Supplementary Materials Part 2 for

Triple, Mutually Orthogonal Bioorthogonal Pairs Through the Design of Electronically Activated Sulfamate-Containing Cycloalkynes

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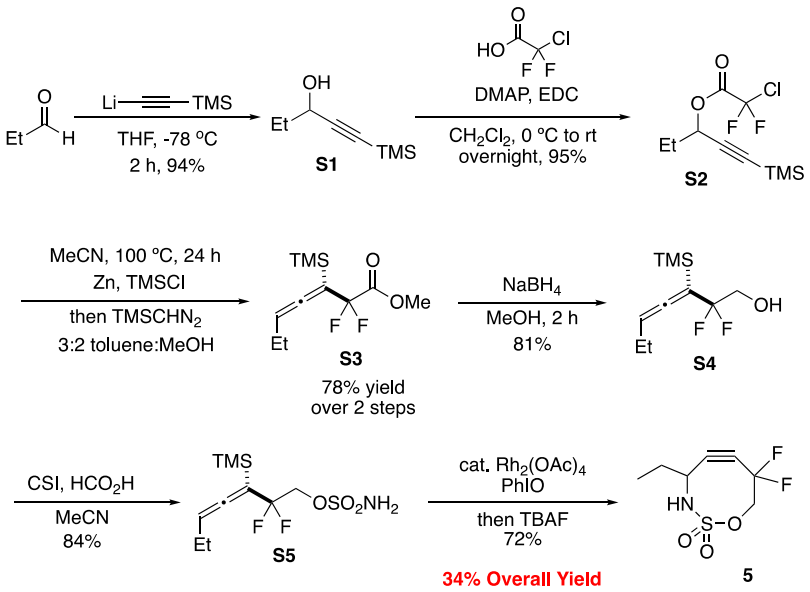
I. General Information.

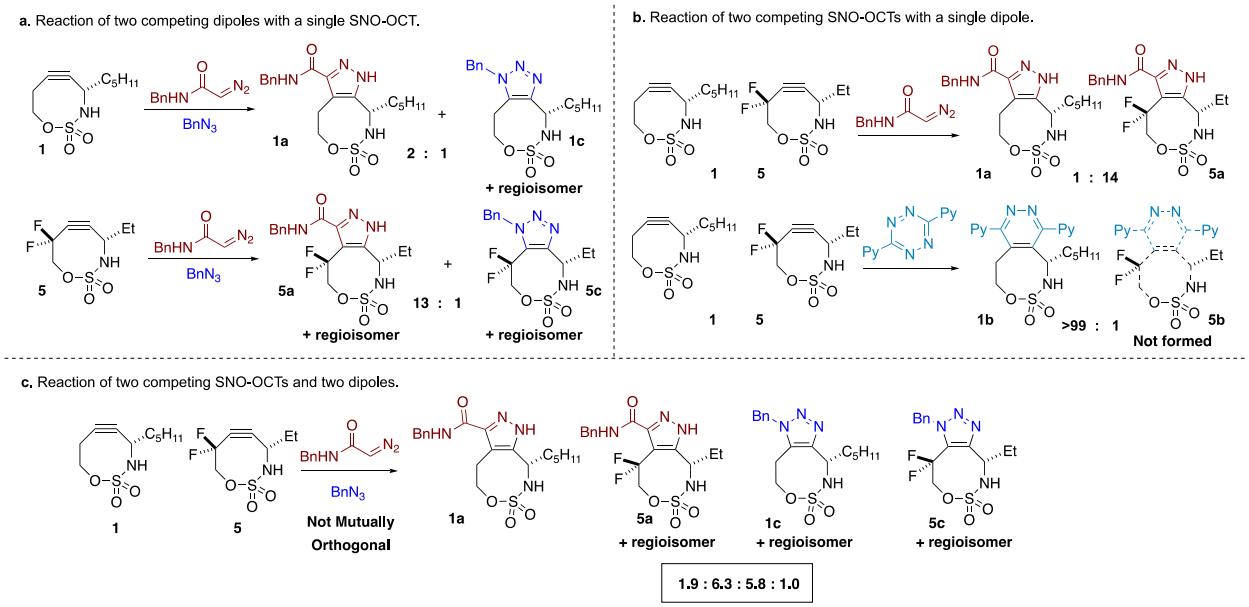
All glassware was either oven-dried overnight at 130ºC or flame dried under a stream of dry nitrogen prior to use. Reagents were used as obtained from the vendor without further purification unless otherwise specified. Dichloromethane, acetonitrile, dimethylformamide were dried over CaH_2 and freshly distilled prior to use. All other solvents and reagents were purified in accordance with "Purification of Laboratory Chemicals".[1a] Air- and moisture sensitive reactions were performed under an atmosphere of nitrogen. Analytical thin layer chromatography (TLC) was performed utilizing pre-coated silica gel 60 F254 plates containing a fluorescent indicator, while preparative chromatography was performed using SilicaFlash P60 silica gel (230-400 mesh) via Still's method.[1b] The mobile phases for column chromatography varied depending on substrate as hexanes/ether, pentane/ether, hexanes/ethyl acetate, or benzene/ethyl acetate were used. Columns were typically run using a gradient method, beginning with 100% of the less polar eluent and gradually increasing the polarity with the other solvent. For reactions producing products without a UV signature, potassium permanganate was employed to visualize the reaction progress.

^{1}H NMR and 13C NMR spectra were obtained using Bruker Avance III 500, Bruker Avance III 400, Bruker Avance III 600, and Varian Mercury-300 NMR spectrometers. For 1H NMR, chemical shifts are reported relative to residual protiated solvent peaks (δ 7.26, 2.49, 7.15 and 4.80 ppm for CDCl3, (CD3)2SO, C6D6 and CD3OD respectively). 13C NMR spectra were measured at either 125 MHz or 150 MHz on the same instruments noted above for recording 1H NMR spectra. Chemical shifts were again reported in accordance to residual protiated solvent peaks (δ 77.1, 39.5, 128.0 and 49.0 ppm for CDCl3, (CD3)2SO, C6D6, and CD3OD, respectively). UPLC/LC-MS data were collected on an Acquity UPLC I-Class PLUS with an Acquity QDA MS detector (Waters) using an ACQUITY UPLC BEH C18 1.7 μm 2.1 X 50 mm column (Waters). Plate reader kinetics were performed on Perkin-Elmer Envision plate reader with a 531 nm filter. Accurate mass measurements were acquired at the University of Wisconsin, Madison using a Thermo Q Exactive™ Plus (electrospray ionization or atmospheric solids analysis probe (ASAP-MS) methods). The NMR facilities are funded by the NSF (CHE-1048642, CHE-0342998), the University of Wisconsin, the NIH (S10 OD012245), and a generous gift from Paul J. and Margaret M. Bender. The purchase of the Thermo Q Exactive™ Plus in 2015 was funded by NIH Award 1S10 OD020022-1 to the Department of Chemistry.

II. Additional chemical schemes and figures.

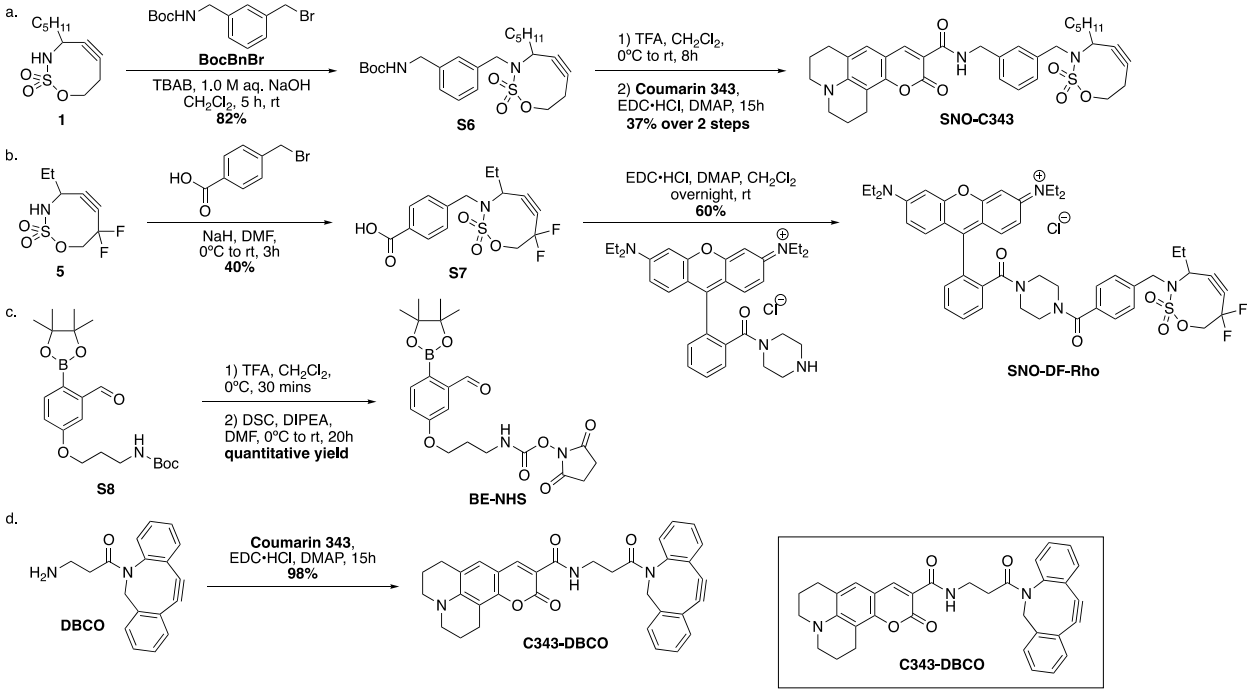


Scheme S2-1. The synthetic scheme for difluorinated SNO-OCT **5**.



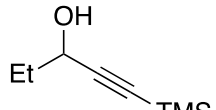
Scheme S2-2. Competition experiments and the extrapolation of chemoselectivity. (a) The competition between benzyl diazoacetamide and benzyl azide showed slight chemoselectivity that favors the formation of the adduction **1a**, while the formation of **5c** is much more favored. However, overall, the selectivity between the two dipoles either the strained alkynes **1** and **5** remains less than ideal. (b) The competition between two competing SNO-OCTs and Type I or III dipole. Benzyl diazoacetamide showed preferred selectivity with **5** as S2A, and an excellent chemoselectivity of dipyridyl tetrazine for **1**. (c) When two SNO-OCTs and two dipoles competing with each other, the poor selectivity is greatly amplified when all

four were combined in one-pot. This demonstrated the non-orthogonality of Type I and II dipoles with the two SNO-OCTs (more details see Figure S2-20).



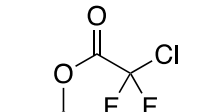
Scheme S2-3. The synthesis of bioorthogonal labeling agents (a) C343-SNO (b) Rho-DF-SNO (c) BE-NHS (d) C343-DBCO.

III. Preparation of SNO-OCT 5.



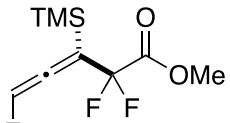
S1

Propargylic alcohol precursor to 5. This compound was prepared based on previously reported procedure with a comparable yield of 94%.[2]



S2

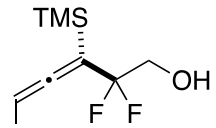
Chlorodifluoro ester precursor to 5. The ester was prepared from the corresponding propargylic alcohol (3.6 mL, 19.85 mmol) in a dry 50 mL round bottom flask. The alcohol was then dissolved in 20 mL dichloromethane under nitrogen atmosphere. Chlorodifluoroacetic acid (3.4 mL, 40.1 mmol) was added to the mixture followed by the addition of DMAP (0.13 g, 1.10 mmol). The mixture was cooled to 0ºC. EDC•HCl (8.30 g , 43.30 mmol) was added in small portions to the reaction mixture at 0ºC and stirred for an additional 5 min at 0ºC. The reaction was allow to warm up to room temperature and to be stirred overnight. The reaction was then quenched with saturated $NaHCO_3$. The aqueous phase was extracted with three portions of dichloromethane. The combined organics were then washed with brine, dried over Na_2SO_4, filtered with cotton and concentrated *in vacuo*. The crude material was purified using Kugelrohr distillation resulting a clear colorless liquid (5.06 g, 18.81 mmol) with a yield of 95%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.43 (t, *J* = 7.1, 6.4 Hz, 1H), 1.915 (p, *J* = 7.3 Hz, 2H), 1.05 (t, *J* = 7.4 Hz, 3H), 0.18 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 158.28 (t, *J* = 34.6 Hz), 116.77 (t, *J* = 300.8 Hz), 99.44, 93.24, 70.11, 27.82, 9.08, -0.40. ^{19}F NMR (377 MHz, $CDCl_3$) δ -64.07. HRMS (ESI) m/z calculated for $C_{10}H_{15}ClF_2O_2Si$ $[M+H]^+$ 269.0571, found 269.0566.



S3

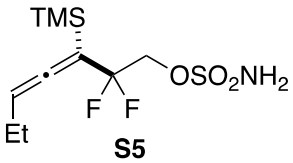
Homoallenic methyl ester precursor to 5. The following reactions were adopted from a modified procedure previously reported.[3,4] The corresponding chlorodifluoro ester (2.00 g, 7.44 mmol) was added to a 50 mL round bottom containing ~5 g activated zinc. Trimethylsilylchloride (1.7 mL, 13.39 mmol) was added followed by the addition of 20 mL acetonitrile. The reaction was refluxed overnight at 100ºC. All volatiles was then concentrated *in vacuo* and the residue was diluted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, and filtered with cotton. The crude was then concentrated again

under reduced pressure. The resulting crude material was carried on directly for methylation. In a 250 mL round bottom, the crude was dissolved in 28 mL methanol, and 42 mL toluene. Trimethylsilyldiazomethane (4.5 mL, 2.0 M in diethyl ether) was added to the reaction mixture at room temperature in a slow and steady stream with vigorous stirring. The mixture was stirred under room temperature for 30 min and was then quenched with acetic acid (0.04 mL, 0.702 mmol). The crude mixture was concentrated under reduced pressure and was purified via column chromatography (0% EtOAc/hexanes to 5% EtOAc/hexanes, gradient) resulting a clear yellow oil (1.45 g, 5.84 mmol) with a yield of 78%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.32 (p, *J* = 6.2 Hz, 1H), 3.84 (s, 3H), 2.03 – 1.98 (m, 2H), 1.00 (t, *J* = 7.4 Hz, 3H), 0.21 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 208.58 (t, *J* = 13.3 Hz), 164.26 (t, *J* = 43.9 Hz), 116.18 (t, *J* = 248.0 Hz), 96.34 (t, *J* = 36.1 Hz), 92.29, 52.68, 20.20, 12.84, -0.99. ^{19}F NMR (377 MHz, $CDCl_3$) δ -93.59 (d, *J* = 256.5 Hz), -93.75 (d, *J* = 257.2 Hz). HRMS (ESI) m/z calculated for $C_{11}H_{18}F_2O_2Si$ $[M+NH_4]^+$ 266.1382, found 266.1378.



