), scanned, and then blocked (50 mL, 5% BSA in 1X TBST buffer, filtered through a 0.22 μm filter, Millipore# SCGP00525) for 1 h at rt or overnight at 4 °C. Antibody incubation (1:50000 dilution,1 μL Peroxidase-conjugated IgG fraction monoclonal mouse anti-biotin added to filtered 50 mL 5% BSA in 1X TBST buffer, Jackson ImmunoResearch# 200-032-211) was performed for 1 h at rt. After washing out the excess antibody with 1X TBST buffer (4×30 mL,10 min each), western ECL substrate was added (10 mL total, 5 min, BIORAD# 170-5060), and the autoradiographic film (BioExcell# 41101002) was developed (ALPHATEK AX300SE).

HPLC-based analysis of no reactivity between tetrazine S9 and cyclopropene 6b for 4 weeks: A reaction mixture consisting of **6b** (2.0 mM) and **S9** (0.5 mM) in 400 μL of 1:1 MeCN/PBS (pH = 7.4, ThermoFisher Scientific# 10010023) was incubated in the dark at rt. At each designated time point (0 h, 3 h, 1 day, 2 days, 7 days, 14 days, and 28 days), a 40 μL aliquot was withdrawn from the reaction mixture, diluted to 200 μL using 1:1 MeCN/ddH_2O, and subjected to reversed phase HPLC (50–100% MeCN + 0.1% TFA over 25 mins, flow rate = 1 mL/min). Each HPLC peak was confirmed to be the expected, unreacted reagent by mass spectrometry analysis.

HPLC-based analysis of no reactivity between L-Glutathione and cyclopropene 6b for 24 h: A reaction mixture consisting of **6b** (2.5 mM) and L-glutathione (10 mM) in 400 µL of 1:1 MeCN/PBS (pH = 7.4, ThermoFisher Scientific# 10010023) was incubated in the dark at rt. At each designated time point (0 h, 3 h, 10 h, and 24 h), a 40 µL aliquot was withdrawn from the reaction mixture, diluted to 200 µL using 1:1 MeCN/ddH$_2$O, and subjected to HPLC (50-100% MeCN + 0.1% TFA over 25 mins, flow rate = 1 mL/min). Each HPLC peak was confirmed to be the expected, unreacted reagent by mass spectrometry analysis.

HPLC-based analysis of stability of cyclopropene 6b at 37 °C: A solution of **6b** (2.5 mM) in 400 µL of 1:1 MeCN/PBS (pH = 7.4, ThermoFisher Scientific# 10010023) was incubated in the dark at 37° C. At each designated time point (0 h, 3 h, 10 h, 24 h, and 7 days), a 40 µL aliquot was withdrawn from the reaction mixture, diluted to 200 µL using 1:1 MeCN/ddH$_2$O, and subjected to HPLC (50–100% MeCN + 0.1% TFA in 25 mins, flow rate = 1 mL/min). No decomposition products were observed for at least 7 days. The HPLC peaks were confirmed to be the expected, unreacted **6b** by mass spectrometry analysis.

HPLC-based analysis of the ligation between cyclopropene 10a and tetrazine S10: A reaction mixture consisiting of **10a** (50 mM) and **S10** (5 mM) in 200 µL of 1:1 MeCN/NH$_4$HCO$_3$ buffer (pH = 8.0, final concentration of buffer = 250 mM) was incubated in the dark at rt. At each designated time point (0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, and 7 h), a 10 µL aliquot was withdrawn from the reaction mixture, diluted to 100 µL using ddH$_2$O + 0.1 % TFA, and subjected to HPLC (30–100% MeCN + 0.1% TFA over 35 mins, flow rate = 1 mL/min). The ligated product peak **S11** was observed as confirmed by mass spectrometry and NMR analyses.

2nd order rate constant determination for reaction between cyclopropene 10a and tetrazine S10: Reaction mixtures consisting of **10a** (50/65/85 mM for pH 8.0 and pH 8.8, and 20/26/34 mM for pH 7.4) and **S10** (5 mM for pH 8.0 and pH 8.8, and 2 mM for pH 7.4.) in 50% MeCN/buffer (total volume = 200 µL, 250 mM NH$_4$HCO$_3$ buffer for pH 7.4 and pH 8.0, and 250 mM Tris buffer for pH 8.8.) were prepared in separate wells of a 96-well plate at rt at the indicated pH and buffer composition. The reaction progress was monitored by recording the disappearance of the characteristic 1,2,4,5-tetrazine absorbance at 520 nm every 10 mins. The pseudo 1st order rates were obtained by plotting the normalized absorbance with respect to the initial absorbance against reaction time (using the pseudo 1st order rate equation A = A$_o$.exp (-k.[**10a**].t) where A = absorbance at time t, A$_o$ = initial absorbance, k.[**10a**] = pseudo 1st order rate constant, and t = time). The 2nd order rate constants were obtained by plotting the pseudo 1st order rates against the concentration of **10a**.

***Fitted equations* for calculation of pseudo 1st order, and 2nd order rate constants**

pH	Obtained *fitted equation* and R^2 (for peudo 1st order rate Constants)	Obtained *fitted equation* and R^2 (for 2nd order rate constants)
7.4	$y = 1.023e^{-2E-05x}$, R² = 0.9986 $y = 1.024e^{-3E-05x}$, R² = 0.9988 $y = 1.023e^{-3E-05x}$, R² = 0.9985	y = 4E-07x + 2E-05, R² = 0.9296
8.0	$y = 1.034e^{-5E-05x}$, R² = 0.9992 $y = 1.044e^{-6E-05x}$, R² = 0.9988 $y = 1.048e^{-7E-05x}$, R² = 0.9974	y = 6E-07x + 2E-05, R² = 0.9932
8.8	$y = 1.001e^{-5E-05x}$, R² = 0.9995 $y = 0.996e^{-6E-05x}$, R² = 0.9998 $y = 0.991e^{-8E-05x}$, R² = 0.9987	y = 9E-07x + 6E-06, R² = 0.9884

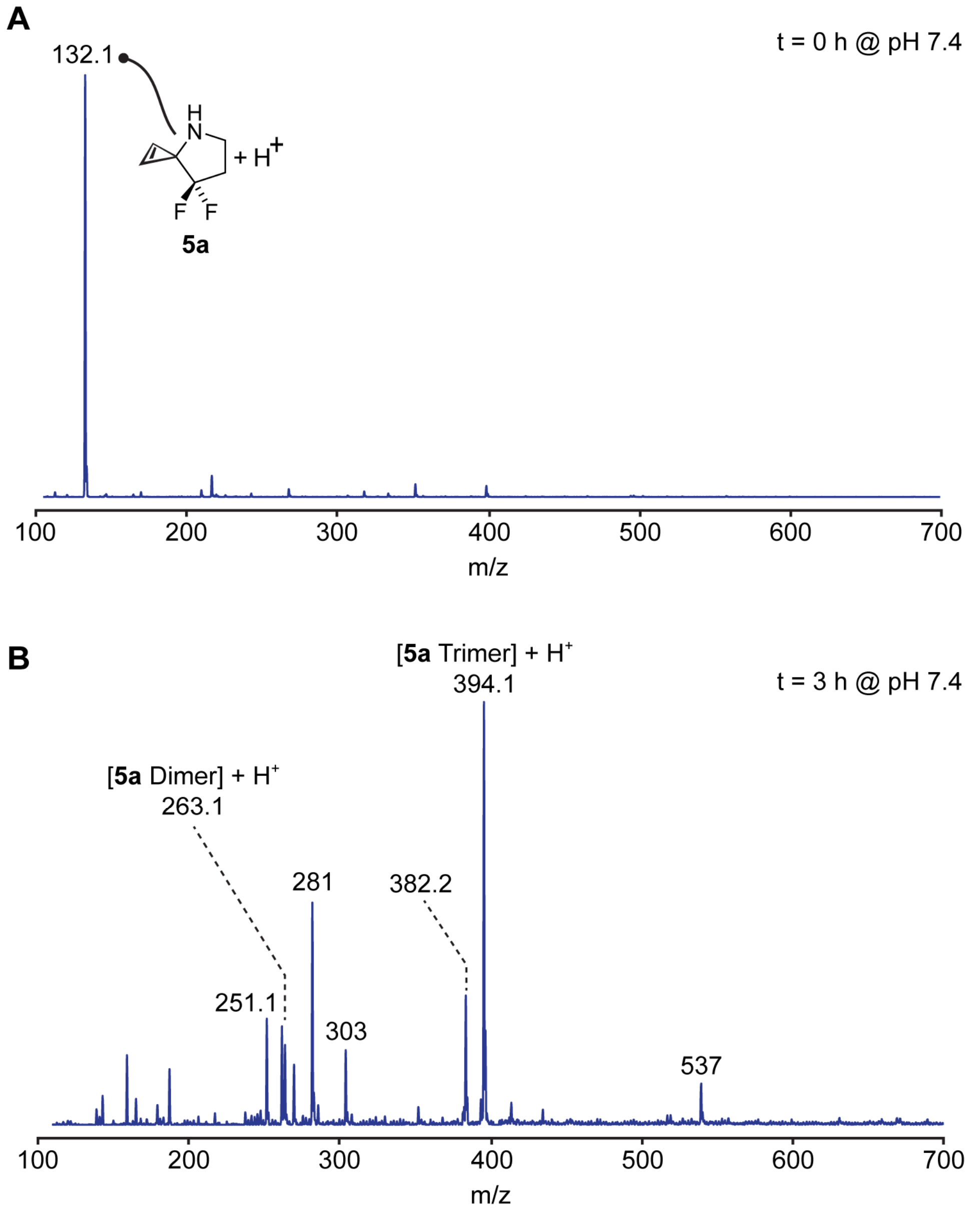


Figure S1: Degradation of cyclopropene 5a at neutral pH. (**A**–**B**) ESI-MS of **5a** after incubation at pH 7.4 for 0 h (A) or 3 h (B).

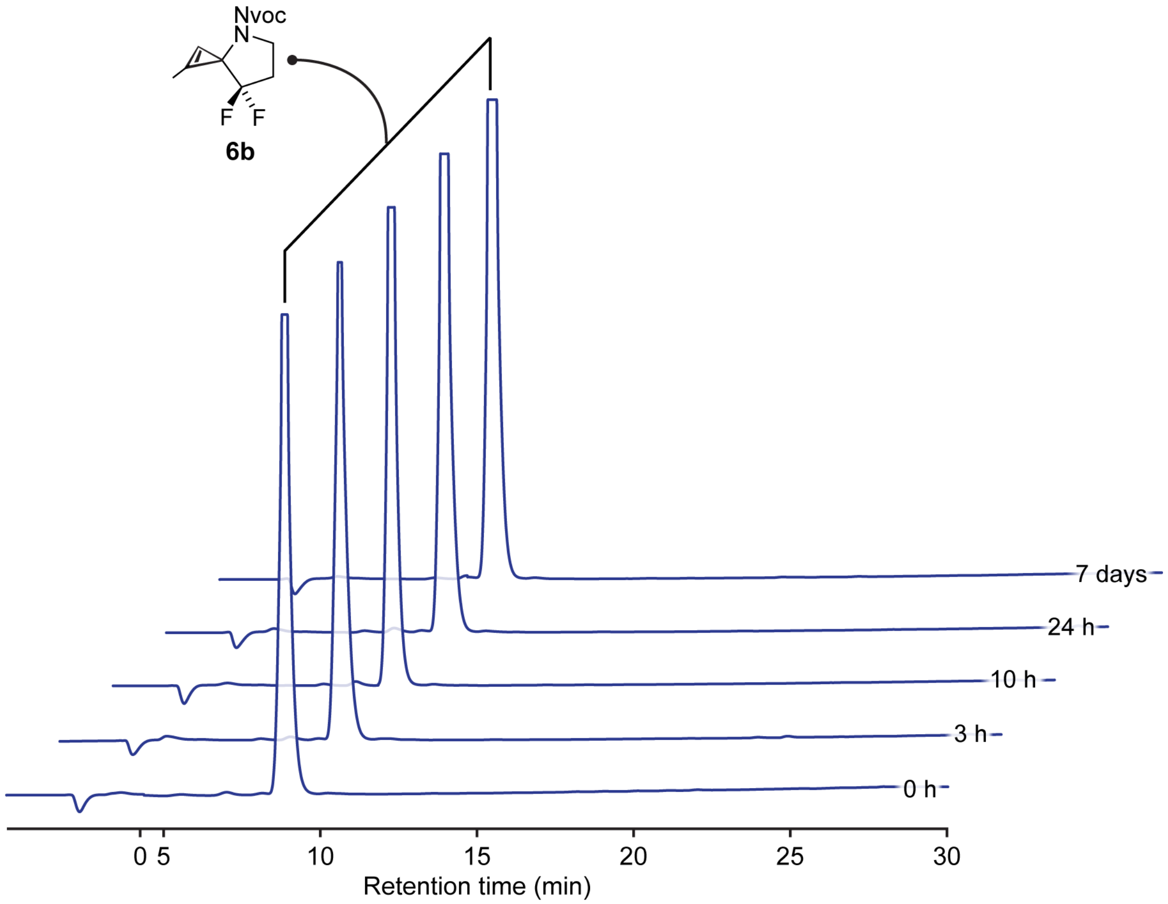


Figure S2: Stability of Nvoc-protected **6b** at 37 °C. HPLC-based assay showed no decomposition products for at least 7 days. The HPLC peaks were confirmed to be the expected, unreacted **6b** by mass spectrometry analysis.

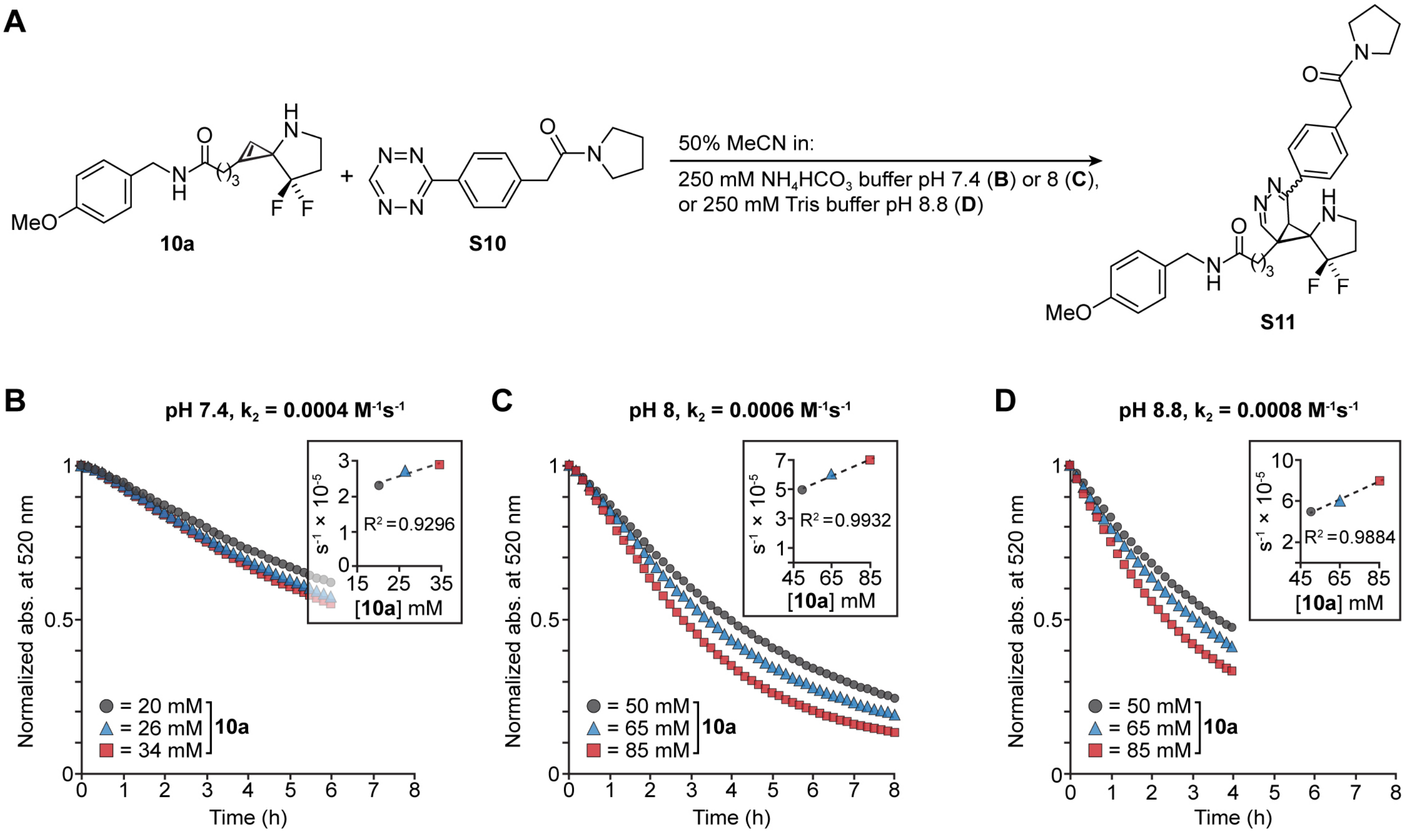


Figure S3: Kinetic evaluation of ligation between cyclopropene **10a** and 1,2,4,5-tetrazine **S10**. (**A**) Incubation of cyclopropene **10a** and 1,2,4,5-tetrazine **S10** in 1:1 MeCN/buffer producted the expected ligated product **S11**. (**B**–**D**) 2nd order rate constants for this ligation reaction were determined at pH 7.4, 8.0, and 8.8.

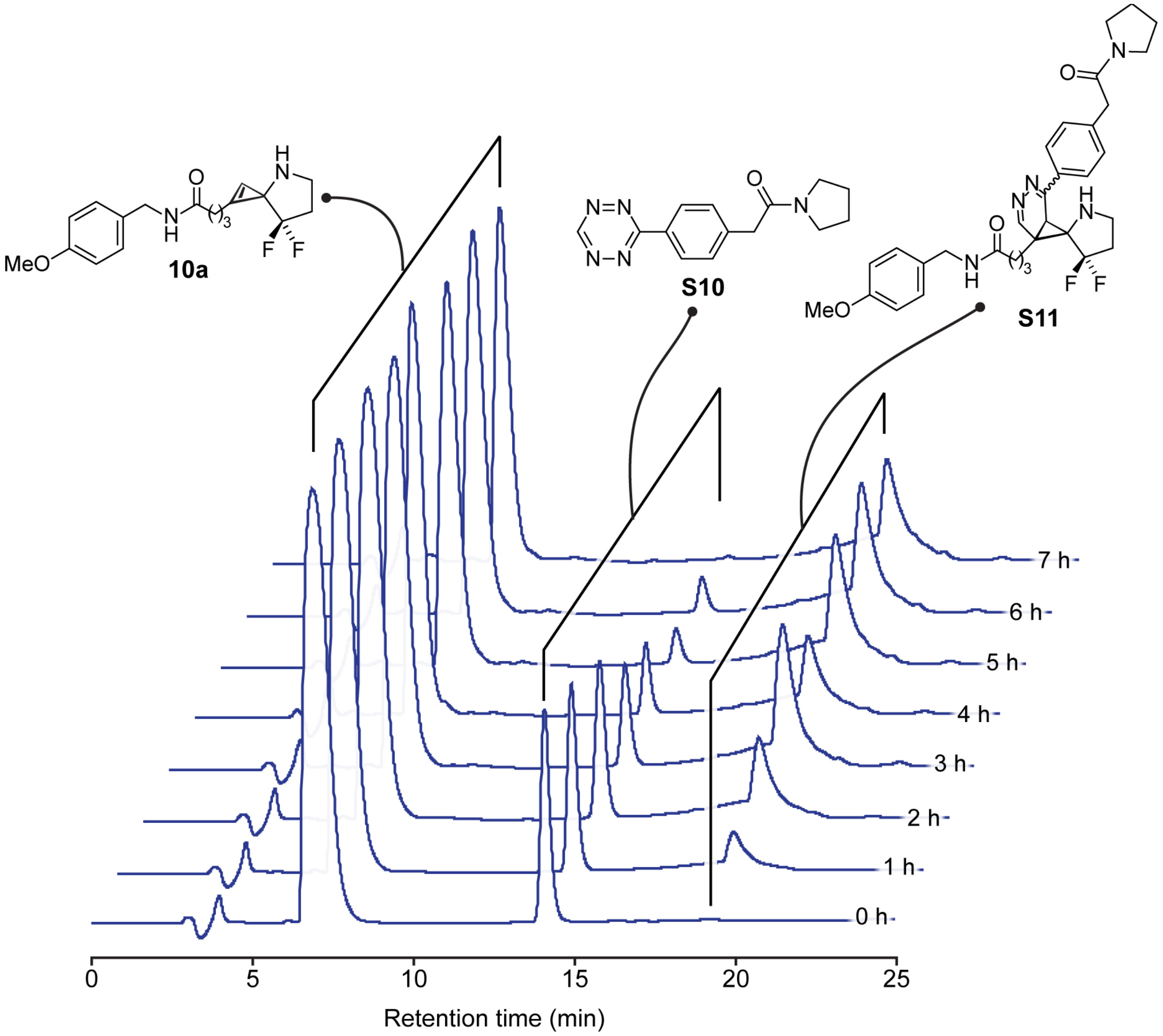
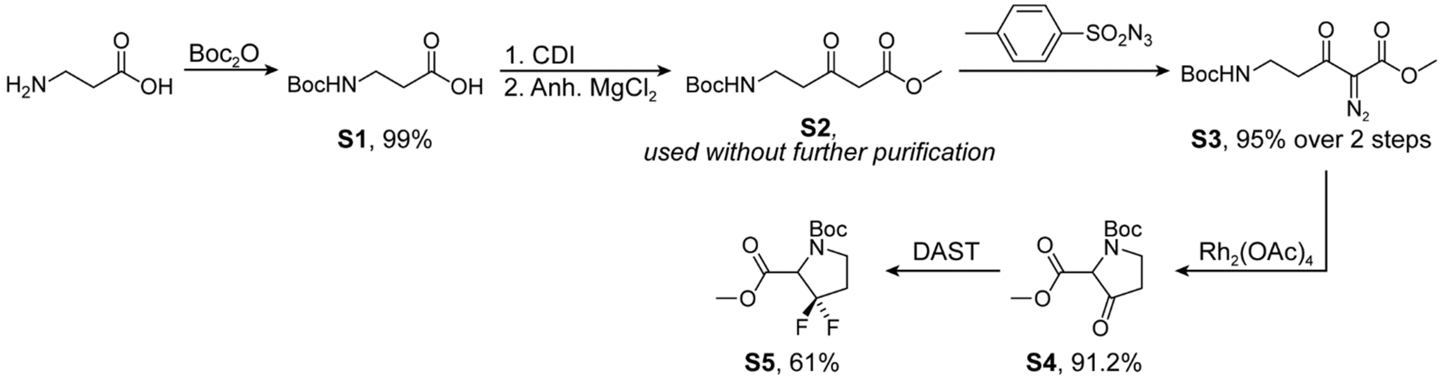
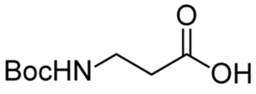


Figure S4: HPLC-based analysis of the ligation between **10a** and **S10**. A reaction mixture consisiting of 1**0a** (50 mM) and **S10** (5 mM) in 1:1 MeCN/NH_4HCO_3 buffer (pH = 8.0) was incubated in the dark at rt. At each designated time point (0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, and 7 h), a 10 µL aliquot was withdrawn from the reaction mixture and subjected to HPLC. The ligated product **S11** peak was observed and confirmed by NMR and mass spectrometry analysis. (Scheme 7, S87).

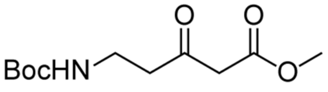
Scheme 1





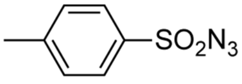
S1

To a solution of β-alanine (20.0 g, 224.49 mmol, 1 eq) in 2M NaOH (200 mL) and THF (200 mL) was added Boc$_2$O (49.98 g, 228.98 mmol,1.02 eq). The reaction mixture was stirred at rt for 16 h, concentrated *in vacuo* to remove THF, and diluted with DCM (50 mL). The organic layer was discarded and the aqueous layer was acidified to pH 1–2 using 2M HCl. The aqueous layer was washed with EtOAc (3×75 mL) and the combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo* to obtain an oil that solidified upon cooling to give **S1** as a white powder (42.2 g, quantitative). R_f = 0.1–0.5 (50% EtOAc/hexanes, visualized w/ Ninhydrin stain). ^{1}H NMR (1:3 mixture of rotamers, 500 MHz, CDCl$_3$): δ= 11.68 (s, 1H), 6.32 (s, 0.27H), 5.16 (s, 0.62H), 3.39–3.32 (m, 2H), 2.56–2.51 (m, 2H), 1.50–1.30 (m, 9H). ^{13}C NMR (rotamers,126 MHz, CDCl$_3$): δ= 177.53, 176.47, 157.69, 156.11, 81.25, 79.79, 37.25, 35.99, 34.54, 28.45. MS (ESI): Calcd for $C_8H_{15}NO_4$ [M]$^+$: 189.1, found: 134.1 [M-C(CH$_3$)$_3$+2H]$^+$, 190.1 [MH]$^+$.



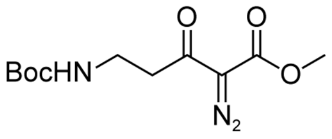
S2

S2 was synthesized based on a modification to a previously described strategy[2]. To a suspension of **S1** (19.78 g, 104.50 mmol, 1 eq) in THF (200 mL) was added CDI (20.49 g, 125 mmol, 1.2 eq) and the mixture was stirred at rt for 4 h during which it turned homogenous. To this activated acid (R_f = 0.25, 50% EtOAc/hexanes, visualized w/ UV) was added a mixture of MgCl$_2$ (9.95 g, 104.50 mmol, 1 eq) and Potassium 3-methoxy-3-oxopropanoate (32.64 g, 209 mmol, 2 eq) in one portion. The suspension was stirred at rt for 16 h, quenched with water, and concentrated *in vacuo* to remove THF. The reaction mixture was then diluted with DCM (150 mL) and 1M HCl (150 mL), and stirred at rt for 15 min to dissolved the white solid. The aqueous layer was acidified to pH 1–2 using 1M HCl and extracted with DCM. The combined organic layers were washed with brine, dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo* to obtain a pale-yellow oil **S2** that was used without further purification (26.2 g). R_f = 0.31 (30% EtOAc/hexanes, visualized w/ UV). ^{1}H NMR (16:84 % mixture of rotamers, 500 MHz, CDCl$_3$): δ= 5.88 (s, 0.16 H), 5.21 (s, 0.82 H), 3.54 (s, 2H), 3.51 (s, 3H), 3.15 (q, J = 6.1 Hz, 2H), 2.59 (t, J = 6.1 Hz, 2H), 1.25–1.20 (m, 9H). ^{13}C NMR (rotamers,126 MHz, CDCl$_3$): δ= 202.22, 168.89, 155.96, 79.27, 52.14, 48.74, 42.71, 40.76, 28.11. MS (ESI): Calcd for $C_{11}H_{19}NO_5$ [M]$^+$: 245.1, found: 146.1 [M-Boc+2H]$^+$, 190.1 [M-C(CH$_3$)$_3$+2H]$^+$, 246.1 [MH]$^+$.



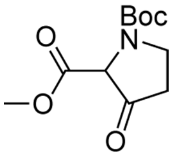
Tosyl azide

To an ice-cold solution of TsCl (10.0 g, 52.46 mmol, 1.0 eq) in acetone (100 mL) and water (50 mL) was added NaN$_3$ (3.58 g, 55.09 mmol, 1.05 eq) and the reaction was allowed to warm to rt over 4 h. Reaction was concentrated *in vacuo* at 27 °C to remove acetone. The aqueous layer was washed with DCM (3×50 mL). The combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo* to obtain **tosyl azide** as a transparent oil that solidified upon freezing (10.3 g, quantitative). R_f = 0.80 (20% EtOAc/hexanes, visualized w/ UV). ^{1}H NMR (700 MHz, CDCl$_3$) : δ= 7.77 (d, J = 8.6 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 2.41 (s, 3H). ^{13}C NMR (175 MHz, CDCl$_3$): δ= 146.24, 135.20, 130.17, 127.27, 21.47. MS (ESI): Calcd for $C_7H_7SO_2N_3$ [M]$^+$: 197.0, found: 220.0 [MNa]$^+$, 417.0 [2M+Na]$^+$.



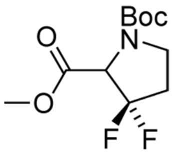
S3

To a solution of crude β-ketoester **S2** (26.2 g, 106.8 mmol, 1 eq) in MeCN (400 mL) was added tosyl azide (21.42 g, 108.9 mmol, 1.02 eq). Et_3N (44.4 mL, 320.4 mmol, 3 eq) was added dropwise and the mixture was stirred at rt for 2 h. The reaction mixture was concentrated *in vacuo*, and diluted with DCM and water. The organic layer was collected and the aqueous layer was further washed with DCM. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by flash chromatography (180 g silica, 30% ethyl acetate/hexanes (v/v)) to obtain **S3** as a yellow oil (27.0 g, 95.2% over two steps). R_f = 0.43 (30% EtOAc/hexanes, visualized w/ UV). ^1H NMR (500 MHz, $CDCl_3$): δ= 5.17 (t, *J* = 6.2 Hz, 1H), 3.51 (s, 3H), 3.08 (q, *J* = 6.2 Hz, 2H), 2.71 (t, *J* = 6.2 Hz, 2H), 1.07 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$): δ= 190.74, 160.95, 155.24, 78.09, 75.30, 51.60, 40.15, 35.03, 27.71. MS (ESI): Calcd for $C_{11}H_{17}N_3O_5$ $[M]^+$: 271.1, found: 272.1 $[MH]^+$, 294.1 $[MNa]^+$.



S4

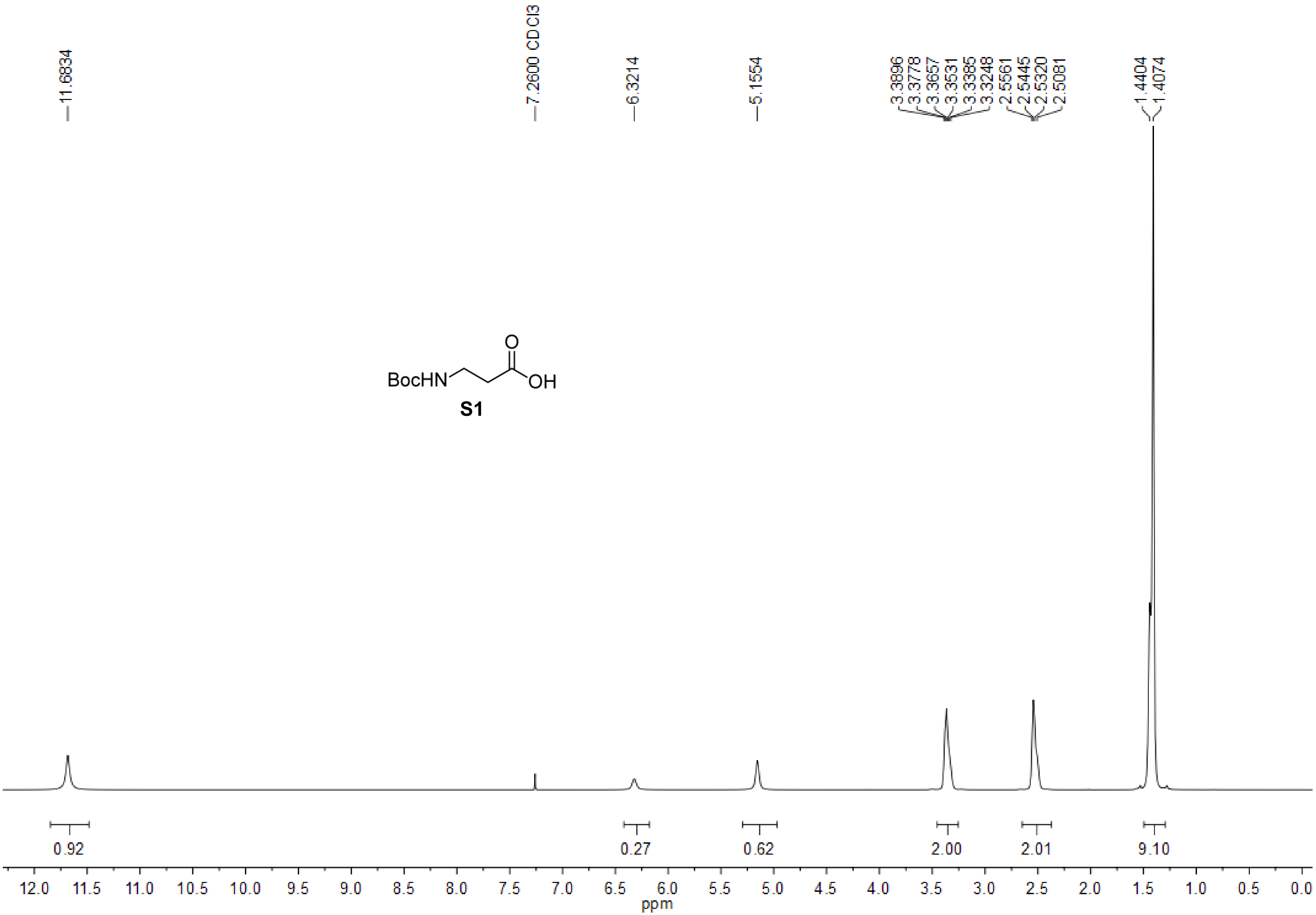
S4 was synthesized based on slight modification of a previous report[2]. To a solution of **S3** (25.5 g, 94.06 mmol, 1 eq) in dry toluene (350 mL) was added Rh_2OAc_4 (208 mg, 0.47 mmol, 0.005 eq) and the mixture was stirred at 90 °C for 1 h. The reaction mixture was concentrated *in vacuo* and passed through silica gel (60 g) using ether to obtain **S4** as an oil that solidified upon cooling (20.85 g, 91.2%). R_f = 0.27 (30% EtOAc/hexanes, visualized w/ UV). ^1H NMR (33:67 % mixture of rotamers, 700 MHz, $CDCl_3$): δ= 4.14 (m, 1H), 3.55–3.48 (m, 1H), 3.45–3.39 (m, 4H), 2.43–2.36 (m, 1H), 2.35–2.31 (m, 1H), 1.14–1.06 (2×s, 9H). ^{13}C NMR (rotamers, 175 MHz, $CDCl_3$): δ= 204.04, 203.51, 166.31, 166.03, 153.42, 153.07, 80.04, 65.05, 64.65, 52.16, 52.12, 41.74, 41.07, 36.40, 35.70, 27.57, 27.44. MS (ESI): Calcd for $C_{11}H_{17}NO_5$ $[M]^+$: 243.1, found: 144.1 $[M\text{-}Boc+2H]^+$, 188.1 $[M\text{-}C(CH_3)_3+2H]^+$, 244.1 $[MH]^+$, 487.2 $[2M+H]^+$.



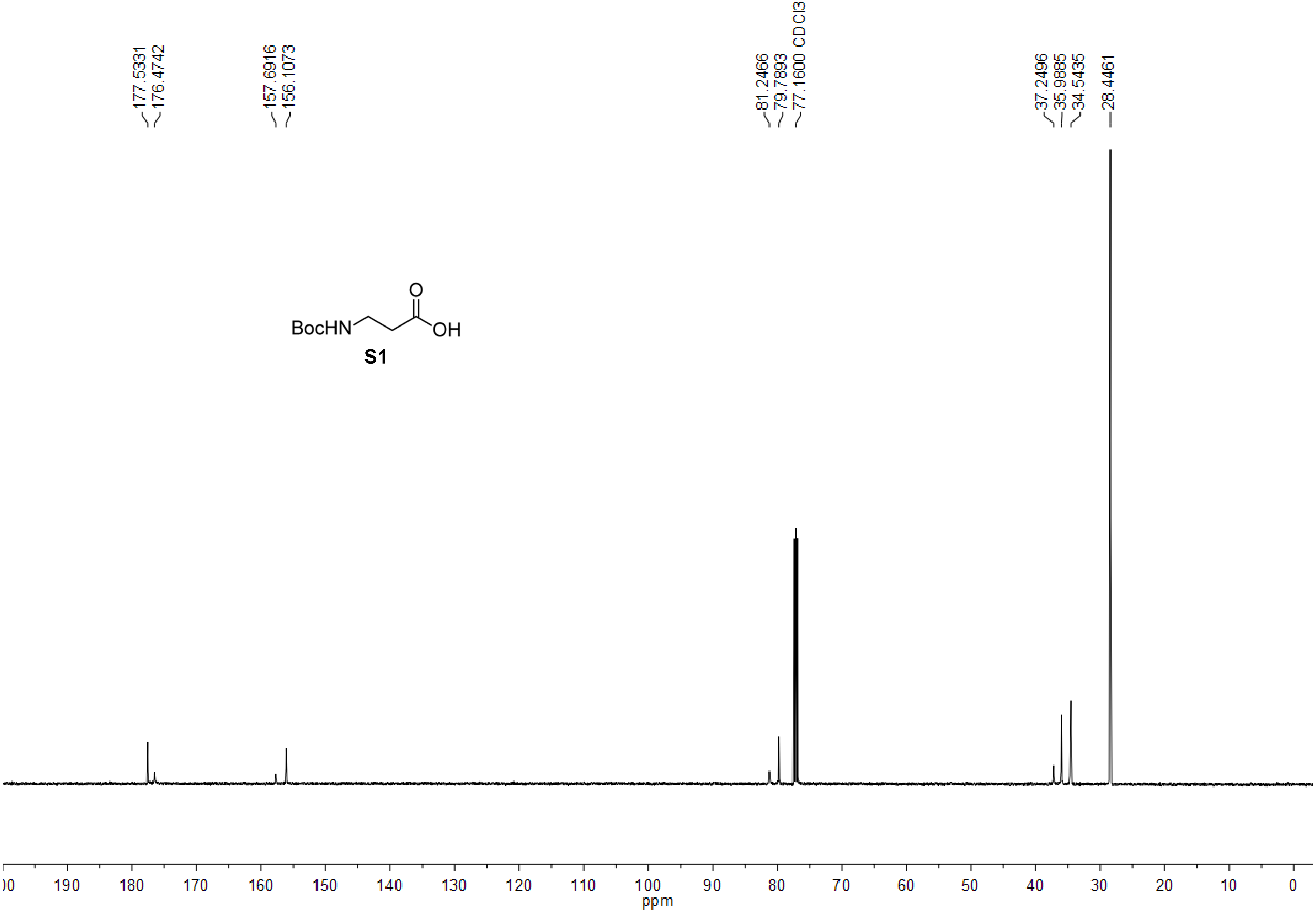
S5

S5 was synthesized based on a modification of a recently described strategy[3]. **S4** (6.5 g, 26.74 mmol, 1 equiv) was added to a flame dried flask under argon and cooled down to 0 °C. DAST (10.6 mL, 80.21 mmol, 3 equiv) was added dropwise using syringe and the resulting solution was allowed to warm to rt overnight. The yellow-orange solution was cooled down to 0°C, diluted with DCM, and quenched using dropwise addition of a saturated $NaHCO_3$ (~200 mL) until pH 8-9. The mixture was stirred for 30 min at 0°C and the resulting biphasic mixture was separated. The aqueous layer was further extracted with DCM. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by flash chromatography (100 g silica, 10% ethyl acetate/hexanes (v/v)) to obtain **S5** as a pale-yellow oil (4.3 g**,** 61%). R_f = 0.27 (10% EtOAc/hexanes, visualized w/ $KMnO_4$). (4.51 g, 16.16 mmol, 64% yield). ^1H NMR (50:60 % mixture of rotamers, 500 MHz, $CDCl_3$): δ= 4.50–4.38 (m, 1H), 3.77 (s, 3H), 3.77–3.68 (m, 1H), 3.55–3.47 (m, 2H), 2.51–2.27 (m, 2H), 1.40 (2×s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ= 167.99, 167.96, 167.92, 167.89, 153.77, 153.16, 128.68, 128.01, 126.65, 125.99, 124.63, 124.00, 81.22, 81.15, 65.41, 65.17, 64.93, 64.83, 64.59, 64.35, 52.90, 52.75, 43.48, 43.44, 42.94, 42.91, 33.62, 33.43, 33.25, 33.02, 32.84, 32.65, 28.33, 28.21. ^{19}F NMR (376 MHz, $CDCl_3$): δ= (-94.24)–(-95.29) (m, 1F), (-105.89)–(-108.12) (m, 1F). MS (ESI): Calcd for $C_{11}H_{17}F_2NO_4$, 265.1; found, 166.1 $[M\text{-}Boc+2H]^+$, 210.1 $[M\text{-}C(CH_3)_3+2H]^+$, 266.1 $[MH]^+$.