S4

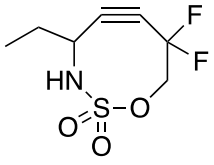
Homoallenic alcohol precursor to 5. The homoallenic alcohol precursor was prepared from the corresponding methyl ester. In a 50 mL round bottom, homoallenic methyl ester (1.45 g, 5.82 mmol) was dissolved in 12 mL methanol. The mixture was cooled to 0ºC before the addition of $NaBH_4$ (0.35 g, 9.25 mmol). The reaction was stirred at 0ºC for 30 min before it was warmed to room temperature. The reaction progression was monitored by TLC. The mixture was then quenched with 1.0 M HCl, and diluted with diethyl ether. The organic layer was washed with 10% Na_2CO_3, then washed with brine, dried over Na_2SO_4, and filtered with cotton. The crude was concentrated *in vacuo*, and was purified via column chromatography (0% EtOAc/hexanes to 20% EtOAc/hexanes, gradient) resulting a clear champagne-color liquid (1.04 g, 4.72 mmol) with a yield of 81%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.26 (p, *J* = 6.1 Hz, 1H), 3.85 (td, *J* = 13.3, 7.2 Hz, 2H), 2.05 (p, *J* = 7.3 Hz, 2H), 1.81 (t, *J* = 7.0 Hz 1H), 1.02 (t, *J* = 7.5 Hz, 3H), 0.19 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 207.70 (t, *J* = 13.7 Hz), 122.09 (t, *J* = 240.0 Hz), 96.86 (t, *J* = 36.7 Hz), 91.40, 65.13 (t, *J* = 30.9 Hz), 20.61, 13.25, -0.74. ^{19}F NMR (376 MHz, $CDCl_3$) δ -95.89 (d, *J* = 254.5 Hz), -97.35 (d, *J* = 254.4 Hz). HRMS (ESI) m/z calculated for $C_{10}H_{18}F_2OSi$ $[M-F]^-$ 201.1106, found 201.1105.



S5

Homoallenic sulfamate precursor to 5. The following procedure was adapted from our previously reported synthesis of SNO-OCT analogues.[5] In a 250 mL three-neck round bottom flask, chlorosulfonyl isocyanate (4.5 mL, 51.70 mmol) in 38 mL acetonitrile was cooled to 0ºC. Formic acid (2.0 mL, 53.15

mmol) was added dropwise at 0ºC under nitrogen atmosphere. The mixture was then allowed to warm up to room temperature and stirred overnight. The reaction was cooled again to 0ºC, to which homoallenic alcohol (4.69 g, 21.26 mmol) was added as a solution in N,N-dimethylacetamide (35.0 mL, 0.6 M) in a dropwise fashion. The reaction was stirred for 1 hour at room temperature and then quenched by the addition of an equal volume of H_2O. The aqueous phase was extracted with three portions of EtOAc and the combined organic phases were washed with five portions of H_2O. The combined organics were dried over Na_2SO_4, filtered with cotton, and concentrated *in vacuo*. The crude material was purified via column chromatography (0% EtOAc/hexanes to 20% EtOAc/hexanes, gradient) resulting a waxy white solid (5.34 g, 17.83 mmol) with a yield of 84%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.33 (p, *J* = 6.1 Hz, 1H), 4.99 (s, 1H), 4.46 – 4.37 (m, 2H), 2.07 (dtd, *J* = 13.7, 7.4, 3.3 Hz, 2H), 1.03 (t, *J* = 7.4 Hz, 3H), 0.19 (s, 9H).^{13}C NMR (126 MHz, $CDCl_3$) δ 208.07 (t, *J* = 13.6 Hz), 120.01 (t, *J* = 241.17 Hz), 96.28 (t, *J* = 36.27 Hz), 92.44, 69.97 (t, *J* = 31.44 Hz), 20.51, 13.18, -0.79. ^{19}F NMR (377 MHz, $CDCl_3$) δ -93.24 (d, *J* = 257.8 Hz), -93.68 (d, *J* = 257.2 Hz). HRMS (ESI) m/z calculated for $C_{10}H_{19}F_2NO_3SSi$ $[M-H]^-$ 298.0750, found 298.0748.

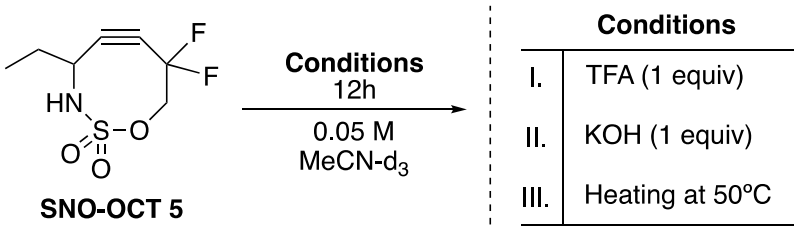


5

Compound 5. The following procedure was adapted from our previously reported synthesis of SNO-OCT analogues.[5] In a 100 mL round bottom, the homoallenic sulfamate (2.67 g, 7.04 mmol) was added and was dissolved in 36 mL dichloromethane. $Rh_2(OAc)_4$ (156.0 mg, 0.35 mmol) was added to the mixture and then stirred for 5 min at room temperature. PhIO (3.19 mg, 14.50 mmol) was added in one portion and the reaction mixture was stirred for 30 min while monitoring by TLC. When the TLC indicated the complete consumption of starting material, the crude mixture was filtered through a layer of celite, diluted with dichloromethane, and concentrated under reduced pressure. The resulting residue was quickly purified via column chromatography (0% EtOAc/hexanes to 10% EtOAc/hexanes, gradient) to yield an oil as the endocyclic methylene aziridine intermediate. Due to the concern of decomposition, the purified product was quickly transferred to a round bottom and diluted with 36 mL of dichloromethane followed by adding a steady stream of TBAF (35.2 mL, 1.0 M in THF). After stirring at room temperature for 30 min, an equal volume of water was added. The phases were extracted with three portions of dichloromethane. The combined organics were dried with Na_2SO_4, filtered with cotton, and concentrated under reduced pressure. The resulting crude was quickly purified via column chromatography (0% EtOAc/hexanes to 20% EtOAc/hexanes, gradient) resulting a white solid (1.14 g, 5.06 mmol) with a yield of 72% over two steps. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.62 (d, *J* = 7.0 Hz, 1H), 4.92 (m, 1H), 4.67 (dt, *J* = 12.2, 6.0 Hz, 1H), 4.24 (tt, *J* = 7.2, 3.3 Hz, 1H), 1.91 – 1.72 (m, 2H), 1.04 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 112.55

(d, *J* = 241.4 Hz), 111.15 (t, *J* = 10.8 Hz), 88.37 (t, *J* = 42.9 Hz), 79.04 (m), 50.08 (t, *J* = 1.7 Hz), 25.03, 10.11. ^{19}F NMR (376 MHz, $CDCl_3$) δ -96.48 (d, *J* = 277.8 Hz), -101.07 (d, *J* = 277.2 Hz). HRMS (ESI) m/z calculated for $C_7H_9F_2NO_3S$ $[M-H]^-$ 224.0198, found 224.0200.

IV. Stability screening of SNO-OCT 5.



General procedure. In a vial equipped with a stir bar, added SNO-OCT (2.3 mg, 0.01 mmol) and then it was dissolved in MeCN-d_3. The mixture is then subjected to a specified reaction condition. The reaction was stirred for 16 hours and then 1.0 equivalence of mesitylene as internal standard was added. The mixture was transferred into an NMR tube and the stability of SNO-OCT under each condition was examined based on integrations against mesitylenes.

Condition I. Trifluoroacetic acid (TFA) stability screening.

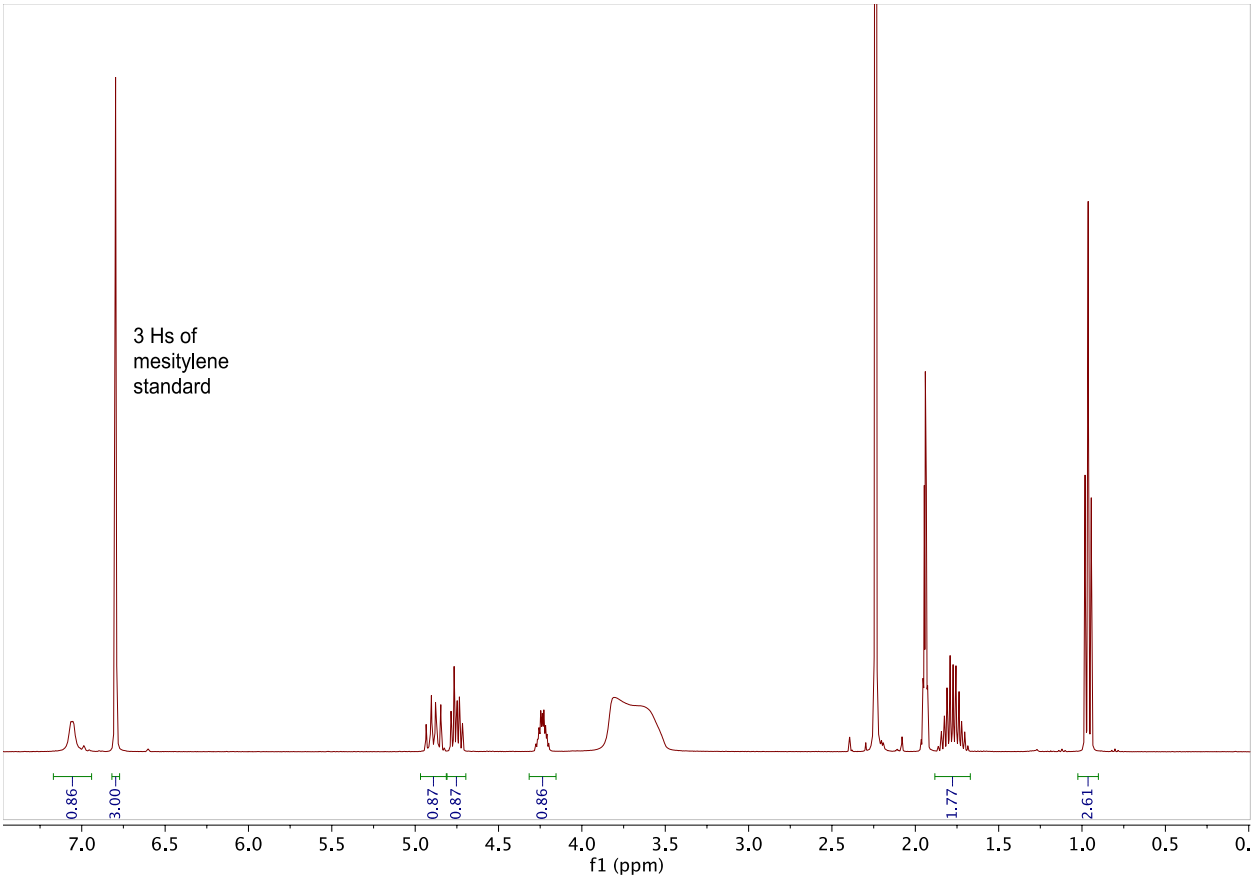


Figure S2-1. SNO-OCT **5** in MeCN-d_3 (0.05 M) with 1 equiv. of TFA for 16 hours at room temperature. Based on the NMR analysis, 87% of the SNO-OCT remained in the solution.

Condition II. Potassium hydroxide (KOH) stability screening.

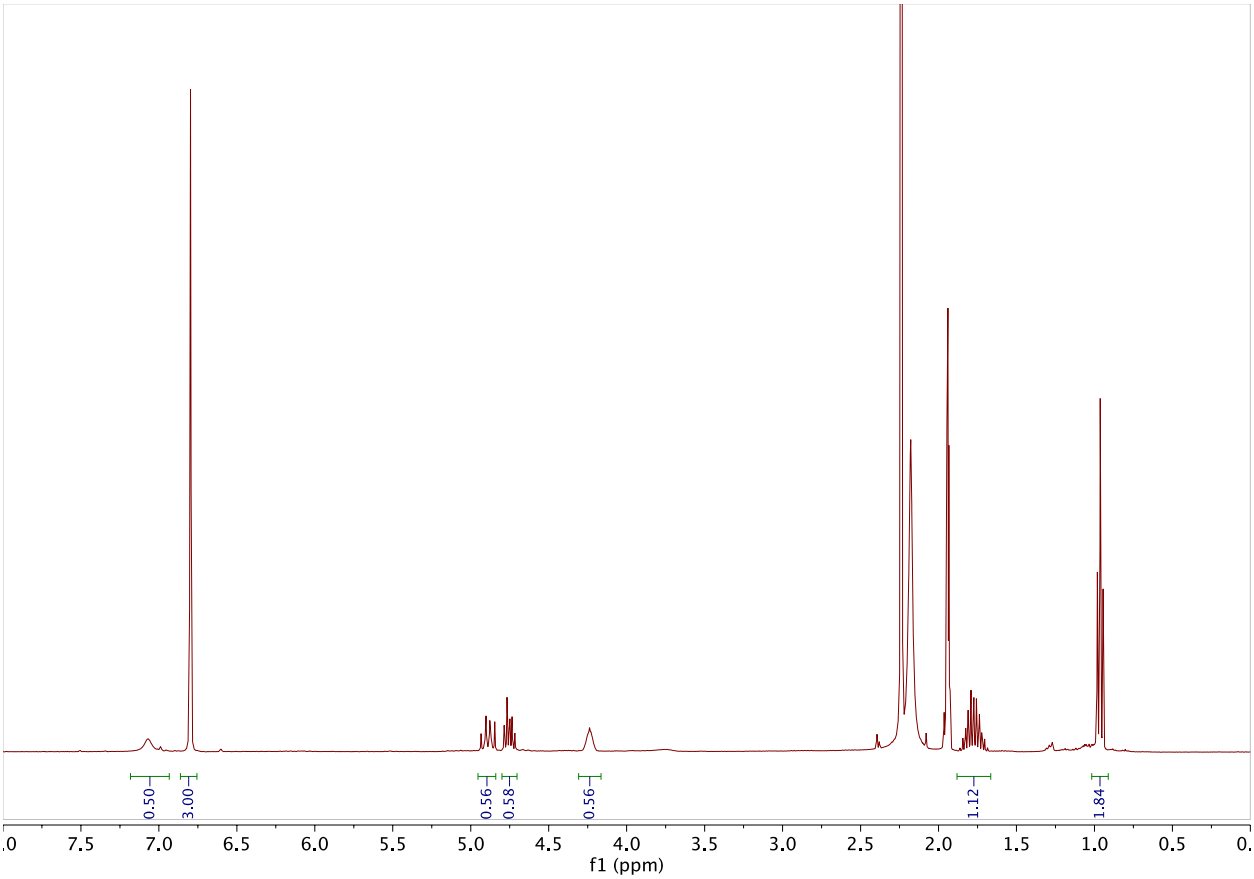


Figure S2-2. SNO-OCT **5** in MeCN-d_3 (0.05 M) with 1 equiv. of KOH for 16 hours at room temperature. Based on the NMR analysis, 56% of the SNO-OCT remained in the solution.

Condition III. Heating at 50ºC stability screening.

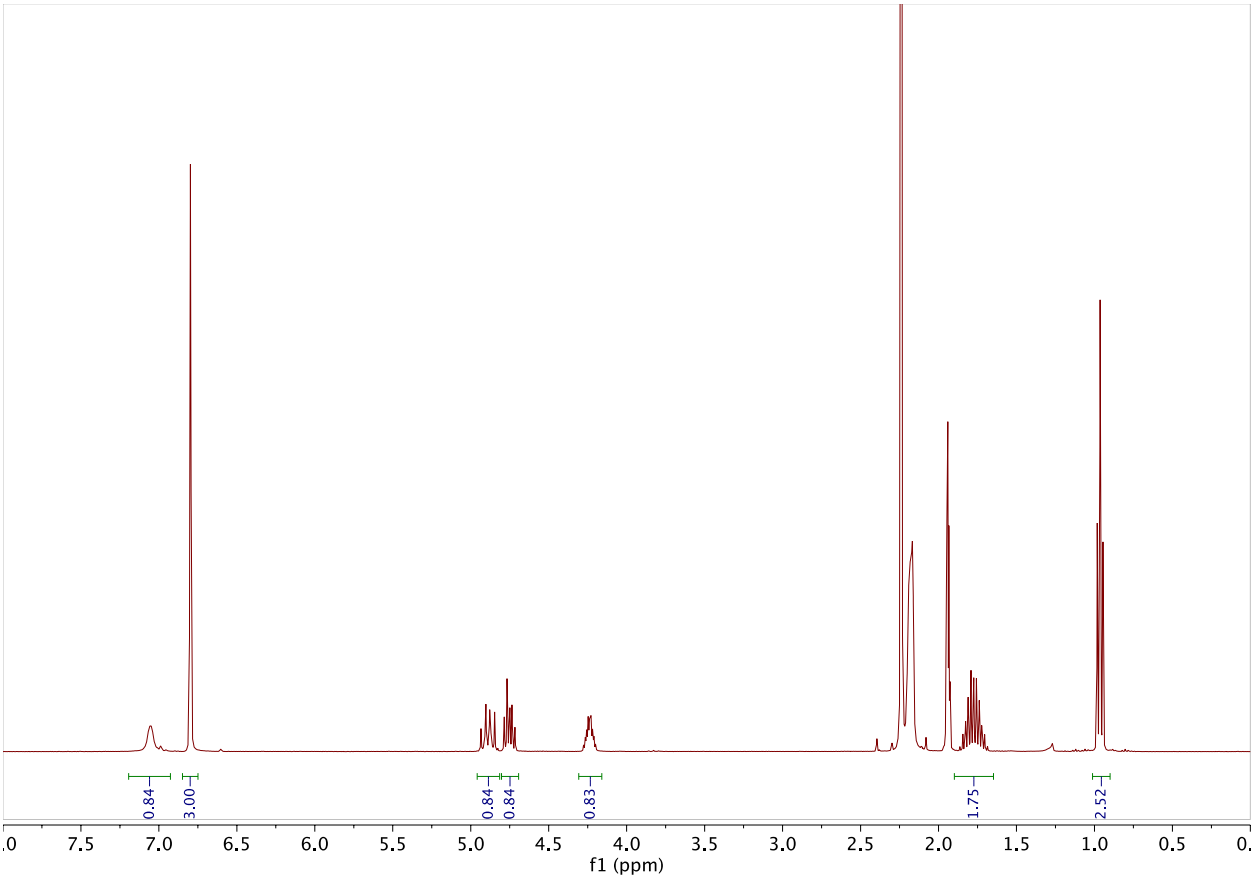
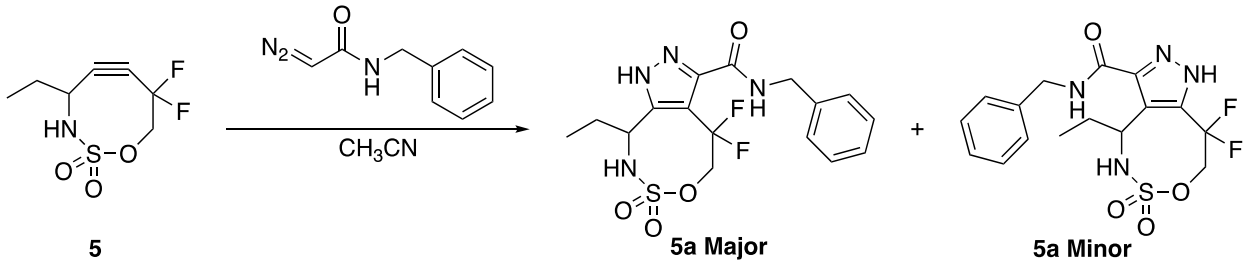


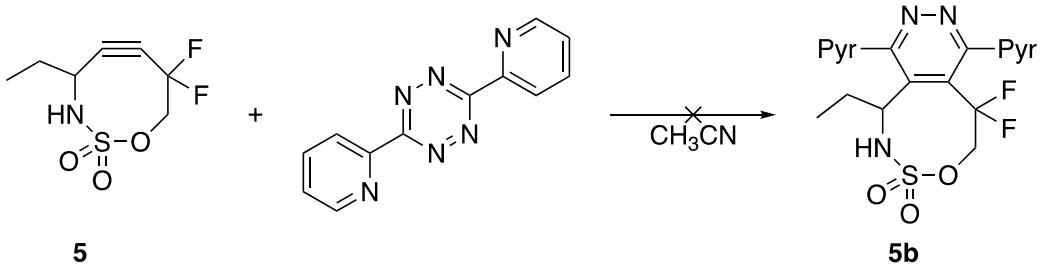
Figure S2-3. SNO-OCT **5** in MeCN-d_3 (0.05 M) was heated at 50ºC for 16 hours. Based on the NMR analysis, 84% of the SNO-OCT remained in the solution.

V. Reactions of SNO-OCTs with dipoles and hydrazide.

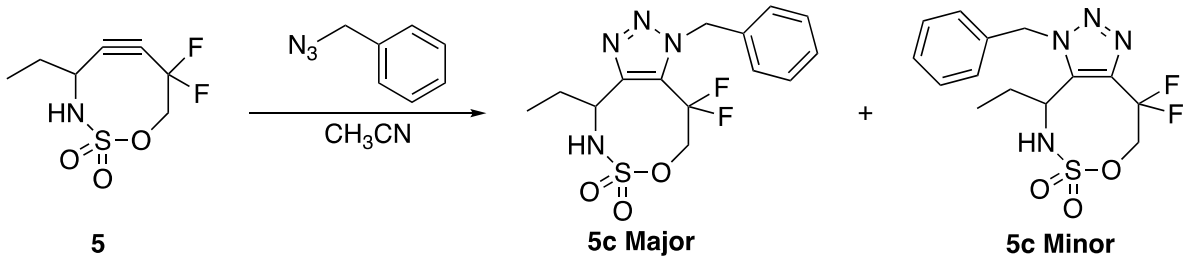
General procedure. The cyclic alkyne (1.0 equiv) was dissolved in CH_3CN (0.1 M). The corresponding dipole (1.0 equiv) was added to the alkyne solution and the mixture was stirred for 1 h to ensure the full conversion. The solvent was then removed under reduced pressure and transferred using dichloromethane.



Diazoles 5a major and 5a minor from reaction of 5 with benzyl diazo acetamide. The diazoles **5a major** and **5a minor** were prepared from alkyne **5** (50.0 mg, 0.222 mmol) according to the general procedure. The crude was purified via column chromatography (0% EtOAc/hexanes to 60% EtOAc/hexanes, gradient) to yield two regioisomers as white solids (**major** 70.3 mg, 0.176 mmol; **minor** 13.0 mg, 0.032 mmol; ratio = 5.4:1) with a yield of 94% in total. ^{1}H NMR (**major**, 500 MHz, $CDCl_3$) δ 12.42 (s, 1H), 7.42 – 7.23 (m, 5H), 7.06 (p, *J* = 5.2 Hz, 1H), 5.76 (s, 1H), 4.90 – 4.73 (m, 1H), 4.68 – 4.45 (m, 4H), 2.12 – 2.00 (m, 1H), 1.97 – 1.86 (m, 1H), 1.04 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (**major**, 126 MHz, $CDCl_3$) δ 159.35, 148.54, 139.65, 136.72, 128.88, 127.92, 127.67, 118.81 (t, *J* = 238.4 Hz), 110.43 (t, *J* = 29.9 Hz), 70.69 (t, *J* = 40.4 Hz), 52.34, 44.26, 26.83, 10.66. ^{19}F NMR (**major**, 376 MHz, $CDCl_3$) δ -77.28 (d, *J* = 274.7 Hz), -84.20. ^{1}H NMR (**minor**, 500 MHz, $CDCl_3$) δ 10.36 (s, 1H), 7.40 – 7.27 (m, 8H), 5.31 – 5.21 (m, 1H), 4.95 – 4.81 (m, 2H), 4.65 (dd, *J* = 14.8, 6.1 Hz, 1H), 4.56 (dd, *J* = 14.8, 5.8 Hz, 1H), 4.30 (ddd, *J* = 23.7, 14.2, 12.4 Hz, 1H), 2.22 (dqd, *J* = 14.9, 7.5, 3.3 Hz, 1H), 1.69 (ddq, *J* = 14.5, 8.7, 7.3 Hz, 1H), 1.07 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (**minor**, 126 MHz, $CDCl_3$) δ 160.85, 144.26, 137.80, 131.62, 128.78, 127.79, 127.63, 124.80 (d, *J* = 6.1 Hz), 118.83 (dd, *J* = 249.6, 239.0 Hz), 71.30 (dd, *J* = 46.4, 36.2 Hz), 51.89 (d, *J* = 1.2 Hz), 43.09, 30.11, 10.18. ^{19}F NMR (**minor**, 376 MHz, $CDCl_3$) δ -70.51 (d, *J* = 268.5 Hz), -101.71 (d, *J* = 269.5 Hz). HRMS (ESI) m/z calculated for $C_{16}H_{18}F_2N_4O_4S$ (**major**) $[M+H]^+$ 401.1090, found 401.1088. HRMS (ESI) m/z calculated for $C_{16}H_{18}F_2N_4O_4S$ (**minor**) $[M+H]^+$ 401.1090, found 401.1090.

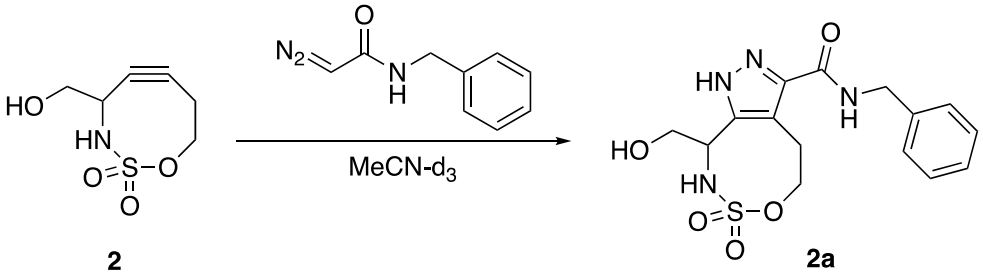


Pyridazine 5b from reaction of 5 with 3,6-di-2-pyridyl-1,2,4,5-tetrazine. The reaction was performed under the general procedure. No reaction progress was observed.



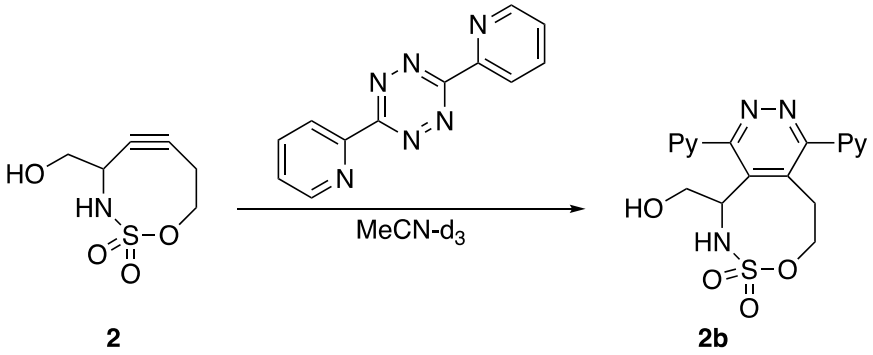
5 **5c Major** **5c Minor**

Triazoles 5c major and 5c minor from reaction of 5 with benzyl azide. The triazoles **5c major** and **5c minor** were prepared from alkyne **5** (40.0 mg, 0.178 mmol) according to the general procedure. The crude was purified via column chromatography (0% EtOAc/hexanes to 45% EtOAc/hexanes, gradient) to yield a clear viscous oil (61.0 mg, 0.171 mmol) as a mixture of regioisomers (**5c major:5c minor** 1.8:1) in 96% yield. ^{1}H NMR (**major**, 500 MHz, $CDCl_3$) δ 7.42 – 7.30 (m, 3H), 7.19 (dd, *J* = 7.0, 2.6 Hz, 2H), 5.78 (d, *J* = 15.1 Hz, 1H), 5.68 (d, *J* = 15.1 Hz, 1H), 4.94 (d, *J* = 9.9 Hz, 1H), 4.62 (q, *J* = 8.7 Hz, 1H), 4.48 – 4.38 (m, 1H), 4.39 – 4.26 (m, 1H), 2.33 (dqd, *J* = 14.7, 7.4, 5.0 Hz, 1H), 2.07 (dqd, *J* = 15.9, 7.3, 5.8 Hz, 1H), 1.12 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (**major**, 126 MHz, , $CDCl_3$) δ 147.19 (t, *J* = 3.8 Hz), 134.22, 128.93, 128.80, 127.55, 125.96 (t, *J* = 29.5 Hz), 116.39 (t, *J* = 242.6 Hz), 69.96 – 69.10 (m), 54.74 (t, *J* = 3.9 Hz), 52.23, 27.07, 10.23. ^{19}F NMR (**major**, 376 MHz, $CDCl_3$) δ -83.51 (d, *J* = 288.1 Hz), -93.18 (d, *J* = 286.4 Hz). ^{1}H NMR (**minor**, 500 MHz, $CDCl_3$) δ 7.43 – 7.35 (m, 3H), 7.17 (dd, *J* = 8.0, 1.9 Hz, 2H), 5.61 (d, *J* = 15.8 Hz, 1H), 5.57 (d, *J* = 15.9 Hz, 1H), 5.05 (s, 1H), 4.84 (dd, *J* = 13.9, 12.1 Hz, 1H), 4.47 (m, 3H), 4.33 (ddd, *J* = 26.5, 13.9, 9.9 Hz, 1H), 1.63 (dqd, *J* = 15.0, 7.5, 3.3 Hz, 1H), 1.52 (ddq, *J* = 14.7, 9.1, 7.4 Hz, 1H), 0.92 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (**minor**, 126 MHz, $CDCl_3$) δ 137.47 (d, *J* = 7.4 Hz), 136.69 (dd, *J* = 34.4, 25.5 Hz), 132.99, 129.49, 129.21, 126.94, 118.76 (dd, *J* = 248.3, 239.3 Hz), 71.87 (dd, *J* = 47.1, 35.8 Hz), 53.21, 50.84 (d, *J* = 1.4 Hz), 28.05, 9.69. ^{19}F NMR (**minor**, 376 MHz, $CDCl_3$) δ -71.62 (d, *J* = 270.2 Hz), -104.51 (d, *J* = 269.7 Hz). HRMS (ESI) m/z calculated for $C_{14}H_{16}F_2N_4O_3S$ (**major**) $[M+H]^+$ 359.0984, found 359.0982. HRMS (ESI) m/z calculated for $C_{14}H_{16}F_2N_4O_3S$ (**minor**) $[M+H]^+$ 359.0984, found 359.0984.



2 **2a**

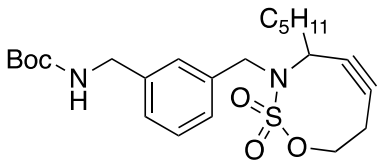
Diazoles 2a from reaction of 2 with benzyl diazo acetamide. The diazoles **2a** were prepared from alkyne **2** (9.6 mg, 0.05 mmol) according to the general procedure, except the deuterated acetonitrile was used and it was stirred under room temperature overnight. Products **2a** was obtained as a single regioisomer in the form of a white solid (13.6 mg, 0.04 mmol) without further purification with a yield of 80%. Due to product's low solubility in acetonitrile, dichloromethane, and chloroform, the eventual NMR spectra were taken in DMSO-d_6. ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.06 (s, 1H), 8.67 (t, J = 6.4 Hz, 1H), 8.40 (d, J = 9.0 Hz, 1H), 7.31 – 7.27 (m, 4H), 7.24 – 7.20 (d, J = 6.7 Hz, 1H), 5.18 (t, J = 5.8 Hz, 1H), 4.48 (q, J = 7.1 Hz, 1H), 4.41 (d, J = 6.0 Hz, 2H), 4.40 (d, J = 6.0 Hz, 2H), 4.35 (ddd, J = 11.2, 5.1, 3.4 Hz, 1H), 4.02 (td, J = 10.9, 5.6 Hz, 1H), 3.79 (dt, J = 10.9, 5.3 Hz, 1H), 3.69 (dt, J = 11.8, 6.1 Hz, 1H), 3.59 – 3.46 (m, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 162.77, 142.54, 141.08, 139.90, 128.13, 127.10, 126.52, 113.71, 70.10, 62.54, 52.44, 41.59, 20.47. HRMS (ESI) m/z calculated for $C_{15}H_{18}N_4O_5S$ $[M+Na]^+$ 389.0890, found 389.0086.