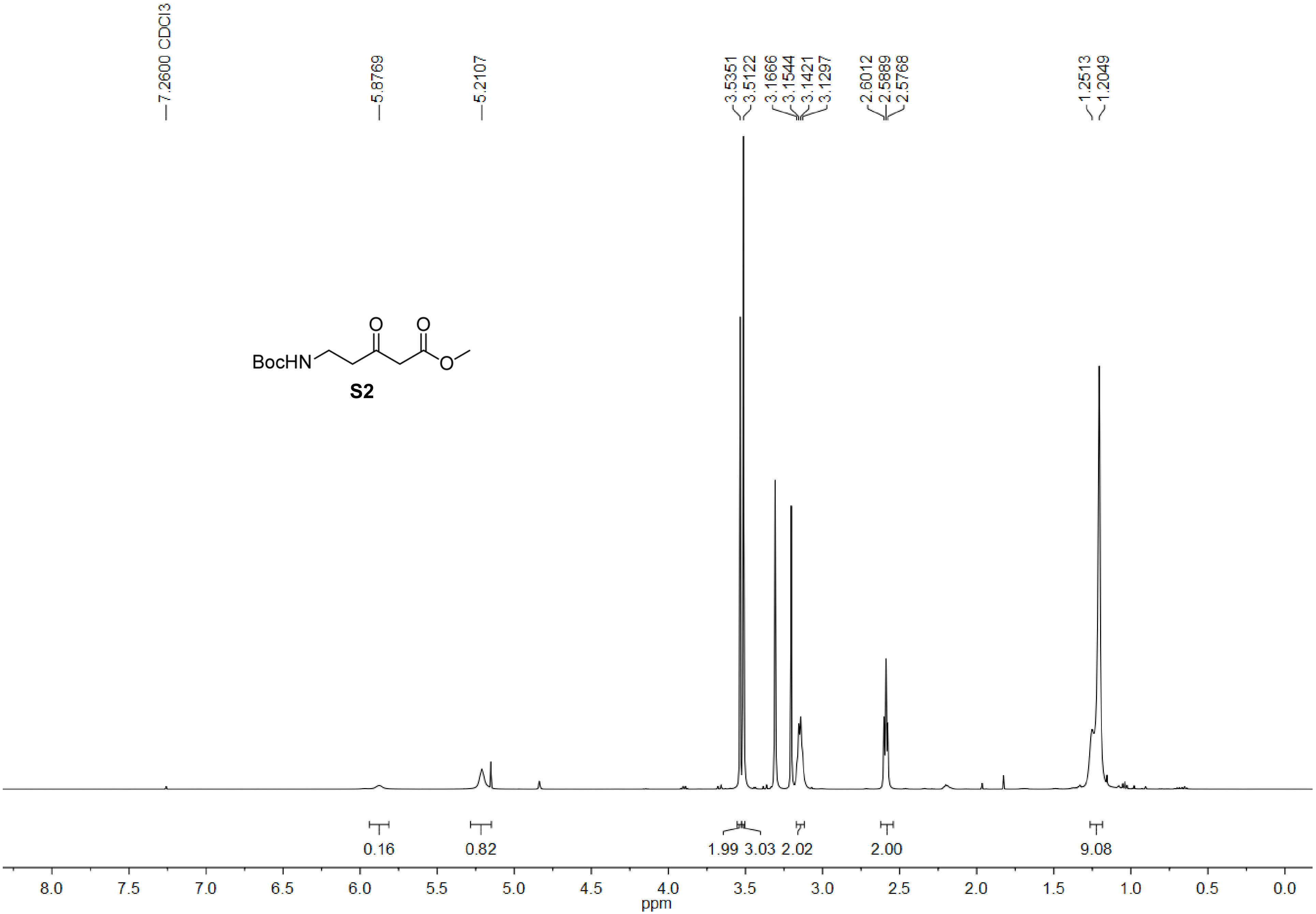
^{1}H NMR (500 MHz)



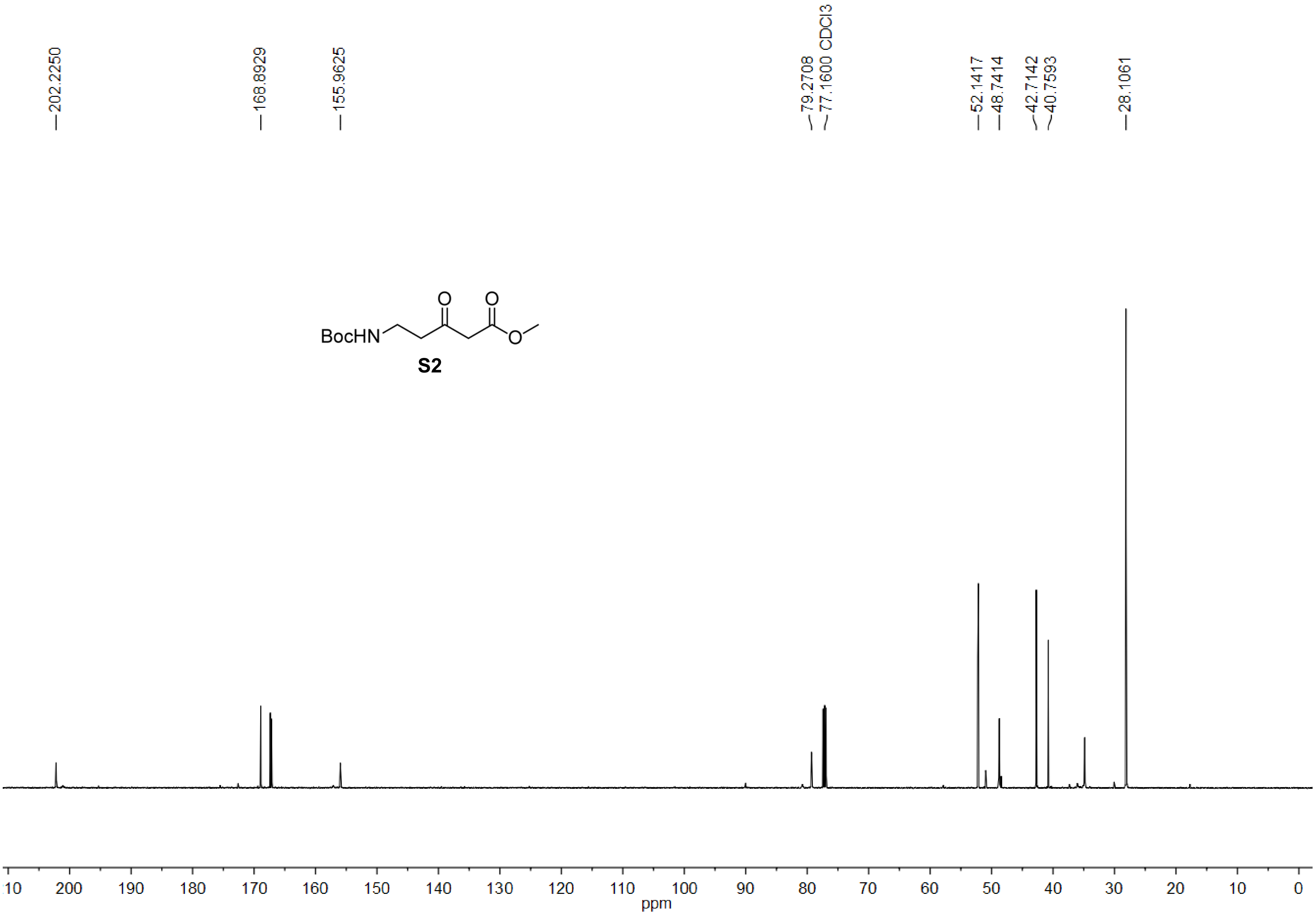
^{13}C NMR (126 MHz)



^{1}H NMR (500 MHz)

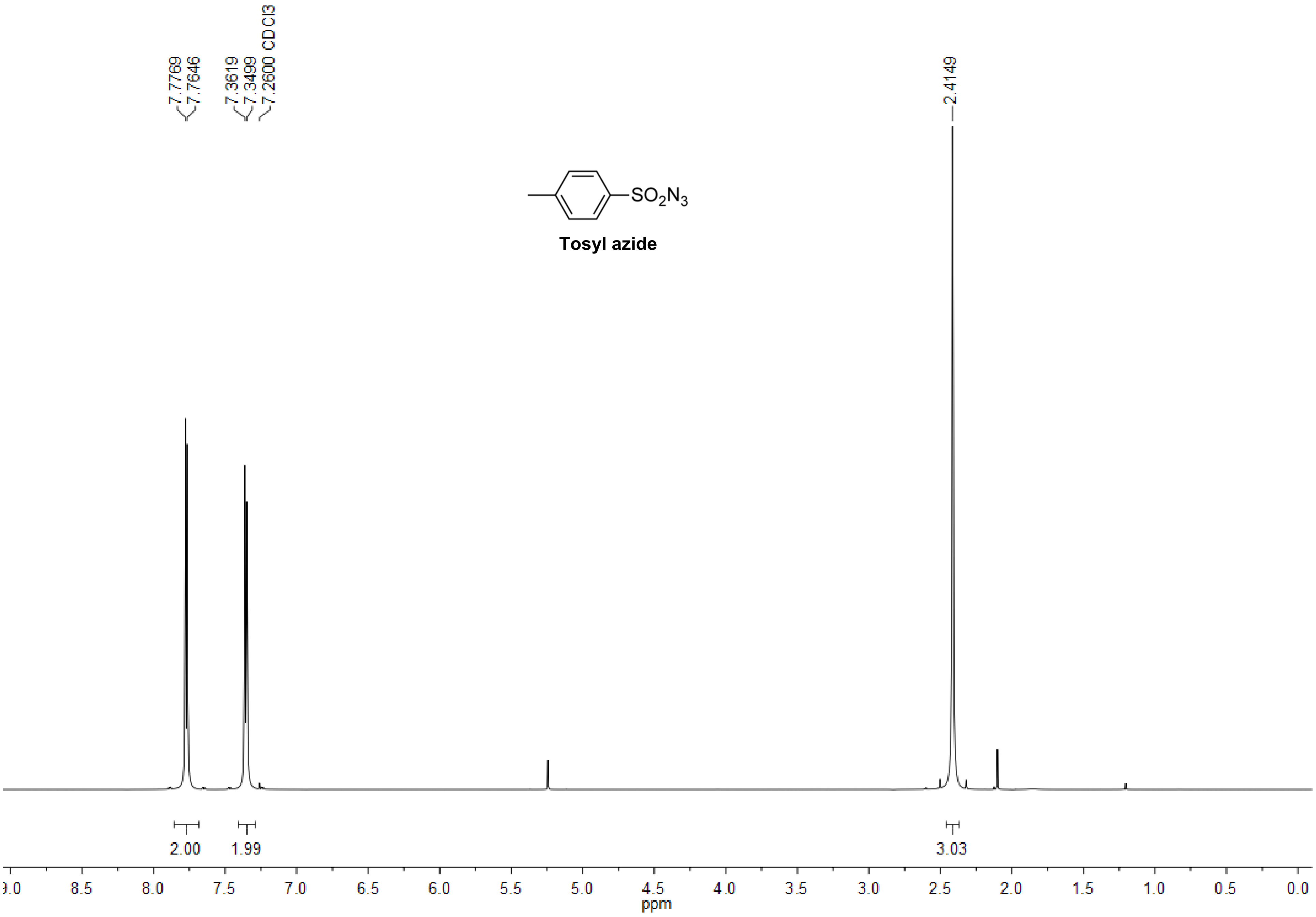


^{13}C NMR (126 MHz)

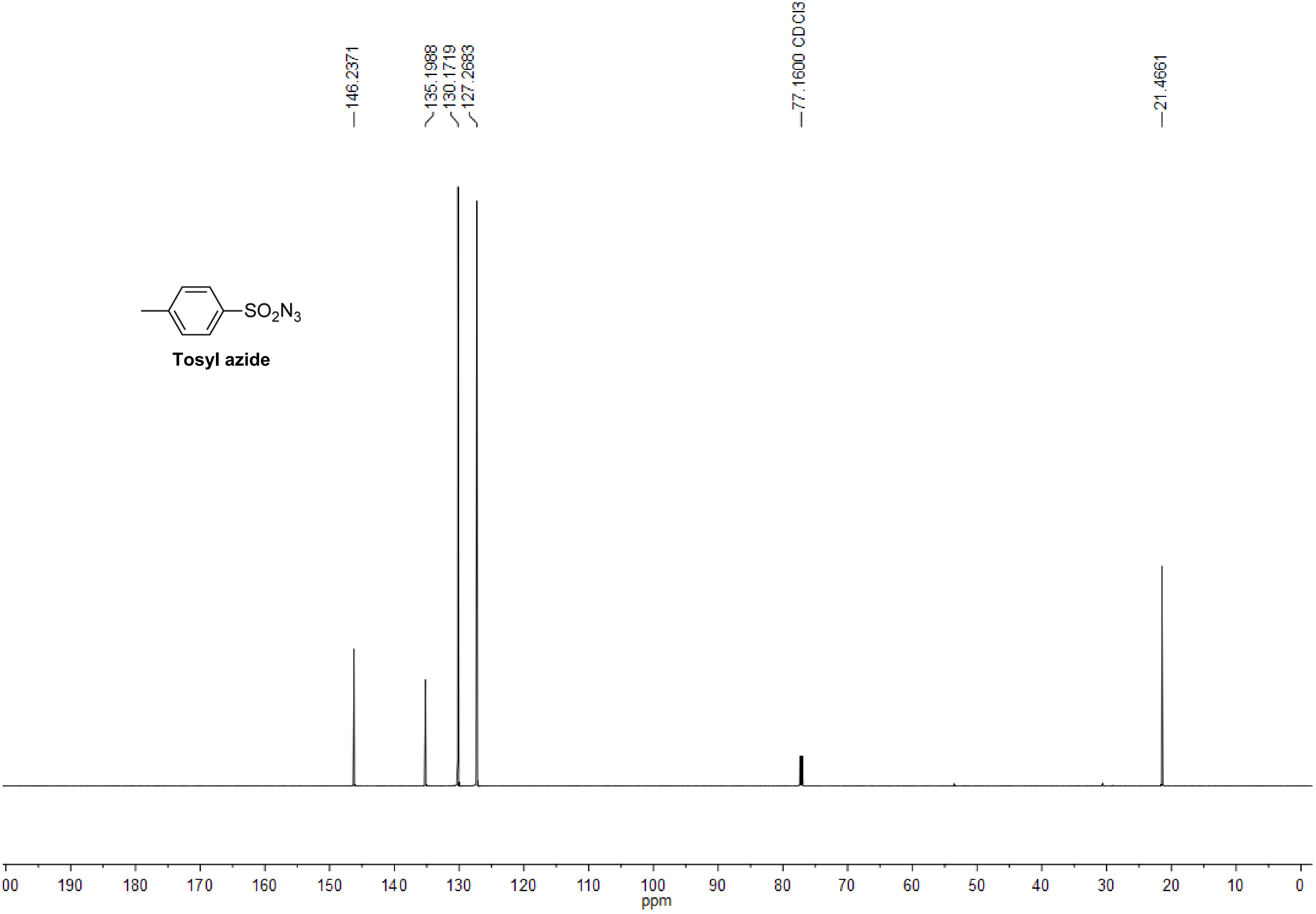


S2

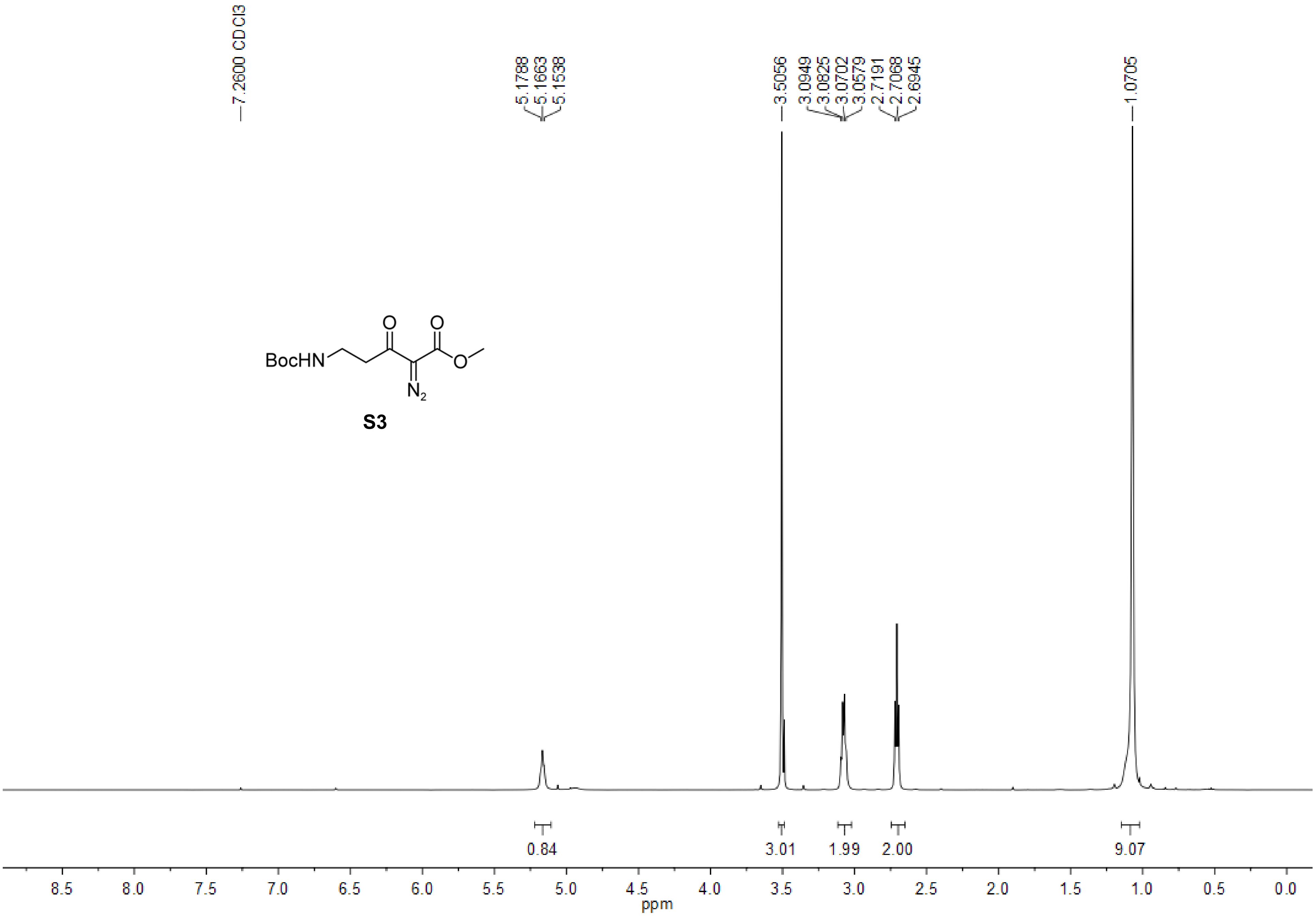
^{1}H NMR (700 MHz)



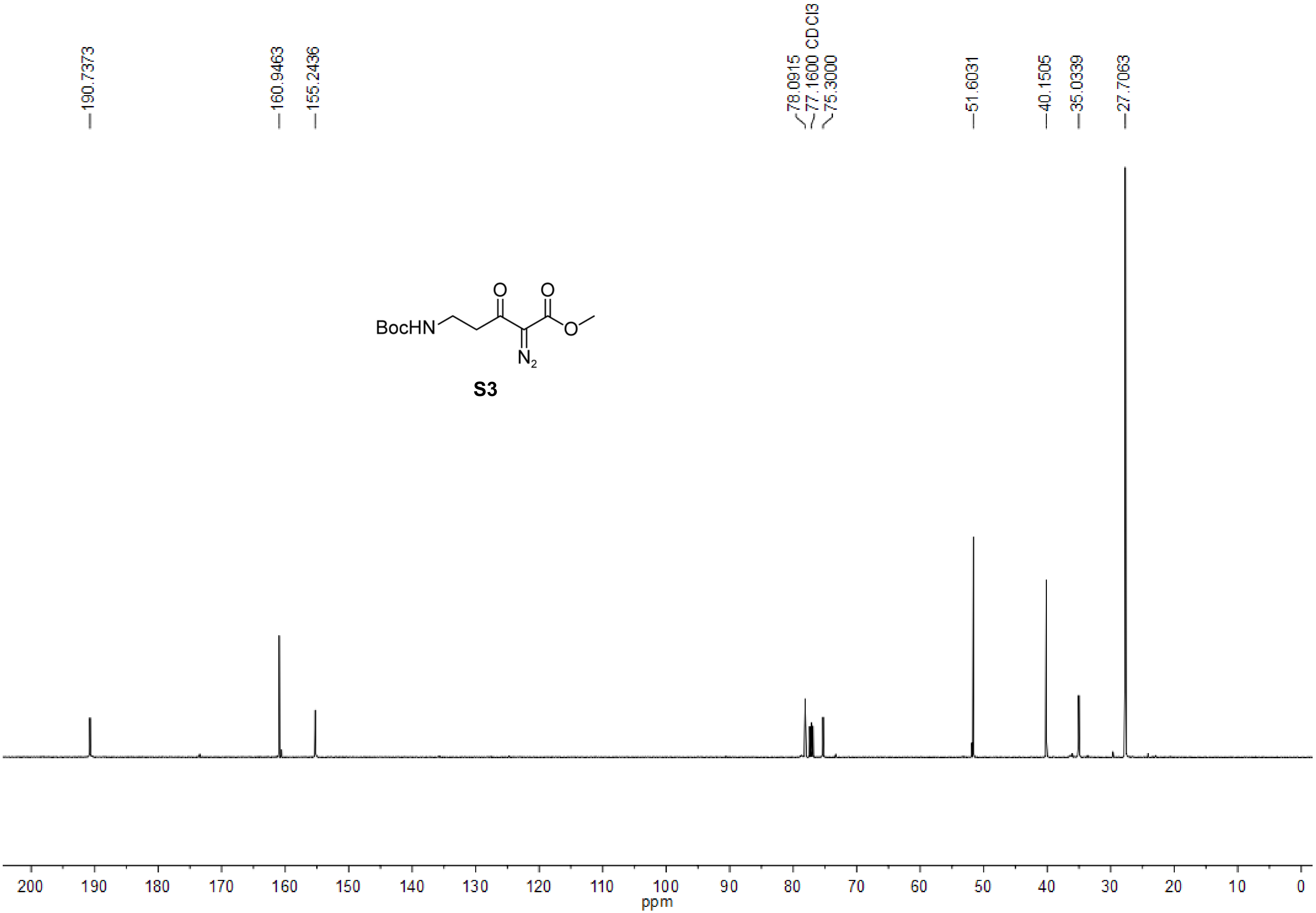
^{13}C NMR (175 MHz)



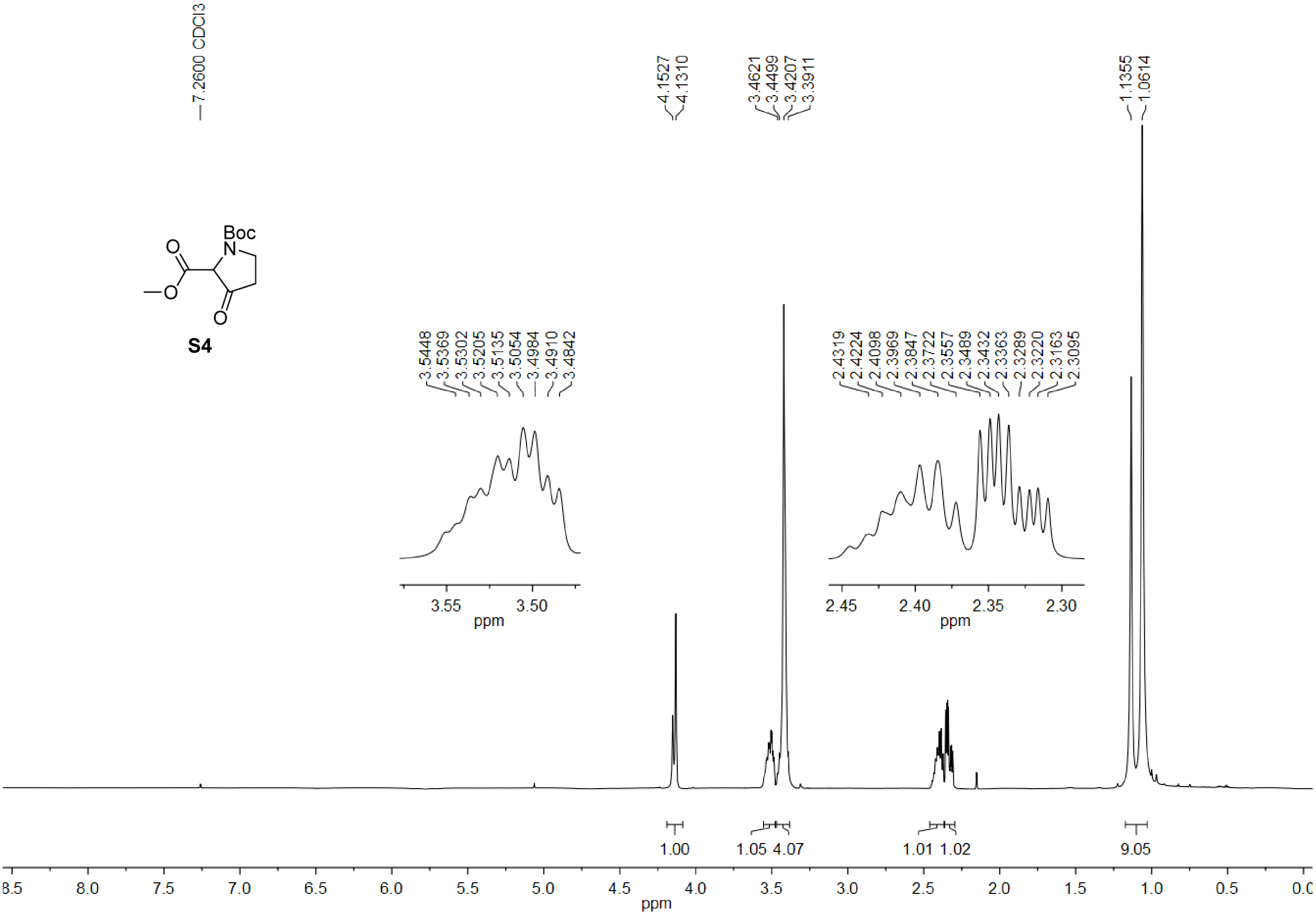
^{1}H NMR (500 MHz)



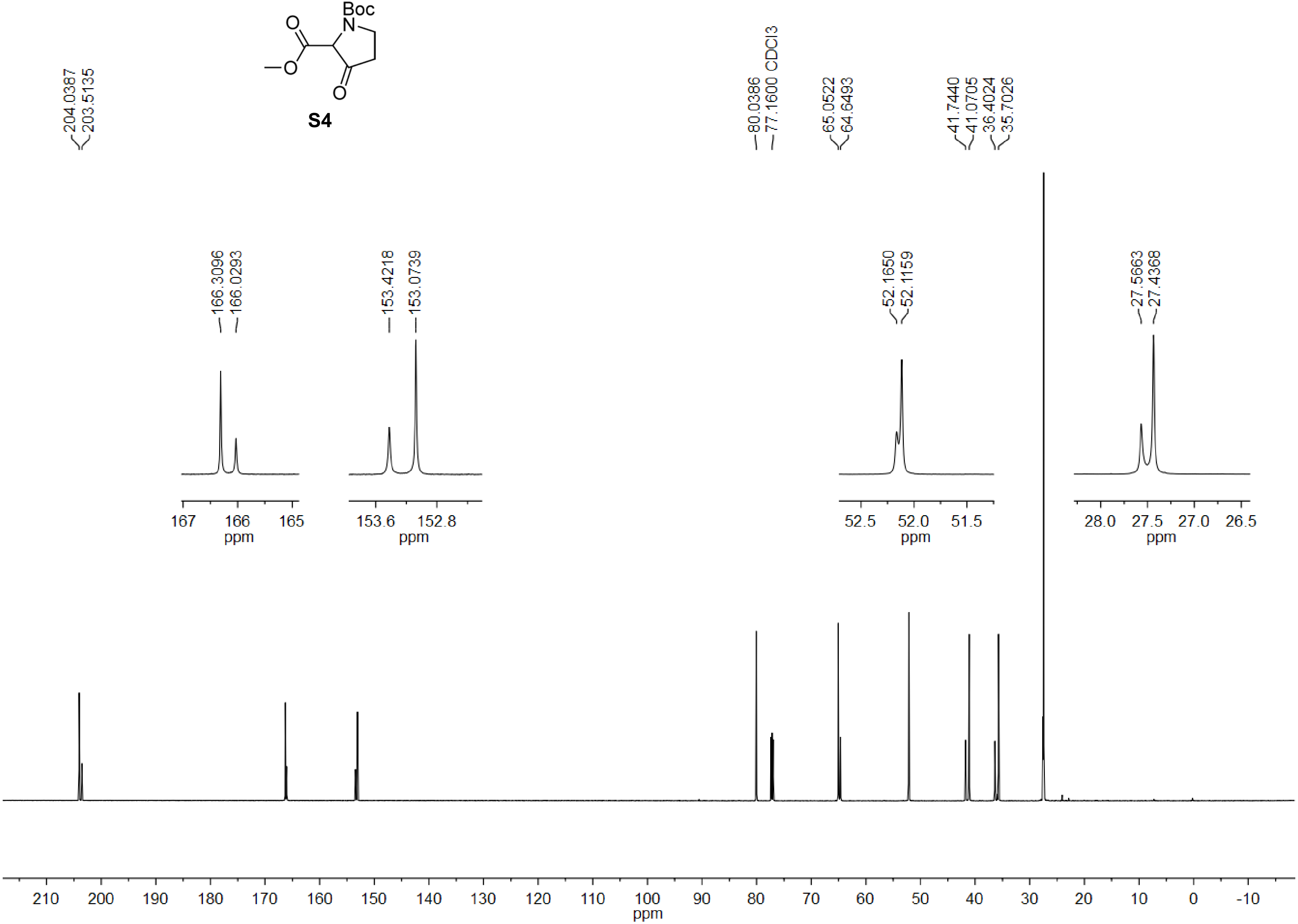
^{13}C NMR (126 MHz)



^{1}H NMR (700 MHz)

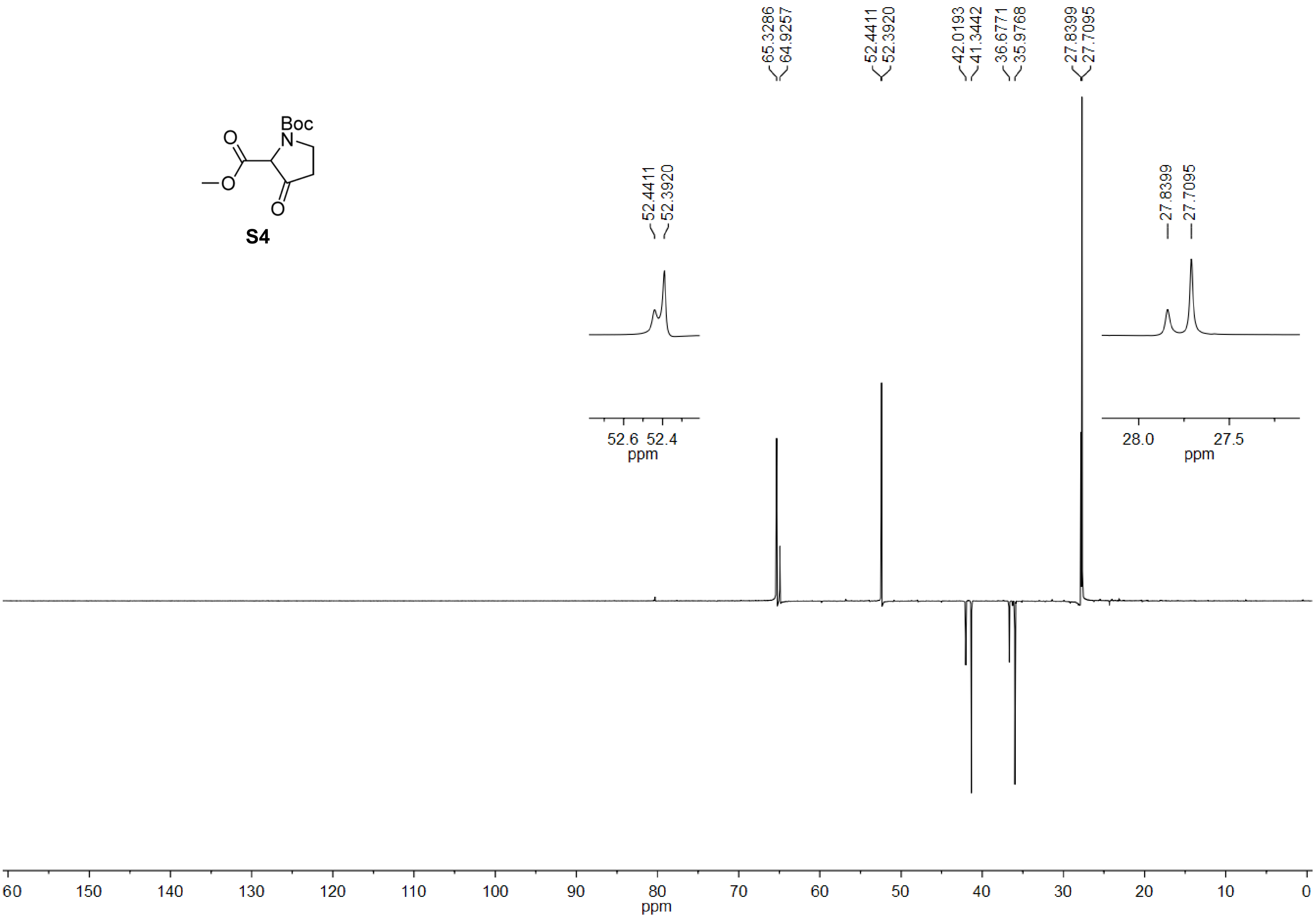


^{13}C NMR (175 MHz)

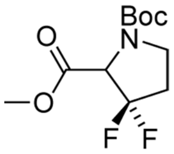


S4

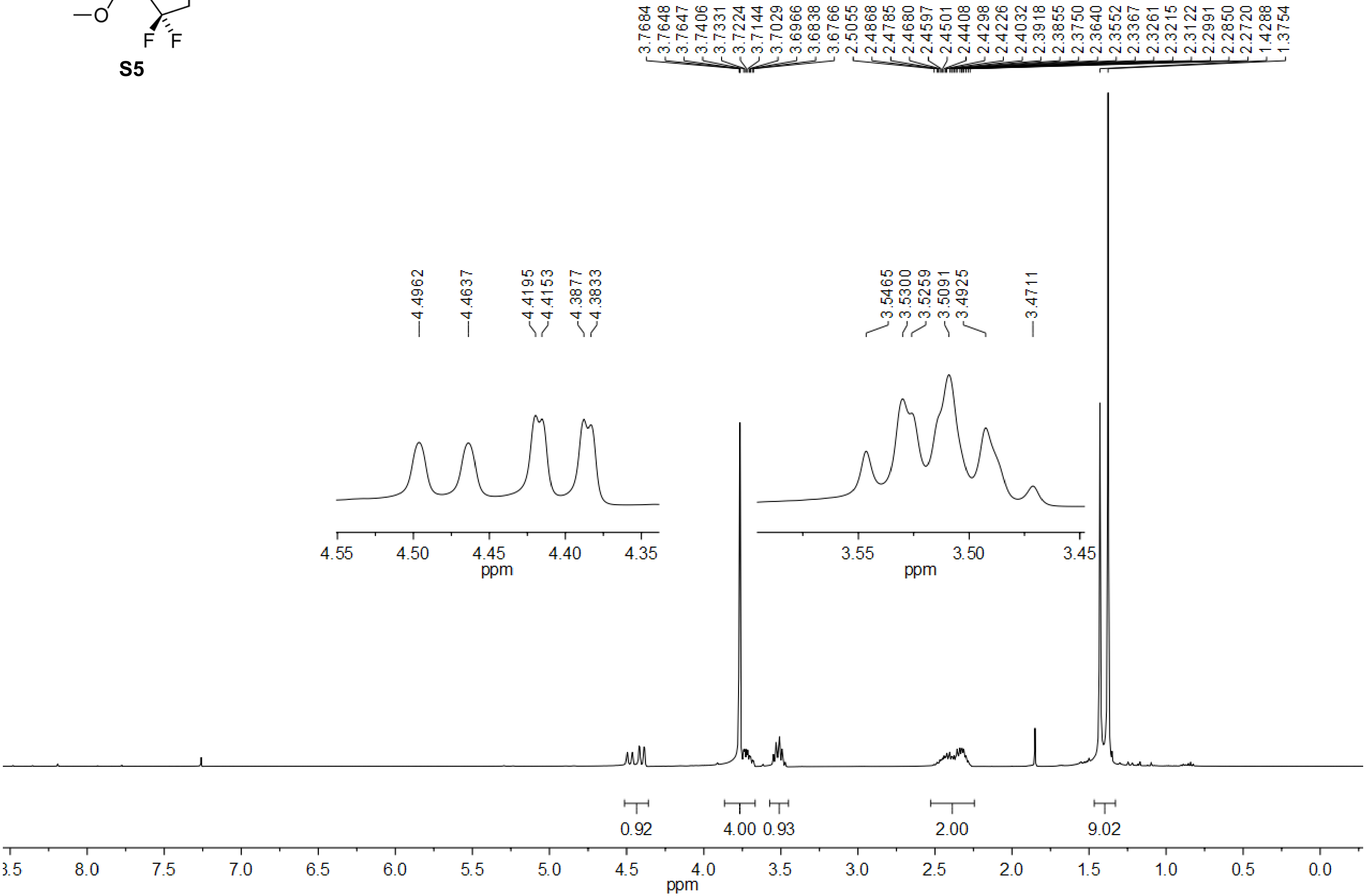
DEPT-135 ^{13}C NMR (175 MHz)



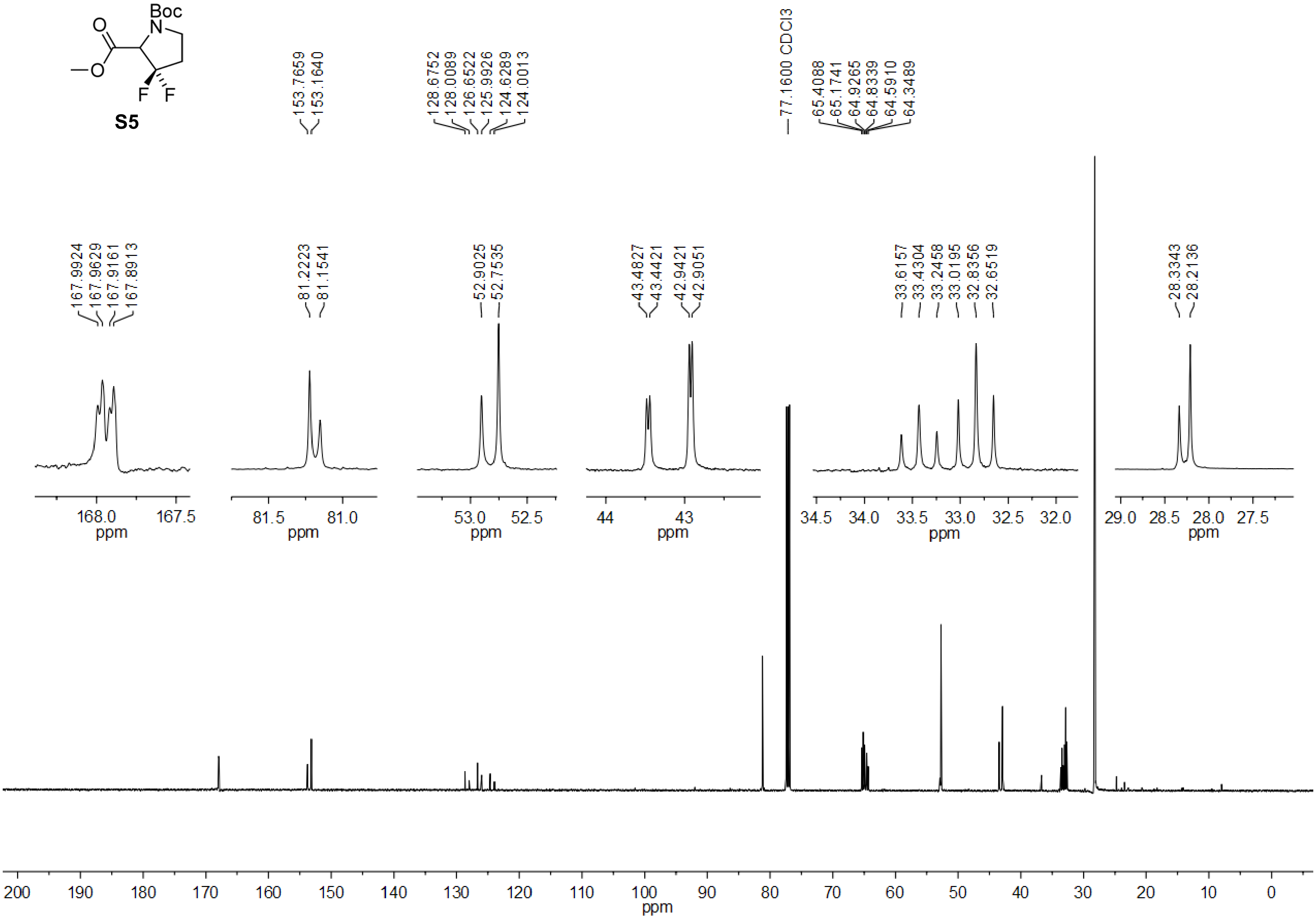
^{1}H NMR (500 MHz)



S5

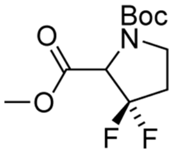


^{13}C NMR (126 MHz)