2 **2b**

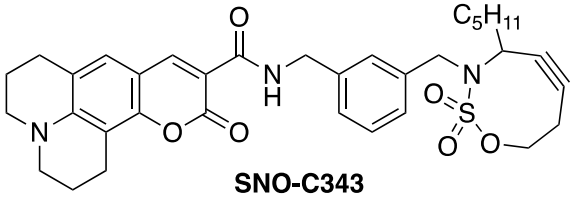
Pyridazine 2b from reaction of 2 with 3,6-di-2-pyridyl-1,2,4,5-tetrazine. The pyrazine **2b** was prepared from alkyne **2** (9.6 mg, 0.05 mmol) and dipyridyl tetrazine (11.8 mg, 0.05 mmol) according to the general procedure, except the deuterated acetonitrile was used and it was stirred under room temperature overnight. Products were obtained without further purification as a white solid with pinkish tints (19.2 mg). Due to products' low solubility in acetonitrile and chloroform, the eventual NMR spectra were taken in DMSO-d_6. The yield was calculated based on ^{1}H NMR peaks ratio between the cycloaddition product and the tetrazine starting material ($\delta_{product}$ = 8.78-8.72 ppm, int. = 2.00. 2H; $\delta_{tetrazine}$ = 8.95 ppm, int. = 0.43, 1H). ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.14 (dd, J = 6.2, 1.4 Hz, 1H), 9.11 (dd, J = 8.1, 1.4 Hz, 1H), 8.78 – 8.72 (m, 2H), 8.23 (ddd, J = 7.7, 6.0, 1.5 Hz, 1H), 8.09 (td, J = 7.7, 1.8 Hz, 1H), 7.94 (dt, J = 7.8, 1.2 Hz, 1H), 7.61 (ddd, J = 7.7, 4.9, 1.2 Hz, 1H), 5.12 (d, J = 6.1 Hz, 1H), 5.00 (s, 1H), 4.95 (ddd, J = 12.6, 6.9, 5.1 Hz, 1H), 4.87 (ddd, J = 13.9, 9.9, 4.6 Hz, 1H), 4.68 (td, J = 6.9, 3.3 Hz, 1H), 4.06 – 3.96 (m, 1H), 3.88 – 3.81 (m, 1H), 3.72 – 3.66 (m, 1H), 3.60 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 160.82, 155.77, 148.39, 147.52, 146.22, 145.77, 145.70, 138.72, 137.78, 137.40, 127.83, 125.35, 124.65, 124.15, 62.50, 55.39, 52.80, 23.13. HRMS (ESI) m/z calculated for $C_{18}H_{17}N_5O_4S$ $[M+Na]^+$ 422.0894, found 422.0892.

VI. Preparation of bioorthogonal conjugates.



S6

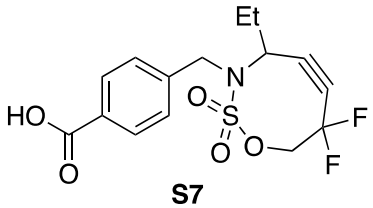
Boc-protected aryl precursor to SNO-C343 (S6). A 50 mL round bottomed flask equipped with a stir bar was charged with SNO-OCT **1** (148.0 mg, 0.64 mmol) and dissolved in dichloromethane (9.0 mL, 0.07 M). BocBnBr (212.7 mg, 0.70 mmol) was added followed by tetrabutylammonium bromide (20.6 mg, 0.06 mmol). 4.5 mL 1.0 M aq. NaOH was then added and stirred under room temperature for 5 hours. Upon the completion indicated by NMR aliquot, equal amount of water was added and the layers extracted with three portions of dichloromethane. The combined organics were dried with sodium sulfate, filtered with cotton, and concentrated under reduced pressure. The crude product was then purified by silica gel chromatography using a 0 to 30% gradient of ethyl acetate in hexane with 5% increments. The product was obtained 82% yield (237.2 mg, 0.53 mmol) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33 – 7.25 (d, 3H), 7.25 – 7.20 (d, *J* = 7.4 Hz, 1H), 5.01 – 4.92 (m, 1H), 4.81 – 4.71 (d, *J* = 14.8 Hz, 1H), 4.67 – 4.57 (m, 1H), 4.35 – 4.25 (t, *J* = 5.4 Hz, 2H), 4.23 – 4.17 (d, *J* = 14.7 Hz, 1H), 3.69 – 3.55 (t, *J* = 7.8 Hz, 1H), 2.87 – 2.72 (dddd, *J* = 16.9, 11.4, 5.6, 2.5 Hz, 1H), 2.36 – 2.23 (dd, *J* = 17.1, 3.8 Hz, 1H), 1.87 – 1.69 (td, *J* = 8.4, 3.9 Hz, 2H), 1.49 – 1.41 (s, 9H), 1.19 – 0.95 (m, 4H), 0.82 – 0.72 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 155.90, 139.60, 136.35, 128.92, 127.54, 127.22, 96.45, 93.75, 79.51, 75.96, 54.92, 54.53, 44.43, 33.82, 30.83, 28.39, 25.79, 22.35, 21.73, 13.86. HRMS (ESI) m/z calculated for $C_{23}H_{34}N_2O_5S$ $[M+NH_4]^+$ 468.2527, found 468.2522.



SNO-C343

Compound SNO-C343. A 25 mL round bottom flask equipped with a stir bar was charged with Boc-protected aryl precursor (118.9 mg, 0.26 mmol) and dissolved dichloromethane (2.6 mL, 0.1 M). The solution was cooled down to 0ºC and then added trifluoroacetic acid (0.4 mL, 5.2 mmol) at 0ºC dropwise. The mixture was stirred for 5 mins before warming it up to room temperature. After 8 hours, the reaction was quenched with equal amount of saturated $NaHCO_3$. The layers were extracted with three portions of dichloromethane, filtered with cotton, and concentrated under reduced pressure. The crude product was directly carried forward. A 10 mL round bottom was charged with the crude product in dichloromethane (2.2 mL, 0.1 M). Coumarin 343 (61.7 mg, 0.22 mmol, 1.0 equiv) and 4-dimethylaminopyridine (DMAP;

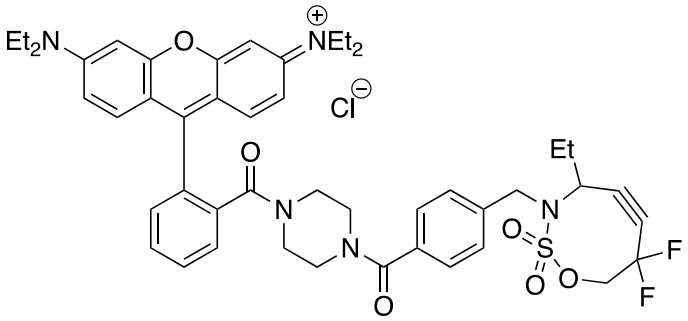
8.0 mg, 0.07 mmol, 0.2 equiv) were then added followed by the addition of EDC•HCl (91.3 mg, 0.48 mmol, 2.2 equiv). The reaction mixture was stirred at room temperature for 15 hours. The reaction was stopped with the addition of water and diluted with dichloromethane. The layers were extracted with three portions of dichloromethane. The pooled organic layer was dried with sodium sulfate, filtered with cotton, and concentrated under reduced pressure. The crude product was then purified by silica gel chromatography using a 40 to 80% gradient of EtOAc in hexane with 10% increments. The product was obtained 37% yield (60.7 mg, 0.098 mmol) over two steps as a dark yellow solid. 1H NMR (500 MHz, $CDCl_3$) δ 9.25 (t, *J* = 6.0 Hz, 1H), 8,63 (s, 1H), 7.41 – 7.28 (m, 4H), 7.01 (s, 1H), 4.97 (td, *J* = 11.2, 4.0 Hz, 1H), 4.76 (d, *J* = 14.7 Hz, 1H), 4.71 – 4.57 (m, 3H), 4.22 (d, *J* = 14.8 Hz, 1H), 3.65 (t, *J* = 8.2 Hz, 1H), 3.33 (q, *J* = 5.7 Hz, 4H), 2.93 – 2.71 (m, 5H), 2.29 (dd, *J* = 17.0, 3.9 Hz, 1H), 1.97 (h, *J* = 6.3, 5.5 Hz, 4H), 1.80 (dh, *J* = 20.4, 7.7, 7.3 Hz, 2H), 1.20 – 0.94 (m, 6H), 0.76 (t, *J* = 7.2 Hz, 3H). HRMS (ESI) m/z calculated for $C_{34}H_{39}N_3O_6S$ $[M+H]^+$ 618.2632, found 618.2639.



S7

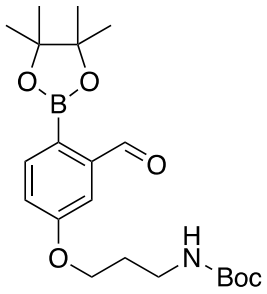
Benzoic acid precursor to SNO-DF-Rho (S7). A 10 ml round bottom equipped with a stir bar was charged with SNO-OCT **5** (128.5 mg, 0.57 mmol) and dissolved in 2.2 mL dimethylformamide. The solution was cooled to 0ºC followed by the addition of NaH 60% in mineral oil (43.3 mg, 1.08 mmol). The mixture was stirred at 0ºC for 5 mins. The reaction mixture was removed from the ice bath and a solution of 4-(bromomethyl)benzoic acid (97.7 mg, 0.45 mmol) in 2.2 mL dimethylformamide (DMF) was then added dropwise over 10 mins. Solid formation was observed during the course of the addition. The reaction mixture was stirred at room temperature for 3 hours. Upon the full consumption of 4-(bromomethyl)benzoic acid, the reaction was cooled to 0ºC, and 4 mL 1.0 M HCl was added dropwise at 0ºC under inert atmosphere. The crude mixture was then quickly transferred to a separatory funnel and diluted ethyl acetate. The layers were extracted with three portions of ethyl acetate. The combined organic layer was dried with sodium sulfate, filtered with cotton, and concentrated under reduced pressure. The crude product was then purified by silica gel chromatography using a 0 to 30% gradient of ethyl acetate in hexane with 10% increments containing a consistent 1% acetic acid. After two round of purifications, the product was obtained 40% yield (64.7mg, 0.18 mmol) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 – 8.06 (d, *J* = 8.2 Hz, 2H), 7.61 – 7.45 (d, *J* = 8.0 Hz, 2H), 5.11 – 4.97 (dd, *J* = 26.4, 12.4 Hz, 1H), 4.95 – 4.83 (d, *J* = 15.3 Hz, 1H), 4.73 – 4.60 (dd, *J* = 12.4, 8.6 Hz, 1H), 4.48 – 4.34 (d, *J* = 15.3 Hz, 1H), 3.80 – 3.61 (s, 1H), 2.02 – 1.90 (dt, *J* = 15.0, 7.7 Hz, 1H), 1.90 – 1.78 (dt, *J* = 14.0, 7.3 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.89, 140.92,

130.82, 129.52, 128.64, 112.50 (t, *J* = 241.3 Hz), 111.06 (t, *J* = 10.9 Hz), 85.79 (t, *J* = 43.4 Hz), 78.77 (m), 56.03, 54.81, 26.19, 10.66. ^{19}F NMR (376 MHz, $CDCl_3$) δ -94.47 (d, *J* = 277.0 Hz), -103.11 (d, *J* = 276.9 Hz). HRMS (ESI) m/z calculated for $C_{15}H_{15}F_2NO_5S$ [M-H]- 358.0566, found 358.0569.



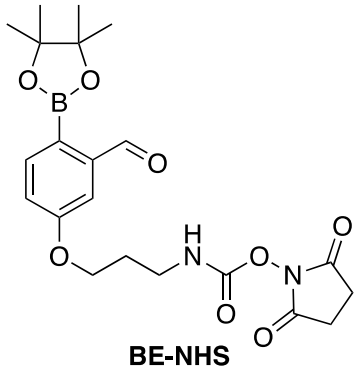
SNO-DF-Rho

Compound SNO-DF-Rho. The following reaction work-up was adopted from a previously reported procedure.[6] A 25 ml round bottom was charged with ArCOOH-Difo-SNO-OCT (5.2 mg, 0.0145 mmol) and dissolved in 4.0 mL dichloromethane. Rhodamine-piperazine (13.2 mg, 0.0258 mmol) and 4-dimethylaminopyridine (0.8 mg, 0.0065 mmol) were then added followed by the addition of EDC•HCl (15.3 mg, 0.0798 mmol). The reaction mixture was stirred at room temperature overnight. The reaction was quenched by the addition of saturated $NaHCO_3$. The aqueous phase was saturated with NaCl and extracted with three portions of dichloromethane. The combined organic layers were dried with sodium sulfate, filtered with cotton, and concentrated under reduced pressure. The crude product was then purified by silica gel chromatography using a 0 to 10% gradient of MeOH in dichloromethane with 5% increments containing a consistent 1% NH_4OH. After two round of purifications, the product was obtained 60 % yield (7.7 mg, 0.0087 mmol) as a purple solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 – 7.31 (m, 10H), 7.27 – 7.15 (m, 2H), 6.91 – 6.74 (m, 2H), 5.02 (ddd, *J* = 23.7, 12.5, 3.1 Hz, 1H), 4.79 (d, *J* = 15.2 Hz, 1H), 4.61 (dd, *J* = 11.4, 7.4 Hz, 1H), 4.41 (d, *J* = 15.2 Hz, 1H), 3.85 – 3.77 (m, 1H), 3.76 – 3.63 (q, *J* = 7.2 Hz, 8H), 3.62 – 3.41 (m, 8H), 1.95 (dq, *J* = 15.4, 7.5 Hz, 1H), 1.84 (dq, *J* = 13.8, 7.3 Hz, 1H), 1.35 (t, *J* = 7.1 Hz, 13H), 0.77 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.21, 167.72, 159.10, 157.74, 155.70, 137.09, 134.97, 134.97, 132.05 (broad singlet), 130.44 (broad singlet), 130.34, 130.20, 128.96, 128.89, 128.67, 128.44, 128.11 (broad singlet), 127.87, 127.63, 113.80, 112.58 (t, *J* = 239.6 Hz, 1C), 96.31(m, 1C), 78.65 (dd, *J* = 46.3, 34.5 Hz, 1C), 55.88, 54.94, 46.24, 26.20, 12.72, 10.67. ^{19}F NMR (376 MHz, $CDCl_3$) δ -94.16 (d, *J* = 275.8 Hz), -103.08 (d, *J* = 275.8 Hz). HRMS (ESI) m/z calculated for $C_{47}H_{52}F_2N_5O_6S^+$ $[M]^+$ 852.3601, found 852.3602.