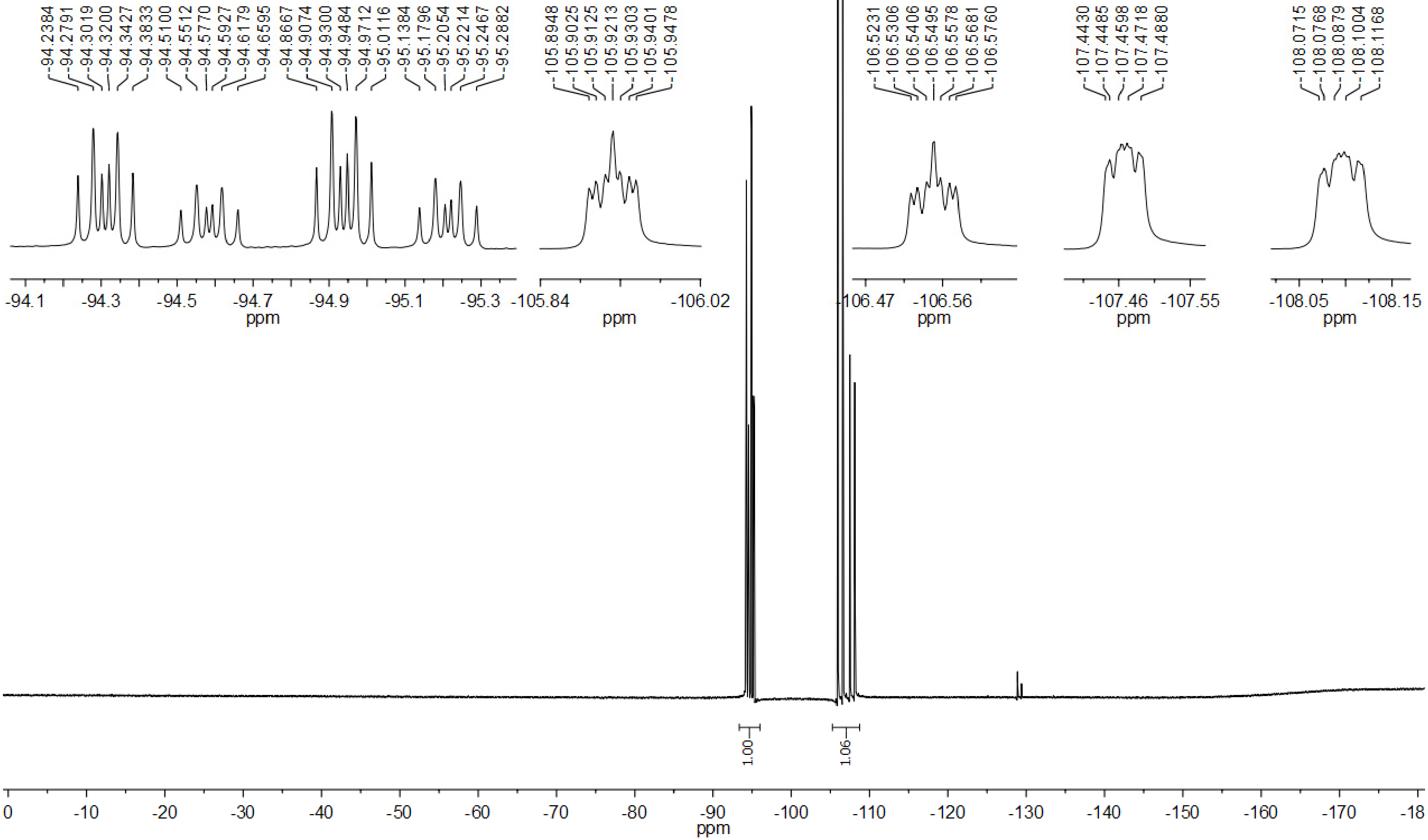


S5

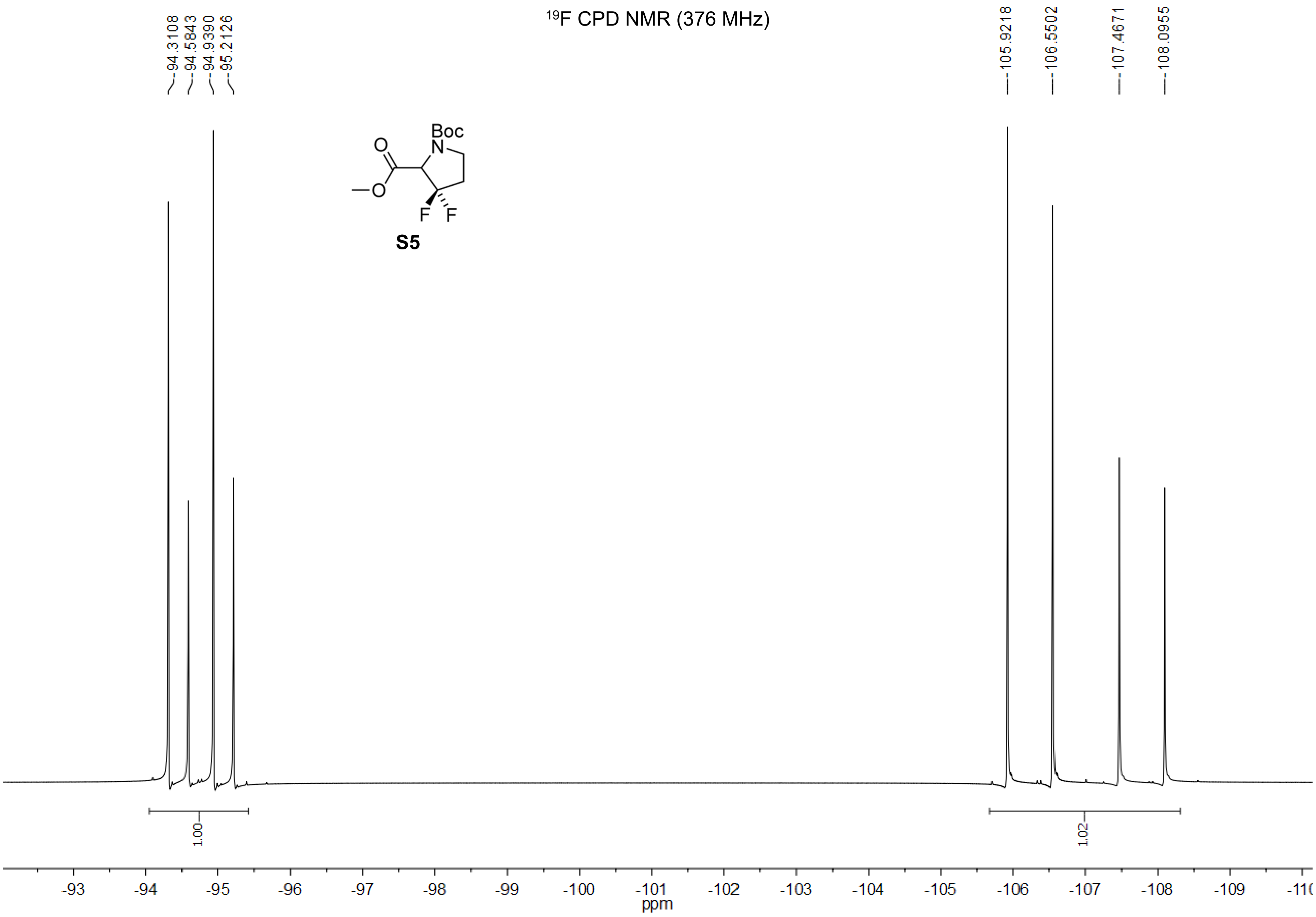
^{19}F NMR (376 MHz)



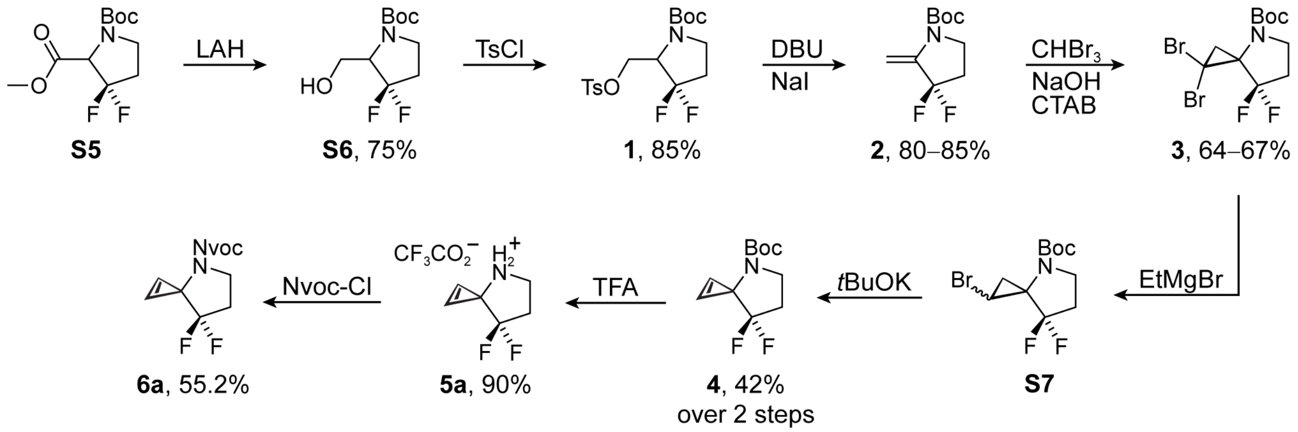
S5

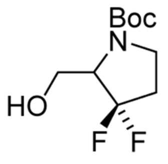


^{19}F CPD NMR (376 MHz)



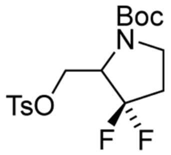
Scheme 2





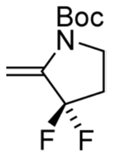
S6

To a solution of **S5** (540 mg, 2.03 mmol, 1 eq) in anhydrous THF (5 mL) at 0 °C was added LAH (85 mg, 2.24 mmol, 1.1 eq) in portions. The resulting suspension was stirred for 30 min at 0 °C. The reaction was quenched with ice cold water, diluted with DCM (10 mL) and separated. The organic layer was collected and the aqueous layer was further washed with DCM. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by flash chromatography (11 g silica, 20% EtOAc/hexanes (v/v)) to obtain **S6** as a colourless oil (474 mg, 99%). A similar reaction starting with 5.0 g of **S5** produced 3.35 g of **S6** (75% yield). R_f = 0.17 (20% EtOAc/hexanes, visualized w/ $KMnO_4$ stain). ^{1}H NMR (16:84 % mixture of rotamers, 400 MHz, $CDCl_3$): δ= 3.98–3.85 (m, 1.5H), 3.85 (s,1H), 3.69–3.67 (m, 1H), 3.51–3.44 (m, 1.5H), 2.57–2.22 (m, 2H), 1.44–1.43 (m, 9H). ^{13}C NMR (rotamers, 100 MHz, $CDCl_3$): δ= 155.88, 155.57, 154.23, 153.39, 128.73, 126.75, 124.72, 99.60, 99.02, 81.15, 80.92, 64.34, 64.12, 63.90, 62.73, 62.50, 61.29, 60.49, 60.05, 49.13, 48.99, 43.48, 42.87, 33.29, 33.11, 32.93, 28.47, 28.40. MS (ESI): Calcd for $C_{10}H_{17}F_2NO_3$ $[M]^+$: 237.1, found: 138.1 $[M\text{-}Boc+2H]^+$, 182.1 $[M\text{-}C(CH_3)_3+2H]^+$, 238.1 $[MH]^+$, 260.1 $[MNa]^+$.



1

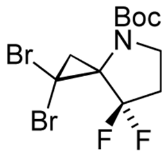
To a solution of **S6** (5.20 g, 21.93 mmol, 1 eq) in DCM (50 mL) was added TsCl (5.01g, 26.32 mmol, 1.2 eq), Et_3N (2.88 g, 28.52 mmol, 1.3 eq), and DMAP (53mg, 0.43 mmol, 0.02 equiv). The reaction mixture was then heated to 45 °C and stirred at this temperature for 16 h. The reaction mixture was diluted with water and the crude product extracted with DCM (4×30 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by flash chromatography (130 g silica, 10% EtOAc/hexanes (v/v)) to obtain **1** as a colourless oil (7.3 g, 85%). R_f = 0.37 (20% EtOAc/hexanes, visualized w/ UV). ^{1}H NMR (47:53 % mixture of rotamers, 500 MHz, $CDCl_3$): δ= 7.70–7.67 (m, 1H), 7.28 (d, *J* = 8.5 Hz, 2H), 5.14 (m, 0.34H), 4.56–4.36 (m, 1H), 4.27–4.25 (m, 0.32H), 4.11–4.04 (m, 1H), 4.00–3.93 (m, 1H), 3.87–3.75 (m, 0.39H), 3.47–3.33 (m, 1.25H), 2.36 (s, 3H), 2.36–2.21 (m, 1.30H), 1.35 (m, 9H). ^{13}C NMR (rotamers, 126 MHz, $CDCl_3$): δ= 153.93, 153.65, 153.55, 153.27, 153.10, 152.90, 151.74, 151.46, 144.98, 144.86, 144.79, 144.60, 132.58, 132.54, 132.27, 129.76, 129.70, 129.63, 127.55, 127.52, 127.50, 80.77, 80.49, 80.41, 80.09, 66.37, 66.35, 65.69, 65.67, 65.58, 65.24, 60.83, 60.59, 60.37, 58.37, 58.14, 48.78, 48.73, 48.28, 48.23, 42.77, 42.30, 3.98, 32.77, 32.34, 32.15, 31.98, 28.00, 27.91, 21.26. ^{13}C DEPT-135 NMR (rotamers, 126 MHz, $CDCl_3$): δ= (up) 129.76, 129.70, 60.97, 60.74, 60.50, 58.51, 58.28, 42.91, 42.43, 28.14, 28.05, 21.40. (down) 66.51, 66.50, 65.83, 65.79, 65.75, 48.92, 48.87, 48.42, 48.37, 33.30, 33.13, 32.95, 32.48, 32.28,32.10. HRMS (ESI): Calcd for $C_{17}H_{23}F_2NO_5S$ $[M+NH_4]^+$: 409.1603, found: 409.1602.



2

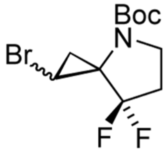
To a solution of **1** (1.10 g, 2.81 mmol, 1 eq) in glyme (30 mL) was added NaI (1.35 g, 9 mmol, 3 eq), and DBU (915 mg, 6 mmol, 2 eq). The reaction mixture was refluxed for 4 h, diluted with water (20 mL), and extracted with Et_2O (3×30 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and used directly without any further purification to obtain **2** as a pale-yellow oil (0.51 g, 82.3%). We found the alkene to be unstable on silica and, therefore, used it without the flash chromatography for future production of **2**. R_f = 0.88 (20% EtOAc/hexanes, visualized w/ UV). ^{1}H NMR (500 MHz, $CDCl_3$): δ= 5.56 (br s, 1H), 4.90 (s, 1H), 3.67

(t, *J* = 7.1 Hz, 2H), 2.31 (m, ,2H), 1.52 (s, 9H). ^{13}C NMR (126 MHz, CDCl$_3$): δ= 152.10, 140.35, 125.06, 123.14, 121.18, 92.84, 83.94, 43.79, 31.97, 31.76, 31.57, 28.40. HRMS (ESI): Calcd for $C_{10}H_{15}F_2NO_5$ [M+H]$^+$: 220.1144, found: 220.1148.



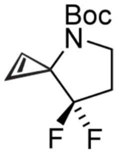
3

To a solution of **2** (2.19 g, 10.00 mmol, 1 eq) in CHBr$_3$ (1.8 mL, 5.1 g, 20.00 mmol, 2 eq) and DCM (3 mL) was added CTAB (0.546 g, 1.00 mmol, 0.15 eq) and the mixture was stirred vigorously. To this was added NaOH (1.75 mL, 50% w/v, 25 M) dropwise. The brown-black reaction mixture was stirred for 13 h at rt. To this reaction was added additional CHBr$_3$ and CTAB based on TLC and the process was repeated until complete consumption of reactant. Complete consumption of reactant was a necessity as the product have same R$_f$ as the reactant. Upon completion, the reaction mixture was diluted with DCM and water. The organic layer was collected, washed with brine, and concentrated under reduced pressure. The crude obtained was purified by flash chromatography (120 g silica, hexanes-2.5% EtOAc/hexanes (v/v)) to obtain **3** as a light-yellow oil (2.07 g, 65%) R$_f$ = 0.28 (2.5% EtOAc/hexanes, visualized w/ UV). ^{1}H NMR (700 MHz, CDCl$_3$): δ= 3.86 (br s, 1H), 3.59 (m, 1H), 2.51–2.45 (m, 1H), 2.40–2.32 (m, 1H), 2.27 (d, *J* = 9.9 Hz, 1H), 1.57–1.52 (m, 1H), 1.45 (s, 9H). ^{13}C NMR (176 MHz, CDCl$_3$): δ= 153.62, 124.93, 123.44, 122.04, 81.50, 52.66, 52.51, 52.48, 52.32, 31.74, 28.44, 22.81, 14.28. HRMS (ESI): Calcd for $C_{11}H_{15}Br_2F_2NO_2$ [M+Na]$^+$: 411.9330, found: 411.9306.



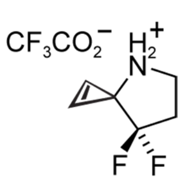
S7

To an ice-cold solution of **3** (800 mg, 2 mmol, 1.0 eq) in anhydrous THF (6.0 mL) under N$_2$ was added 3M EtMgBr (67 µL, 0.4 mmol, 0.2 eq) and Ti(OiPr)$_4$ (60 mg, 0.064 mL, 0.2 mmol, 0.1 eq) and the mixture was stirred at the same temperature for 5 mins. The ice-bath was removed and additional 3M EtMgBr (733 µL, 2.0 mmol, 1.0 eq) was added and the reaction was stirred at rt for 1 hour. The reaction was quenched by ice-water and diluted with DCM. The organic layer was collected and the aqueous layer was further washed with DCM. The combined organic layers were dried over anhydrous Na$_2$SO$_4$, concentrated *in vacuo*, and purified by flash chromatography (18 g silica, 2% EtOAc/hexanes (v/v)) to give crude **S7** as a mixture of diastereomers which was used for the next step without further purification. R$_f$ = 0.50 (5% EtOAc/hexanes, visualized w/ UV). ^{1}H NMR (500 MHz, CDCl$_3$): δ= 3.89 (br s, 1H), 3.62 (t, *J* = 7.4 Hz, 0.56H), 3.55 (td, *J* = 10.5, 4.5 Hz, 0.78H), 3.21 (m, 0.65H), 2.50–2.39 (m, 0.82H), 2.37–2.21 (m, 1.53H), 1.75 (t, *J* = 8.8 Hz, 0.79H), 1.54 (2×s, 1.16 H), 1.46 (2×s, 9H). MS (ESI): Calcd for $C_{11}H_{16}BrF_2NO_2$ [M]$^+$: 311.0, found: 256.0 [M-C(CH$_3$)$_3$+2H]$^+$, 258.1 [M+2-C(CH$_3$)$_3$+2H]$^+$, 312.0 [MH]$^+$, 314.0 [M+2+H]$^+$. Alternatively, to a solution of **3** (3.2 g, 8.18 mmol, 1.0 eq) in anhydrous DCM (130.0 mL) under N$_2$ at -78 °C was dropwise added 2M iPrMgCl (4.91 mL, 9.82 mmol, 1.2 eq) and the mixture was stirred at the same temperature for 15 mins. The reaction was quenched with by saturated NH$_4$Cl. The organic layer was collected and the aqueous layer was further washed with DCM. The combined organic layers were dried over anhydrous Na$_2$SO$_4$, concentrated *in vacuo*, and purified by flash chromatography (100 g silica, 2.5% EtOAc/hexanes (v/v)) to give crude **S7** (1.55 g) which was used without further purification.



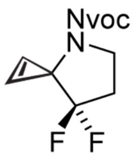
4

The crude **S7** (1.55 g, 4.98 mmol, 1.0 eq) was dissolved in THF (100.0 mL) and cooled down to -78 °C. To this solution was added KOtBu (0.839 g, 7.47 mmol, 1.5eq) and the reaction was allowed to gradually warm to rt. Further, KOtBu was added if necessary. The reaction was quenched with water, concentrated *in vacuo*, and diluted with DCM. The organic layer was collected and the aqueous layer was further washed with DCM. The combined organic layers were dried over anhydrous Na$_2$SO$_4$, concentrated *in vacuo*, and purified by flash chromatography (75 g silica, 5% EtOAc/hexanes(v/v)) to obtain **4** (0.79 g, 41.8% over two steps) as a pale-yellow oil that solidified upon storage at -20 °C. R$_f$ = 0.23 (5% EtOAc/hexanes, visualized w/ KMnO$_4$). ^{1}H NMR (500 MHz, CDCl$_3$): δ= 7.28 (br s, 2H), 3.56 (t, *J* = 6.0 Hz), 2.35 (m, 2H), 1.40 (s, 9H). ^{13}C NMR (126 MHz, CDCl$_3$): δ= 154.20, 126.57, 124.63, 122.68, 111.59, 80.12, 47.82, 47.55, 47.27, 41.02, 32.14, 31.95, 31.75, 28.59. HRMS (ESI): Calcd for $C_{11}H_{15}F_2NO_2$ [M+H]$^+$: 232.1144, found: 232.1143.



5a

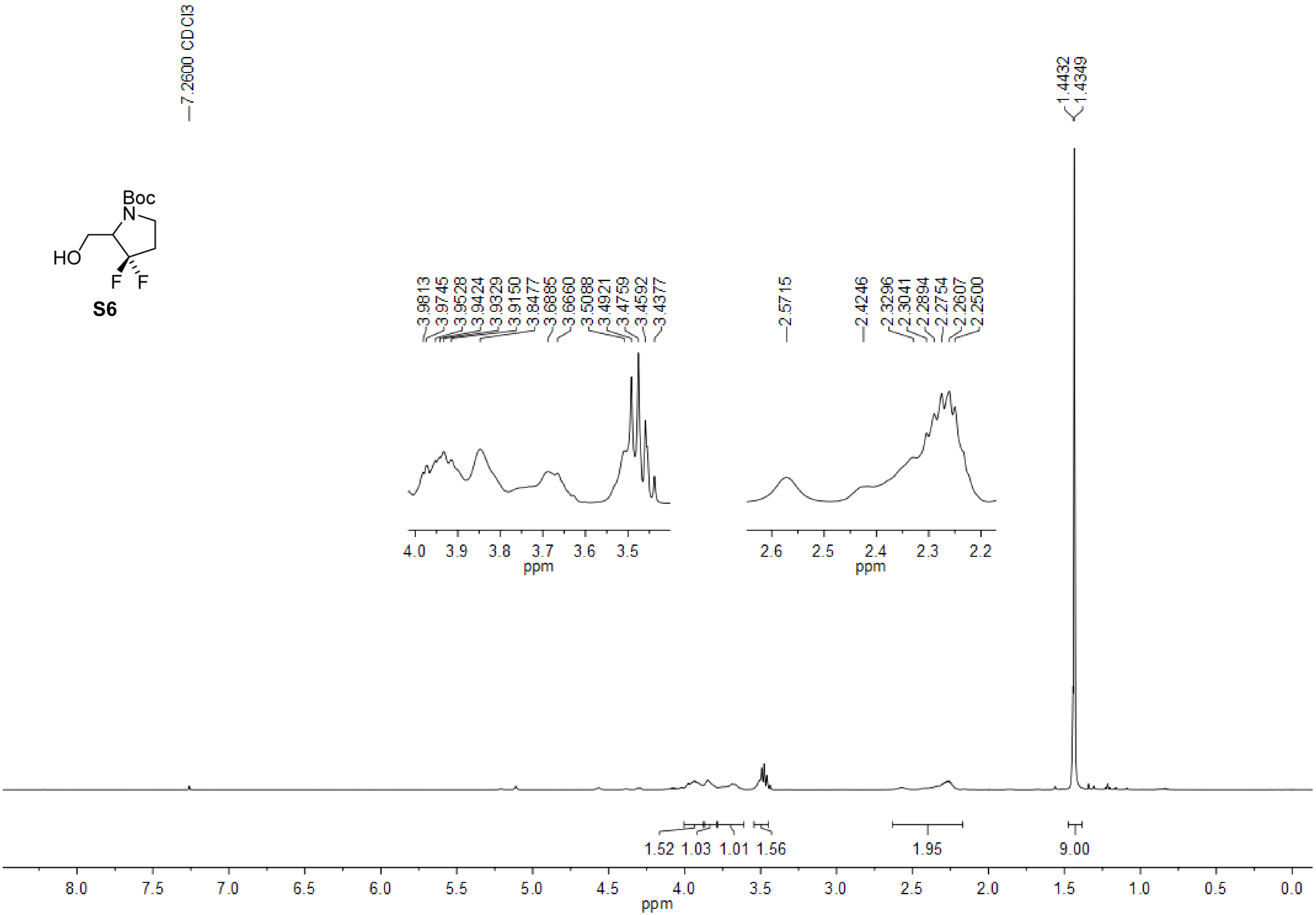
To an ice-cold solution of **4** (40 mg, 0.17 mmol) in DCM (2.0 mL) was added TFA (0.4 mL). After stirring the reaction at the same temperature for 1 h, it was concentrated *in vacuo* (<25 °C) to obtain **5a** as a TFA salt (38 mg, 90%). ^{1}H NMR (700 MHz, CD$_3$OD): δ= 7.79 (s, 2H), 3.53 (t, *J* = 4.3 Hz, 2H), 2.69 (m, 2H). ^{13}C NMR (176 MHz, CD$_3$OD): δ= 161.45, 161.24, 161.02, 160.81, 125.70, 124.31, 122.92, 119.69, 118.04, 116.41, 114.77, 108.98, 54.81, 40.84, 40.82, 40.79, 34.17, 34.02, 33.88, 28.67. HRMS (ESI): Calcd for $C_6H_7F_2N$ [M+H]$^+$: 132.0619, found: 132.0621.



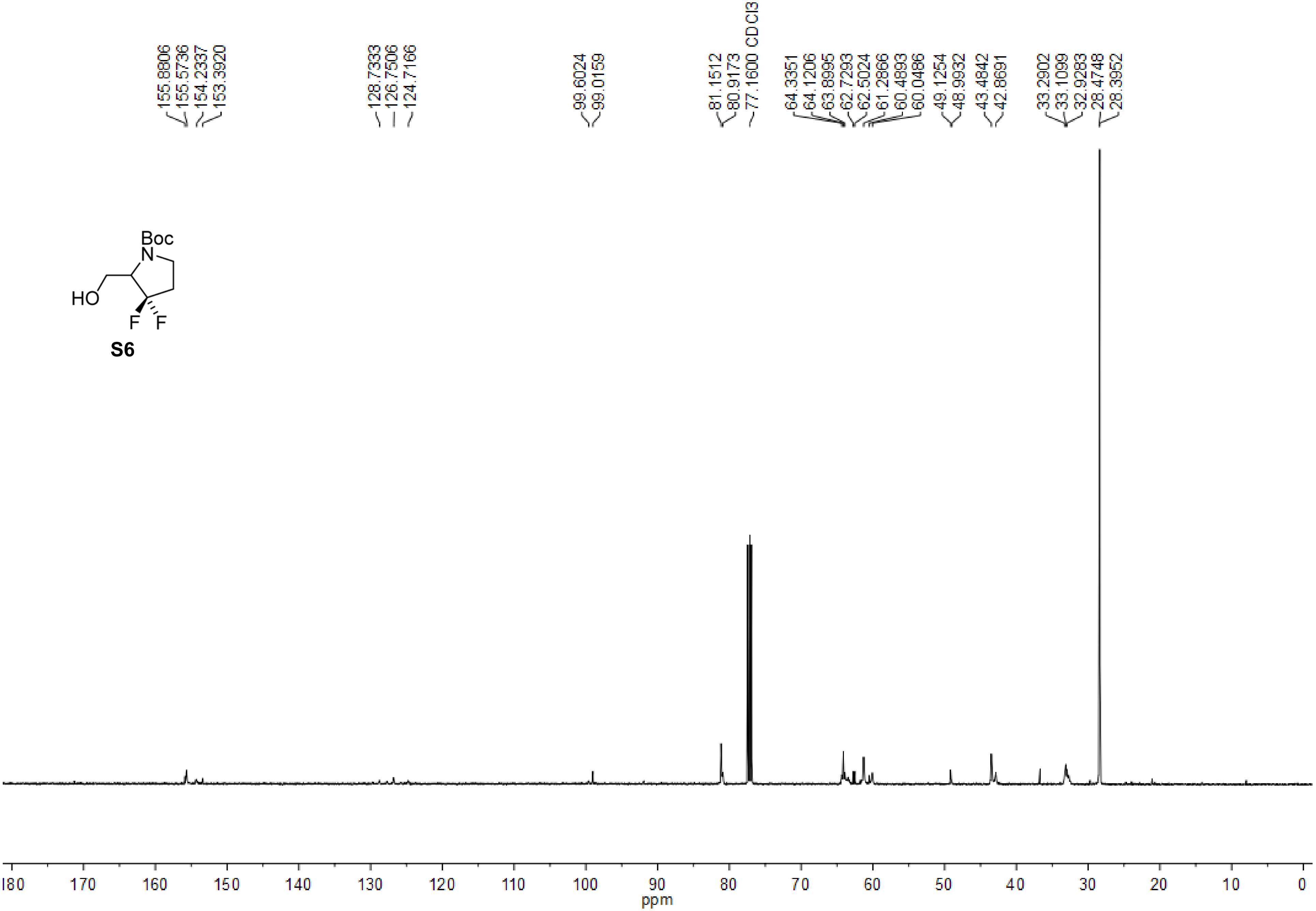
6a

To a solution of **5a** (7 mg, 0.03 mmol, 1.0 eq) in $CHCl_3$/ Et_2O (2 mL, 9:1 v/v) at 0 °C was added solid $NaHCO_3$ (pH ~8.5) and 4,5-Dimethoxy-2-nitrobenzyl chloroformate (9.45 mg, 0.03 mmol, 1.2 eq) respectively. The reaction was allowed to warm to rt and stirred for 15 h. It was then diluted with DCM and water. The organic layer was collected and the aqueous layer was further washed with DCM. The combined organic layers were dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by HPLC (R_t = 13.5 min, 30%–100% MeOH over 25 mins) to give **6a** (5.8 mg, 55.2 %). R_f = 0.19 (25% EtOAc/hexanes, visualized w/ UV). ^1H NMR (700 MHz, $CDCl_3$): δ= 7.68 (s, 1H), 7.32 (s, 2H), 6.93 (s, 1H), 5.45 (s, 2H), 3.97 (s, 3H), 3.95 (s, 3H), 3.67 (t, *J* = 6.0 Hz, 2H), 2.41 (m, 2H). ^{13}C NMR (176 MHz, $CDCl_3$): δ= 153.44, 148.39, 140.29, 127.51, 111.45, 110.90, 108.40, 63.74, 56.56, 53.57, 41.21, 32.11, 31.92, 31.74, 29.58, 29.50, 29.39, 29.22. HRMS (ESI): Calcd for $C_{16}H_{16}F_2N_2O_6$ $[M]^+$: 370.3088, found: 370.3089.

^{1}H NMR (400 MHz)

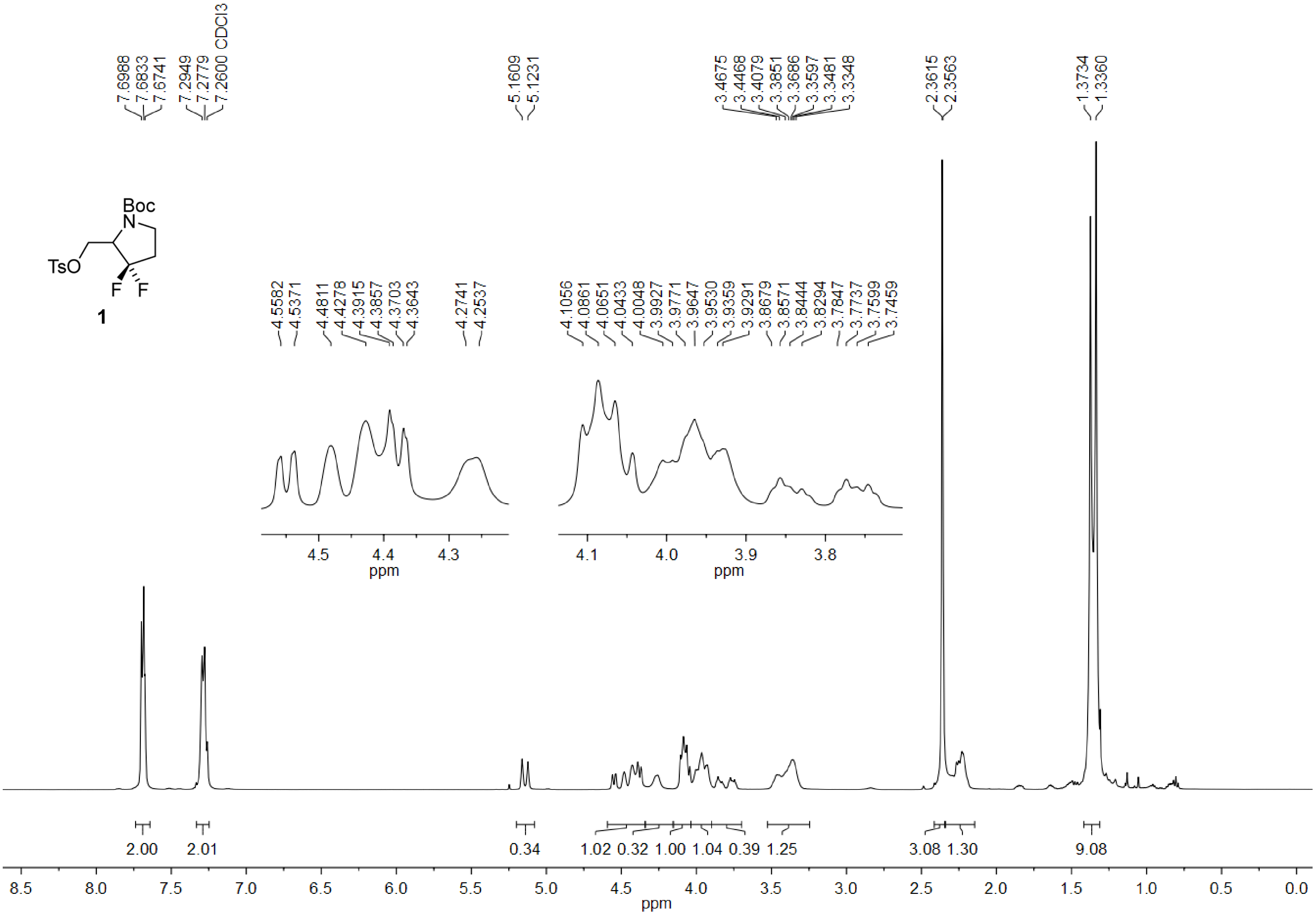

S6

^{13}C NMR (100 MHz)

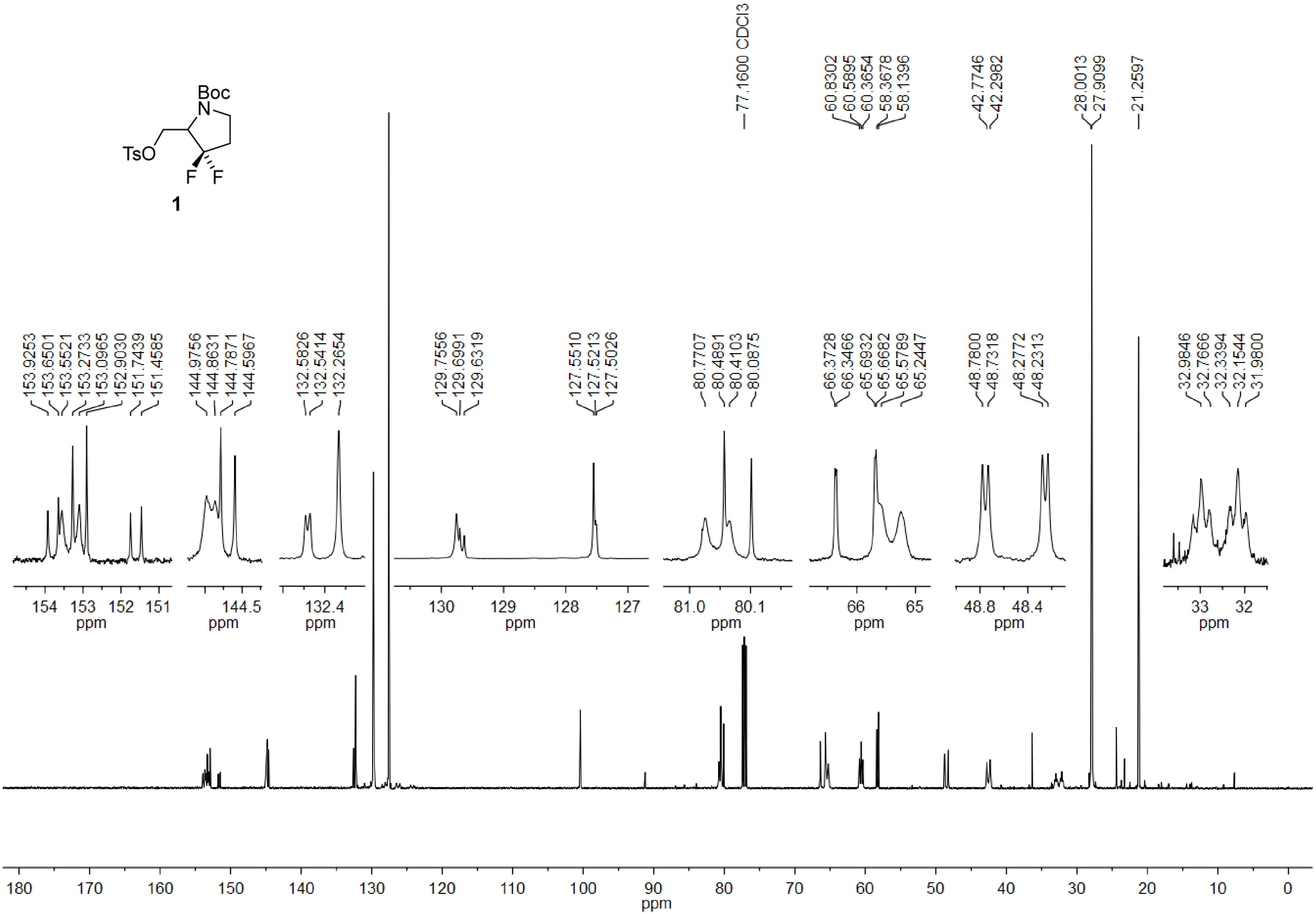


S6

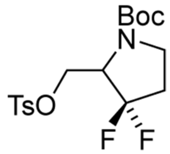
^{1}H NMR (500 MHz)



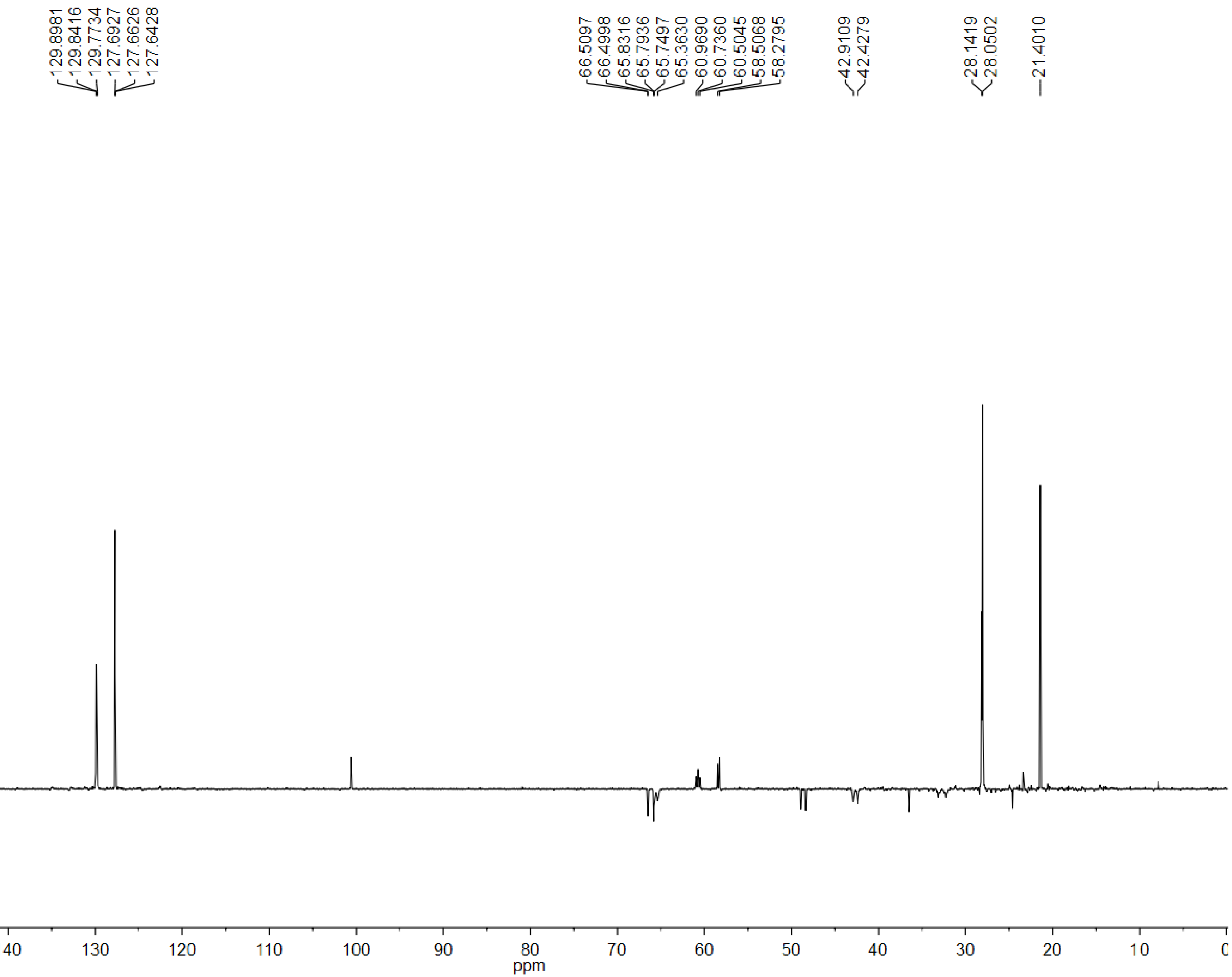
^{13}C NMR (126 MHz)



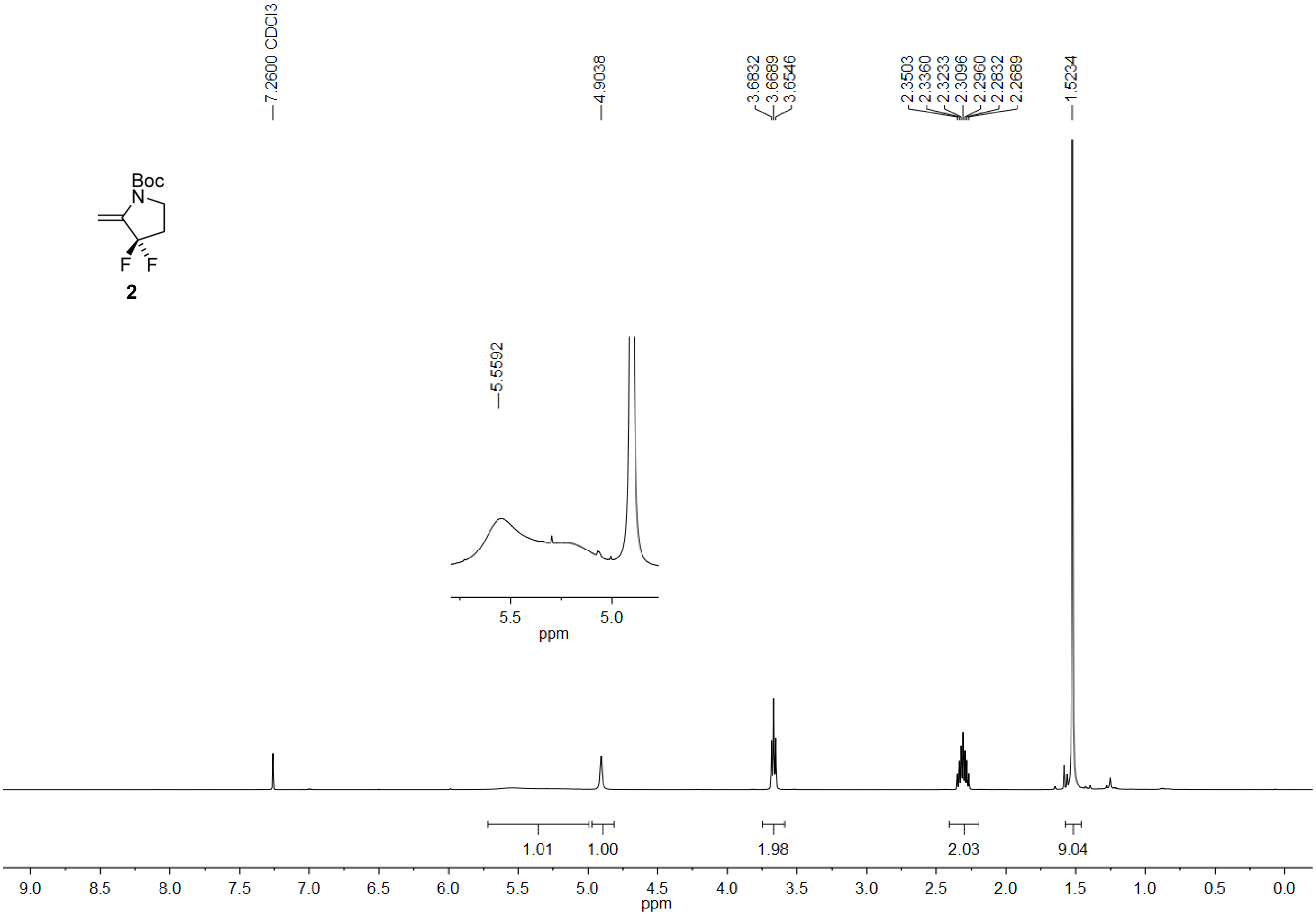
DEPT-135 ^{13}C NMR (500 MHz)



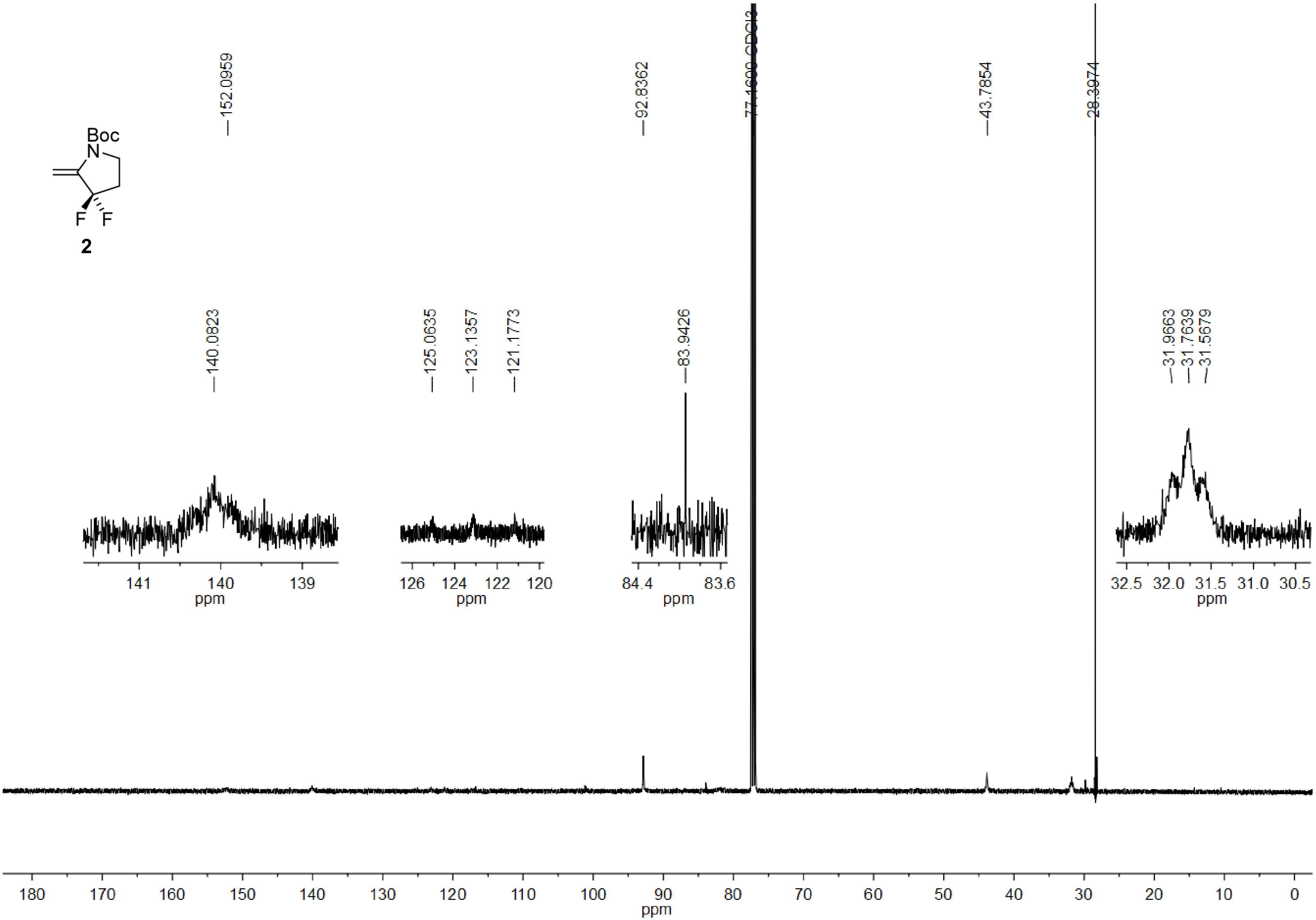
1



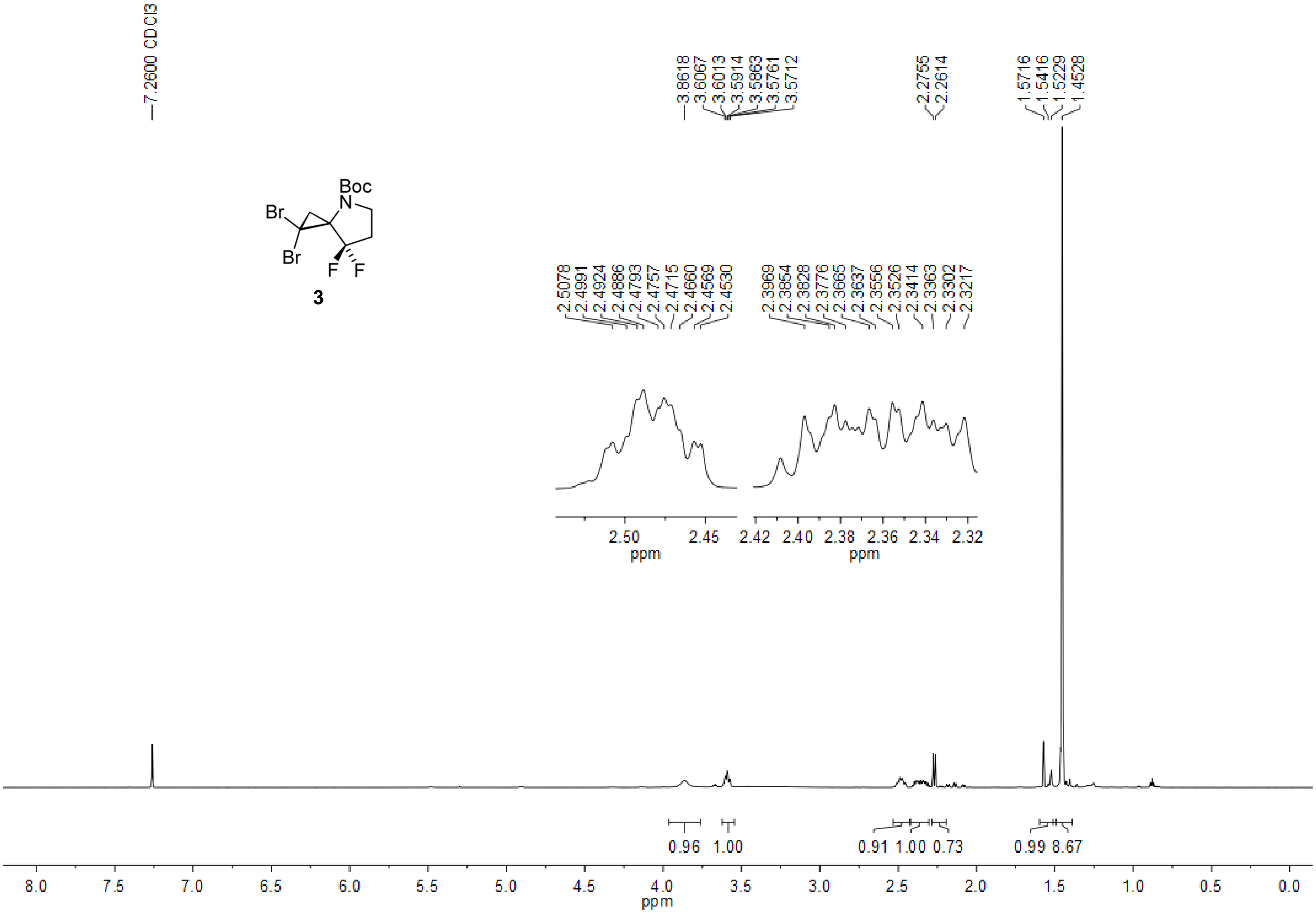
^{1}H NMR (500 MHz)



^{13}C NMR (126 MHz)

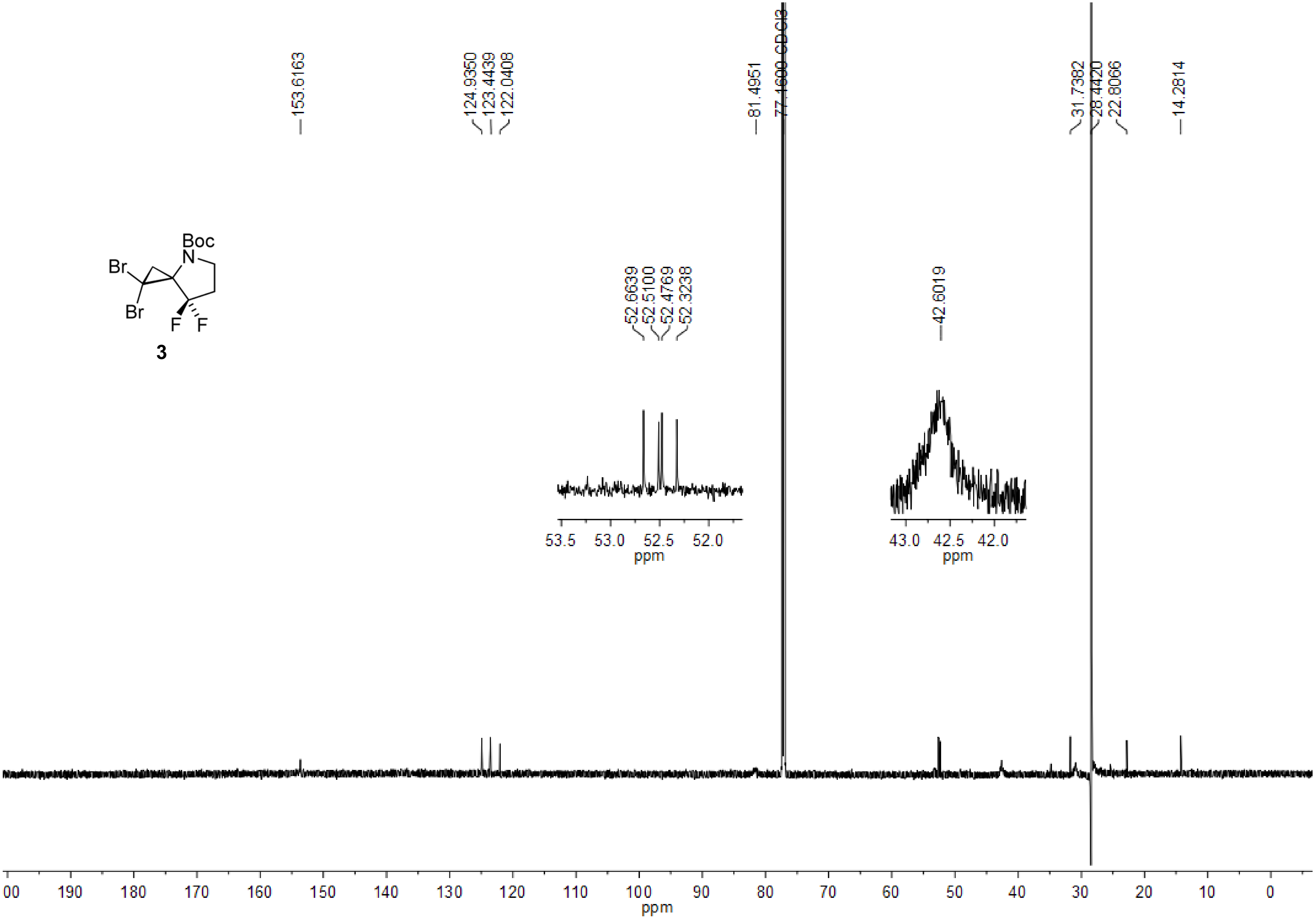


^{1}H NMR (700 MHz)

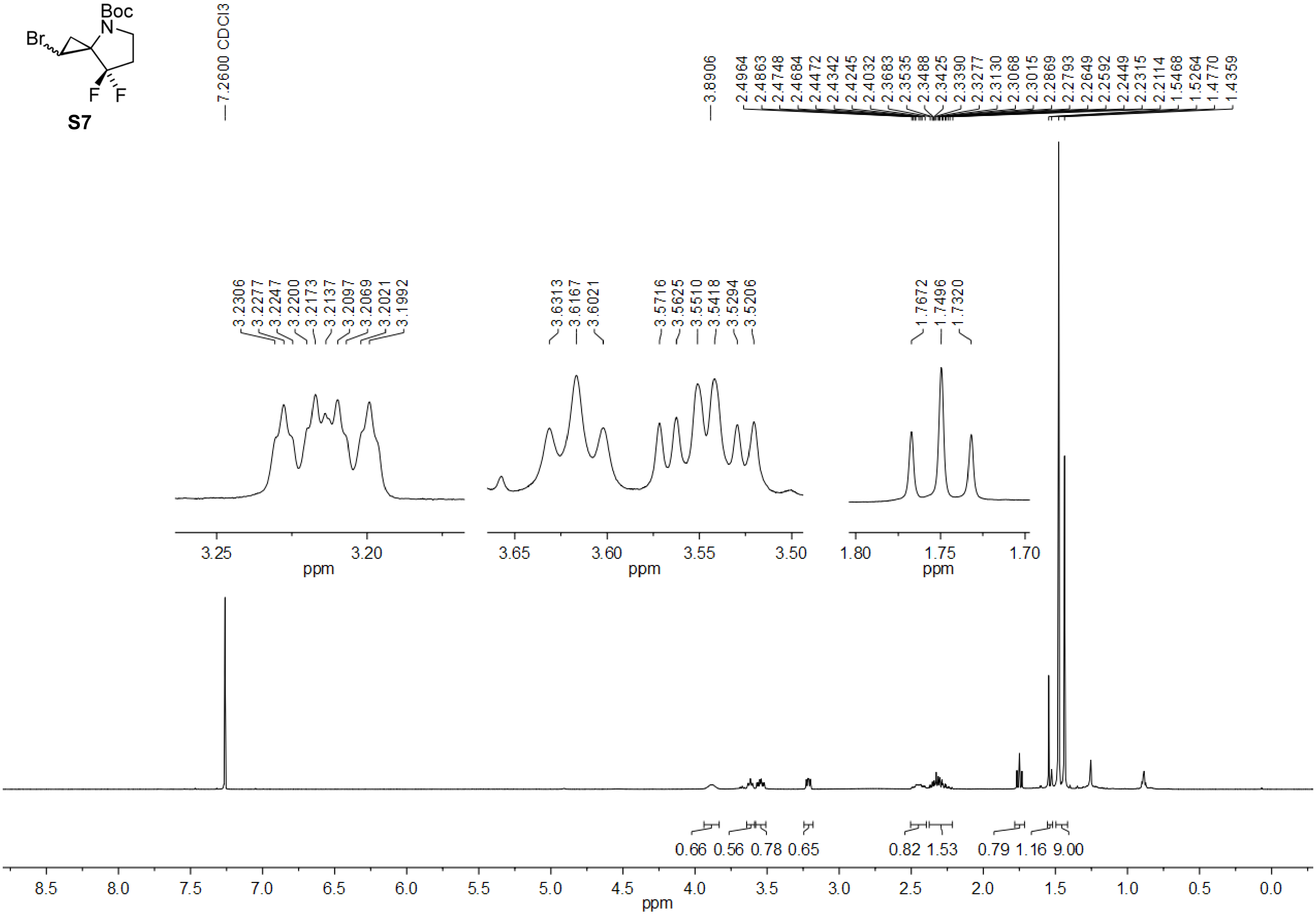


3

^{13}C NMR (176 MHz)

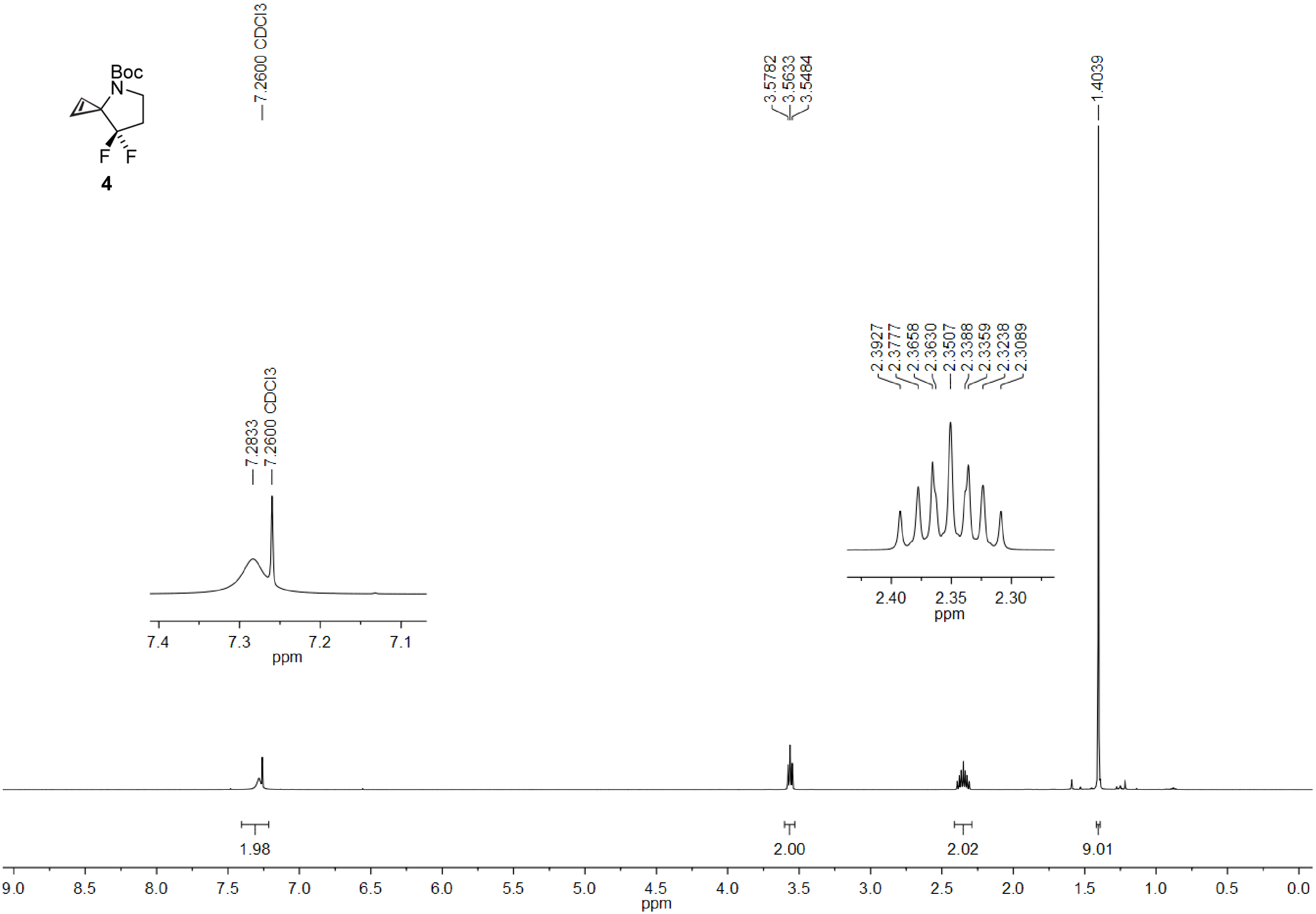


^{1}H NMR (500 MHz)

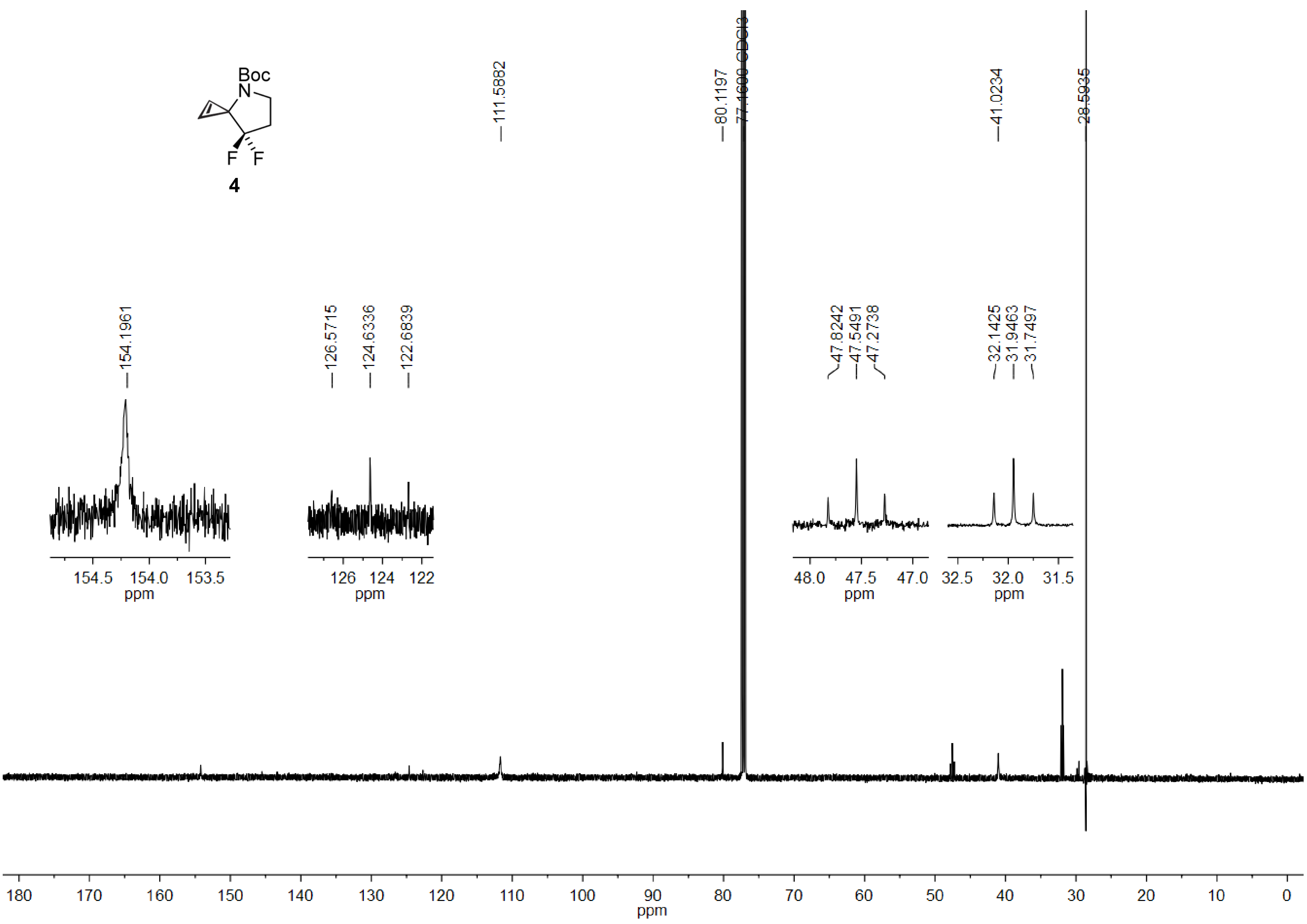


^{1}H NMR (500 MHz)

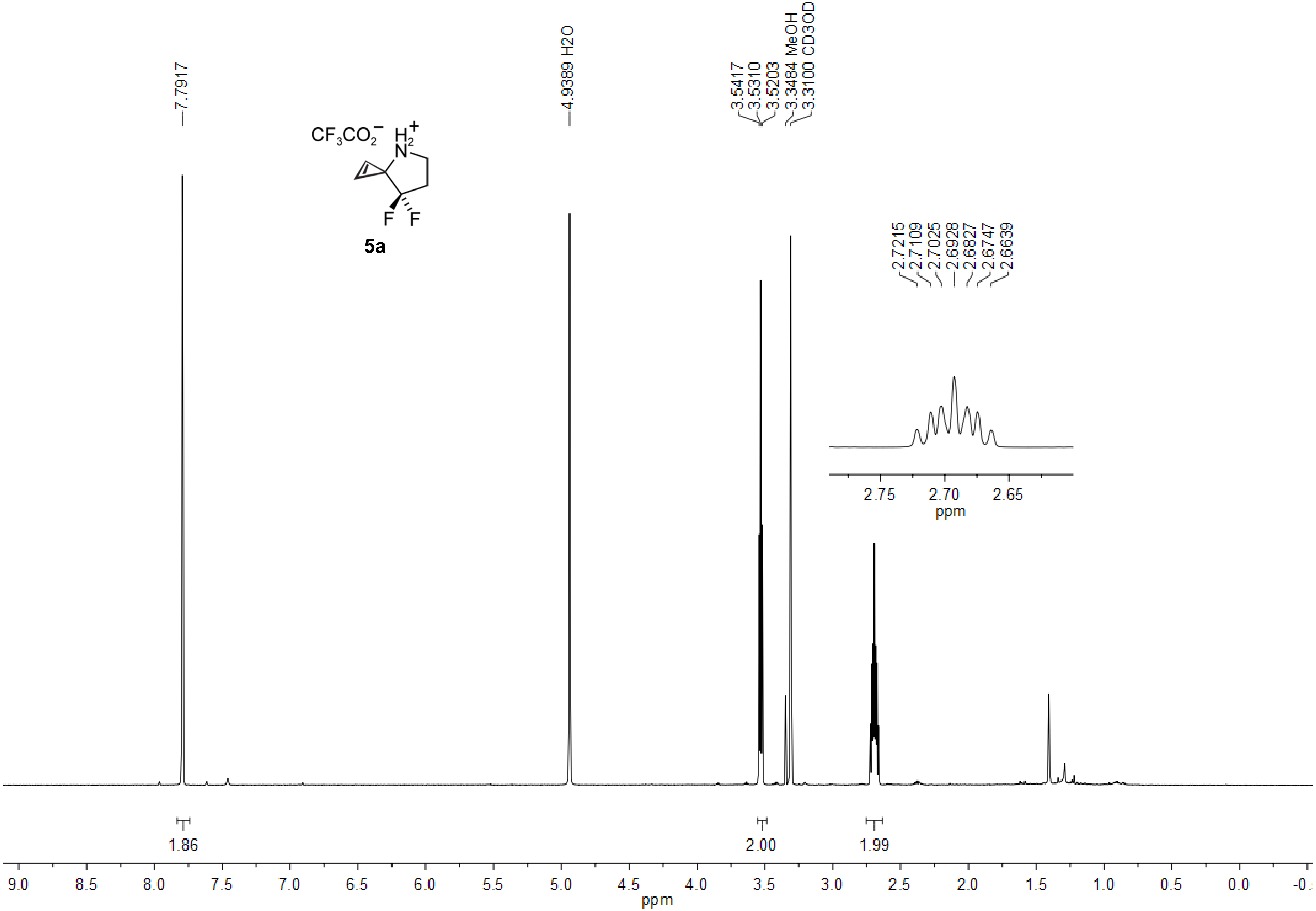
4



^{13}C NMR (126 MHz)

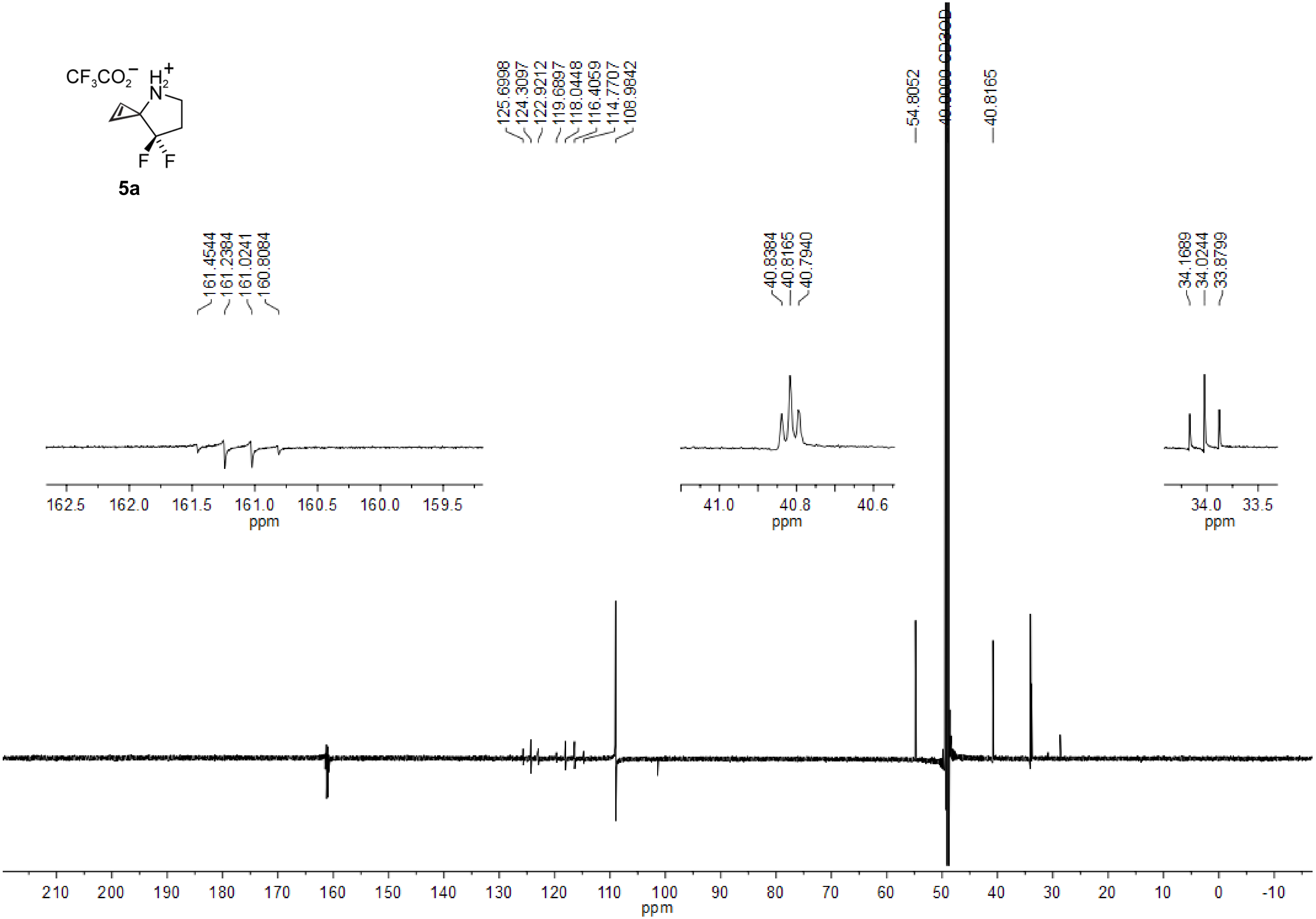


^{1}H NMR (700 MHz)

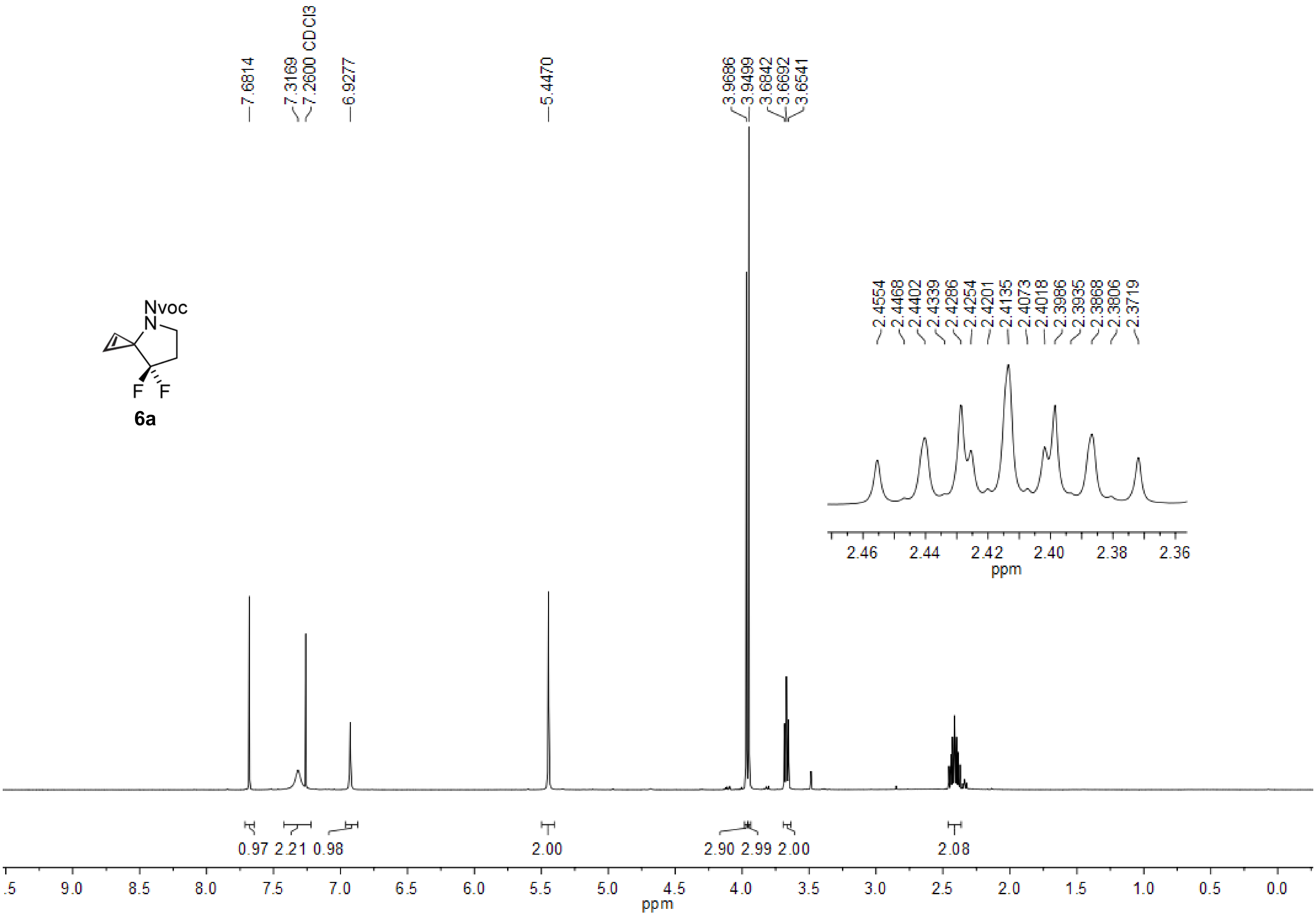


^{13}C NMR (176 MHz)

5a

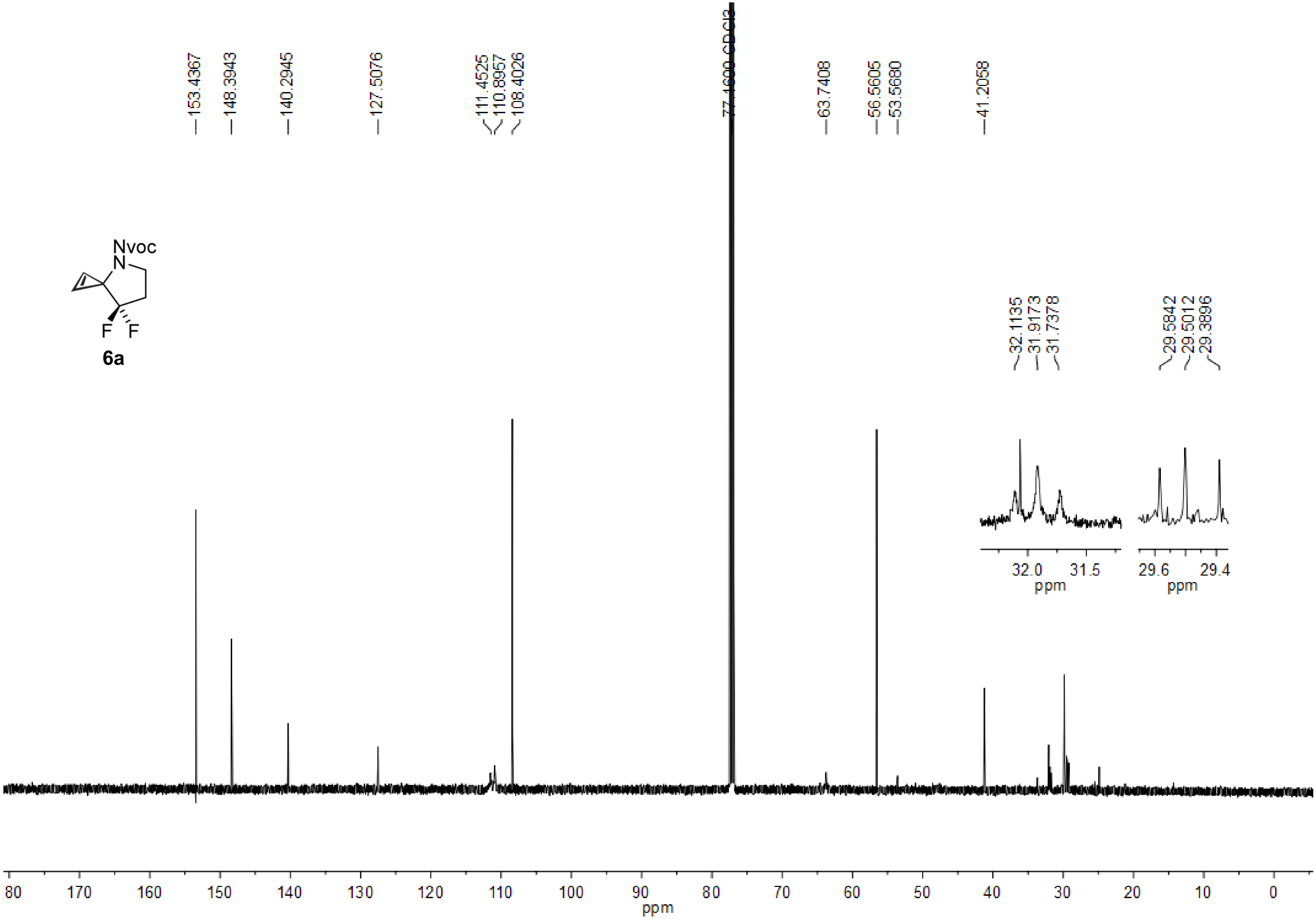


^{1}H NMR (700 MHz)

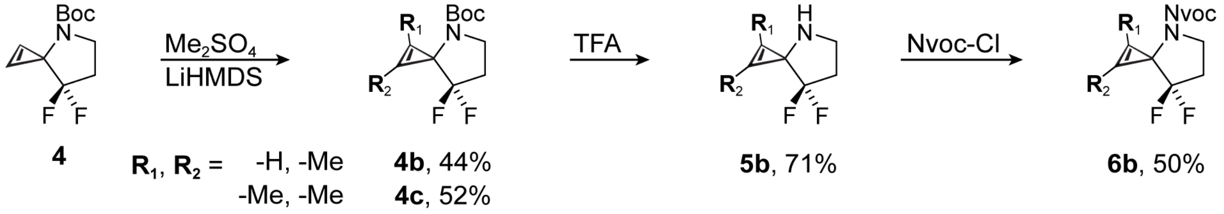


6a

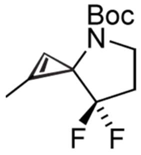
^{13}C NMR (176 MHz)



Scheme 3

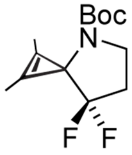


General procedure for C1 and/or C2 functionalization of cyclopropenes. The mono/bis methylation reactions using LiHMDS were accomplished using a modification to a previuosuly described strategy modifying cyclopropenes[4]. To a solution of LiHMDS (1.2 eq for mono-substitutions and 5.0 eq for disubstitutions) in dry THF at -30 °C under N_2 was added **4** (as a solution in dry THF) in one portion. The orange reaction mixture was stirred at the same temperature for 10 mins, followed by addition of Me_2SO_4 (1.05–1.15 eq for mono-substitutions and 10–20 eq for disubstitutions). The reaction was followed using MS (approx. 1–1.5 h for mono-substitutions and 2 h for disubstitutions). The reaction was quenched by dropwise addition of saturated NH_4Cl. The reaction was diluted with DCM and the organic layer was collected. The aqueous layer was further extracted with DCM and the combined organic layers were dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by flash chromatography over silica gel (2.5–5% EtOAc/Hexanes) to obtain pale yellow oil **4b** and **4c**. The purified products were dissolved in DCM (~10 mL) and cooled down using an ice-bath. TFA (20% v/v) was added and the reaction mixture was gradually allowed to warm to rt. The reaction was monitored using TLC. Upon complete deprotection of Boc, reaction was concentrated *in vacuo*, and purified using HPLC to obtain **5b**. The purified TFA salt was dissolved in $CHCl_3/Et_2O$ (9:1 (v/v)) and cooled down in an ice-bath. To this solution was added solid $NaHCO_3$ (pH ~8.5) and Nvoc-Cl (1.2 eq). The reaction was stirred at rt overnight. The reaction mixture was diluted with DCM and water and the organic layer was separated. The combined organic layer was dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by HPLC to obtain **6b**.