S8

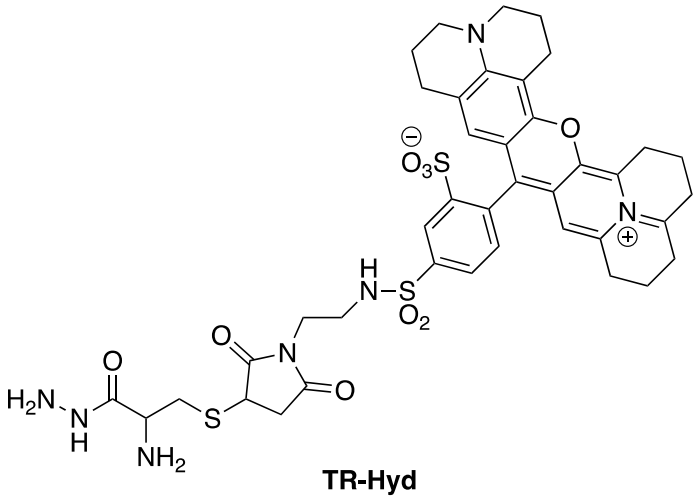
Compound S8. This compound was prepared based on a previously published procedure.[7]



BE-NHS

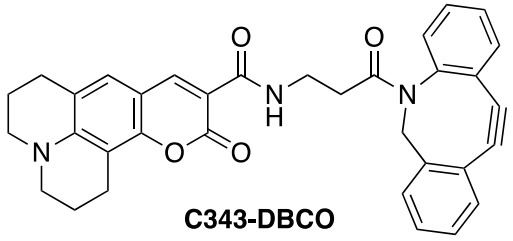
Compound BE-NHS (Boronic ester-NHS). The following reactions was prepared based on previously published procedures.[7,8] In a glass vial equipped with a stir bar, Compound **S6** (118.1 mg, 0.29 mmol) was dissolved in dichloromethane (3.0 mL). The solution homogeneous solution was then cooled to 0ºC and then trifluoroacetic acid (0.4 mL, 5.80 mmol) was added dropwise. At 0ºC, the mixture was then stirred under nitrogen atmosphere for 30 mins. All volatiles were then removed under reduced pressure, resulting a viscus yellow oil. The crude material was carried forward without any purification. In a vial equipped with a stir bar, the crude was diluted with DMF (0.64 mL). The mixture was then cooled to 0ºC in an ice bath followed by the dropwise addition of disuccinimidyl carbonate (DSC; 148.6 mg, 0.58 mmol) in DMF (0.58 mL). Diisopropylethylamine (DIPEA; 0.1 mL, 0.58 mmol) was then added at 0ºC. The mixture was allowed to warm up to room temperature and stirred under nitrogen atmosphere for 20 hours. The reaction was diluted with 50 mL ethyl acetate and then washed with 50 mL of 1M HCl. The organic phase was washed with 3 x 50 mL of water. The organics was then dried with Na_2SO_4, filtered through cotton, and concentrated under reduced pressure. A brown viscus oil (152.7 mg) was obtained without purification. Due to the presence of DMF as the only impurity, we determined a quantitative yield based on ^{1}H-NMR integration between the desired product and DMF (ratio = 1 : 0.8; DMF = 0.24 mmol; Product = 0.30 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.64 (s, 1H), 7.86 (d, *J* = 8.2 Hz, 1H), 7.47 (d, *J* = 2.6 Hz, 1H), 7.13 (dd, *J* = 8.2, 2.6 Hz, 1H), 6.03 (s, 1H), 4.13 (t, *J* = 6.0 Hz, 2H), 3.49 (td, *J* = 6.7, 6.0 Hz, 2H), 2.81 (s, 4H), 2.09 (m, 3H), 1.37 (s, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 195.03, 170.18, 160.98, 151.63, 143.53, 138.08,

120.36, 111.23, 84.32, 65.75, 39.58, 37.46, 25.55, 24.95. ^{11}B NMR (128 MHz, $CDCl_3$) δ 30.97. HRMS (ESI) m/z calculated for $C_{21}H_{27}BN_2O_8$ $[M + Na]^+$ 469.1753, found 469.1757. (Note: ^{13}C signal of the **$^{13}C_{Aryl}$**-B at 124.53 ppm was missing from the ^{13}C- NMR spectra due to the quadrupolar effect of ^{11}B, thus the shift of **$^{13}C_{Aryl}$**-B was determined by HSQC and HMBC correlations; see NMR spectra section for more details)



TR-Hyd

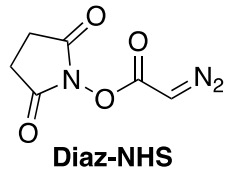
Compound TR-Hyd. The following reactions was prepared from previously published procedures.[7]



C343-DBCO

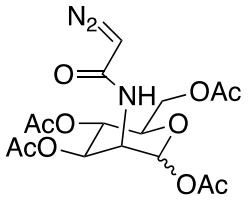
Compound C343-DBCO. In a dried vial equipped with a stir bar, dibenzocyclooctyne-amine (DBCO; 10.0 mg, 0.036 mmol) and coumarin 343 (13.0 mg, 0.045 mmol) were dissolved in dichloromethane. EDC•HCl (9.3 mg, 0.049 mmol) was then added to the mixture followed by the addition of DMAP (0.6 mg, 0.005 mmol). The reaction was stirred under room temperature overnight. The next day, the reaction mixture was diluted with dichloromethane, and then washed with brine. The organics were then dried over Na_2SO_4, filtered through cotton, and concentrated under reduced pressure. The crude product was then purified by silica gel chromatography using a 0 to 100% gradient of ethyl acetate in hexane with 20% increments. The product was obtained 98% yield (19.2 mg, 0.035 mmol) as a brown solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8,86 (t, *J* = 6.1 Hz, 1H), 8.50 (s, 1H), 7.71 (d, *J* = 7.5 Hz, 1H), 7.39 – 7.31 (m, 5H), 7.29 (dd, *J* = 7.5, 1.3 Hz, 1H), 7.19 (dd, *J* = 7.5, 1.4 Hz, 1H), 6.97 (s, 1H), 5.18 (d, *J* = 13.8 Hz, 1H), 3.64 (d, *J* = 13.8 Hz, 1H), 3.53 (qd, *J* = 6.7, 3.5 Hz, 2H), 3.32 (q, *J* = 5.4 Hz, 4H), 2.87 (t, *J* = 6.5 Hz, 2H), 2.76 (t, *J* = 6.3 Hz, 2H), 2.62 (dt, *J* = 16.2, 7.1 Hz, 1H), 2.11 (dt, *J* = 16.2, 6.4 Hz, 1H), 1.97 (h, *J* = 6.0 Hz, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.52, 163.44, 162.63, 152.62, 151.40, 148.01, 147.99, 147.79, 132.27, 129.12, 128.47, 128.27, 128.03, 127.67, 127.05, 126.92, 125.46, 123.11, 122.58, 119.47, 115.00, 109.22, 108.18, 107.73,

105.67, 55.30, 50.20, 49.79, 35.77, 34.74, 27.45, 21.16, 20.23, 20.12. HRMS (ESI) m/z calculated for $C_{34}H_{29}N_3O_4$ $[M + H]^+$ 544.2231, found 544.2231.



Diaz-NHS

Compound Diaz-NHS. The following reactions was prepared from previously published procedures.[9]

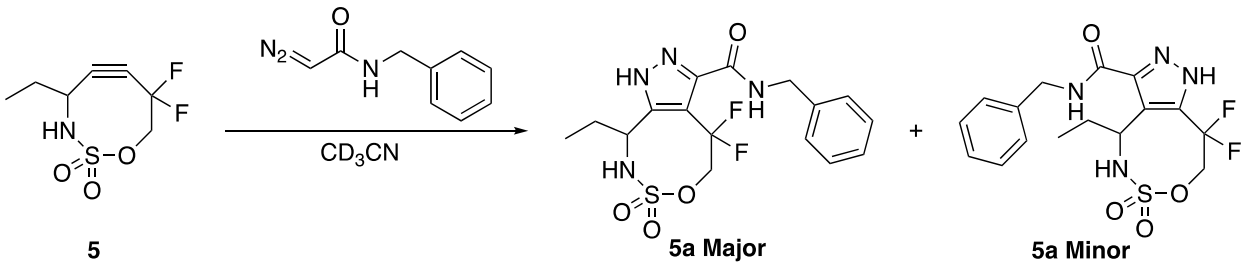


Ac_4ManDiaz

Compound Ac_4ManDiaz. The following reactions was prepared from previously published procedures.[10]

VII. NMR kinetics for SNO-OCT/dipole cyclization.

NMR kinetics general procedure. We employed previous published procedure on the study of SNO-OCT analogues.[5]



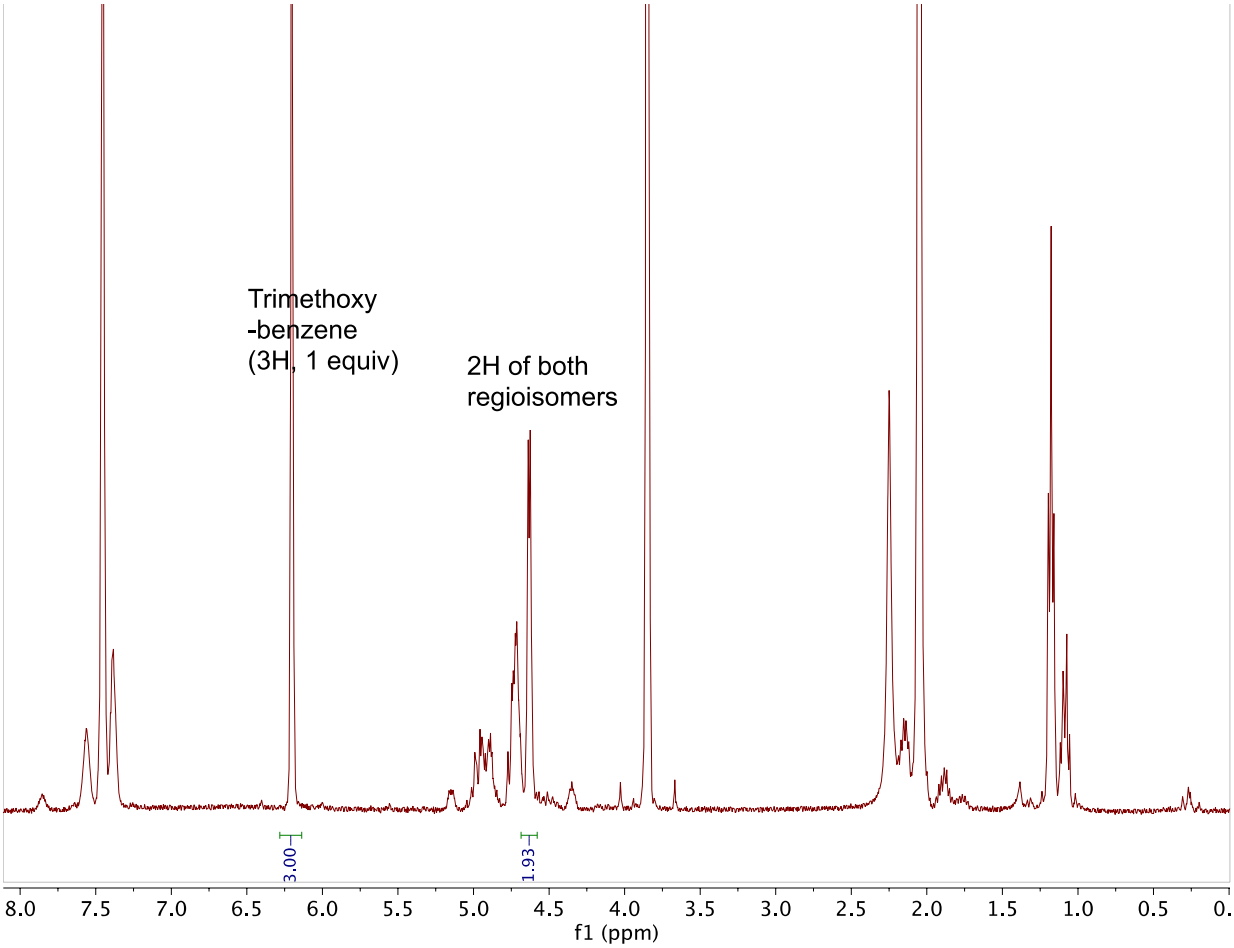


Figure S2-4 Reaction between **5** and benzyl diazoacetamide after 1 min and 45 seconds at **24ºC**,we observed around 93% conversion.

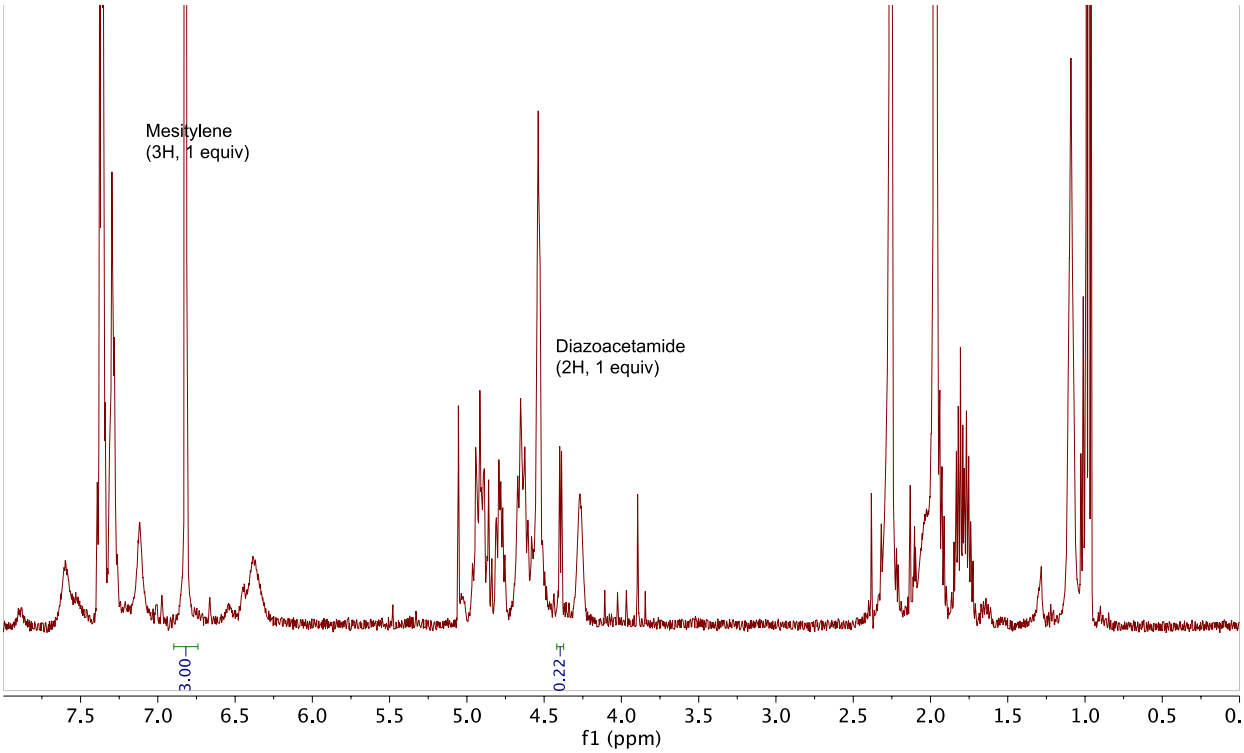


Figure S2-5. Reaction between **5** and benzyl diazoacetamide after 2 min and 15 seconds at 0ºC, we observed around 89% conversion.