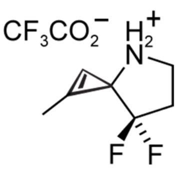
4b

Yield = 44%. ^1H NMR (500 MHz, $CDCl_3$): δ= 6.71 (br s, 1H), 3.54 (t, *J* = 7.6 Hz, 2H), 2.30 (m, 2H), 2.17 (s, 3H), 1.40 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$): δ= 154.00, 127.82, 125.76, 123.81, 119.49, 119.42, 109.99, 109.95, 109.90, 102.73, 102.69, 102.65, 79.74, 50.14, 49.87, 49.60, 41.15, 32.25, 32.06, 31.86, 28.59, 10.54. HRMS(ESI): Calcd for $C_{12}H_{18}F_2NO_2$ $[M+H]^+$:246.1300, found: 246.1303. We found it difficult to separate this molecule from the dimethyl version even after multiple rounds of silica gel flash chromatography.



4c

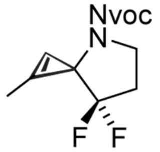
Yield = 51.6%. ^1H NMR (500 MHz, $CDCl_3$): δ= 3.53 (t, *J* = 7.6 Hz, 2H), 2.31–2.22 (m, 2H), 2.02 (s, 6H), 1.40 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$): δ= 110.00, 109.96, 109.91, 79.31, 41.04, 36.78, 28.62, 24.83, 23.48. Peaks for three quaternary carbons (CO, sp^3 C, and CF_2) were not observed. HRMS(ESI): Calcd for $C_{13}H_{19}F_2NO_2$ $[M+H]^+$:260.1457, found: 260.1458.



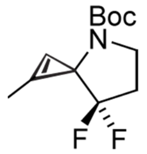
5b

Yield = 71%. ^1H NMR (500 MHz, CD_3OD): δ= 7.18 (dt, *J* = 3.5, 1.0 Hz, 1H), 3.51 (t, *J* = 7.5 Hz, 2H), 2.71–2.62 (m, 2H), 2.28 (d, *J* = 1.1 Hz, 3H). ^{13}C NMR (126 MHz, CD_3OD): δ= 118.35, 118.31, 118.28, 100.47, 100.44, 100.43, 100.40, 40.80, 40.77, 40.74, 34.42, 34.22, 34.01, 8.65. Peaks for two quaternary carbons (CO and CF_2) and TFA were not observed. HRMS (ESI): Calcd for $C_7H_9F_2N$ $[M+H]^+$:146.0776, found: 146.0775. We observed that this molecule has extremely low absorbance on HPLC (220 nm and 365 nm), therefore, we recommend using it directly for the next step.

HPLC (R_t = 11.5 min, 50–100% MeCN over 25 mins, flow rate = 1 mL/min). Yield = 50%.^{1}H NMR (700 MHz, $CDCl_3$): δ= 7.69 (s, 1H), 6.94 (s, 1H), 6.74 (s, 1H), 5.44 (s, 2H), 3.96 (2×s, 6H), 3.66 (m, 2H), 2.38 (m, 2H), 2.18 (s, 3H). ^{13}C NMR (176 MHz, $CDCl_3$): δ= 153.47, 148.35, 140.21, 119.19, 108.38, 102.52, 56.57, 56.51, 41.25, 41.22, 41.20, 32.10, 31.95, 31.88. HRMS(ESI): Calcd for $C_{17}H_{18}F_2N_2O_6$ $[M+H]^+$:385.1206, found: 385.1212.

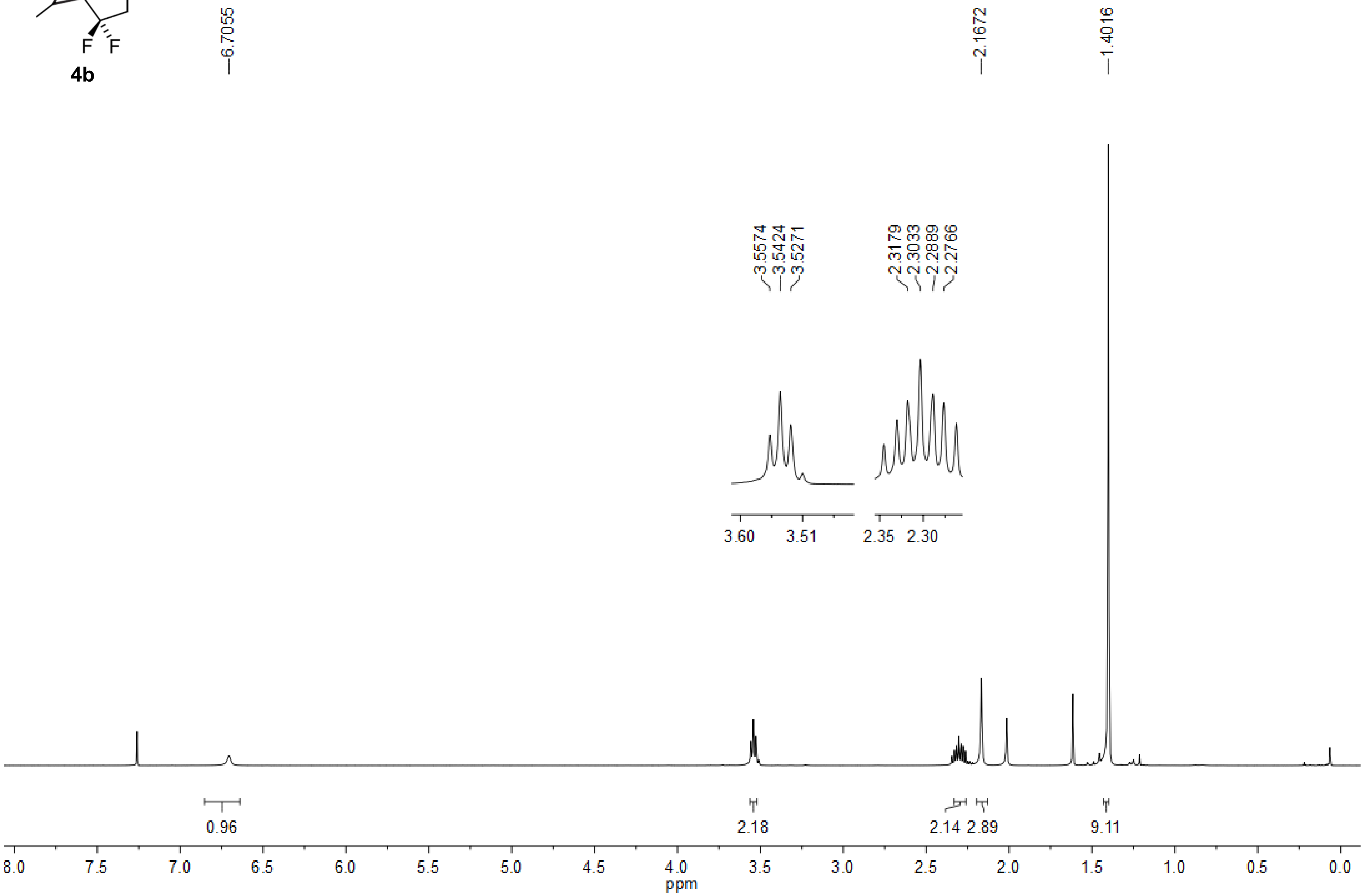


6b

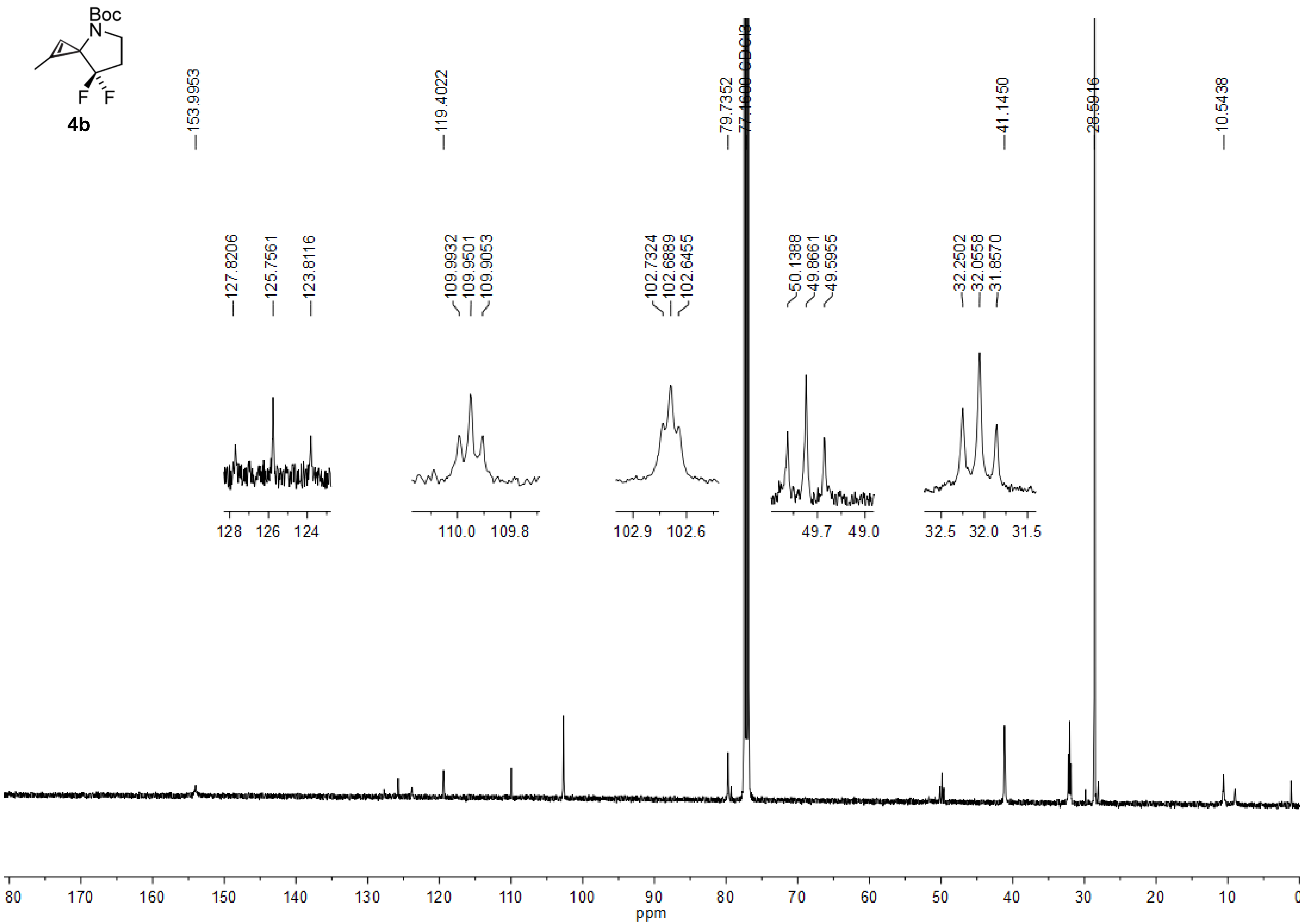


4b

^{1}H NMR (500 MHz)

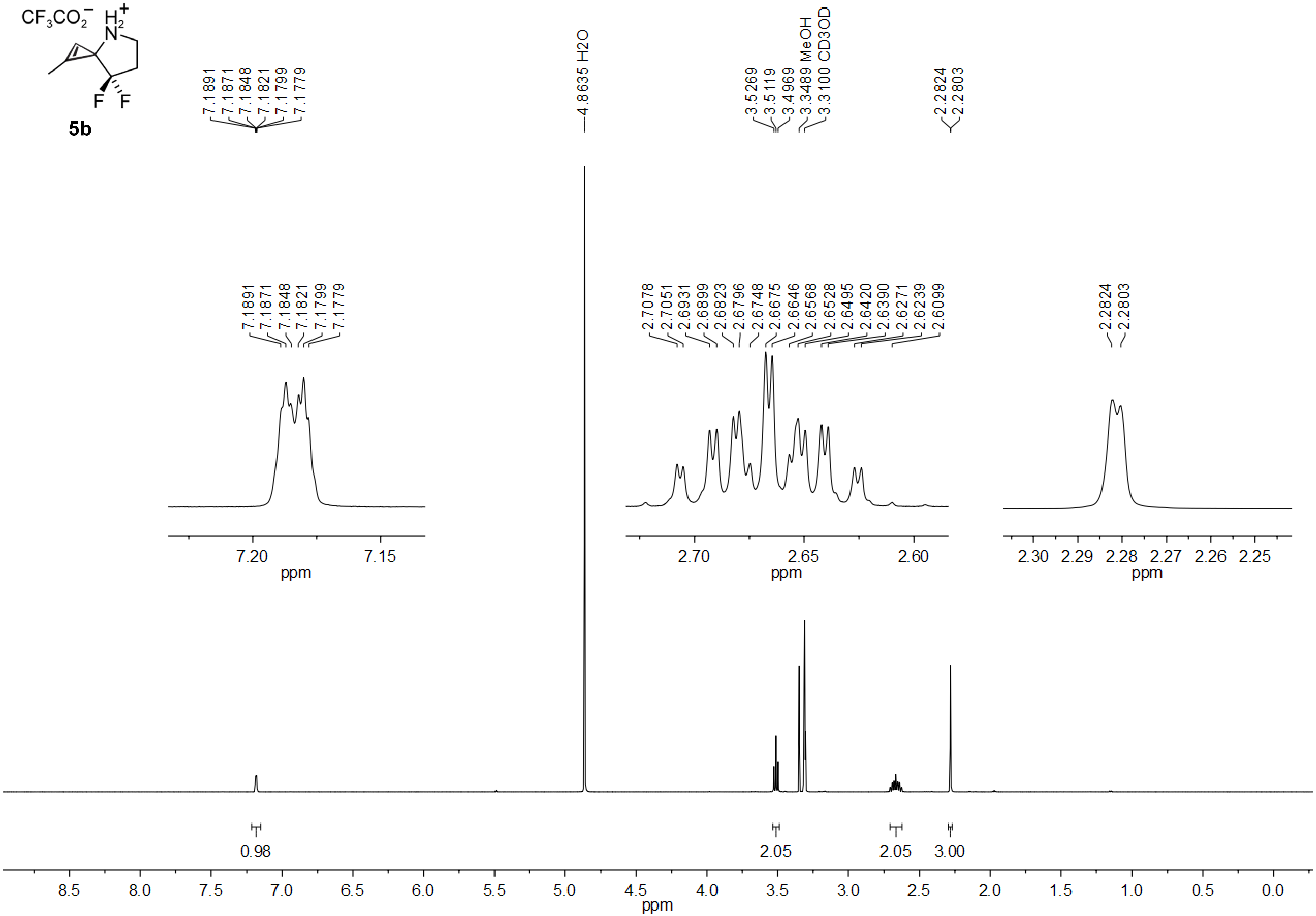


^{13}C NMR (126 MHz)

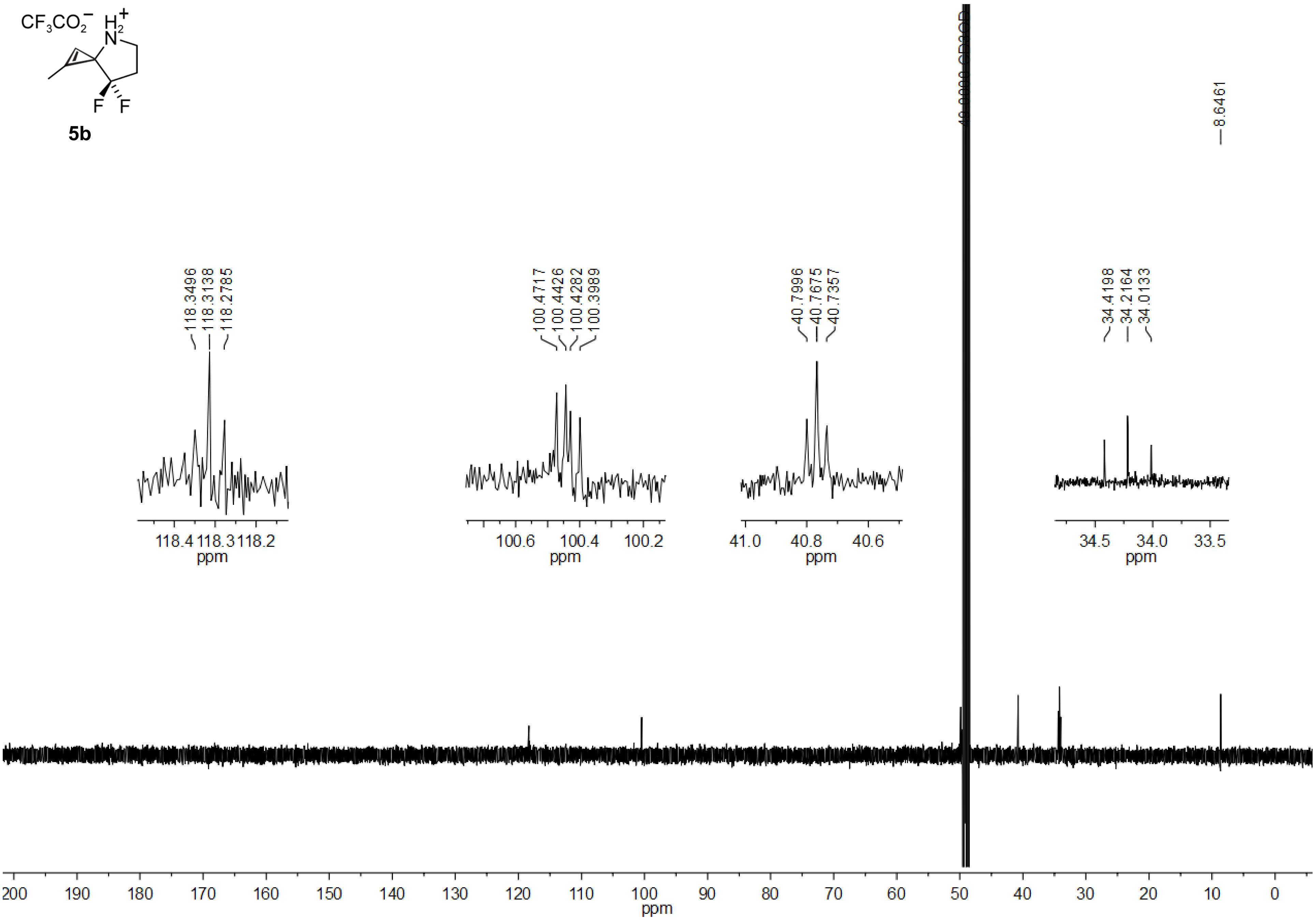


4b

^{1}H NMR (500 MHz)

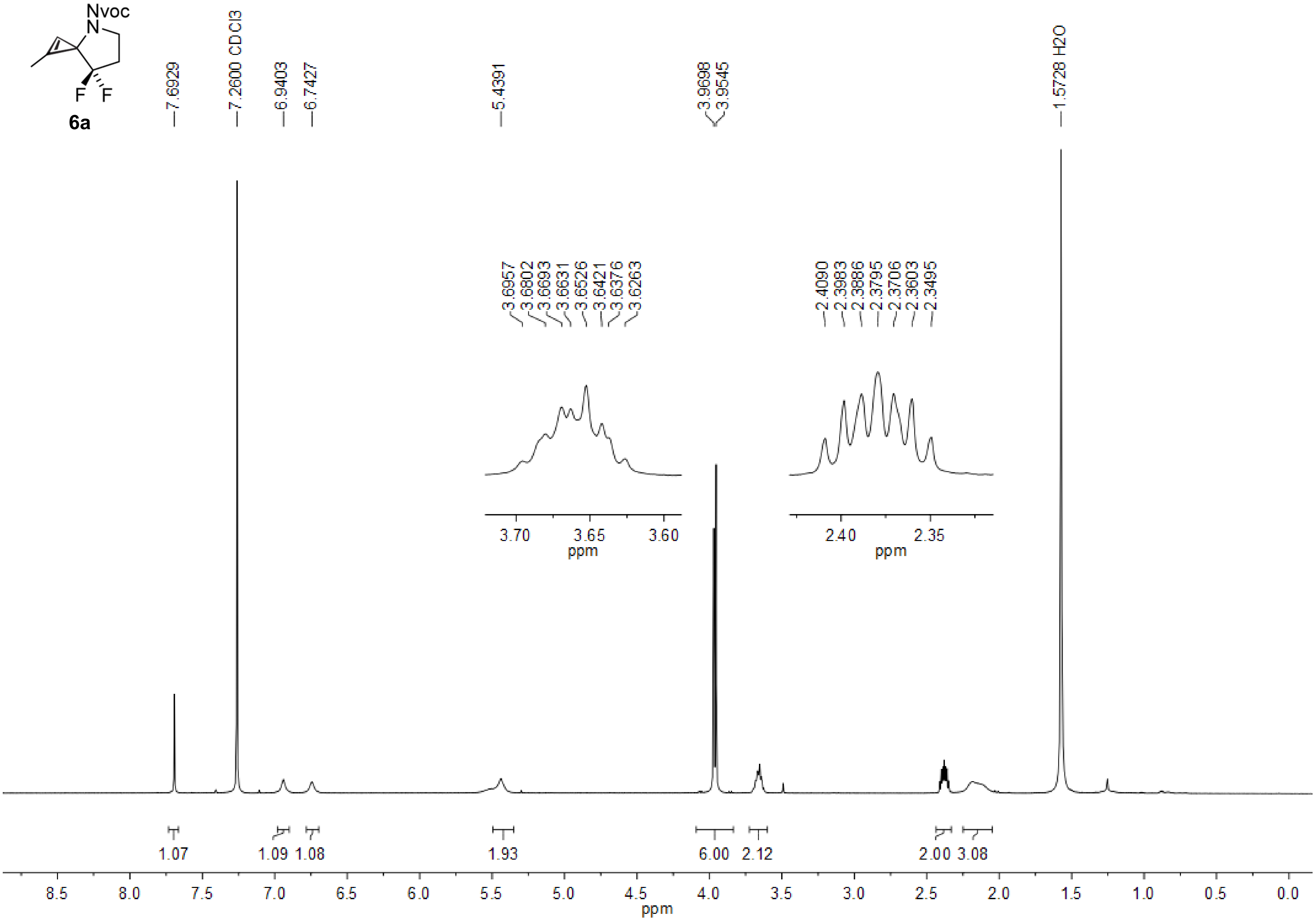

5b

^{13}C NMR (126 MHz)

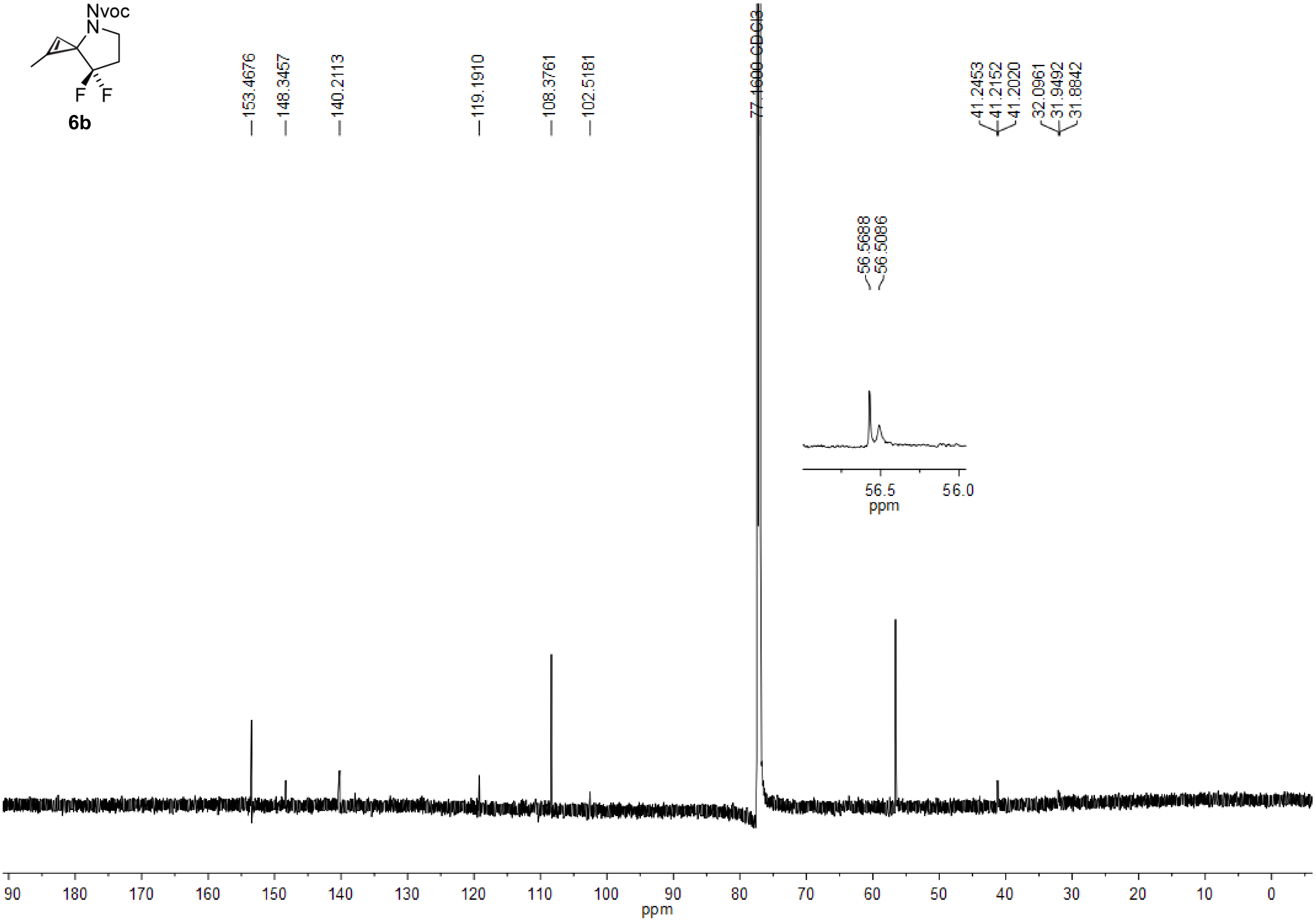


5b

^{1}H NMR (700 MHz)

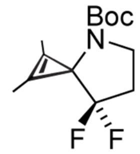


^{13}C NMR (176 MHz)

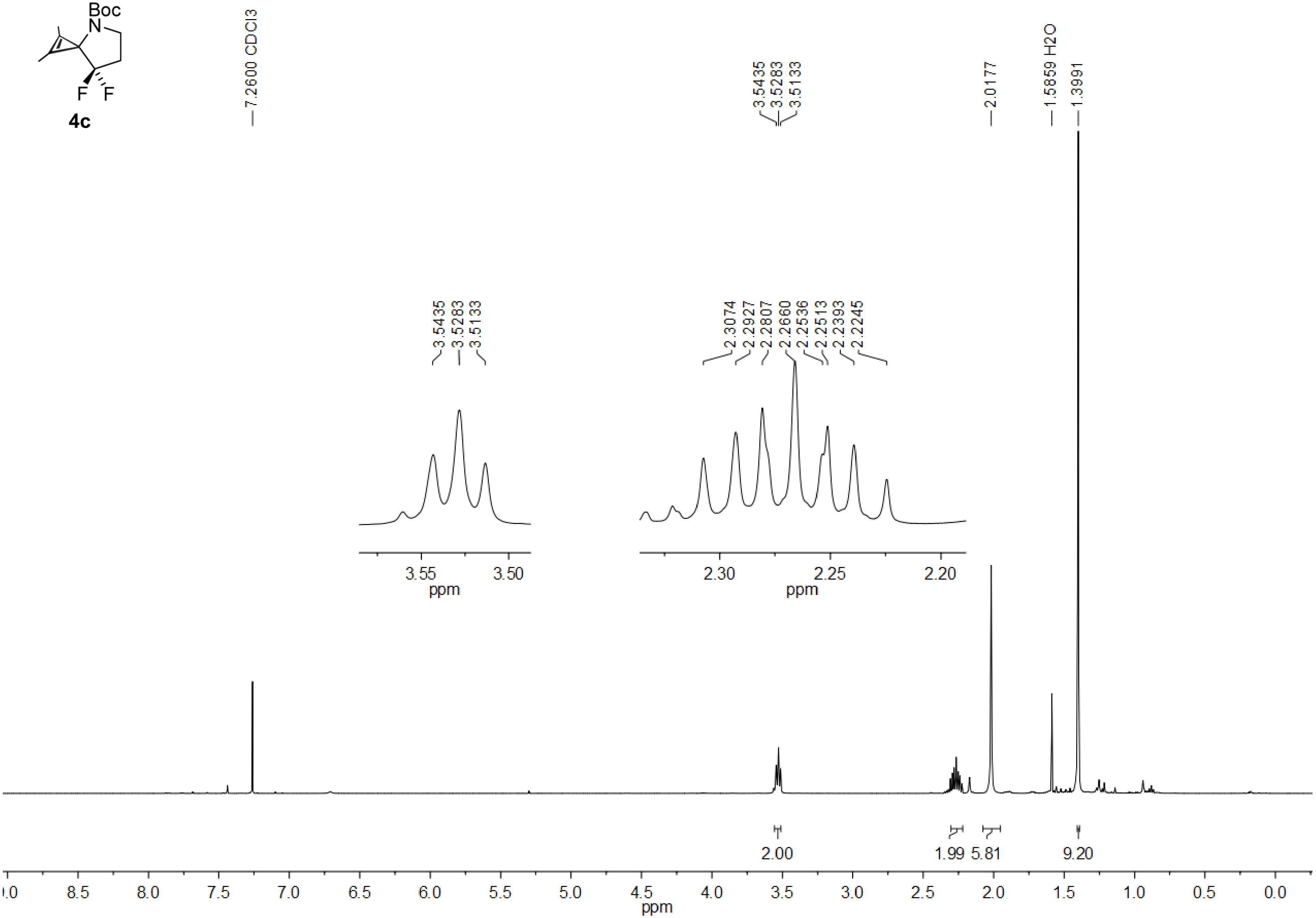


6b

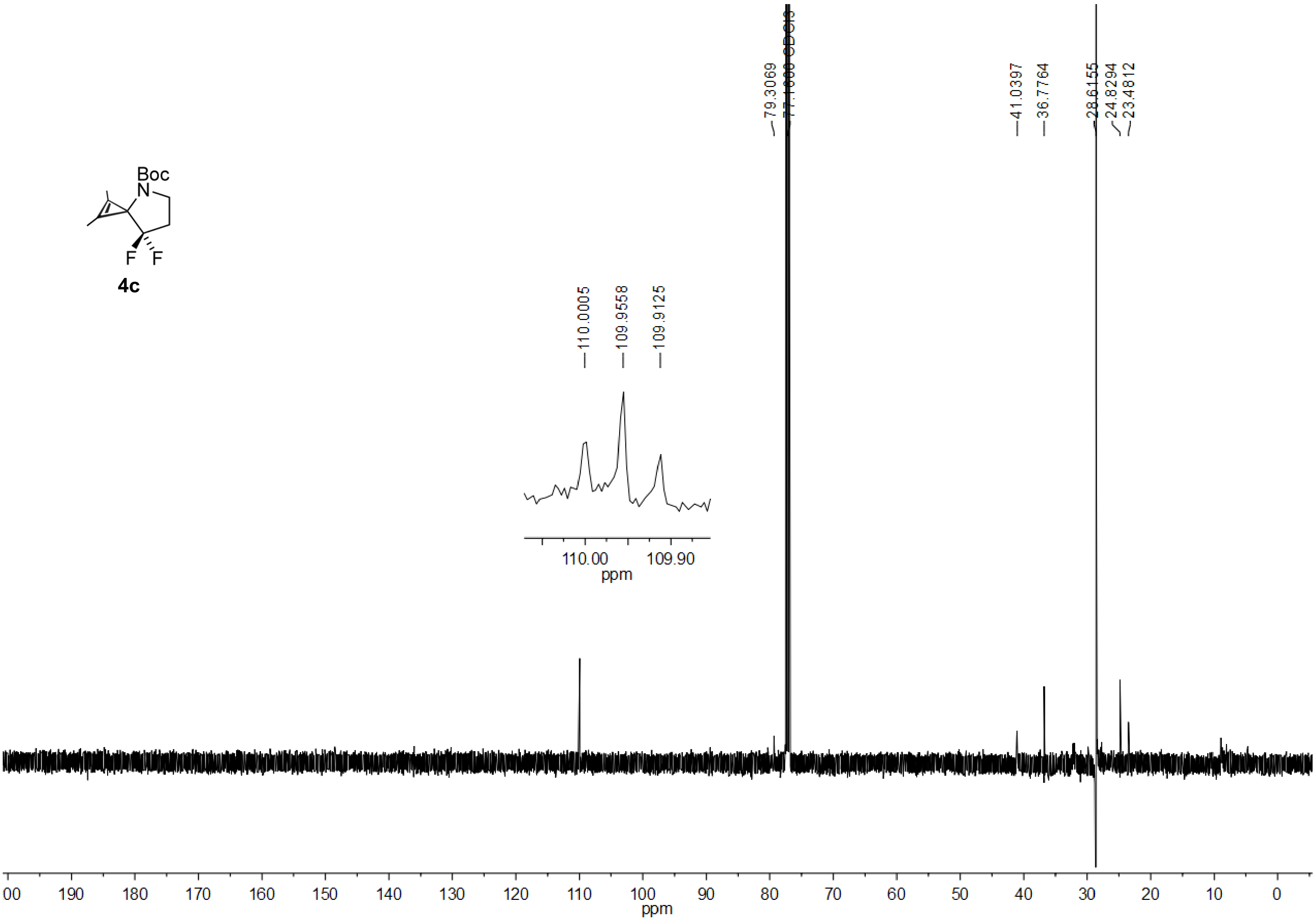
^{1}H NMR (500 MHz)



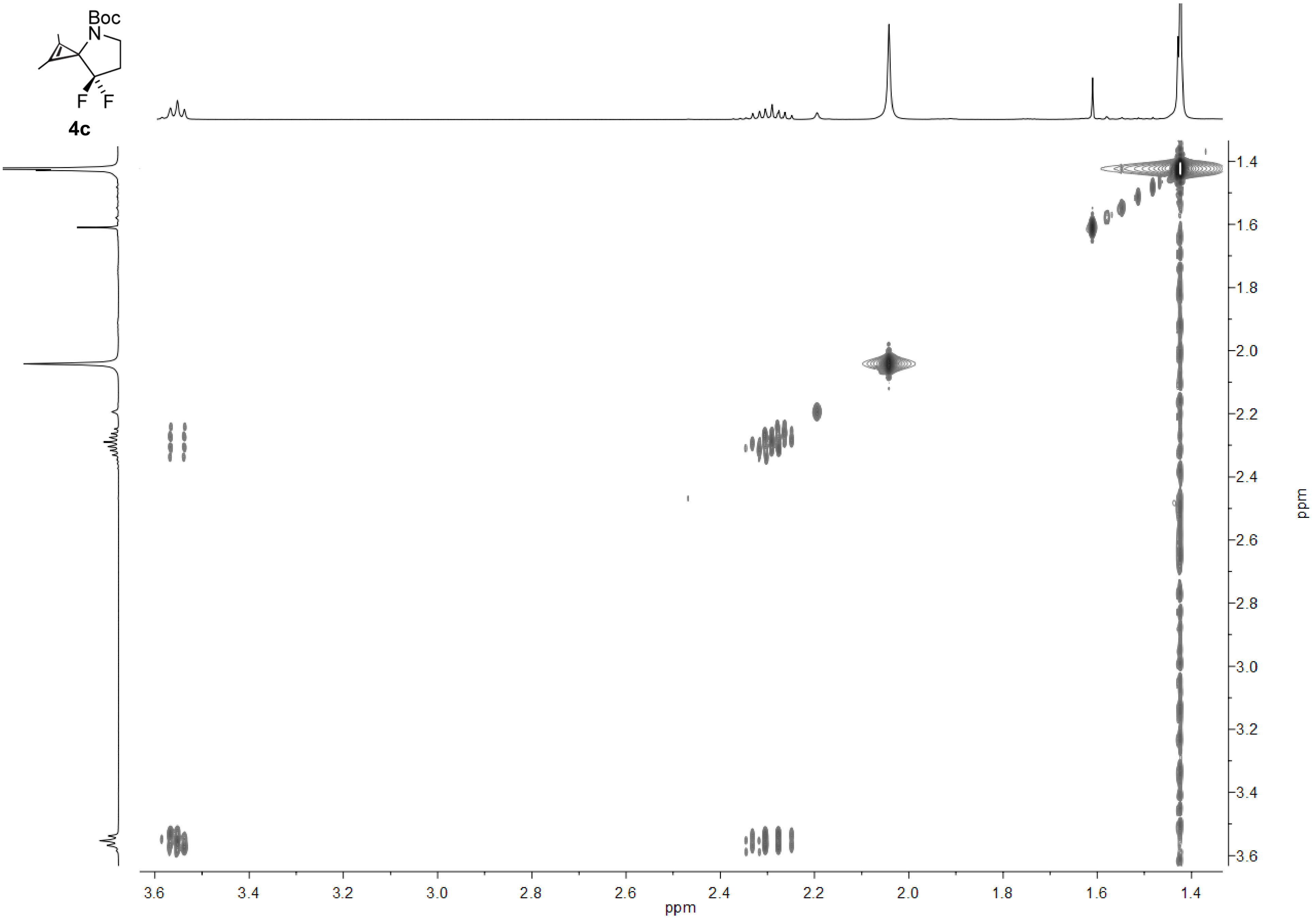
4c



^{13}C NMR (126 MHz)

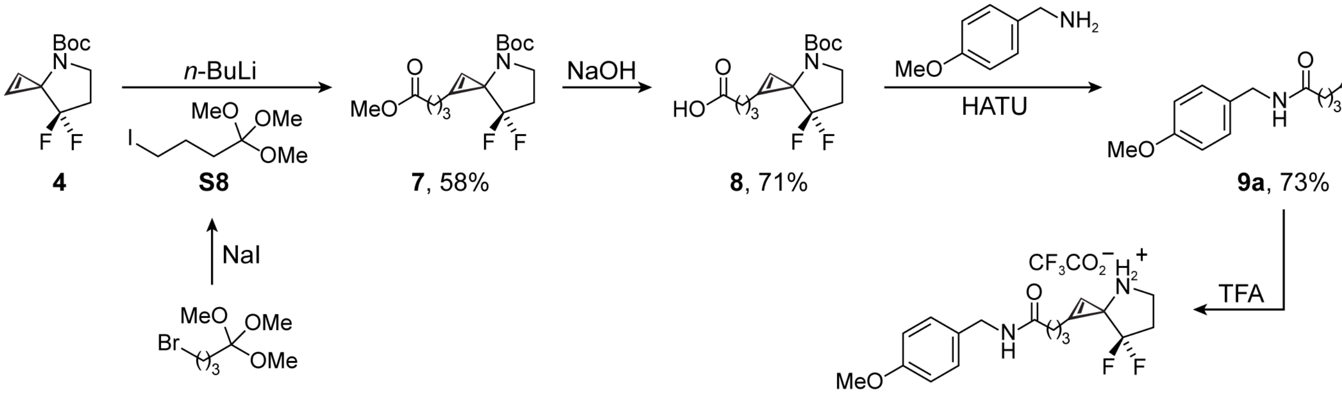


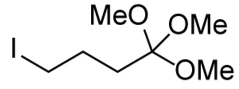
COSY NMR (500 MHz)



4c

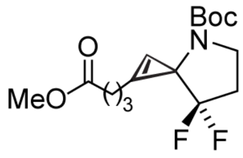
Scheme 4





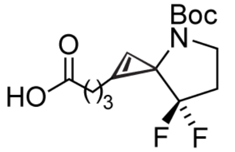
S8

To a solution of trimethyl 4-bromoorthobutyrate (105 mg, 0.46 mmol, 1.0 eq) in acetone (2.5 mL) was added NaI (346 mg, 2.32 mmol, 5.0 eq) in one portion. The resulting ivory suspension was stirred at rt and the reaction progress was monitored using mass spectrometry (~20 h). The reaction mixture was concentrated *in vacuo* and extracted with DCM and water. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to obtain **S8** (117.6 mg) as a pale-yellow oil that was used immediately without further purification as it was highly prone to decomposition. Attempts to purify **S8** using silica gel chromatography resulted in conversion of the trimethyl orthoformate group to the methyl ester. ^{1}H NMR (700 MHz, $CDCl_3$): δ= 3.23 (s, 9H), 3.22–3.19 (m, 2H), 1.86–1.83 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 115.33, 49.56, 31.35, 27.18, 7.15. MS (ESI): Calcd for $C_7H_{15}IO_3$ $[M-OMe]^+$: 242.9882, found: 242.9876.



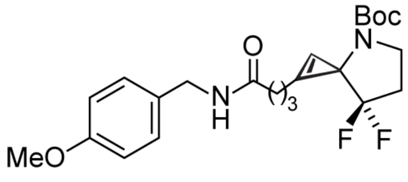
7

To a solution of **4** (30 mg, 0.13 mmol, 1.0 eq) in anhydrous THF (4.0 mL) was added HMPA (46.5 mg, 45 µL, 0.259 mmol, 2 eq) under an atmosphere of N_2. The reaction mixture was stirred at rt for 10 mins and then cooled to -78 °C. To this pale-yellow solution was added n-BuLi (97 µL, 1.6 M in hexane, 0.156 mmol, 1.2 eq) dropwise. The reaction turned orange and then deep red. **S8** (117.6 mg) as solution in anhydrous THF (0.5 mL) was added to the reaction mixture at -78 °C. The resulting yellow mixture was stirred for 3 h while allowing the reaction temperature to warm gradually to rt, at which point it was quenched with saturated NH_4Cl and extracted with DCM and 2M HCl. The combined organic layers were dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by flash chromatography (5 g silica, 10% EtOAc/hexanes (v/v)) to obtain **7** as a pale-yellow oil (25 mg, 58%, and 73% based on recovery of 6 mg of starting material **4**). R_f = 0.12 (10% EtOAc/hexanes, visualized w/ $KMnO_4$). ^{1}H NMR (500 MHz, $CDCl_3$): δ= 6.75 (s, 1H), 3.65 (s, 3H), 3.58–3.43 (m, 2H), 2.56 (s, 2H), 2.38 (t, *J* = 7.5 Hz, 2H), 2.29 (m, 2H), 1.93 (dd, *J* = 10.8, 7.0 Hz, 2H), 1.38 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$): δ= 173.63, 153.90, 127.20, 125.25, 123.30, 122.73, 102.99, 79.77, 51.65, 50.22, 49.95, 49.67, 41.10, 33.29, 32.11, 31.92, 31.72, 29.79, 24.49, 22.26. HRMS (ESI): Calcd for $C_{16}H_{23}F_2NO_4$ $[M+Na]^+$: 354.1493, found: 354.1494.