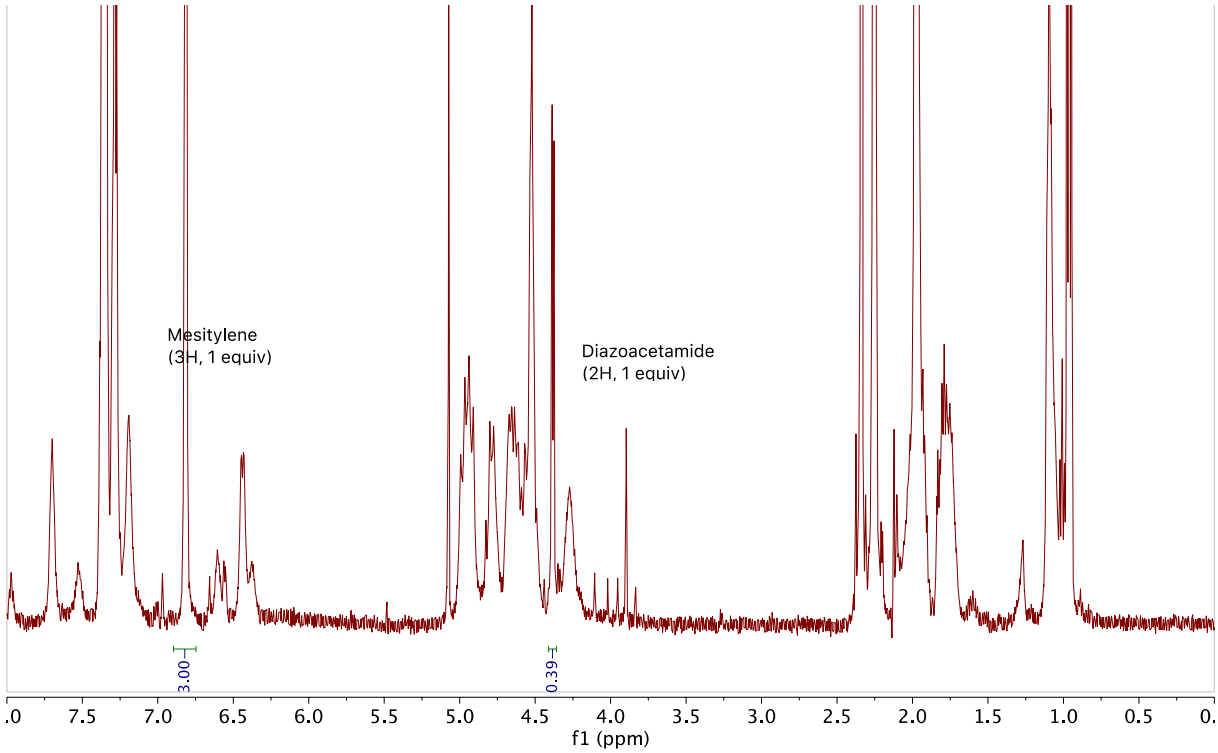


Figure S2-6. Reaction between **5** and benzyl diazoacetamide after 2 min and 13 seconds at -25ºC, we observed around **81%** conversion.

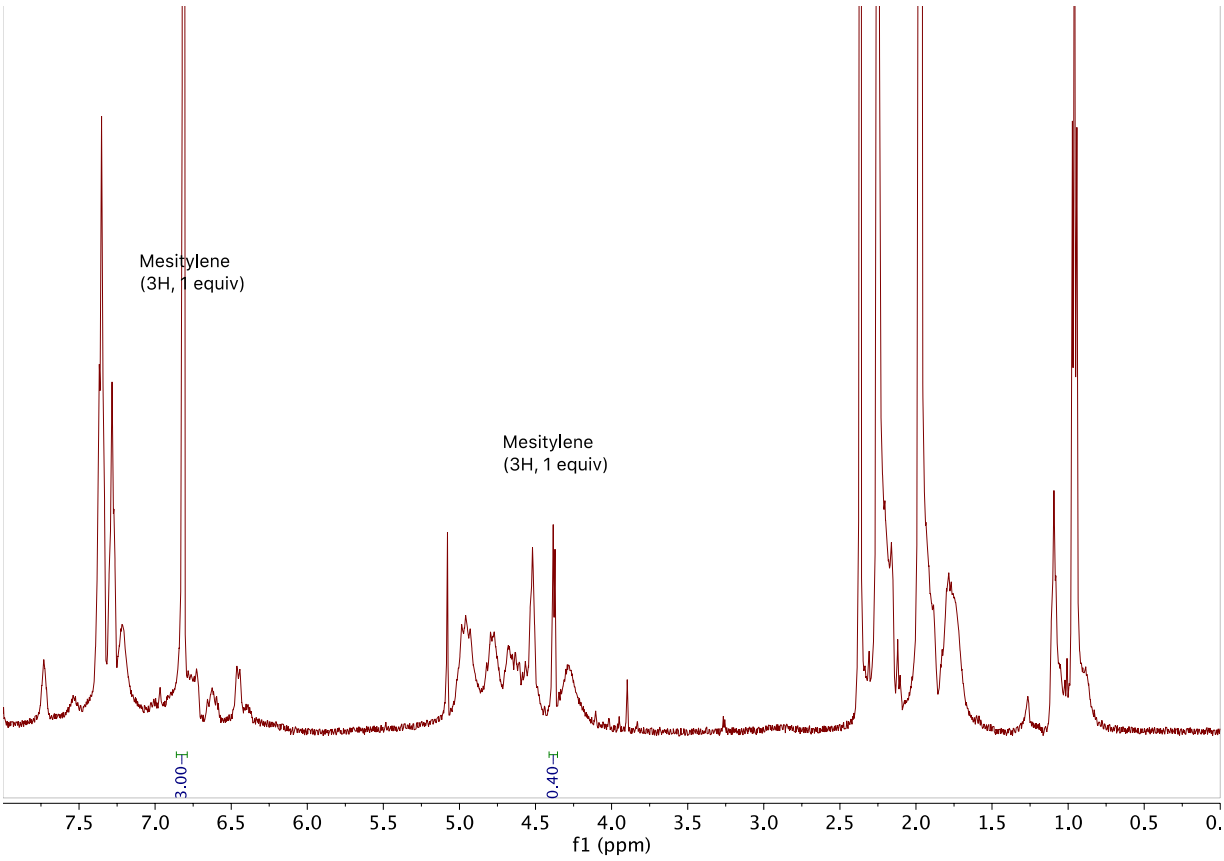
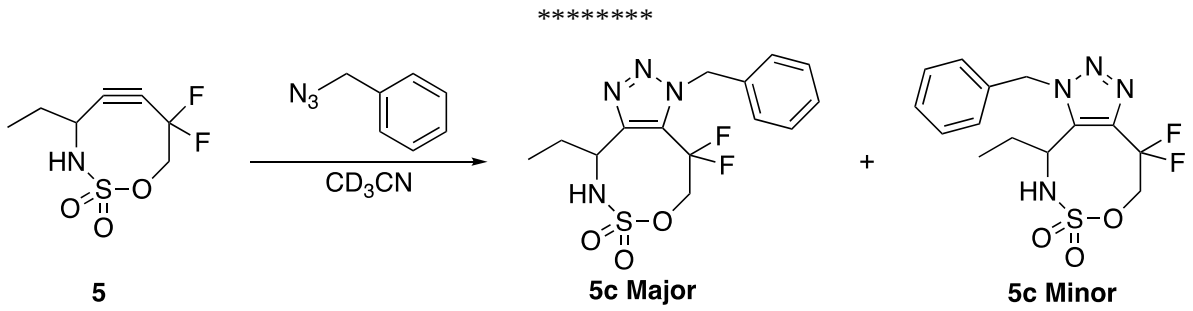
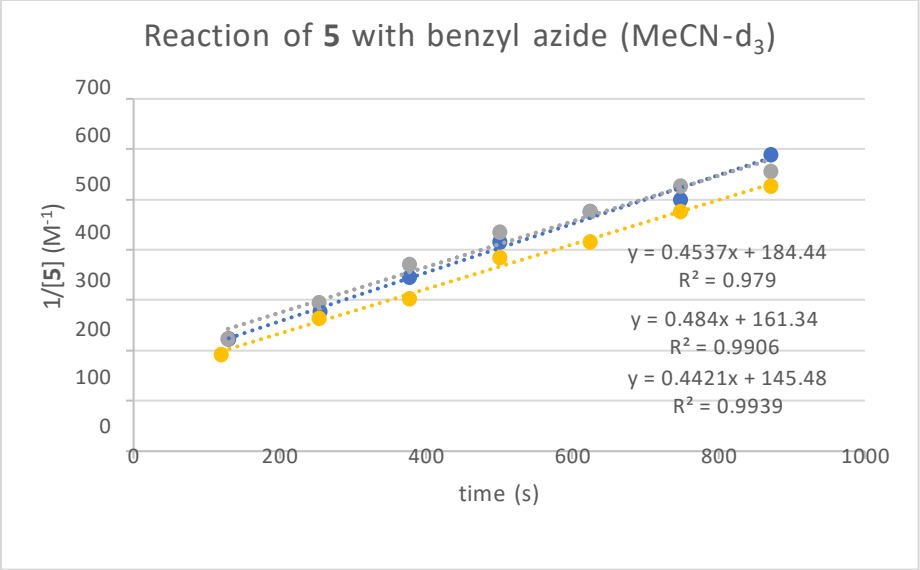


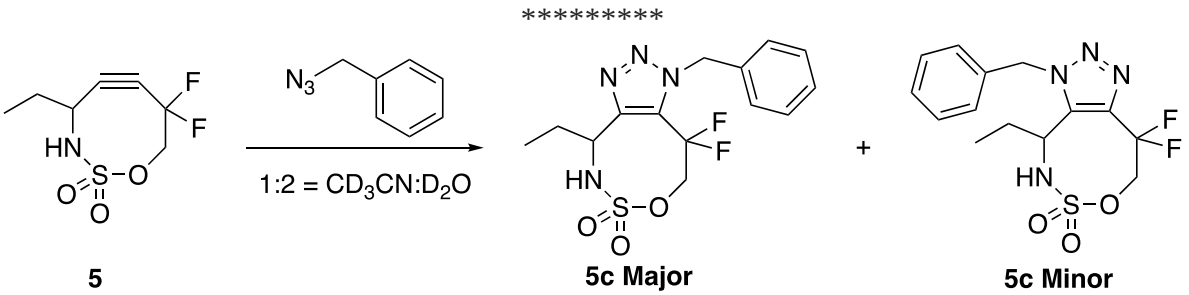
Figure S2-7. Reaction between **5** and benzyl diazoacetamide after 2 min and 4 seconds at -32ºC, we observed around 80% conversion.

Note: We also performed a VT-NMR kinetics experiment on the OH-SNO-OCT **2** (see below) using the same method as **5** + benzyl diazoacetamide to show case that our method is valid. We believe that **5**'s kinetics is too fast for us to achieve an accurate kinetics data at a reasonable temperature, but here we would like to provide as much information as possible to showcase the high kinetics rate of **5**. We performed a series of competition experiments to estimate the rate of this reaction to be > 5.13 $M^{-1}•s^{-1}$ (more detail see Figure S2-16 and S2-17).





$k_2 = 0.459 \pm 0.02\ M^{-1}\ s^{-1}$



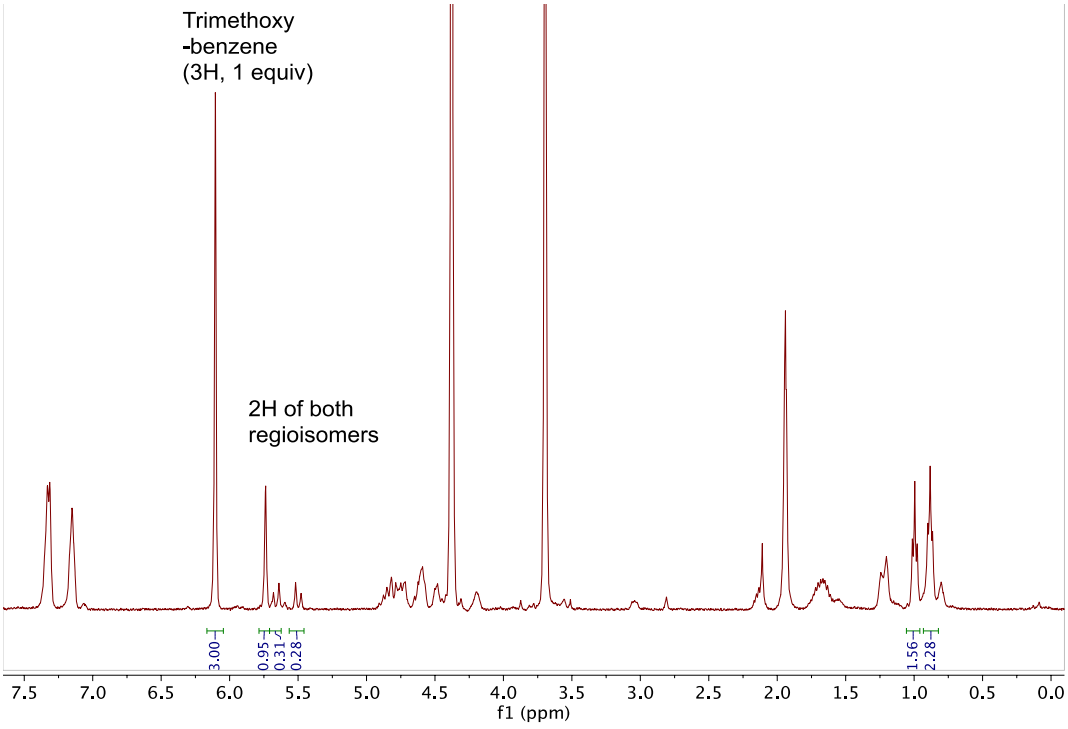


Figure S2-8. In the reaction between **5** and benzyl azide in CD_3CN : D_2O (1 : 2 ratio) after 1 min and 37 seconds, we observed around **77%** conversion, thus we conclude the kinetics rate of this reaction is too fast for NMR to measure the initial kinetics rate at 23ºC, when the reaction takes place in a more aqueous environment. We believe this is rate increase is due to the stabilization effect of a more polar transition state that was also observed in other cycloaddition reactions.[11]

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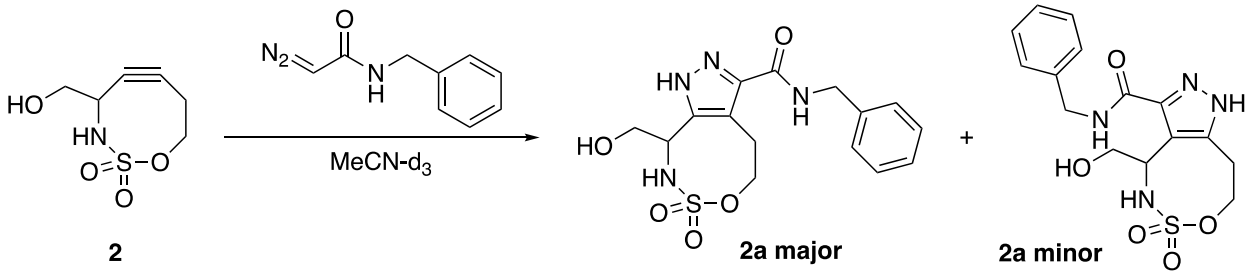

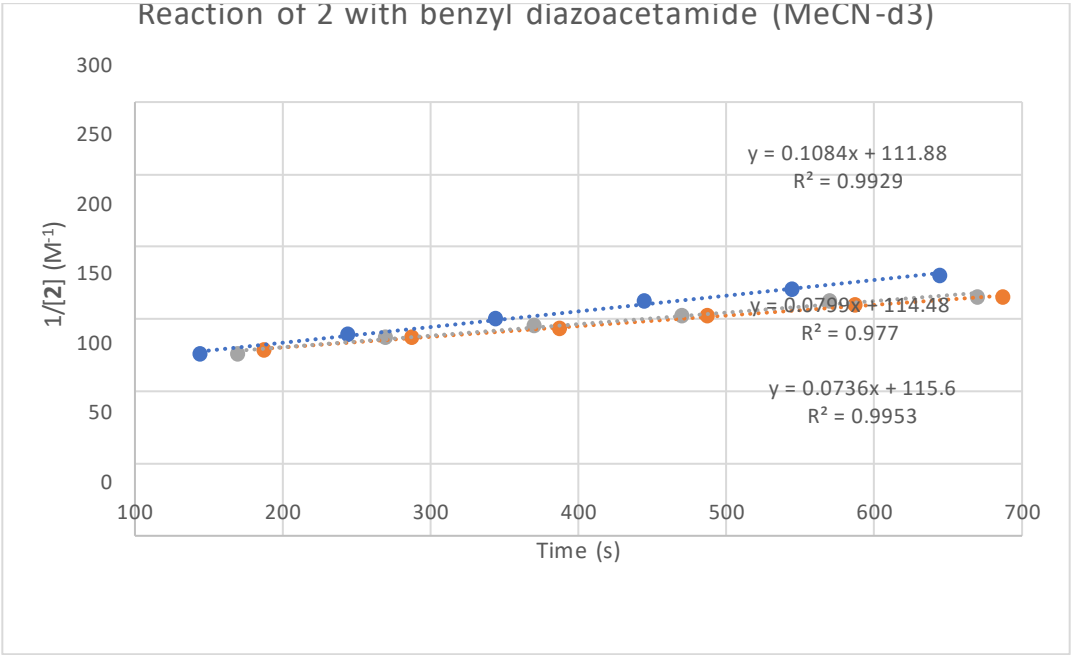

$k_2 = 0.087 \pm 0.011\ M^{-1}\ s^{-1}$

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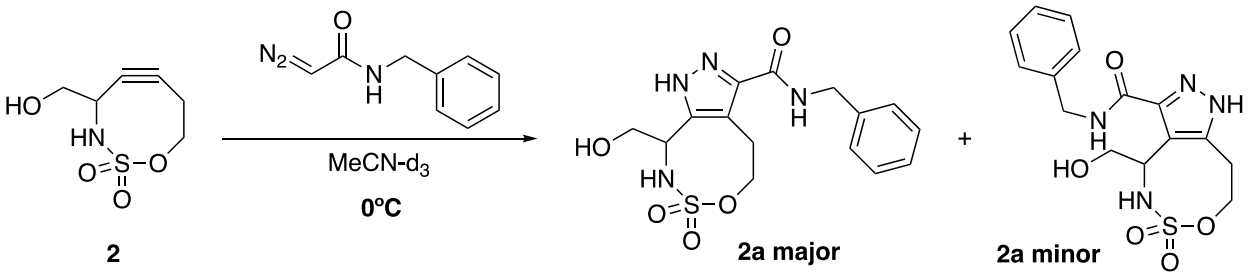
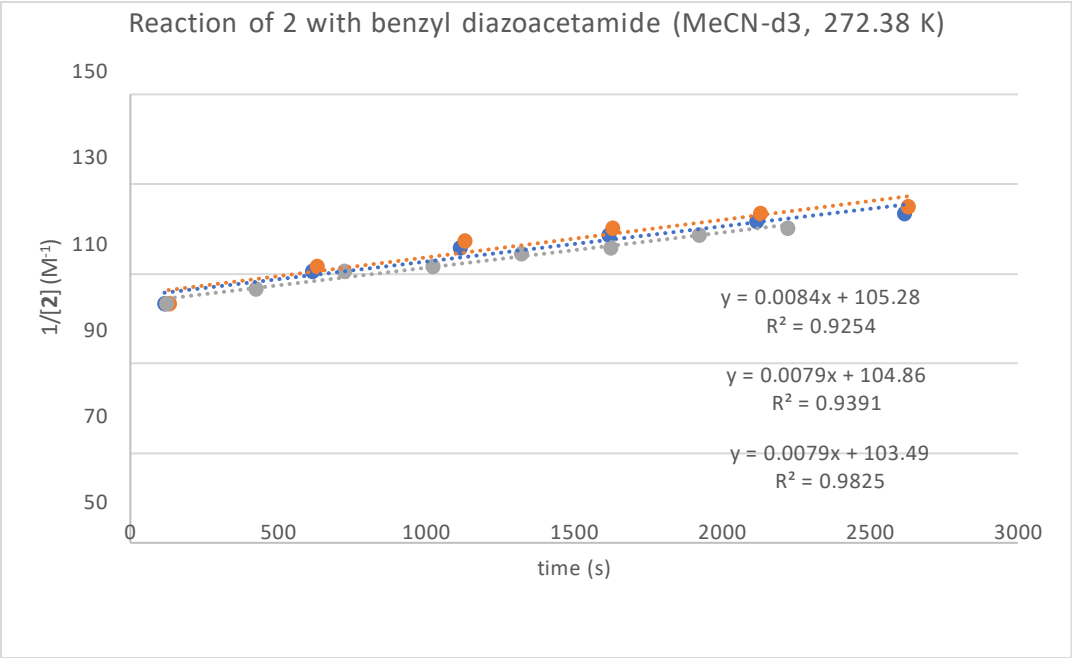




$k_2 = 0.0081 \pm 0.0002\ M^{-1}\ s^{-1}$

VIII. Plate reader kinetics for SNO-OCT/tetrazine cyclization.

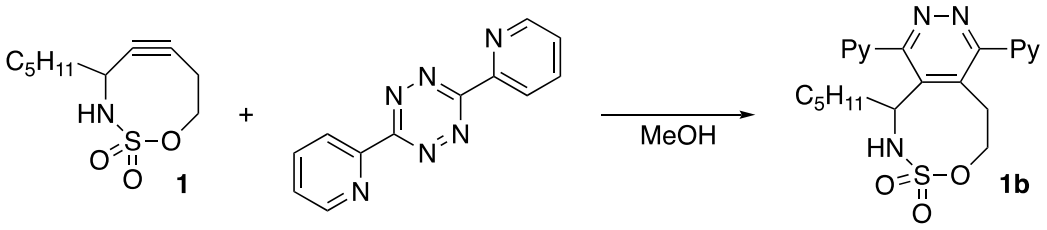
General procedure. A stock solution of SNO-OCT (0.02 M) and a stock solution of 3,6-di-2-pyridyl-1,2,4,5-tetrazine (0.002 M) both in methanol were prepared. The kinetics between SNO-OCTs and tetrazine was monitored using on Perkin-Elmer Envision plate reader in clear flat-bottomed 96-well pates. 100 µL of tetrazine solution was first added to a well and the initial absorption was measured ($\mathbf{A_{int}}$). After the addition of SNO-OCT solution to the tetrazine containing well, the decrease of tetrazine absorbance was measured at 531 nm over a period of 9,000 seconds with an interval of 300 seconds. All experiments were performed in triplicates. The second order kinetic rate ($\mathbf{k_2}$) was derived from the equation below:

(1) $$\mathbf{\varepsilon l = A_{int} / 0.002}$$

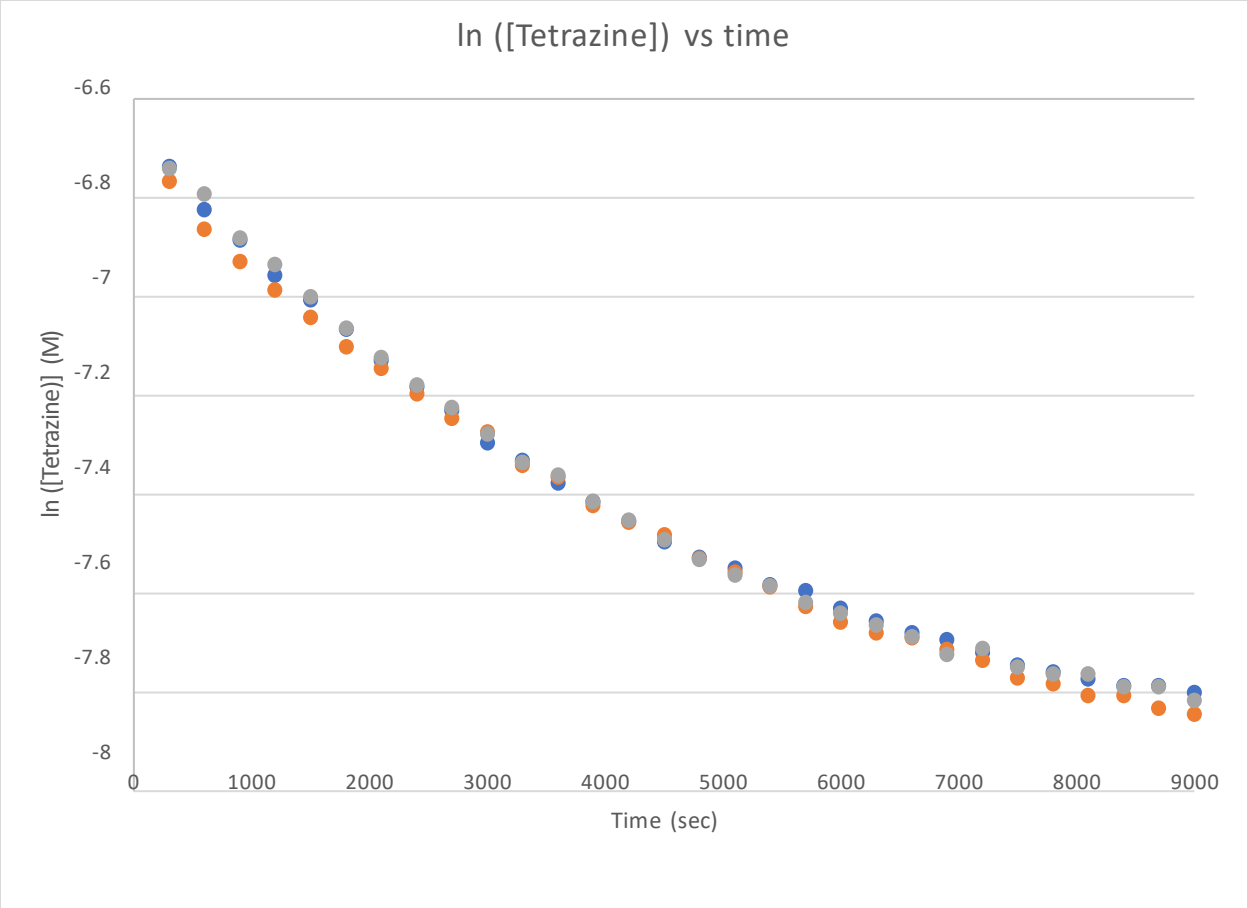
(2) $$\mathbf{\ln [tetrazine] = \ln (A_{1\ to\ 300} / (\varepsilon l \times 2))}$$

(3) $$\mathbf{k_{obs} = slope\ of\ \ln([tetrazine])\ vs\ time}$$

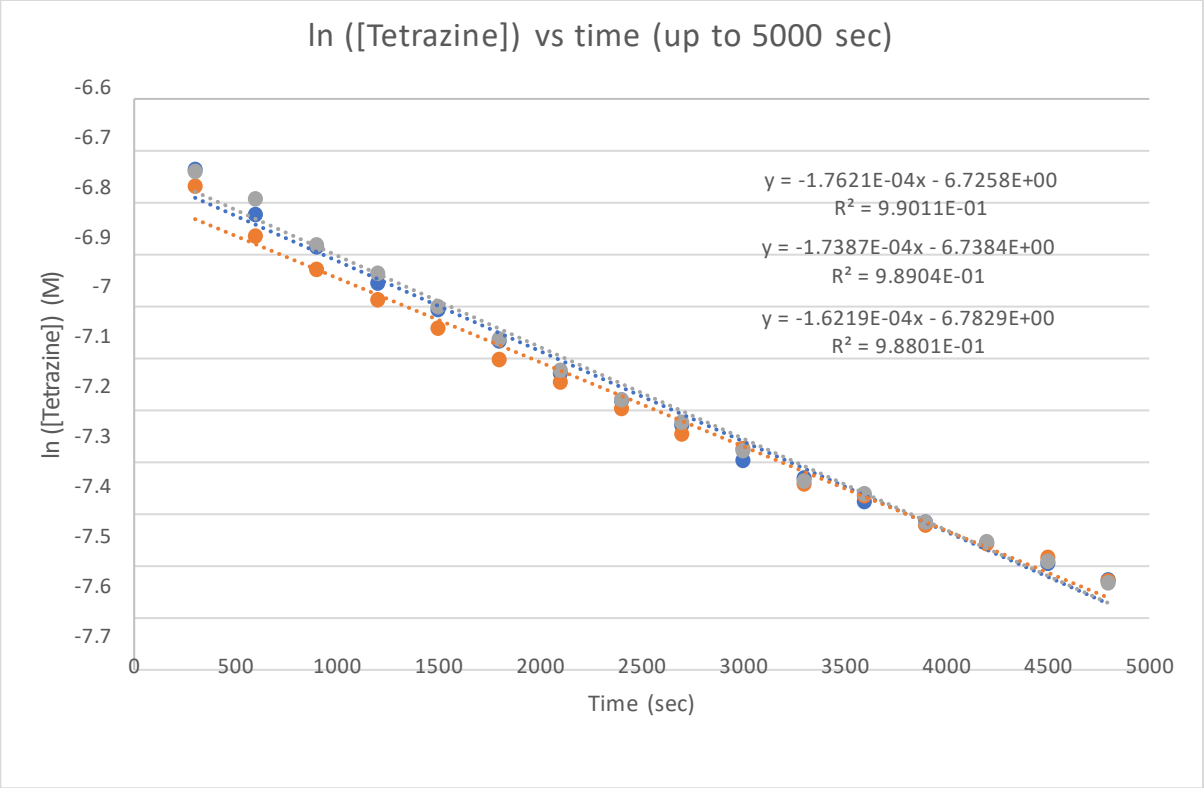
(4) $$\mathbf{k_2 = k_{obs} / 0.01}$$



Well No.	Abs 1	Abs 2	Abs 3	Average
A int	0.171	0.199	0.181	0.184
εl	85.5	99.5	90.5	91.83



Note: Due to the change of the slope pass 5000 seconds, we decided to use data points before 5000 seconds to achieve a more accurate initial kinetics data. A reduced version of data points up to 5000 seconds can be seen below.



Well No.	Abs 1	Abs 2	Abs 3	Average
kobs	1.7621E-04	1.6219E-04	1.7387E-04	1.7076E-04
k2	1.7621E-02	1.6219E-02	1.7387E-02	1.7076E-02

$k_2 = 0.0171 \pm 0.0004\ M^{-1}\ s^{-1}$

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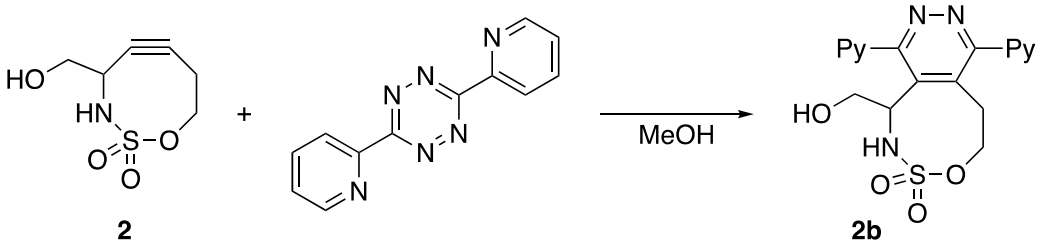
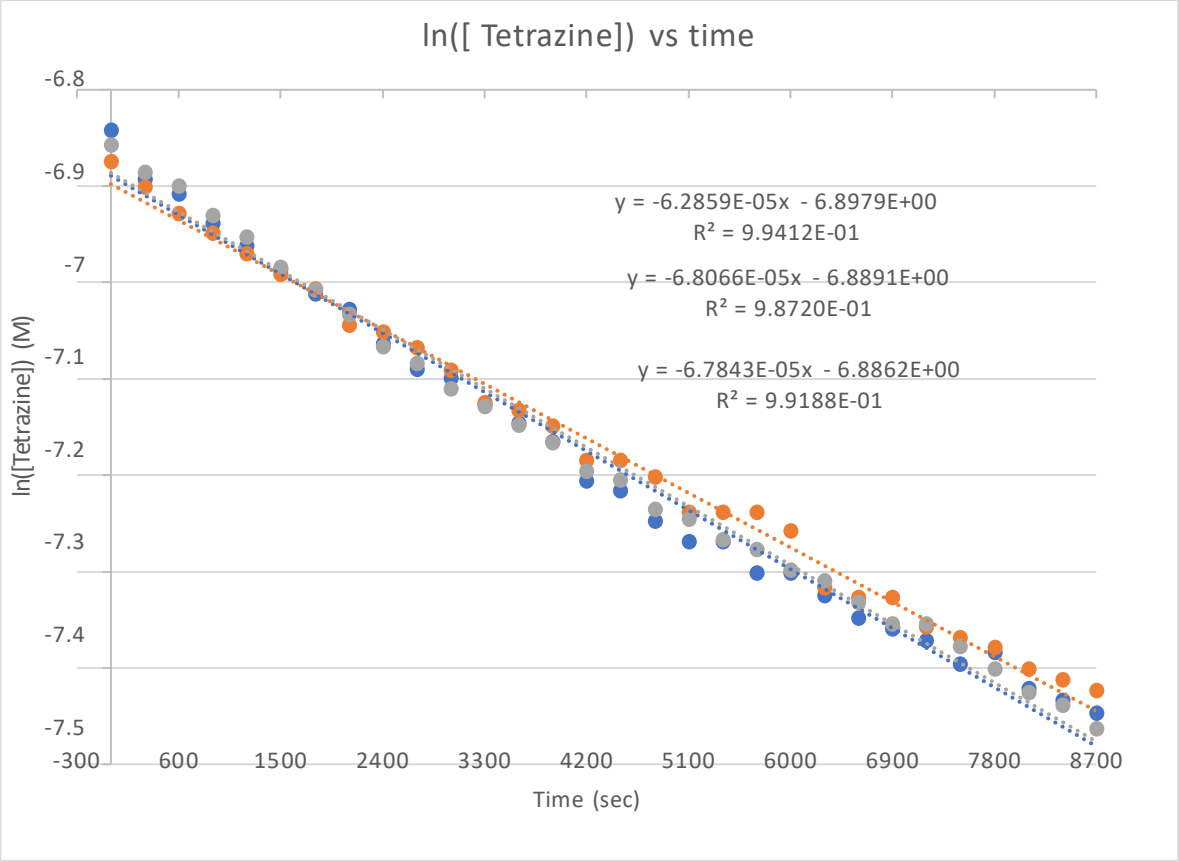