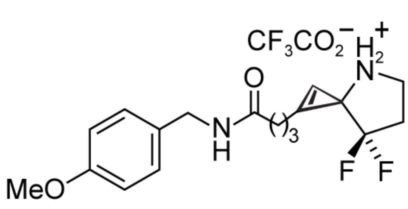
8

To a solution of **7** (25 mg, 0.075 mmol) in MeOH (5.0 mL) was added 1M NaOH (2.0 mL) dropwise at rt and the mixture was allowed to stir overnight. The reaction was concentrated *in vacuo*, diluted with DCM, water, and 1M NaOH (2.0 mL). The aqueous layer was washed with DCM. The aqueous layer was acidified to pH 1–2 using 2M HCl and extracted with DCM. The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to obtain **8** as a pale-yellow oil (17 mg, 71%). R_f = 0. 2 (30% EtOAc/hexanes, visualized w/ $KMnO_4$).^{1}H NMR (500 MHz, $CDCl_3$): δ= 6.78 (s, 1H), 3.66–3.39 (m, 2H), 2.66–2.55 (m, 2H), 2.44 (t, *J* = 7.4 Hz, 2H), 2.38– 2.29 (m, 2H), 2.01– 1.95 (m, 2H), 1.39 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$): δ= 178.54, 153.96, 125.31, 123.39, 122.62, 103.13, 80.06, 50.31, 50.04, 49.76, 41.16, 33.26, 32.12, 31.92, 31.72, 28.57, 24.43, 22.07. HRMS (ESI): Calcd for $C_{15}H_{21}F_2NO_4$ $[M+Na]^+$: 340.1336, found: 340.1343.



9a

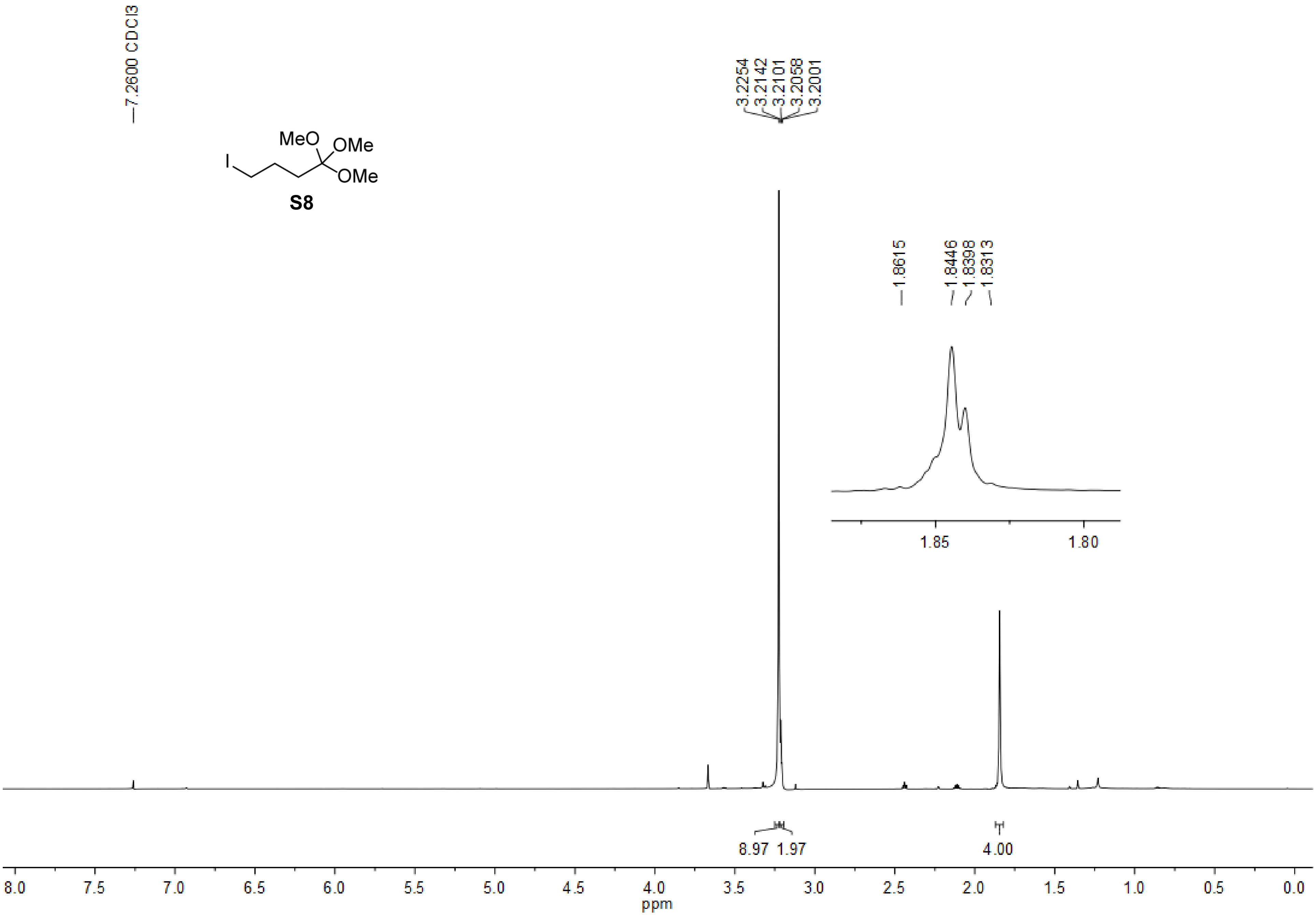
To an ice-cold solution of **8** (11 mg, 0.035 mmol, 1.0 eq) in dry DMF (1.0 mL) under an inert atmosphere was added DIPEA (5.38 mg, 7.25 uL, 0.042 mmol, 1.2 eq). Further, HATU (15.84 mg, 0.042 mmol, 1.2 eq) as a solution in dry DMF (0.4 mL) was added dropwise and the reaction was continued stirring at the same temperature for 30 mins. 4-methoxybenzylamine (10.4 mg, 0.028 mmol, 1.1 eq) as a solution in dry DMF (0.4 mL) was added to this activated acid and the resultant yellowish mixture was allowed to gradually warm to rt overnight. The reaction mixture was extracted using DCM and water. The combined organic layer was dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by flash chromatography (4.0 g silica, 30%-50% EtOAc/hexanes (v/v)) to obtain **9a** (11 mg, 73%). R_f = 0.11 (30% EtOAc/hexanes, visualized w/ UV). ^{1}H NMR (500 MHz, $CDCl_3$): δ= 7.20–7.19 (d, *J* = 8.5 Hz, 2H), 6.85– 6.84 (d, *J* = 8.5 Hz, 2H), 6.76 (s, 1H), 4.40– 4.31 (m, 2H), 3.79 (s, 3H), 3.54–3.50 (m, 2H), 2.60–2.58 (t, *J* = 6.5 Hz, 2H), 2.34–2.21 (m, 4H), 2.04– 1.98 (m, 2H), 1.36 (s, 10H). ^{13}C NMR (126 MHz, $CDCl_3$): δ= 172.47,159.05, 130.78, 129.22, 128.43–127.83, 125.36–119.94, 114.13, 103.32, 79.91, 55.44, 50.41–49.86, 43.09, 41.27, 35.52, 32.11, 31.92, 29.85, 28.59, 24.32, 23.15. HRMS (ESI): $C_{23}H_{30}F_2N_2O_4$ $[M+H]^+$: 437.2252, found: 437.2237.



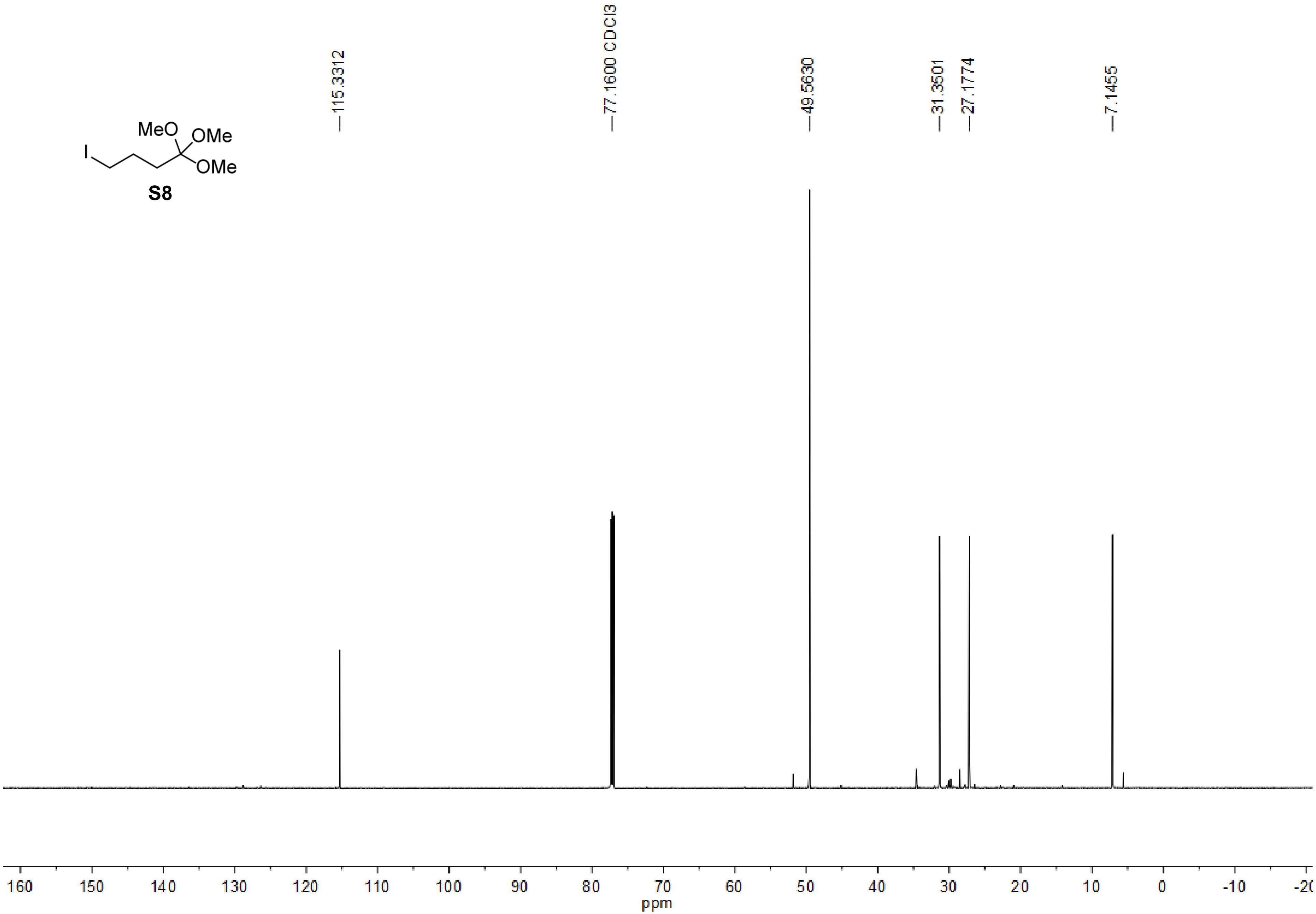
10a

To an ice-cold solution of **9a** (10 mg, 0.023 mmol) in DCM (3.0 mL) was added TFA (0.6 mL). The reaction was allowed to gradually warm to rt over 2 h. The reaction was concentrated *in vacuo*, re-suspended in MeOH (0.2 mL), and purified by HPLC (R_t = 23.8 min, 10-90% MeOH in 25 mins, flow rate = 1 mL/min) to obtain **10a** (5.1 mg, 66.2%) as a TFA salt. ^{1}H NMR (400 MHz, CD_3OD): δ= 7.26 (d, *J* = 3.8 Hz, 1H), 7.20 (d, *J* = 8.6 Hz, 2H), 6.87 (d, *J* = 8.7 Hz, 2H), 4.29 (s, 2H), 3.77 (s, 3H), 3.52 (t, *J* = 7.5 Hz, 2H), 2.68 (m, 4H), 2.34 (m, 2H), 1.99 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD): δ= 174.76, 160.47, 131.91, 129.95, 125.00, 121.51, 119.14, 114.93, 101.01, 55.69, 43.66, 40.82, 40.80, 40.76, 35.72, 34.41, 34.16, 33.90, 23.93, 23.89. HRMS (ESI): Calcd for $C_{18}H_{22}F_2N_2O_2$ $[M+H]^+$: 337.1728, found: 337.1710.

^{1}H NMR (700 MHz)

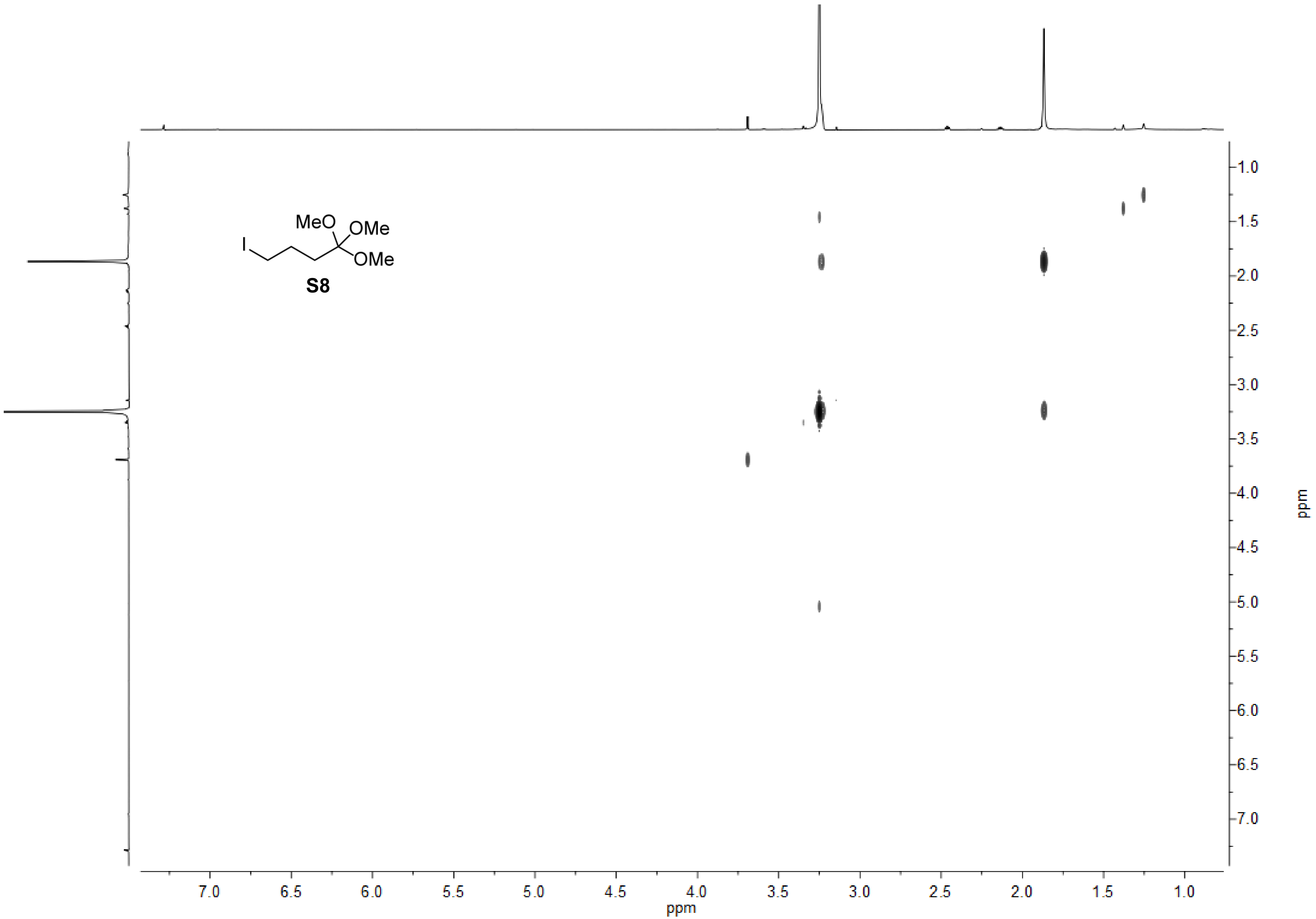


^{13}C NMR (176 MHz)



S8

COSY (700 MHz)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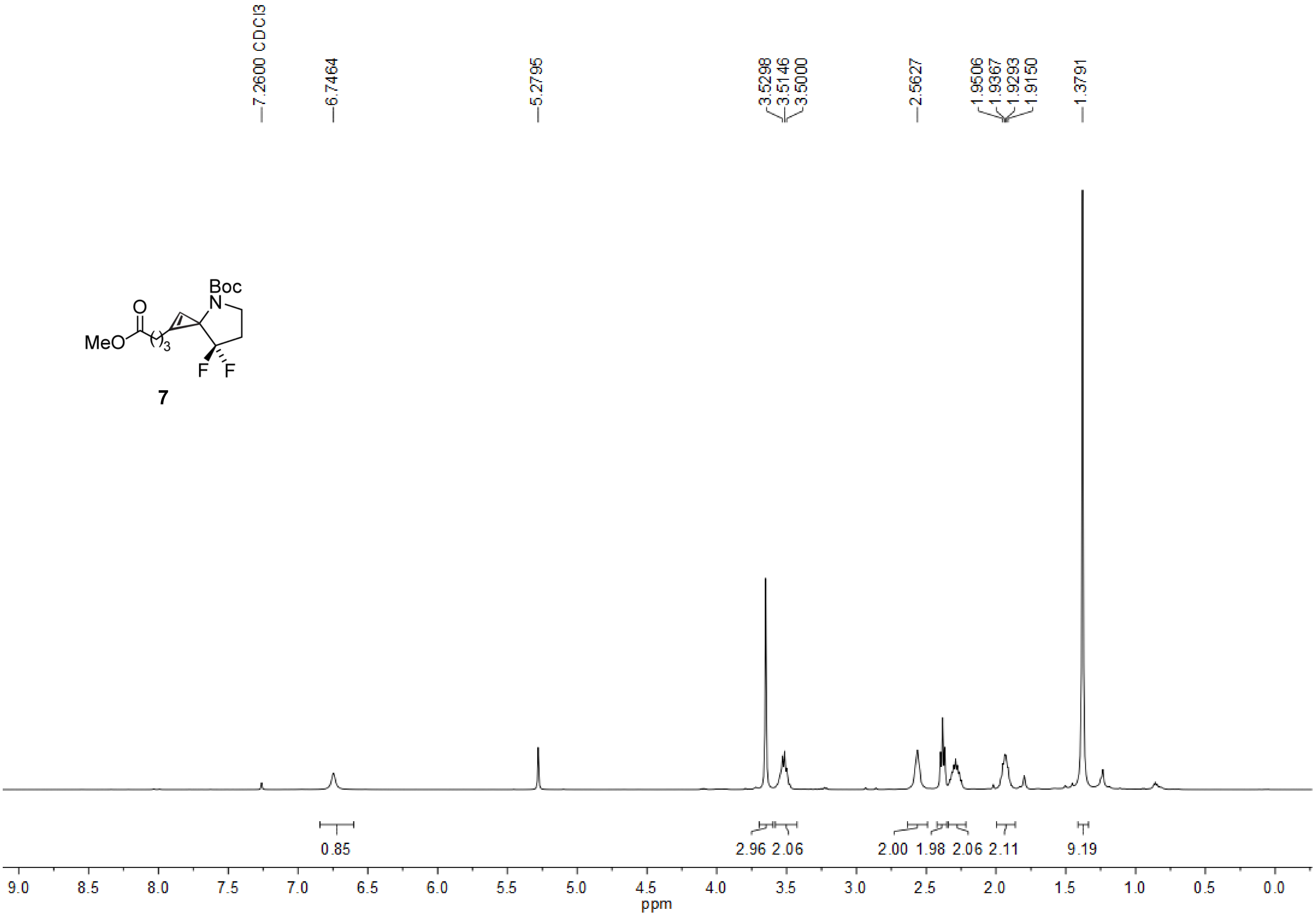
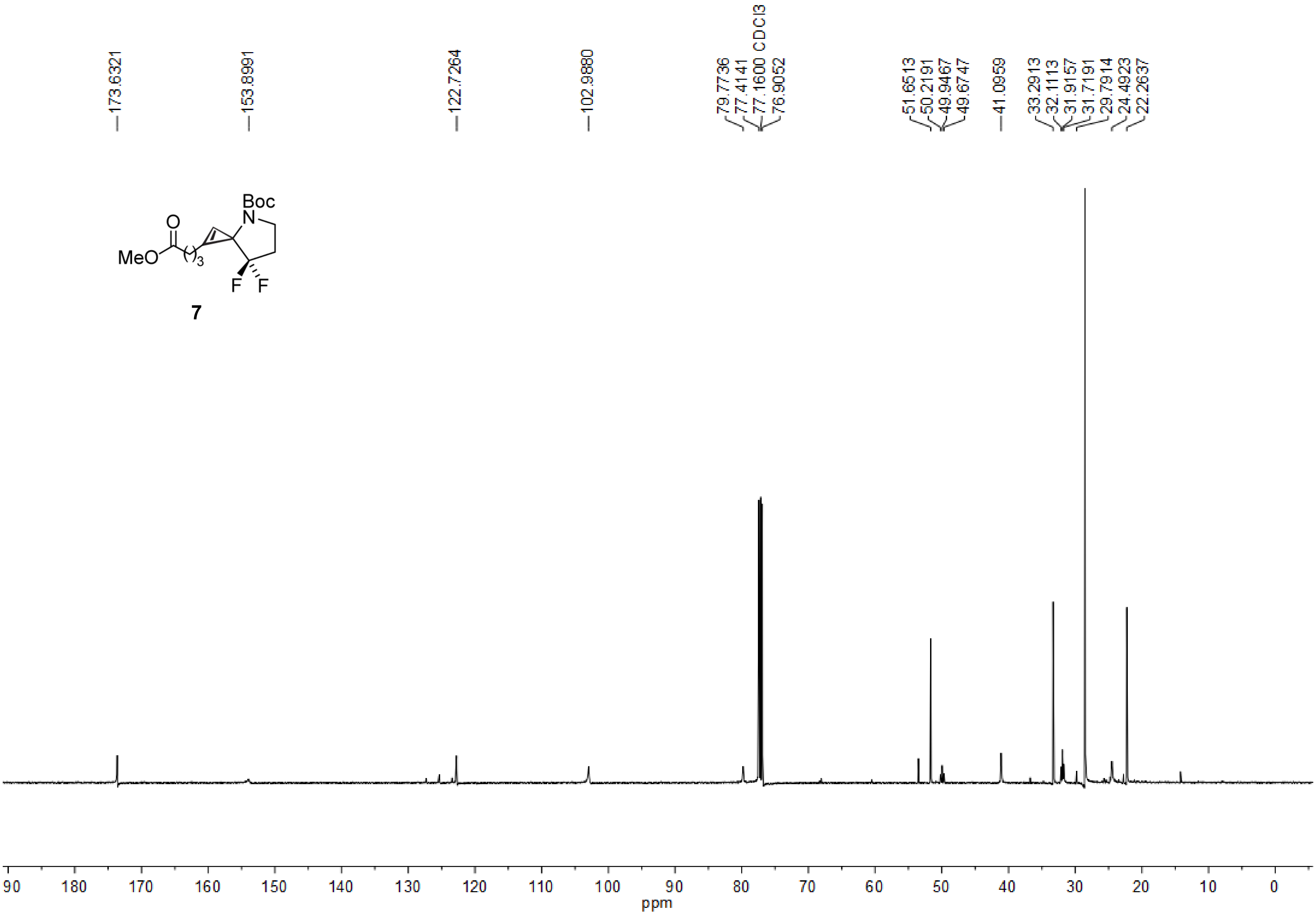
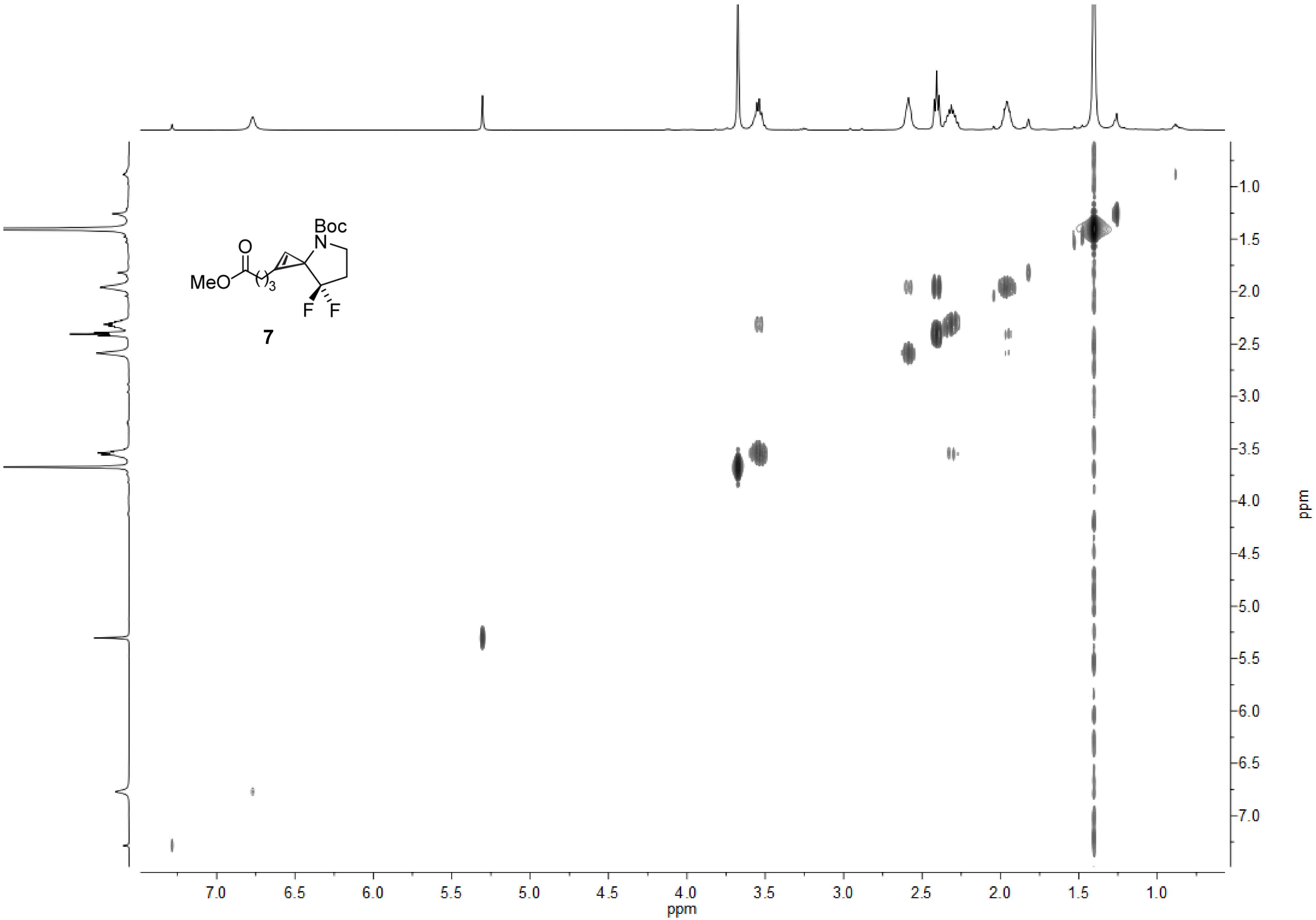
^{1}H NMR (500 MHz)



^{13}C NMR (126 MHz)



COSY (500 MHz)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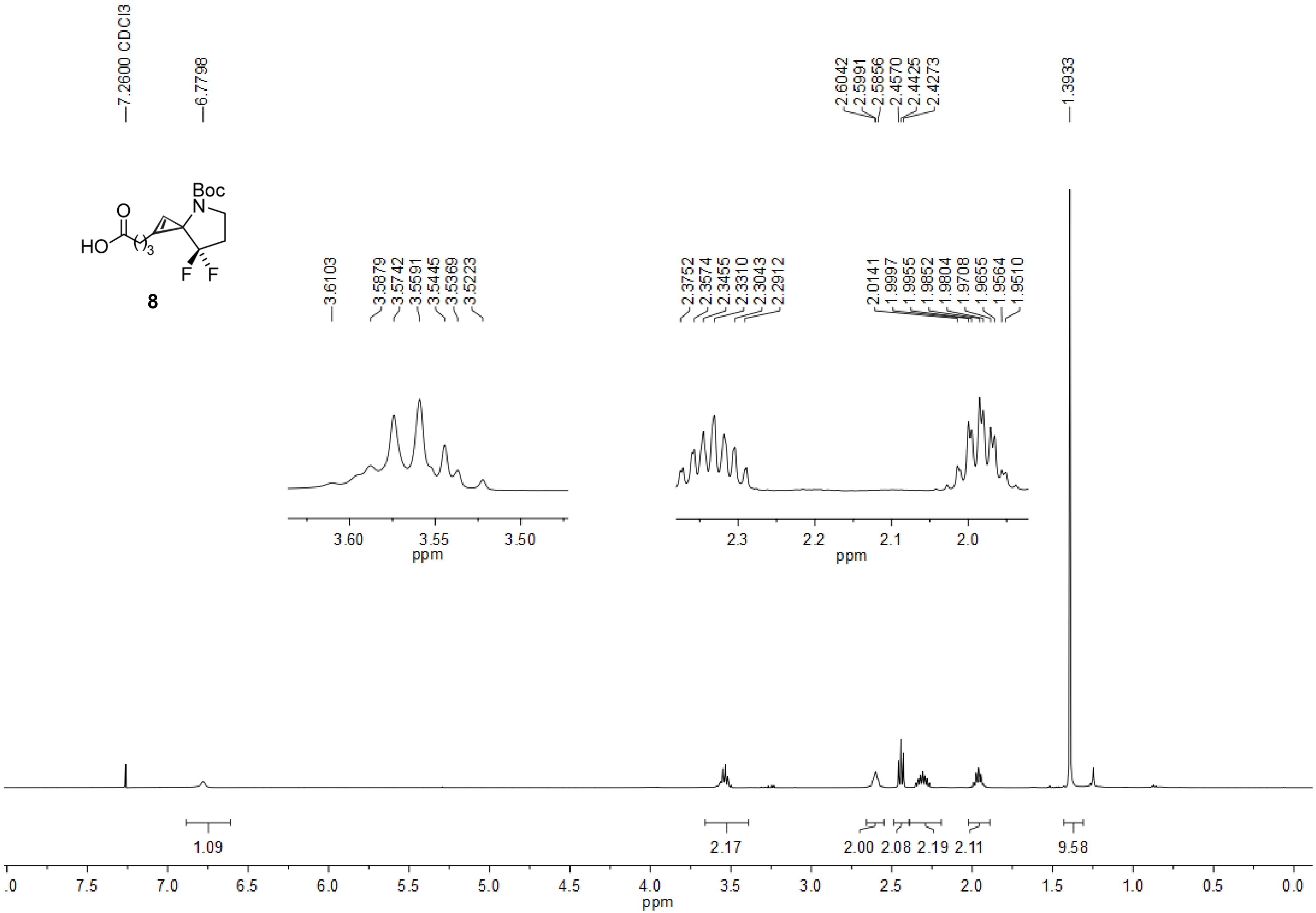
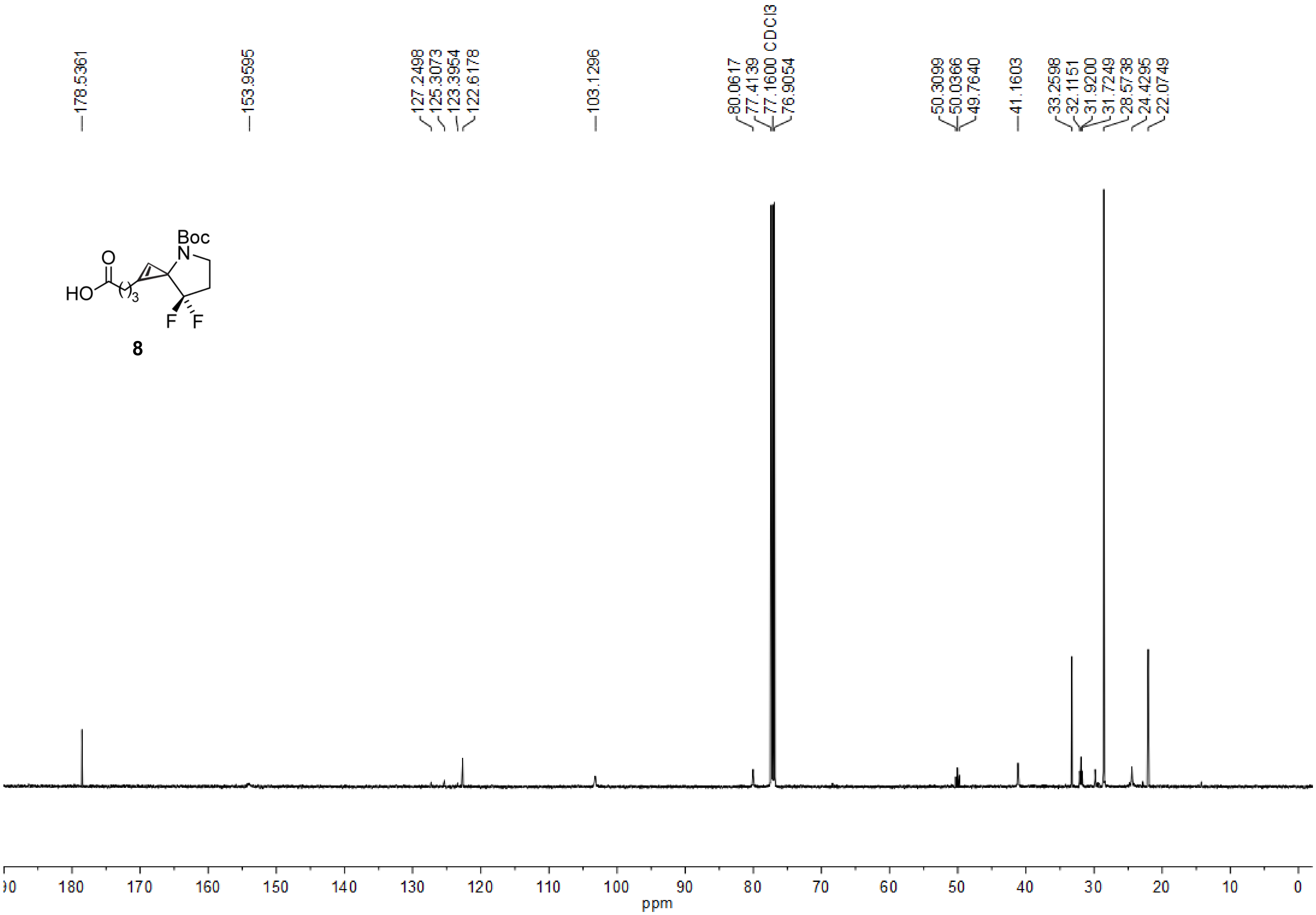
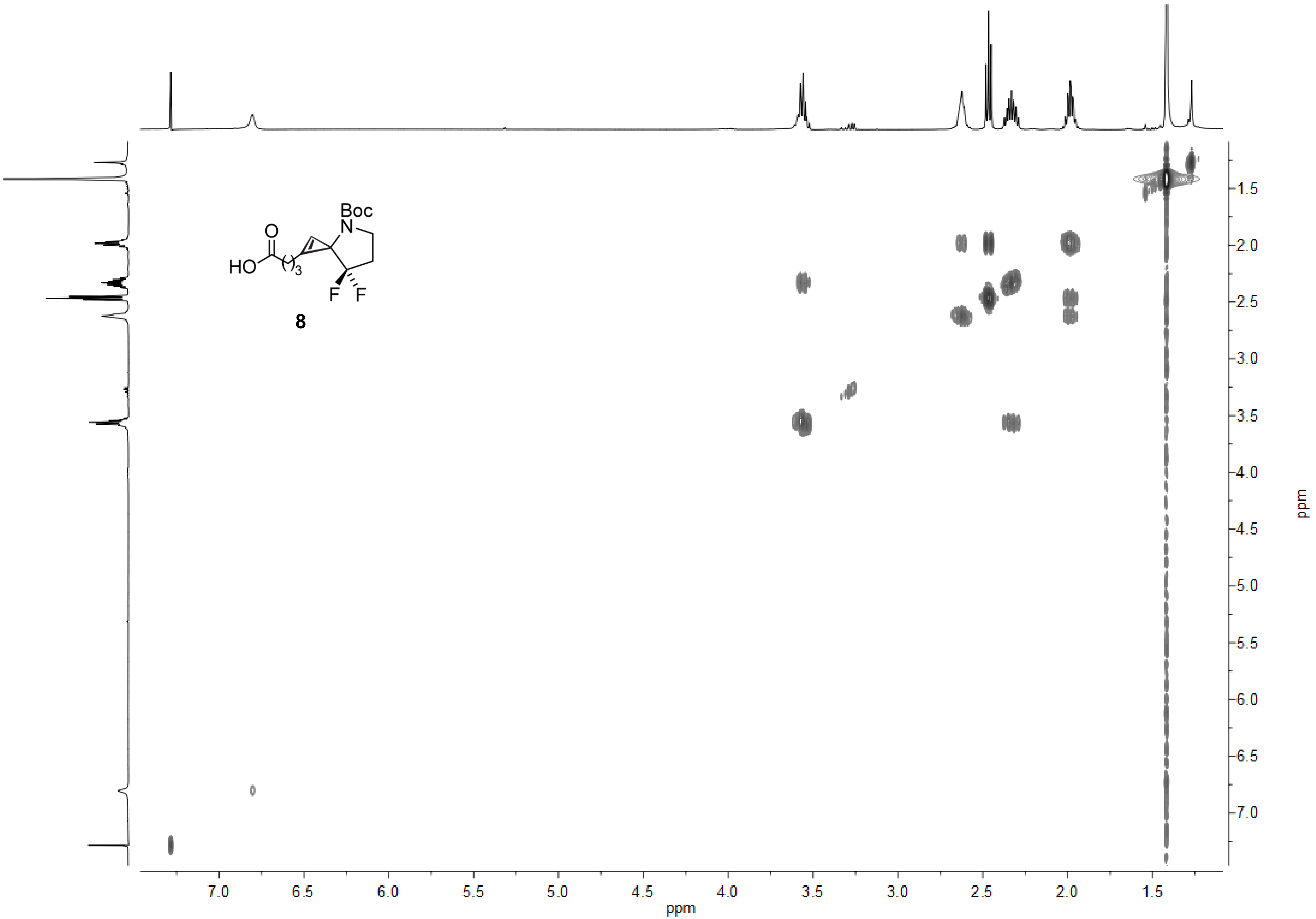
^{1}H NMR (500 MHz)



^{13}C NMR (126 MHz)



COSY (500 MHz)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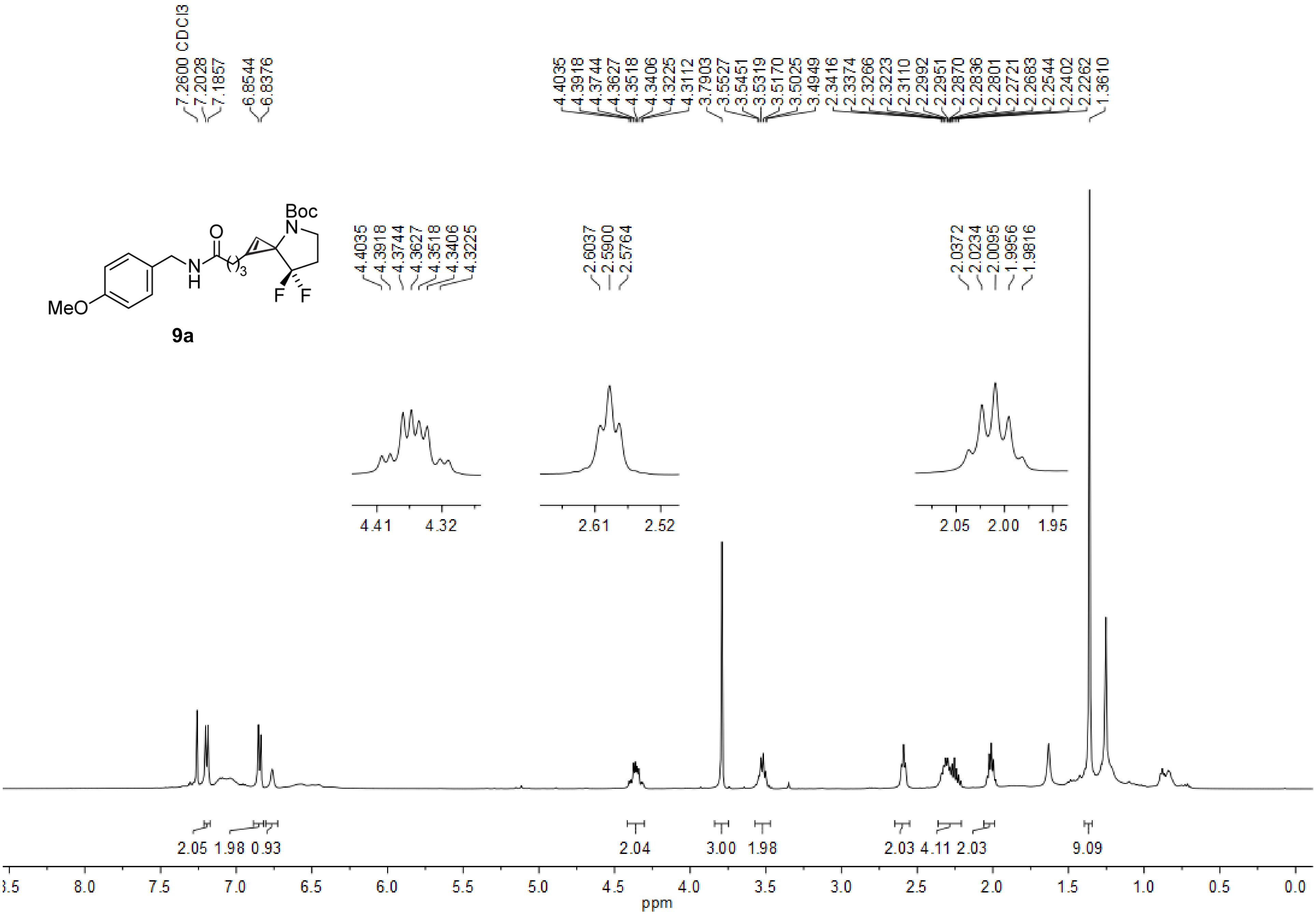
ppm

^{1}H NMR (500 MHz)



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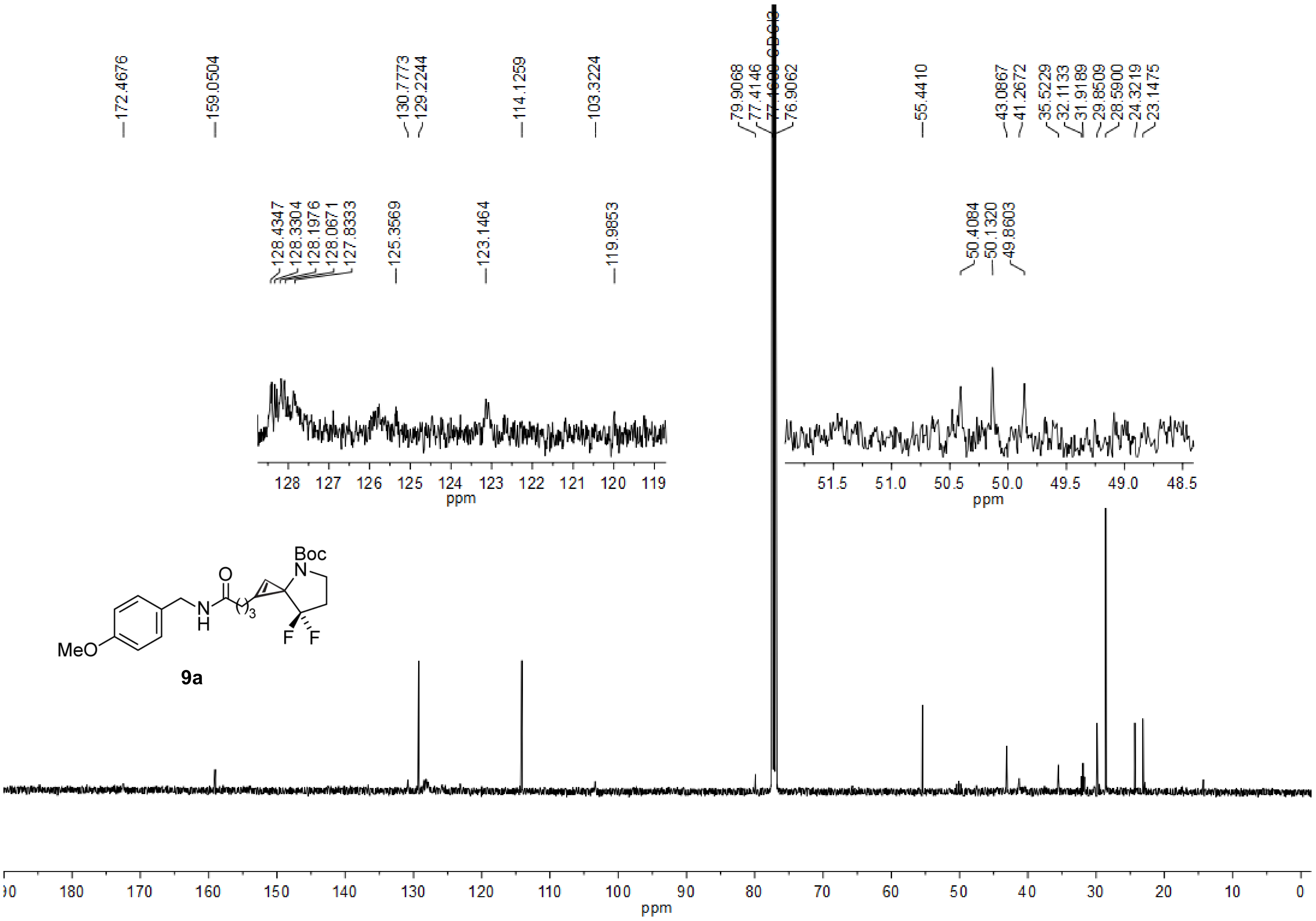
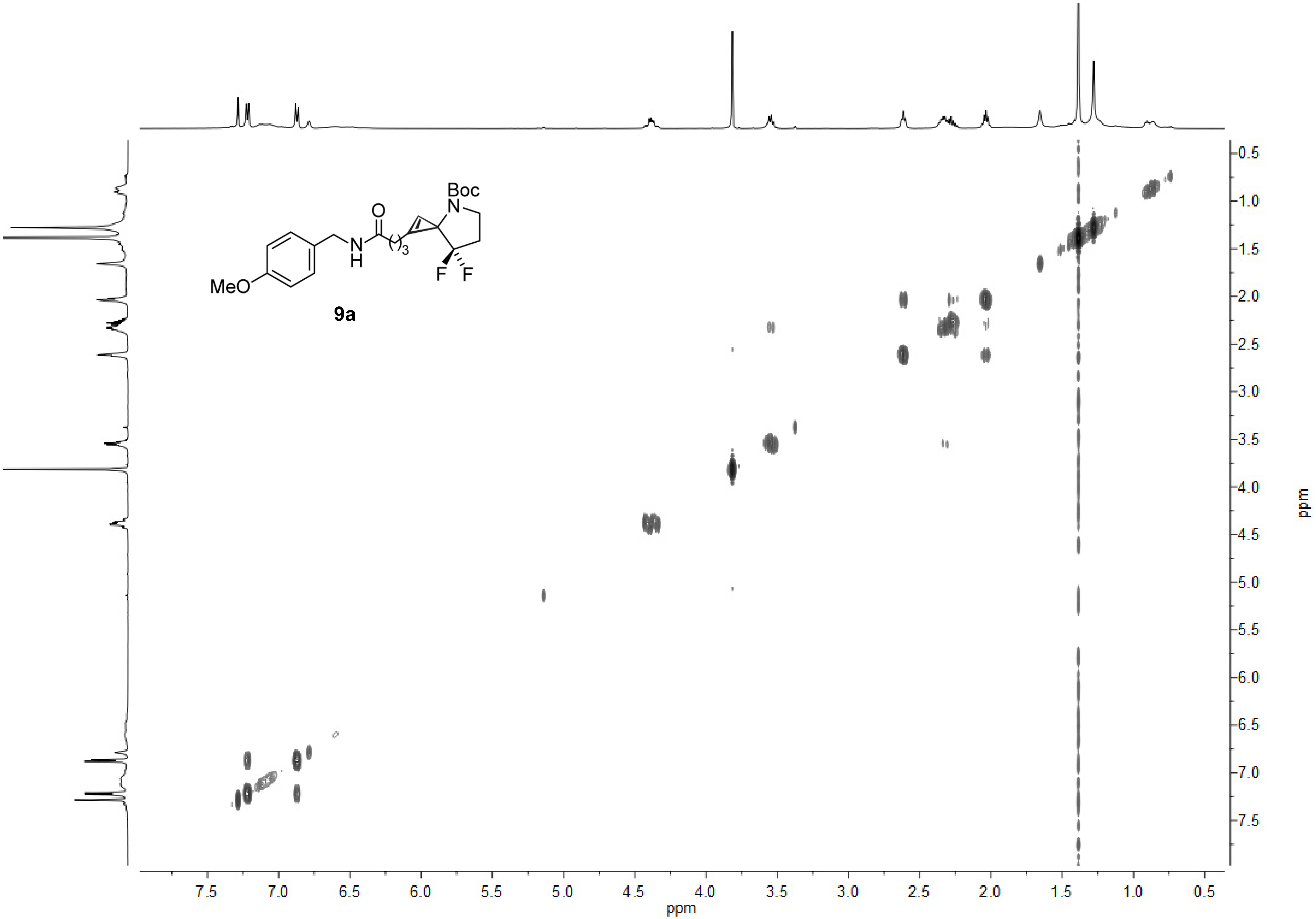
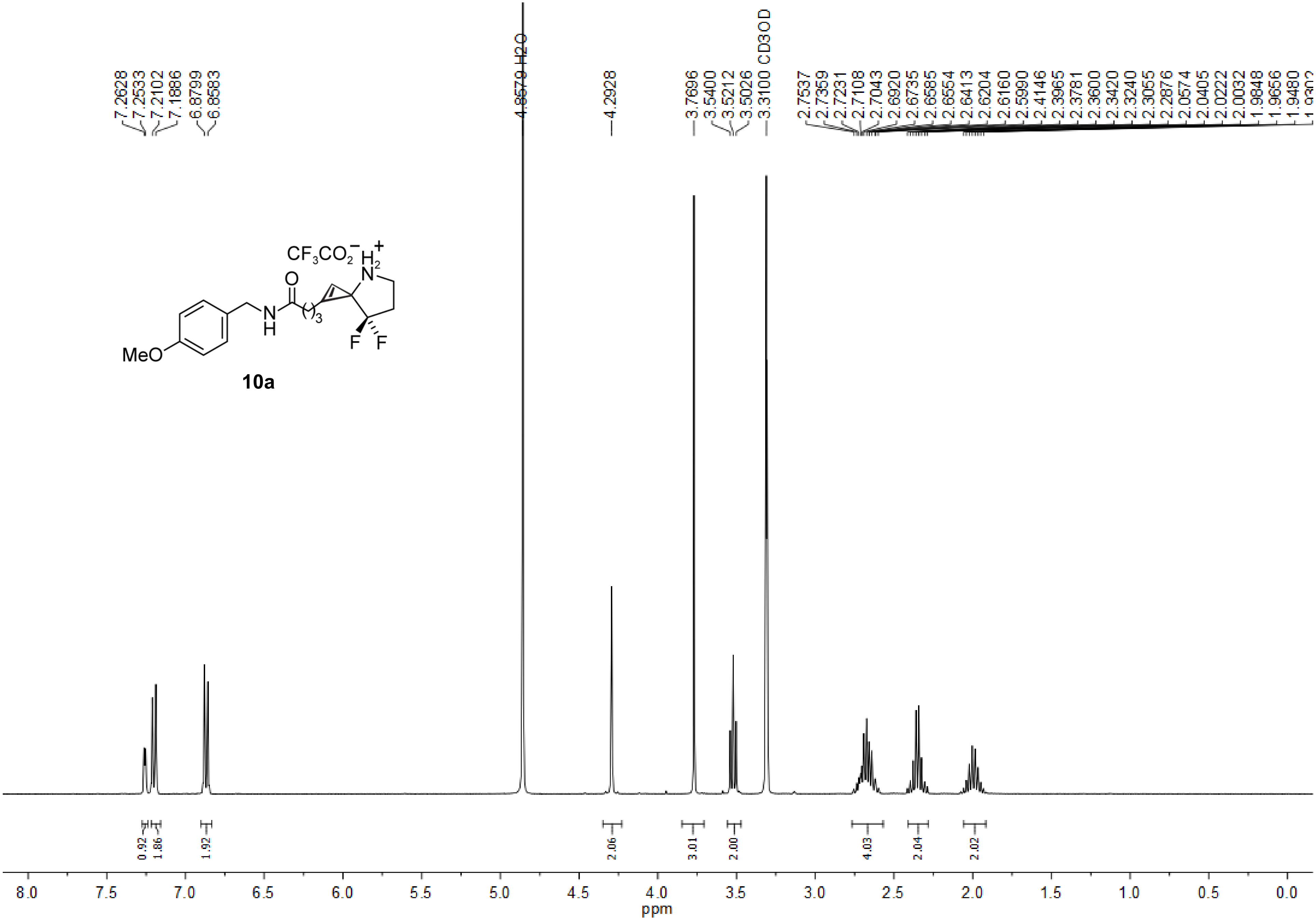


9a

COSY NMR (500 MHz)

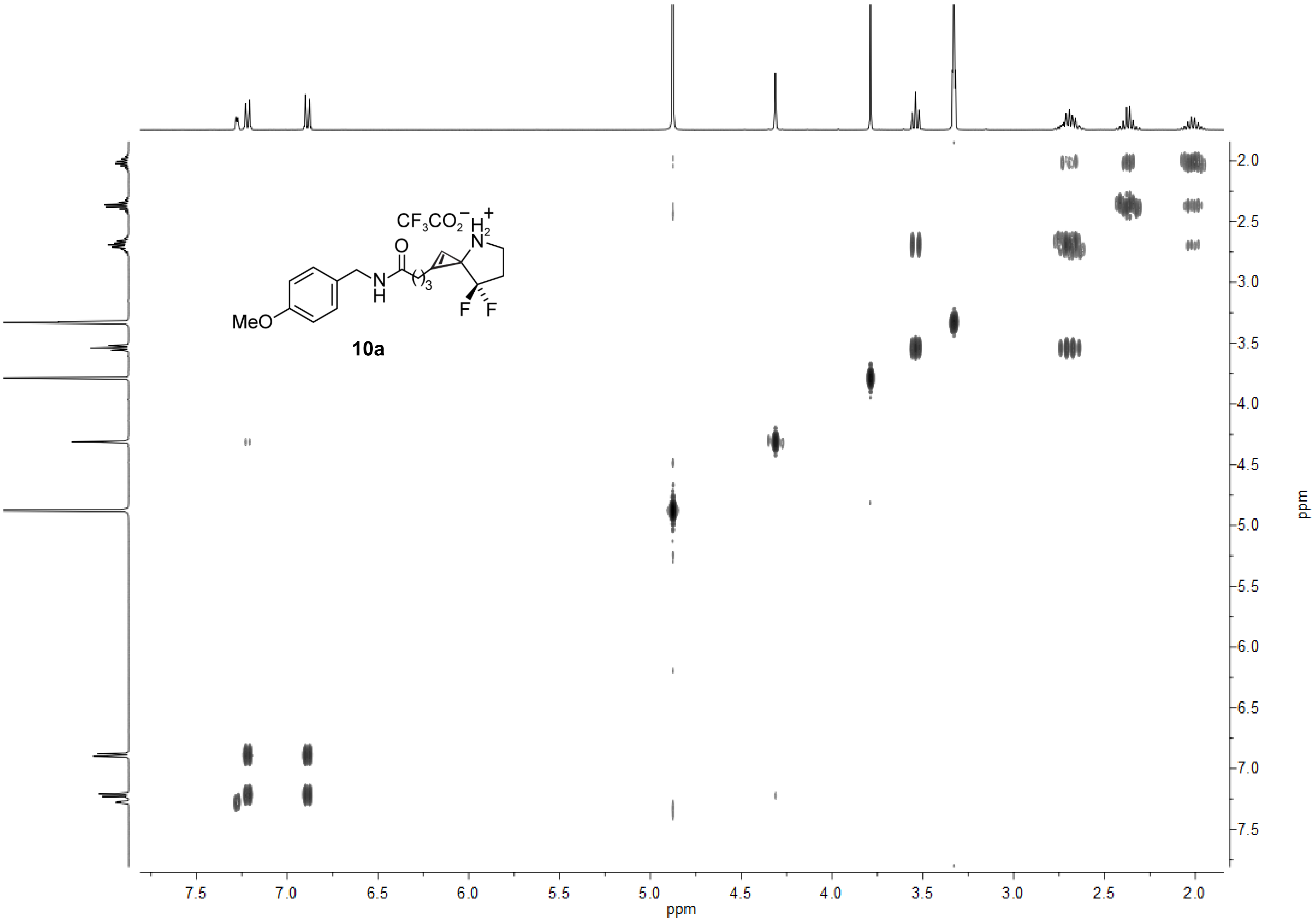


^1H NMR (400 MHz)



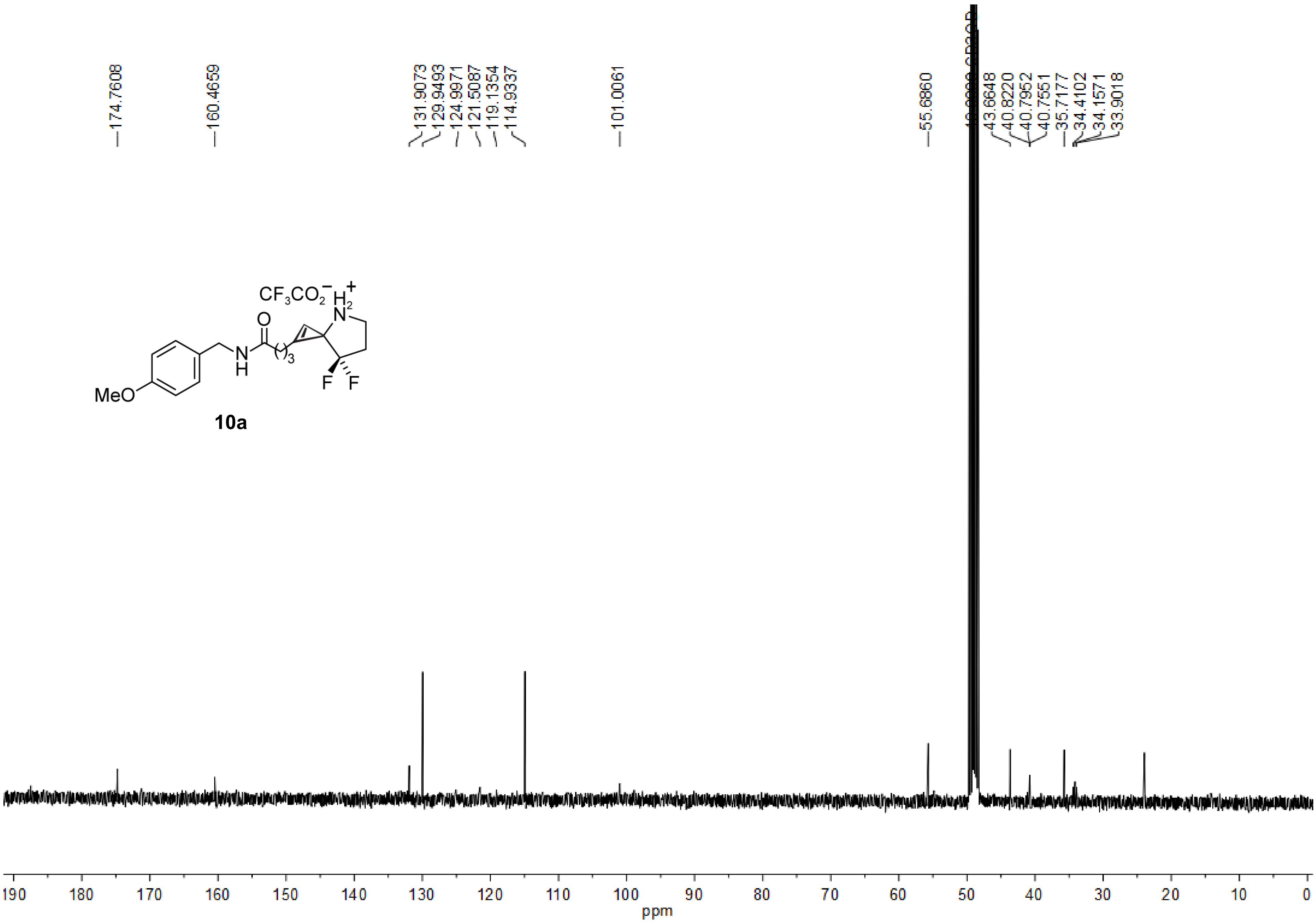
10a

COSY NMR (400 MHz)

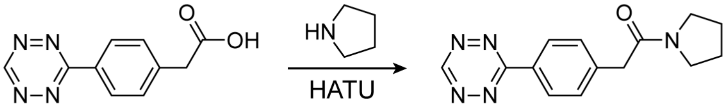


10a

^{13}C NMR (101 MHz)



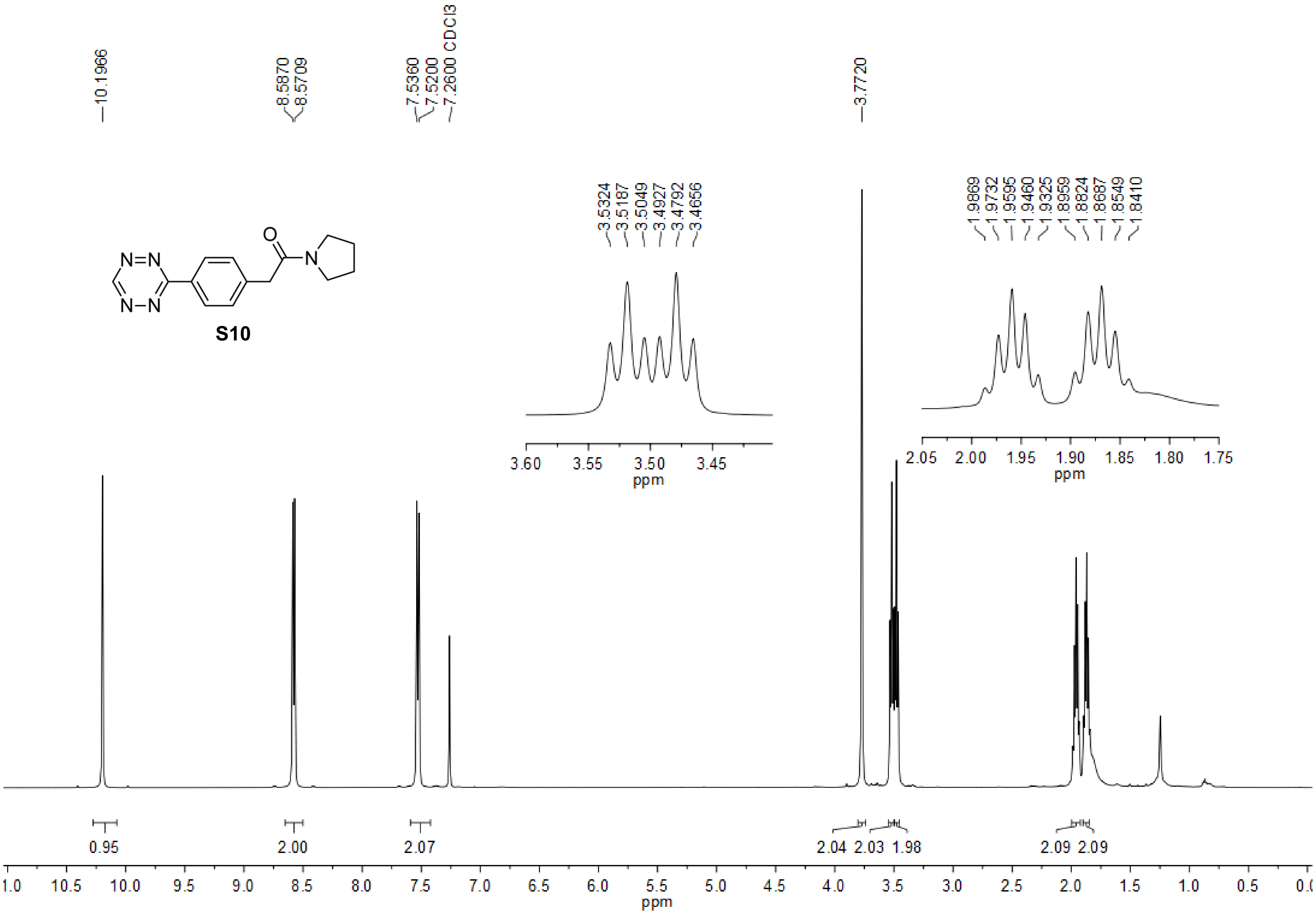
Scheme 5



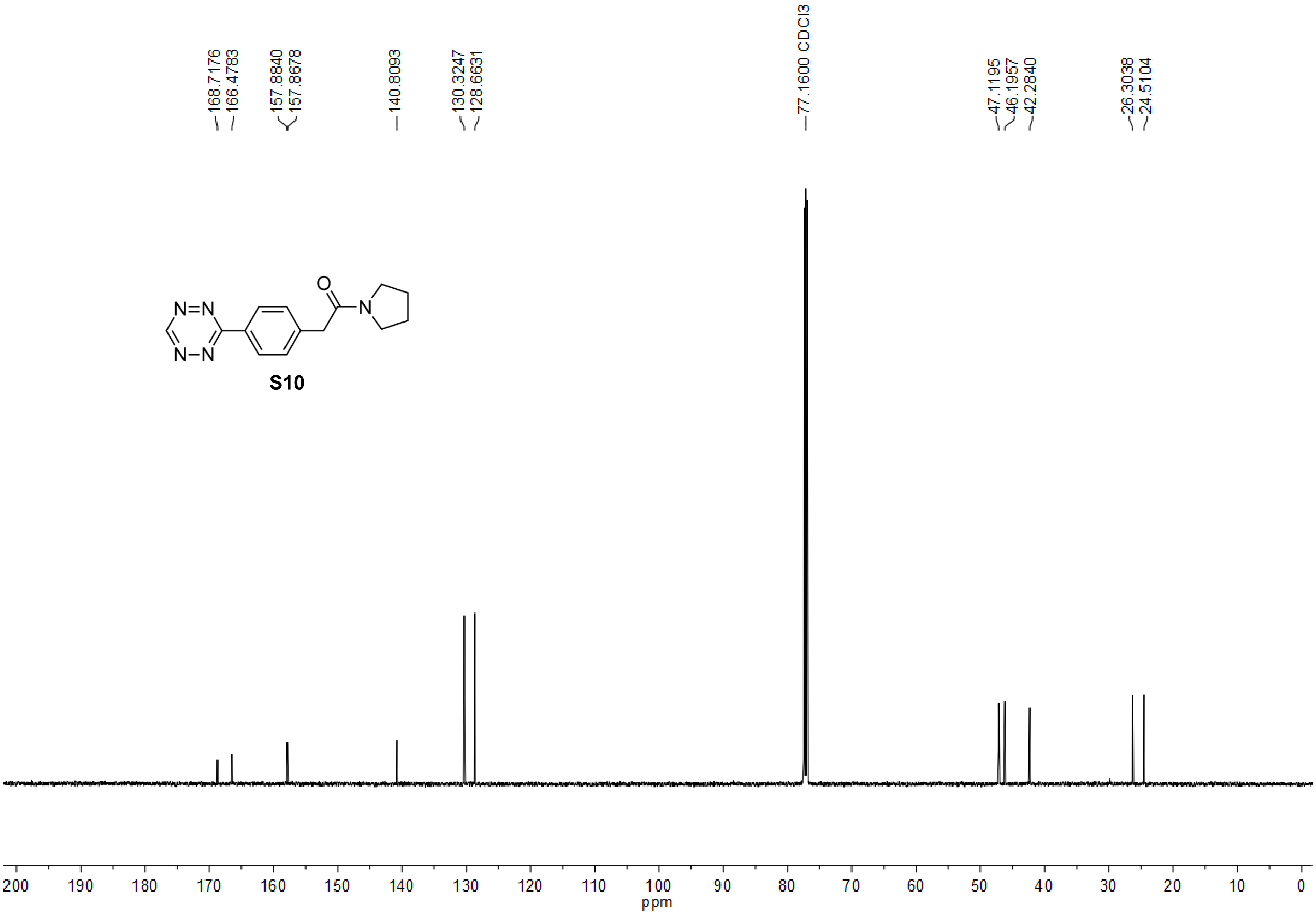
S9 **S10**, 66%

To an ice-cold solution of 1,2,4,5-tetrazine acid **S9**, which was synthesized as previously described[5], (60 mg, 0.28 mmol, 1.0 eq) in dry DMF (3.0 mL) under N_2 atmosphere was added 2,6-Lutidine (65 µL, 59.5 mg, 0.56 mmol, 2.0 eq). HATU (211.1 mg, 0.56 mmol, 2.0 eq) was added and the reaction was stirred at the same temperature for 0.5 h. To this mixture was dropwise added pyrrolidine (53 µL, 45.4 mg, 0.64 mmol, 2.3 eq). The resultant yellowish mixture was allowed to gradually warm to rt overnight. It was then quenched with saturated $NaHCO_3$ and extracted with DCM and water. The combined organic layer was dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by flash chromatography (10.5 g silica, 1% MeOH/DCM (v/v)) followed by HPLC (R_t = 16.8 min, 35–100% MeOH in 35 mins, flow rate = 20 mL/min) to obtain **S10** (64 mg, 85%). ^{1}H NMR (500 MHz, $CDCl_3$): δ= 10.20 (s, 1H), 8.58 (d, J = 8.0 Hz, 2H), 7.53 (d, J = 8.0 Hz, 2H), 3.77 (s, 2H), 3.52 (t, J = 6.9 Hz, 2H), 3.48 (t, J = 6.8 Hz, 2H), 1.96 (p, J = 6.8 Hz, 2H), 1.87 (p, J = 6.8 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$): δ= 168.72, 166.48, 157.88, 140.81, 130.32, 128.66, 47.12, 46.20, 42.28, 26.30, 24.51. HRMS (ESI): Calcd for $C_{14}H_{15}N_5O$ $[M+H]^+$: 270.1349, found: 270.1352.

^{1}H NMR (500 MHz)

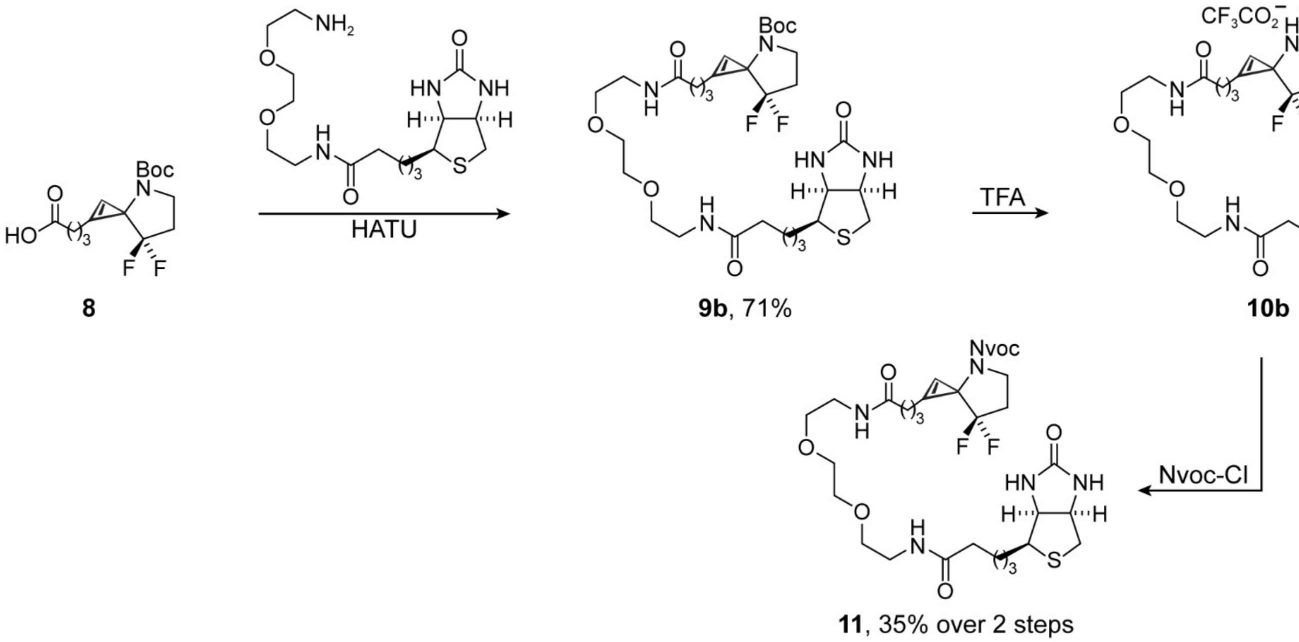


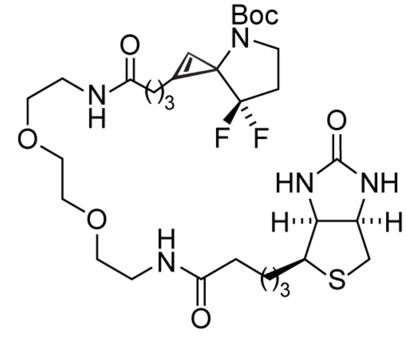
^{13}C NMR (126 MHz)



S10

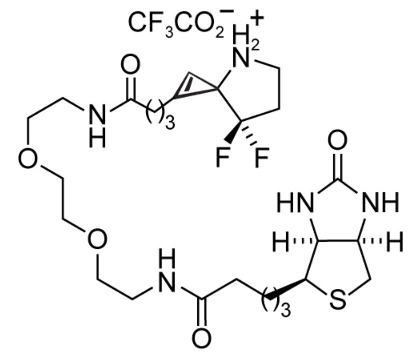
Scheme 6





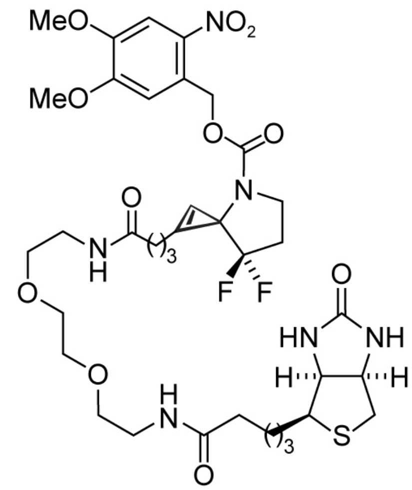
9b

To an ice-cold solution of **8** (8 mg, 0.025 mmol, 1.0 eq) in dry DMF (1.0 mL) under an inert atmosphere was added DIPEA (3.9 mg, 5.3 µL, 0.030 mmol, 1.2 eq). Further, HATU (11.52 mg, 0.030 mmol, 1.2 eq) as a solution in dry DMF (0.3 mL) was added dropwise and the reaction was continued stirring at the same temperature for 30 mins. PEG$_2$-aminoethyl ether biotin (10.4 mg, 0.028 mmol, 1.1 eq) as a solution in dry DMF (0.3 mL) was added to this activated acid and the resulting yellowish mixture was allowed to gradually warm to rt overnight. The reaction mixture was extracted using DCM and water. The combined organic layer was dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by flash chromatography (3.5 g silica, 5% MeOH/DCM (v/v) to 100% MeOH) to obtain **9b** (8 mg, 47%). R_f = 0.42 (10% MeOH/DCM, visualized w/ $KMnO_4$). ^{1}H NMR (700 MHz, CD_3OD): δ= 6.93 (d, *J* = 3.6 Hz, 1H), 4.49 (dd, *J* = 7.8, 5.0 Hz, 1H), 4.30 (dd, *J* = 7.9, 4.5 Hz, 1H), 3.62 (s, 4H), 3.56–3.51 (m, 6H), 3.38–3.34 (m, 4H), 3.21 (ddd, *J* = 8.9, 5.8, 4.5 Hz, 1H), 2.93 (dd, *J* = 12.7, 5.0 Hz, 1H), 2.71 (d, *J* = 12.7 Hz, 1H), 2.61–2.52 (m, 2H), 2.35 (dq, *J* = 13.5, 7.0, 6.5 Hz, 2H), 2.31 (t, *J* = 7.5 Hz, 2H), 2.22 (t, *J* = 7.4, 2H), 1.98–1.90 (m, 2H), 1.76–1.57 (m, 4H), 1.45 (q, *J* = 7.6 Hz, 2H), 1.41 (s, 9H). ^{13}C NMR (176 MHz, CD_3OD): δ= 176.15, 175.50, 166.10, 148.54, 126.46, 124.10, 105.91, 103.76, 71.31, 71.29, 70.63, 70.62, 63.36, 61.62, 57.01, 42.19, 41.05, 40.31, 40.28, 36.74, 36.25, 32.46, 28.76, 26.85, 25.42, 24.29. MS (ESI): Calcd for $C_{31}H_{49}F_2N_5O_7S$ $[M+H]^+$: 674.3, found: 674.3.



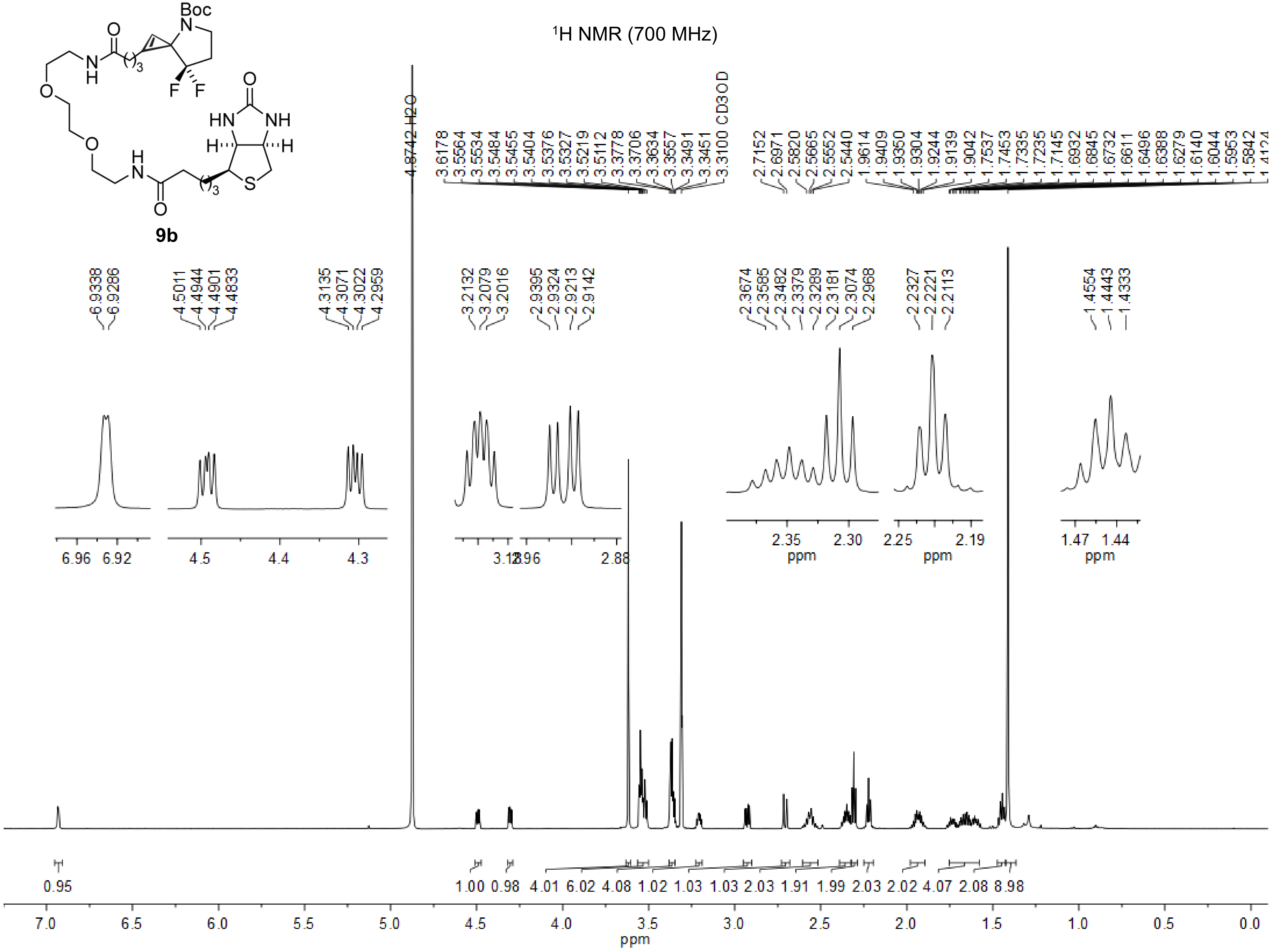
10b

To an ice-cold solution of **9b** (7 mg, 0.010 mmol) in DCM (1.5 mL) was added TFA (0.3 mL) and the reaction was allowed to gradually warm to rt over 2 h. The reaction was concentrated *in vacuo* to obtain crude **10b** as a TFA salt that was used in the next step without further purification. An analytically pure sample was obtained by re-suspending the reaction mixture in water (0.3 mL) and purifying it by HPLC (R_t = 30.9 mins, 0–50% MeOH in 20 mins, 50 – 100% from 20 – 25 mins, and then 100% MeOH from 25 – 35 mins, flow rate = 1 mL/min). ^{1}H NMR (500 MHz, CD_3OD): δ= 7.29 (d, *J* = 3.8 Hz, 1H), 4.50 (ddd, *J* = 7.9, 5.0, 1.0 Hz, 1H), 4.31 (ddd, *J* = 7.9, 5.0, 1.0 Hz, 1H), 3.62 (s, 3H), 3.57–3.51 (m, 5H), 3.37 (q, 4H), 3.23–3.19 (m, 1H), 2.93 (dd, *J* = 12.8, 5.0 Hz, 1H), 2.77–2.61 (m, 5H), 2.22 (t, *J* = 7.4 Hz, 2H), 2.06–1.92 (m, 3H), 1.78–1.56 (m, 5H), 1.48–1.42 (m, 3H). HRMS (ESI): Calcd for $C_{26}H_{41}F_2N_5O_5S$ [M+H]$^+$: 574.2875, found: 574.2875.



11

To an ice-cold suspension of crude **10b** in MeCN (3.0 mL) and water (2.0 mL) was added solid $NaHCO_3$ (pH ~ 8.5). To this reaction mixture was added Nvoc-Cl (3.44 mg, 0.0125 mmol, 1.2 eq) and the reaction was allowed to warm up to rt overnight under dark. The reaction was extracted using DCM and water. The combined organic layer was dried over anhydrous Na_2SO_4, concentrated *in vacuo*, re-suspended in methanol, filtered through a 0.45 μm filter, and purified by HPLC (R_t = 18.5 mins, 50–100% MeOH over 10 mins, and then 100% MeOH from 10–35 mins, flow rate = 1 mL/min) to obtain **11** (2.95 mg, 35% over 2 steps). R_f = 0.31 (10% MeOH/DCM, visualized w/ UV). ^{1}H NMR (700 MHz, CD_3OD): δ= 7.74 (s, 2H), 6.99 (s, 1H), 4.49–4.48 (m, 1H), 4.31–4.29 (dd, *J* = 7.9, 4.5 Hz, 1H), 3.97 (s, 3H), 3.91 (s, 3H), 3.69 (d, *J* = 12.4 Hz, 2H), 3.60 (s, 4H), 3.54–3.52 (q, *J* = 5.5 Hz, 4H), 3.37–3.33 (m, 4H), 3.21–3.18 (m, 1H), 2.93–2.91 (dd, *J* = 12.7, 5.0 Hz, 1H), 2.71–2.69 (d, *J* = 12.7 Hz, 1H), 2.45–2.39 (m, 3H), 2.30–2.20 (m, 4H), 1.76–1.56 (m, 6H), 1.45–1.41 (m, 3H). ^{13}C NMR (176 MHz, CD_3OD): δ= 176.15, 166.10, 109.49, 104.03, 101.19, 71.29, 70.62, 70.60, 63.36, 61.62, 57.59, 57.48, 57.35, 57.00, 56.87, 42.22, 41.05, 40.28, 36.74, 36.13, 29.76, 29.50, 26.84, 24.20, 17.29. Peaks for quaternary carbons were not observed. HRMS (ESI): Calcd for $C_{36}H_{50}F_2N_6O_{11}S$ [M+H]$^+$: 813.3305, found: 813.3309.



9b

Boc
N
O
N
H
3
F
F
O
HN
NH
H
H
O
O
H
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O
3
S
1H NMR (700 MHz)
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3.5376
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3.12
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ppm
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ppm
0.95
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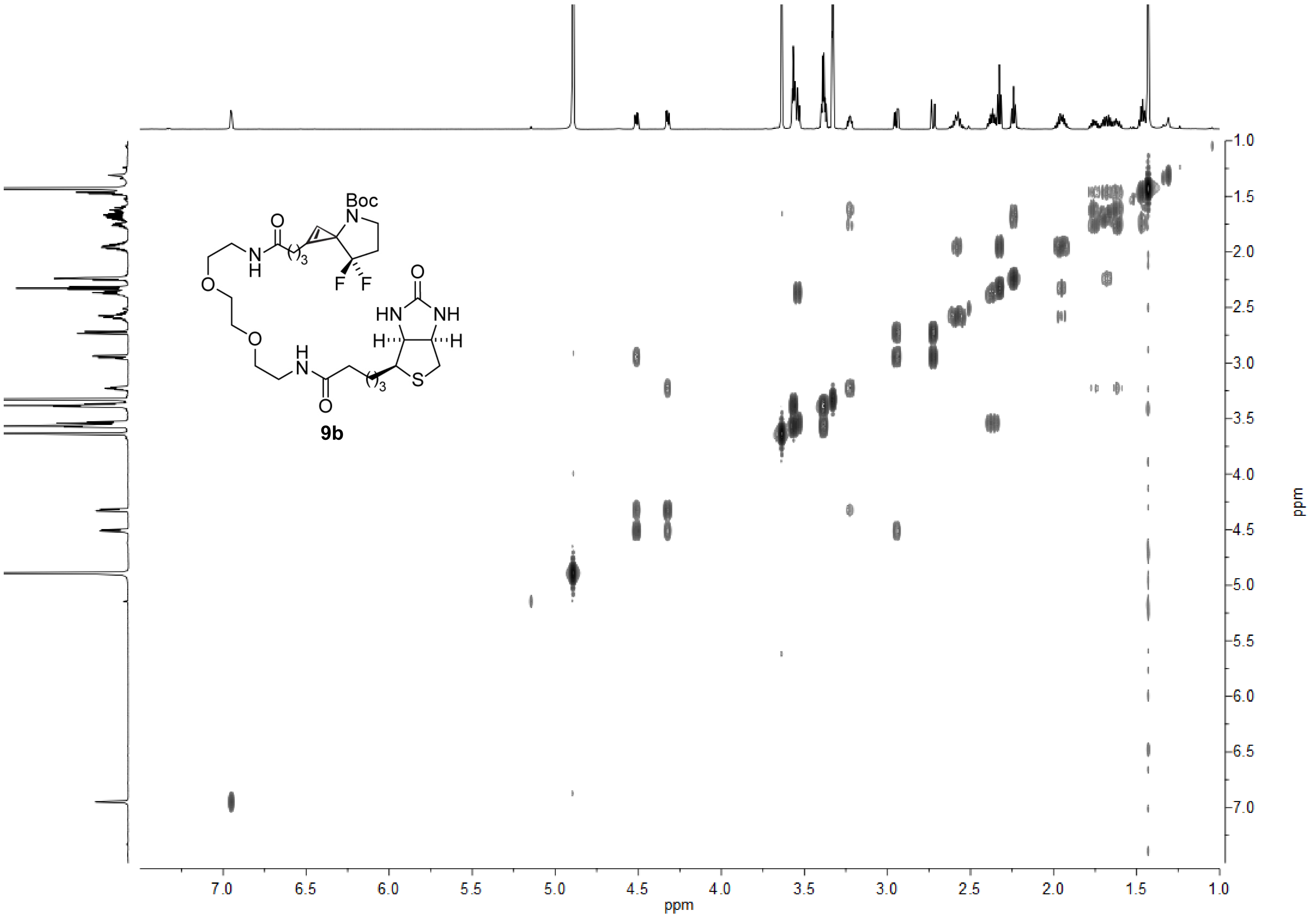
ppm

COSY (700 MHz)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NH
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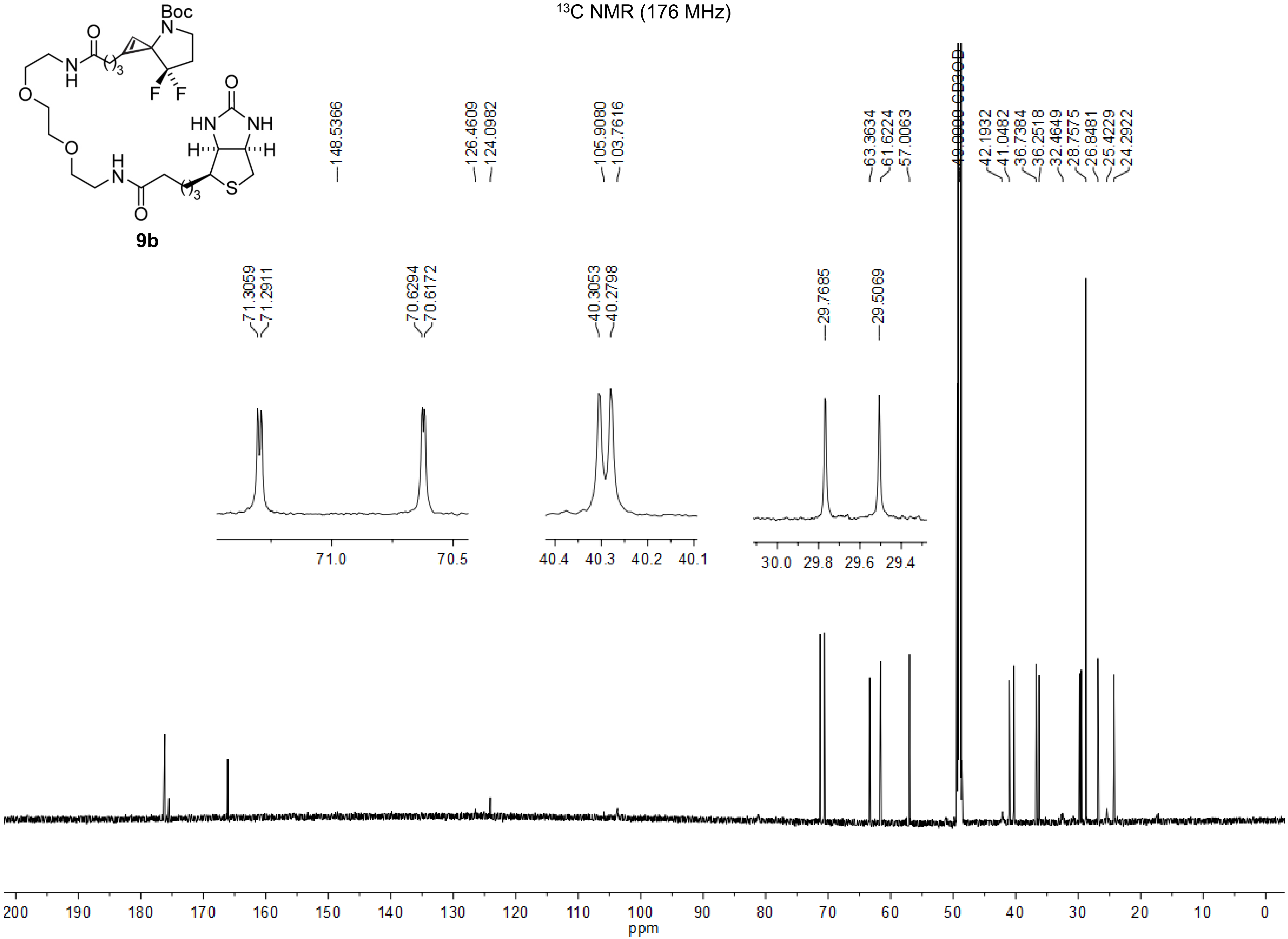
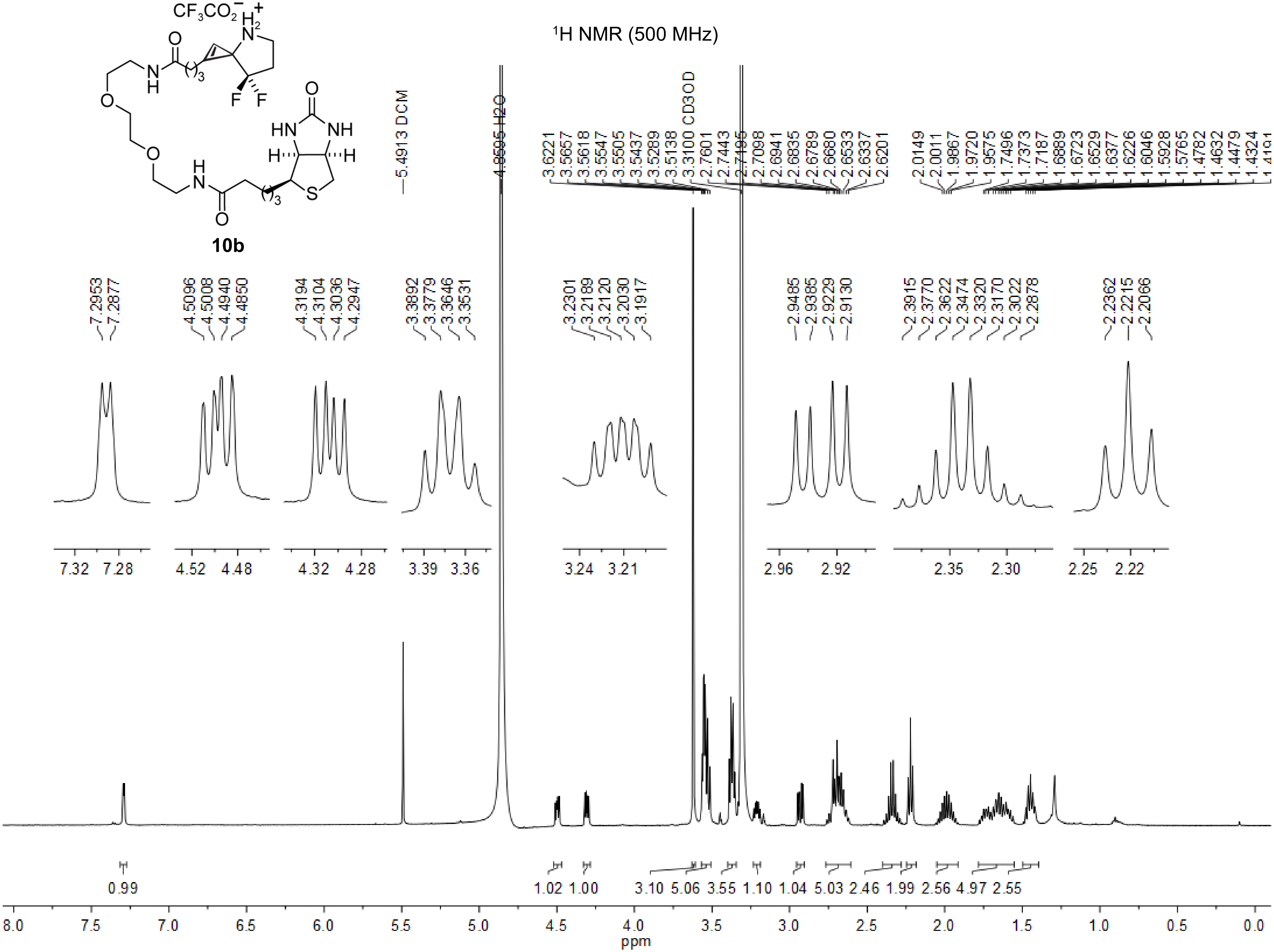
^{13}C NMR (176 MHz)



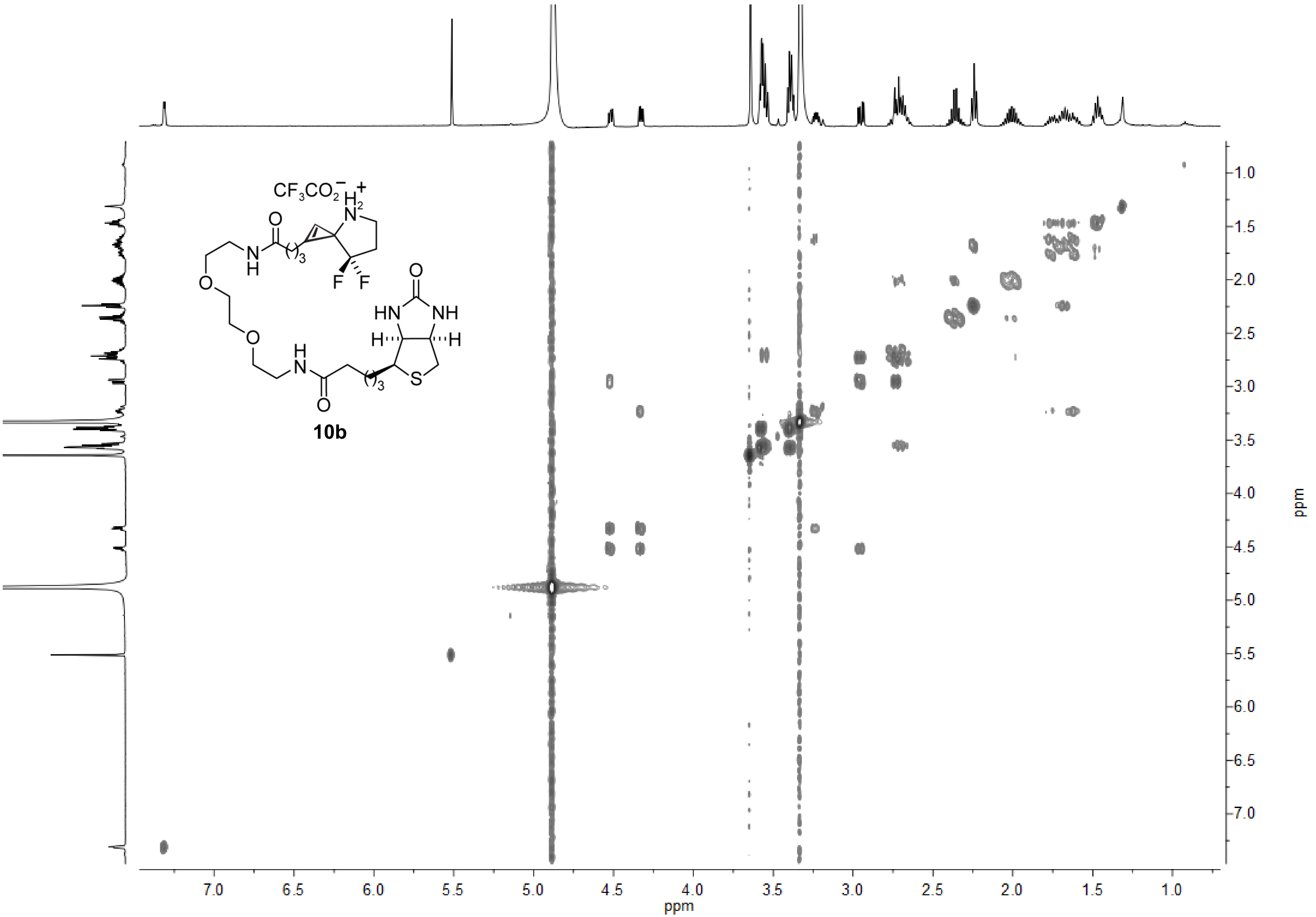
9b



10b

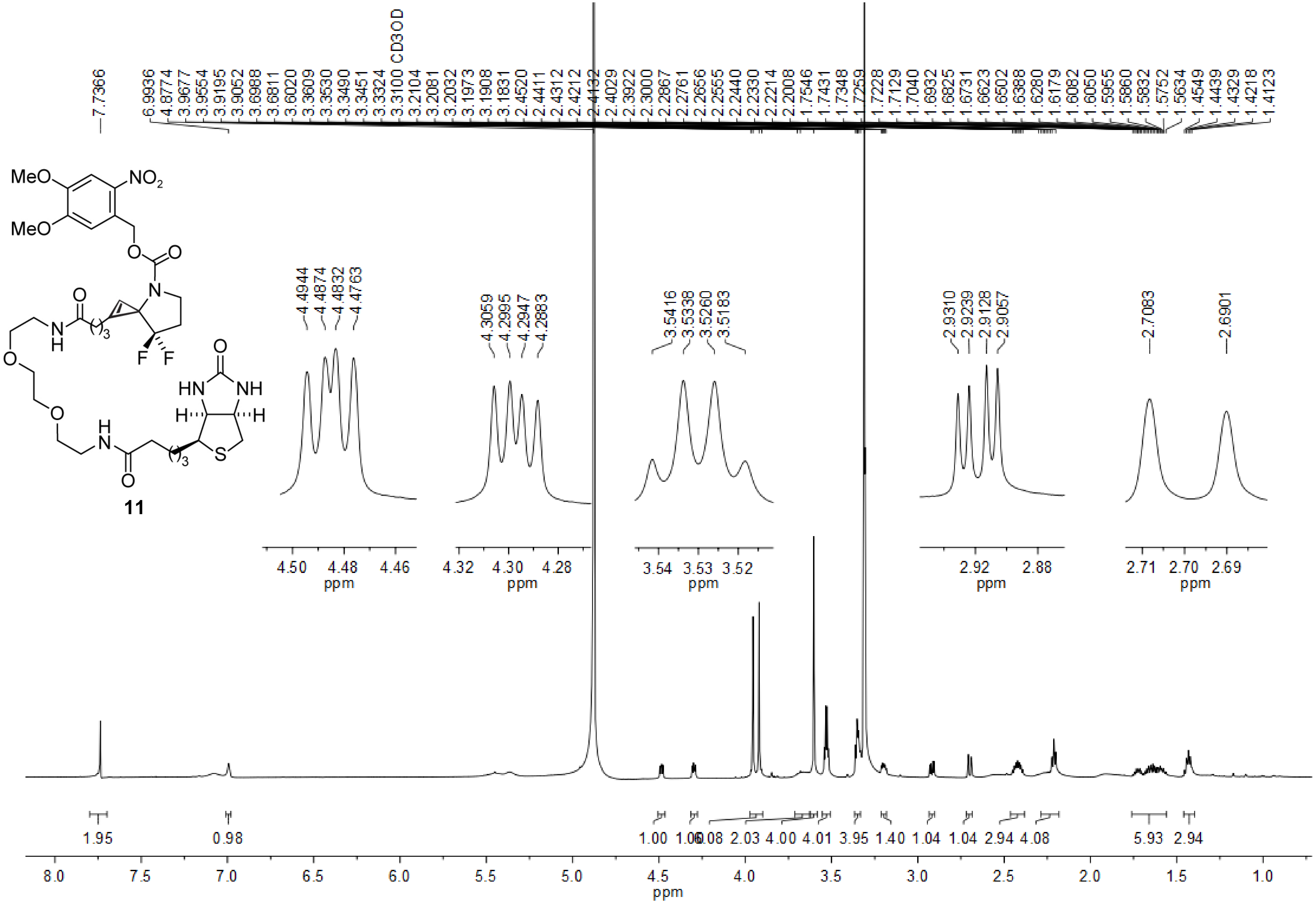
^{1}H NMR (500 MHz)

COSY (500 MHz)



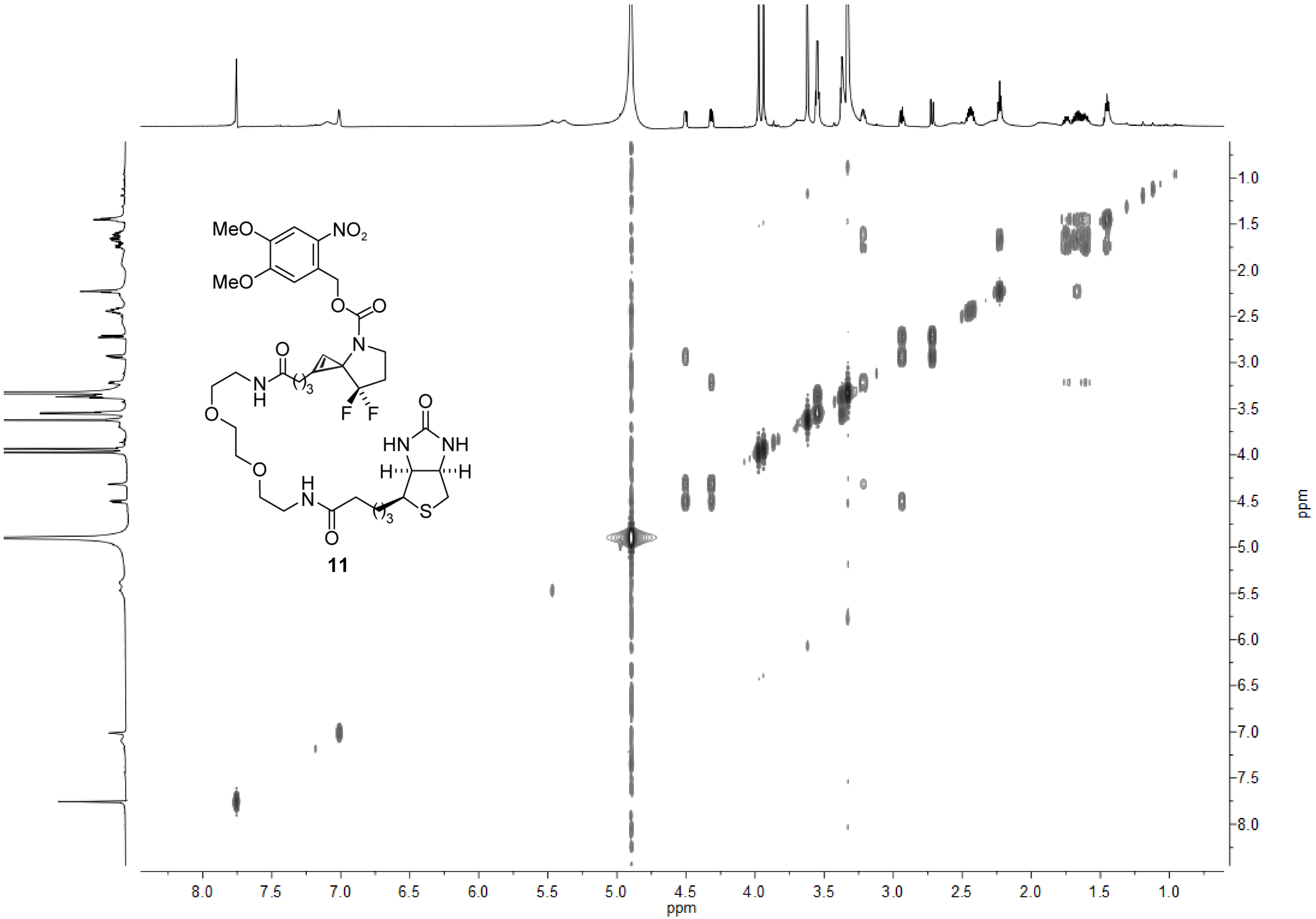
10b

¹H NMR (700 MHz)



11

COSY (700 MHz)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S
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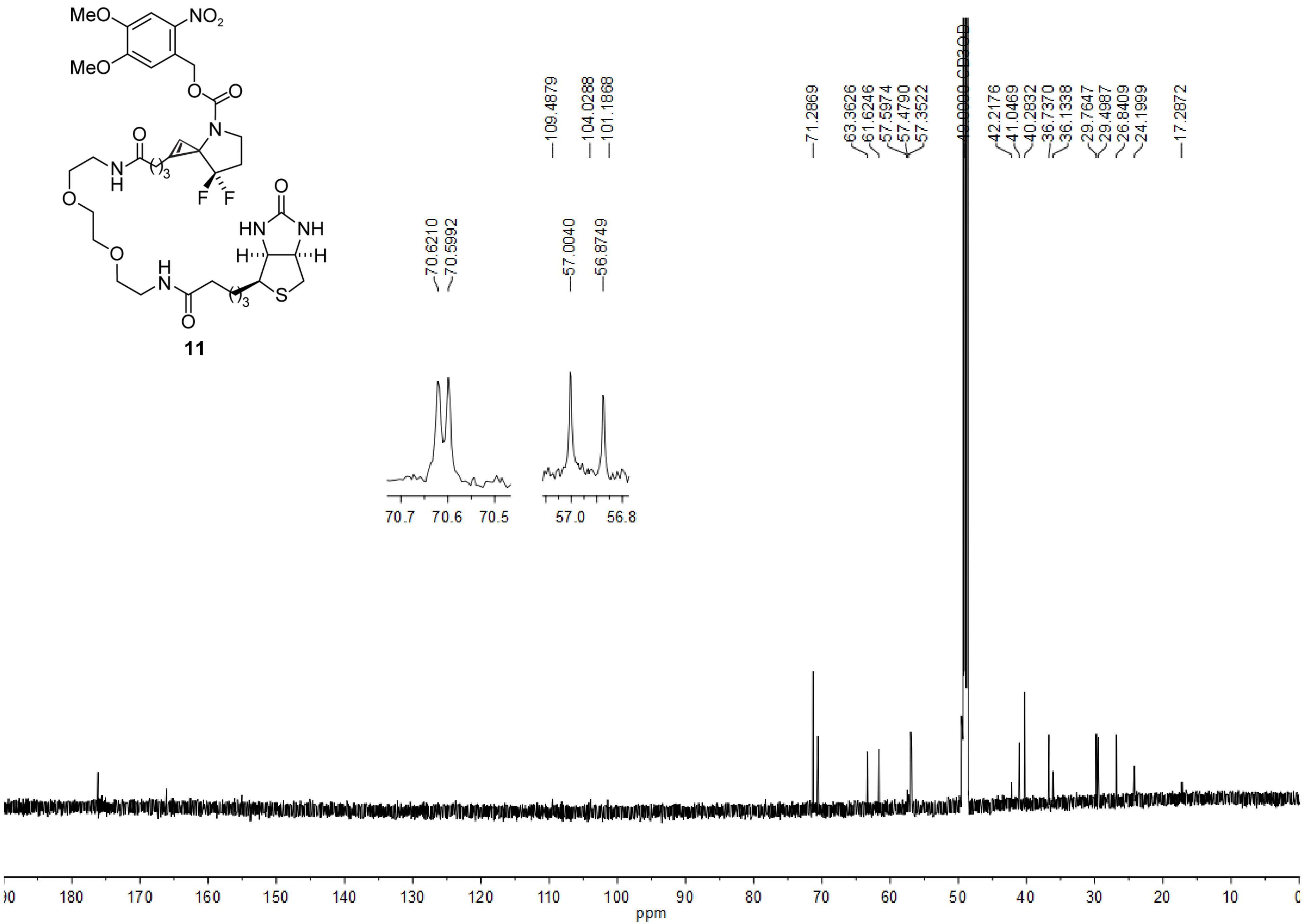
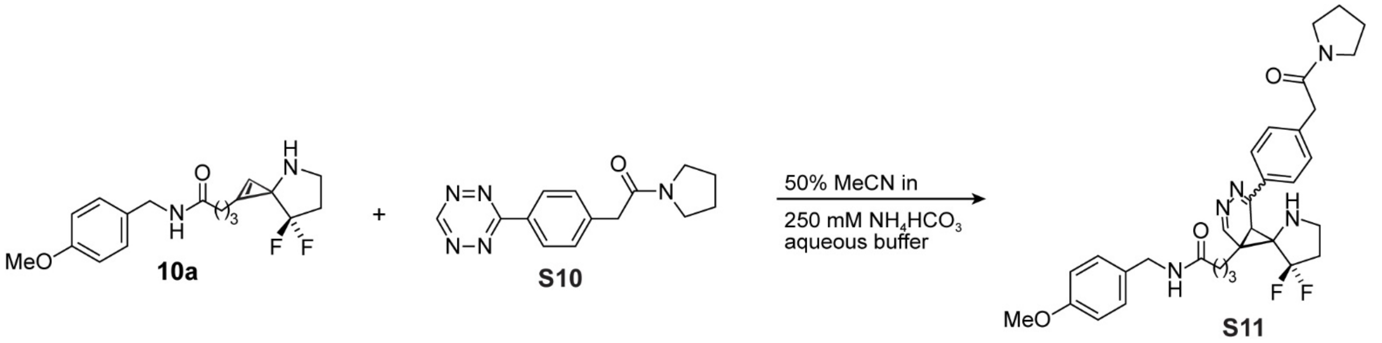
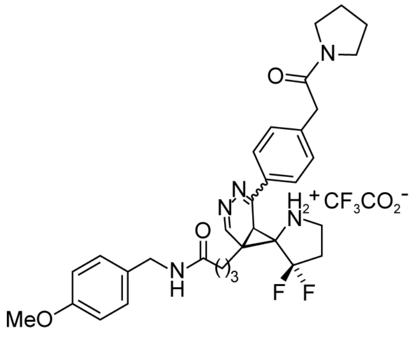
^{13}C NMR (176 MHz)



11

Scheme 7

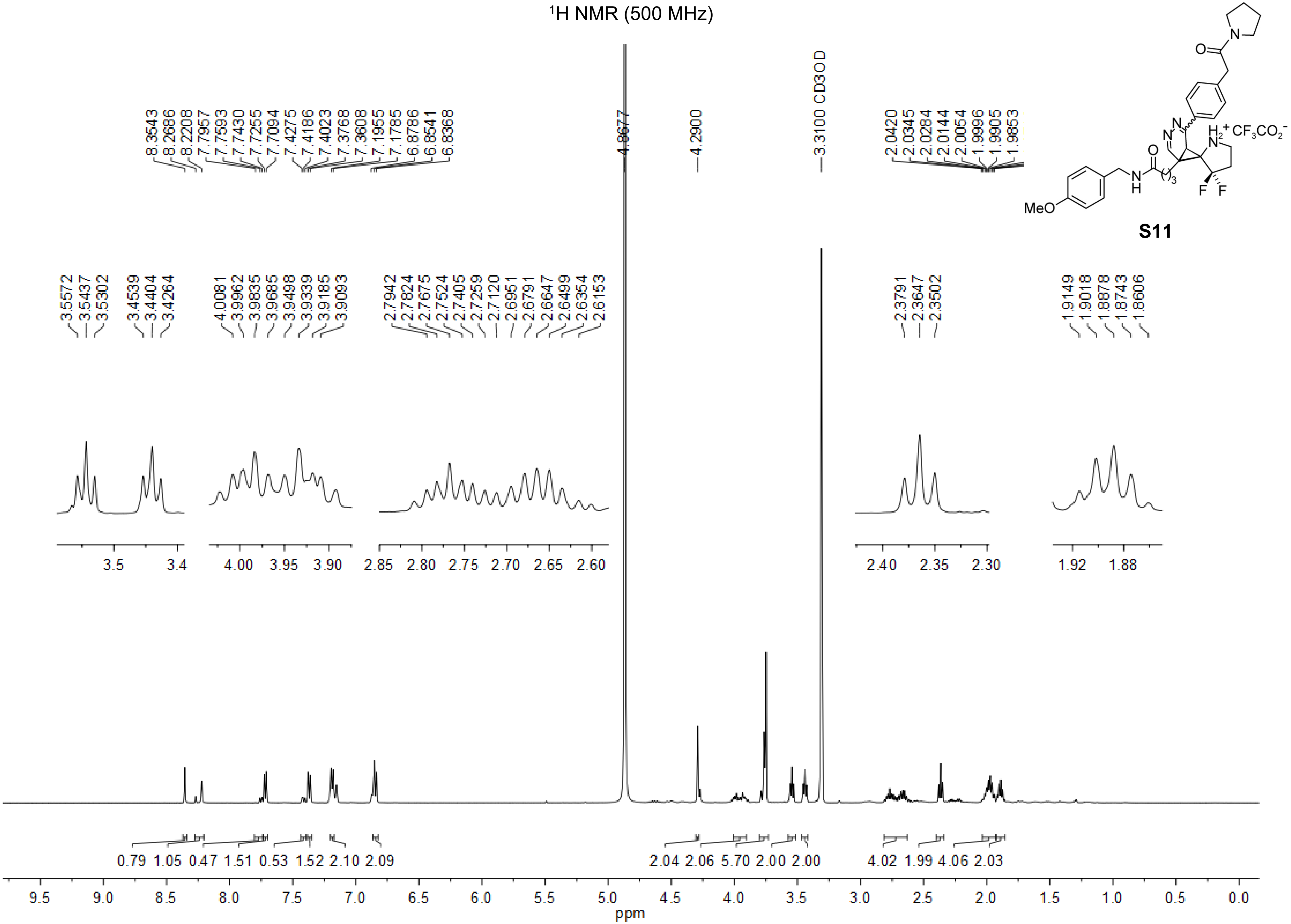




S11

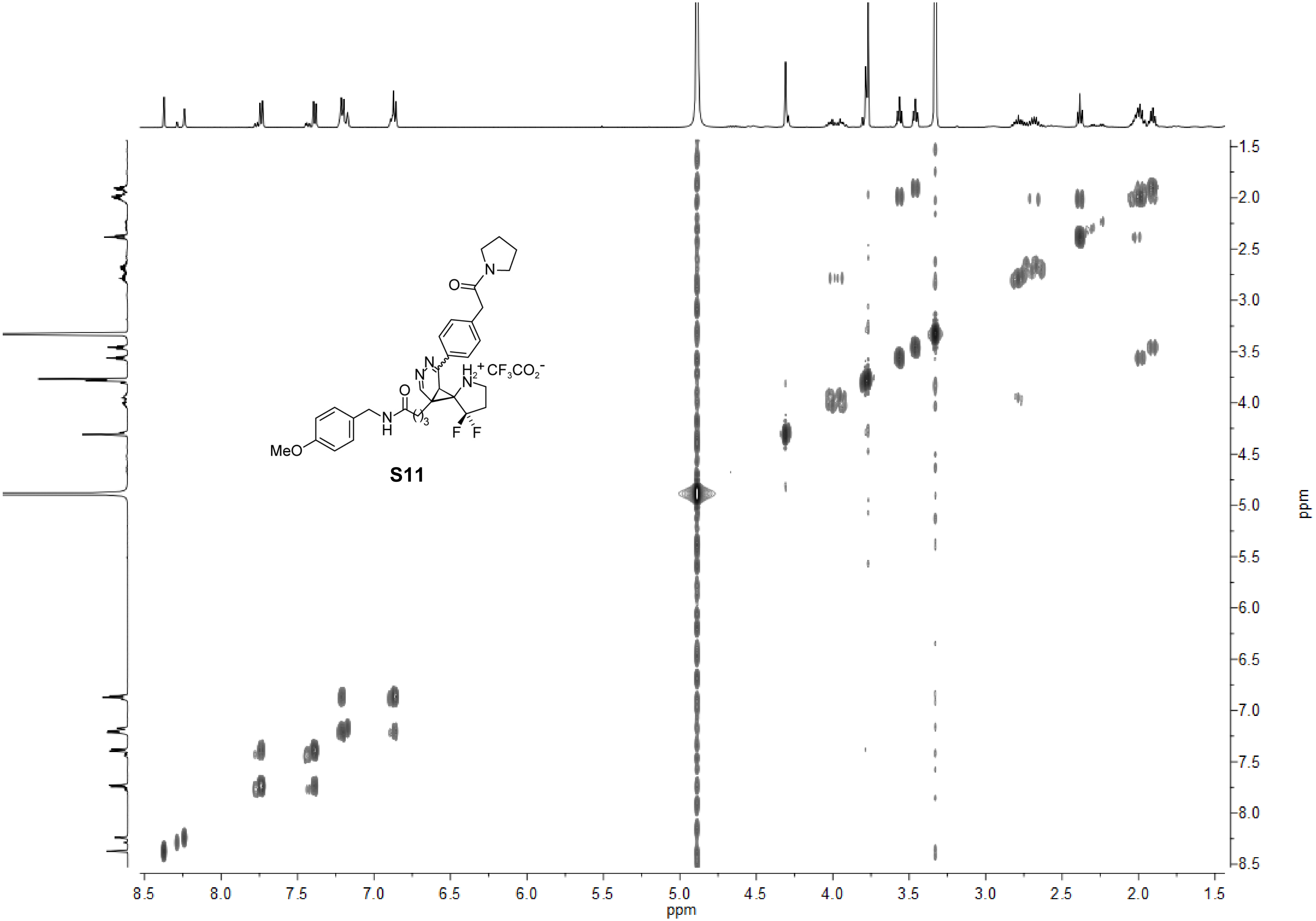
To a solution of **10a** (final concentration = 50 mM) and **S10** (final concentration = 5 mM) in MeCN (100 µL) was added NH_4HCO_3 buffer (100 µL) and the reaction was left standing at rt. The reaction was quenched by adding TFA (10 µL, resulting pH = 1) after 7–8 h. The reaction mixture was concentrated *in vacuo*, re-dissolved in 30–50% MeCN/H_2O and purified by HPLC (R_t = 19.1 min, 30–100% MeCN over 35 mins) to obtain **S11**. Nine reactions, used initially for the determination of the reaction's 2nd order rate constant, were separated by HPLC and the fractions for S11 combined to obtain a sufficient amount of material for NMR characterization. Yield (calculated based on the combined reactions and with S10 as the limiting reagent) = 89%. ^{1}H NMR (500 MHz, CD_3OD): δ= 8.35 (s, 1H), 8.24 (2×s, 1H), 7.81–7.73 (m,0.5H), 7.72 (d, *J* = 8.1 Hz, 1.5H), 7.44–7.39 (m, 0.5H), 7.37 (d, *J* = 8.0 Hz, 1.5H), 7.20–7.18 (m, 2H), 6.86–6.84 (m, 2H), 4.29 (s, 2H), 4.01–3.90 (m, 2H), 3.80–3.73 (m, 6H), 3.54 (t, *J* = 6.7 Hz, 2H), 3.44 (t, *J* = 6.9 Hz, 2H), 2.81–2.61 (m, 4H), 2.36 (t, *J* = 7.2 Hz, 2H), 2.01–1.94 (m, 4H), 1.89 (p, *J* = 6.8 Hz, 2H). ^{13}C NMR (176 MHz, CD_3OD): δ= 174.68, 171.48, 160.43, 154.64, 150.56, 140.58, 132.48, 131.98, 131.06, 130.02, 129.99, 129.94, 129.62, 129.58, 129.54, 129.51, 114.94, 114.92, 55.69, 47.23, 44.24, 43.64, 42.31, 36.26, 35.83, 32.39, 26.97, 25.33, 23.89, 23.51. HRMS (ESI): Calcd for $C_{32}H_{38}F_2N_5O_3$ $[M+H]^+$: 578.2937, found: 578.2925.

^{1}H NMR (500 MHz)



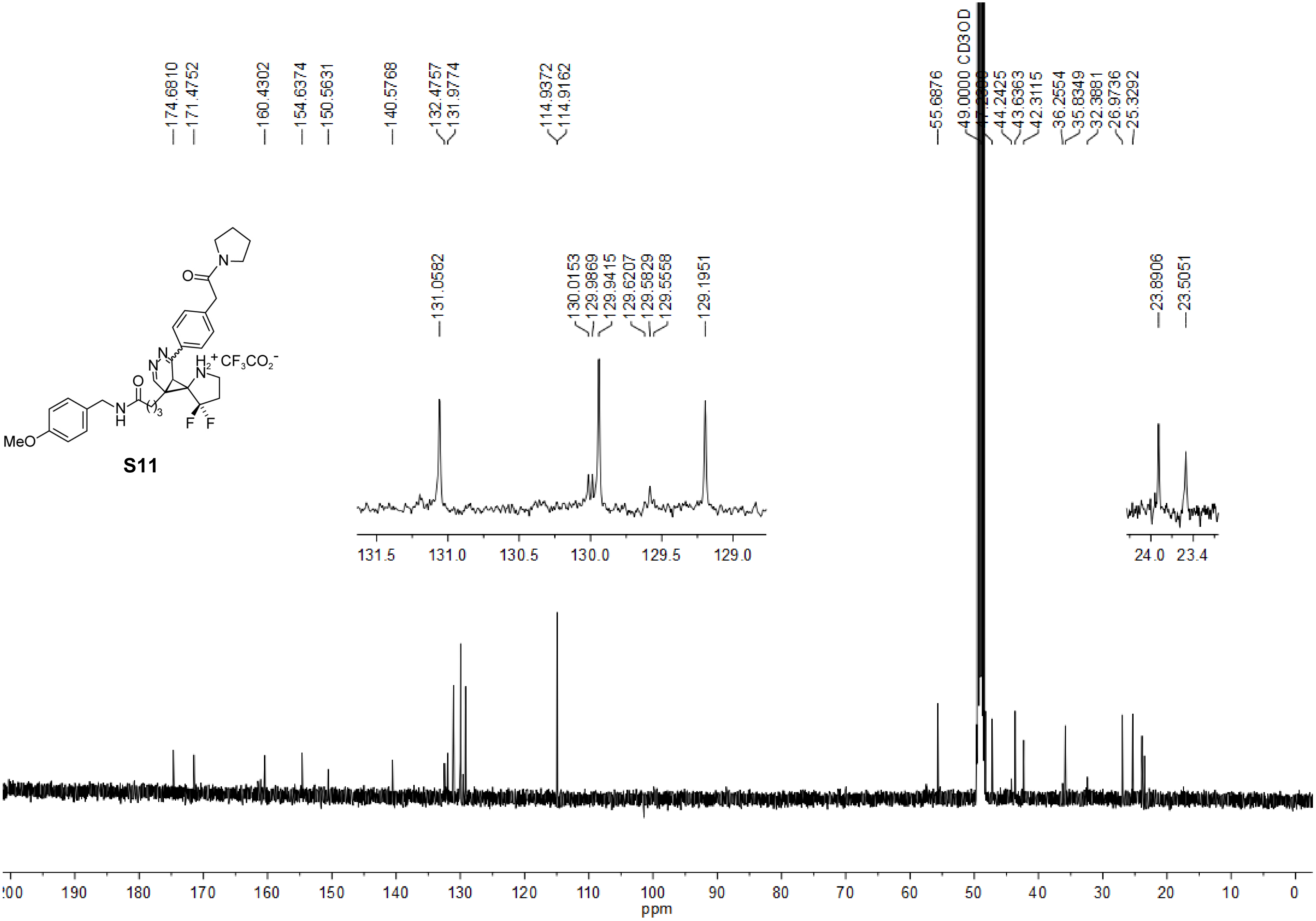
S11

COSY NMR (500 MHz)



S11

^{13}C NMR (126 MHz)



S11

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