2 **2b**

Well No.	Abs 1	Abs 2	Abs 3	Average
A int	0.132	0.149	0.136	0.139
εl	66.0	74.5	68.0	69.5

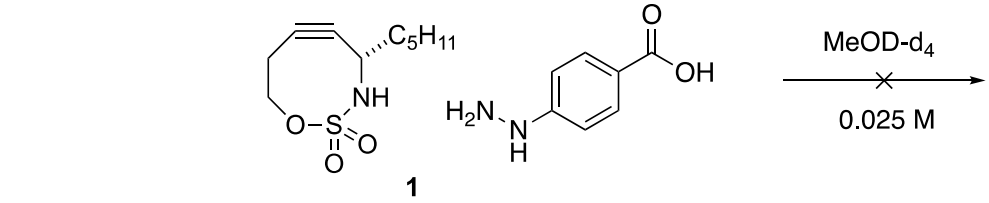


Well No.	Abs 1	Abs 2	Abs 3	Average
kobs	6.8066E-05	6.2859E-05	6.7843E-05	6.6256E-05
k2	6.8066E-03	6.2859E-03	6.7843E-03	6.6256E-03

$k_2 = 0.0066 \pm 0.0002\ M^{-1}\ s^{-1}$

IX. SNO-OCTs' reactivity study with hydrazide.

General procedure. In a vial equipped with a stir bar, SNO-OCT (1 equiv) was dissolved in MeOD-d_4 and then hydrazine (1 equiv) was added to the mixture. The mixture was stirred under room temperature overnight. After 24 hours, one equivalence of mesitylene internal standard was added to the reaction mixture and the sample was then transferred to an NMR-tube to be analyzed.



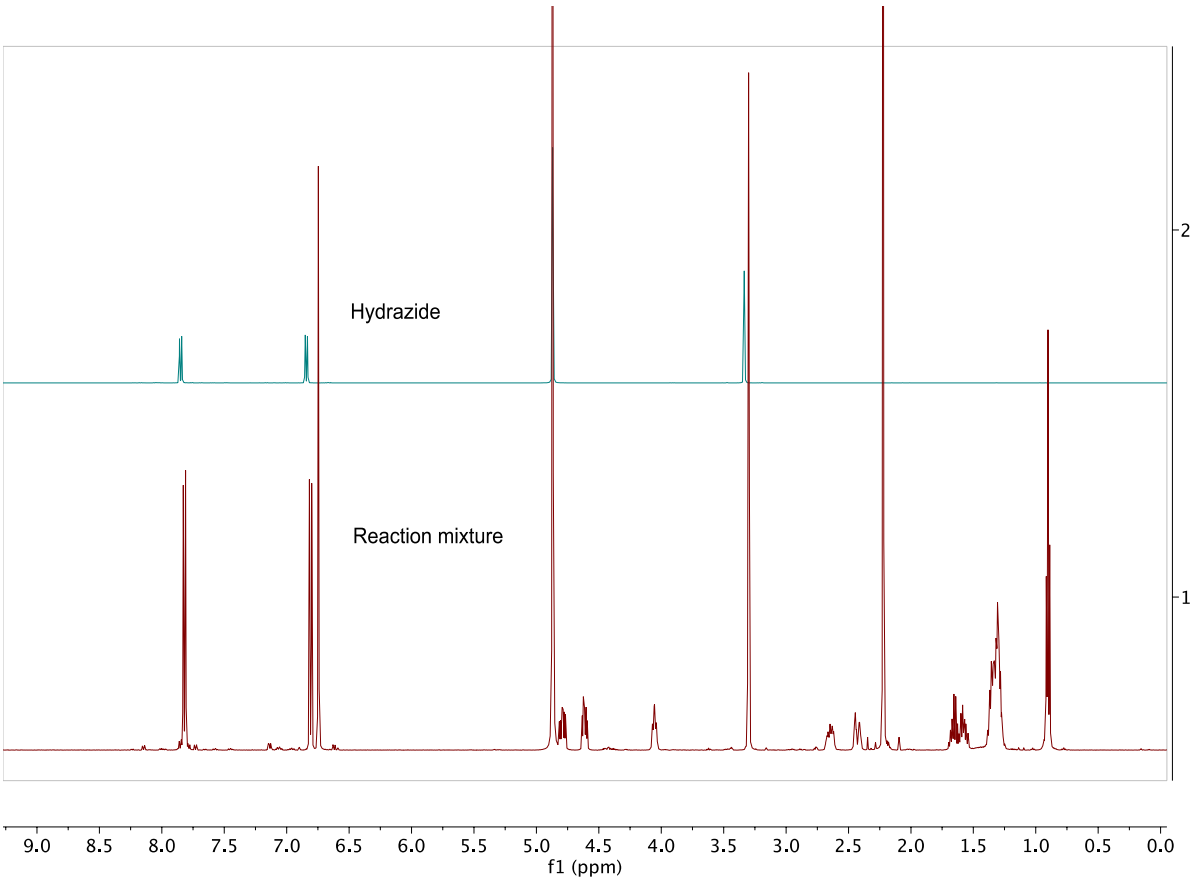


Figure S2-9A. Overlaid spectra shows hydrazide remains in the mixture unreacted.

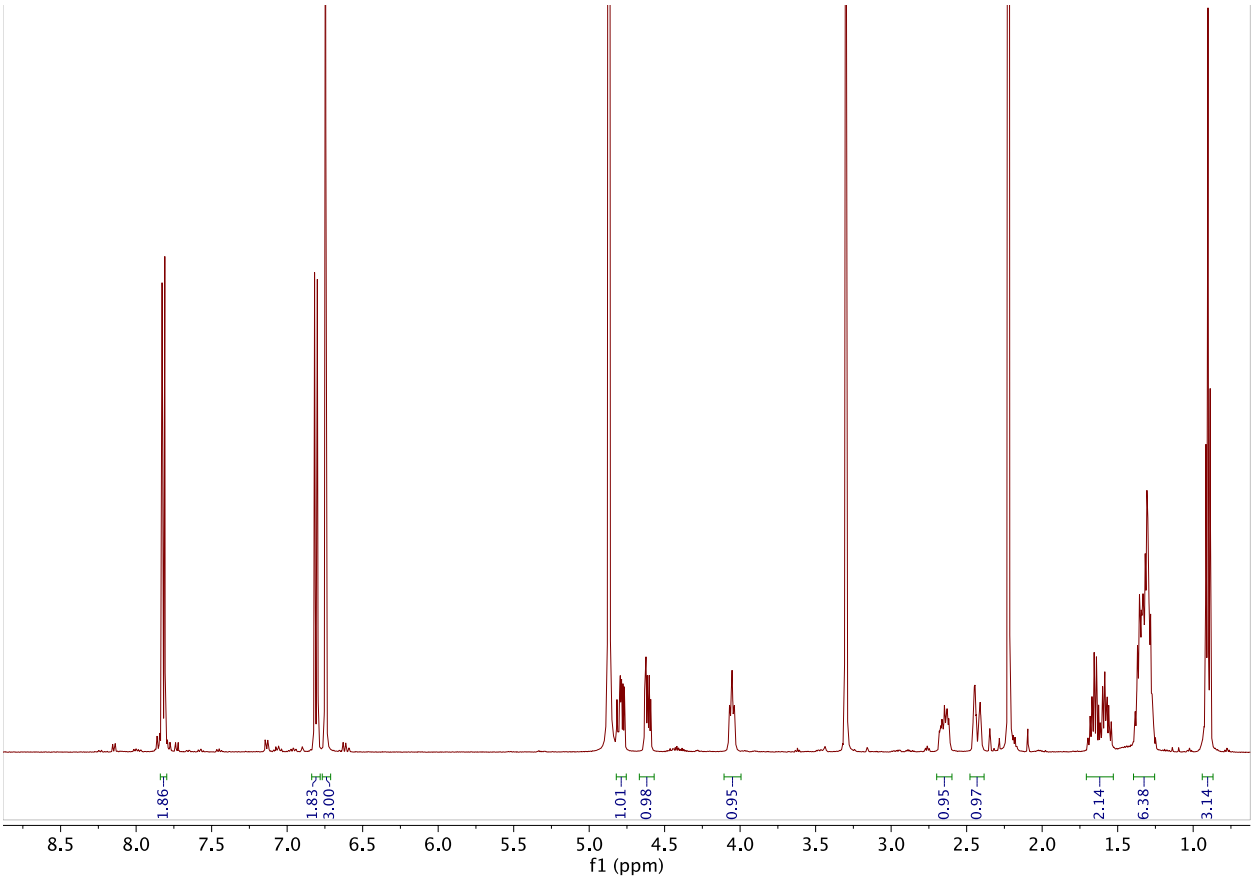


Figure S2-9B. Due to the observed low solubility of hydrazine, the integration appears to be lower. On the other hand, SNO-OCT **1** has mostly remained unreacted based on the integration.

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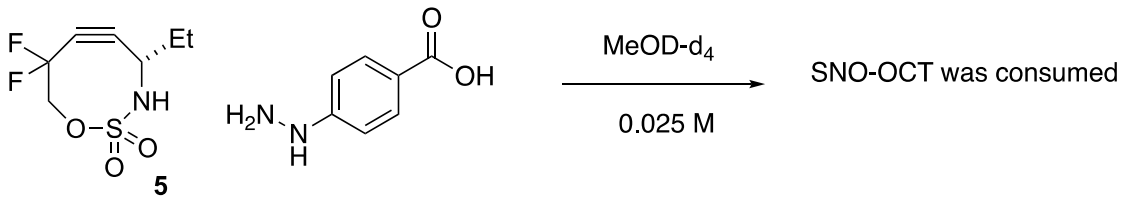


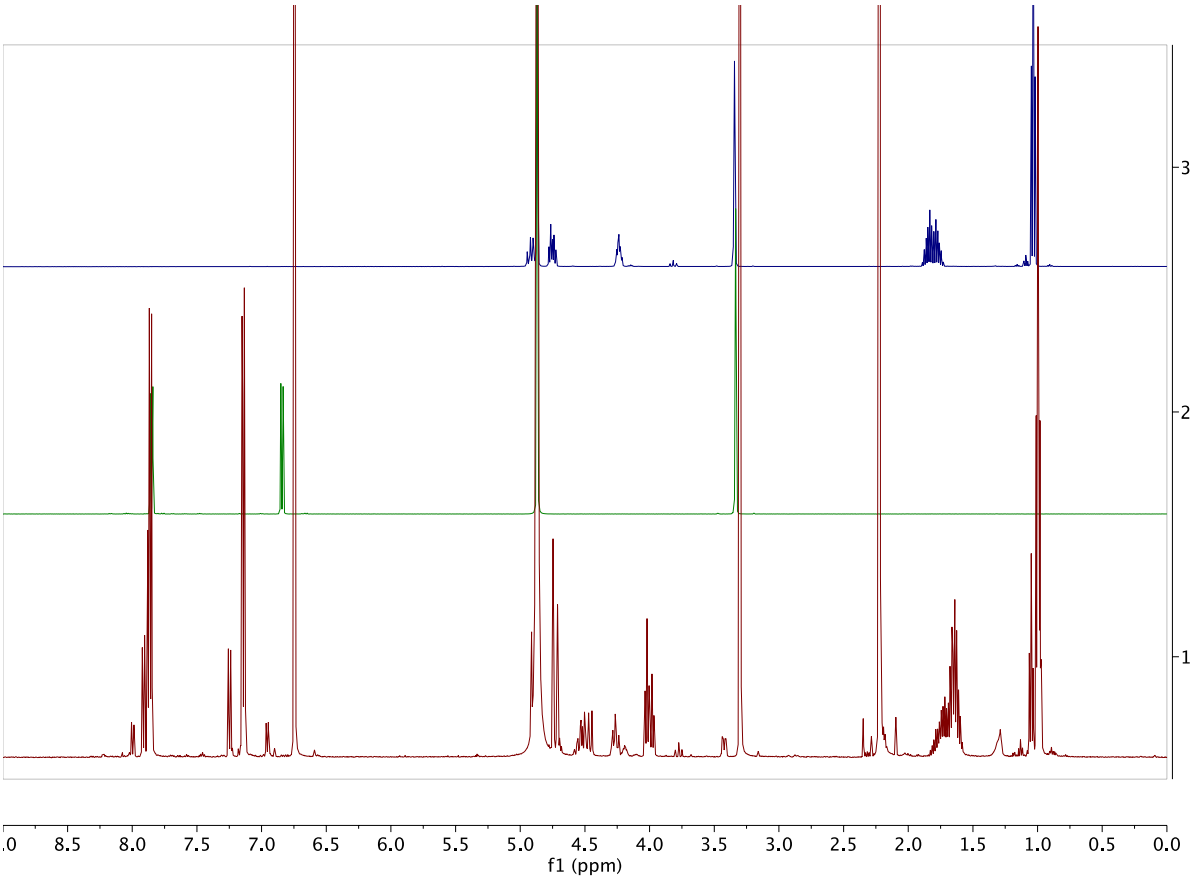
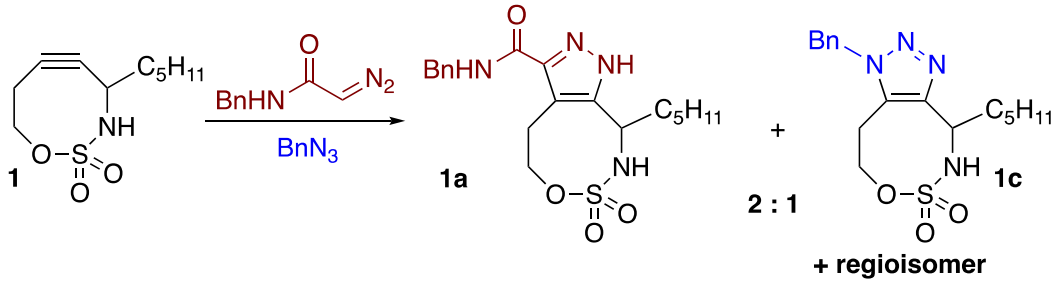


Figure S2-10. Overlaid spectra shows both SNO-OCT **5** and hydrazine were consumed. Therefore, we conclude that SNO-OCT **5** is reactive to hydrazine. The resulting products' isolation on silica was attempted, but this resulted an insoluble white solid when we tried to dissolve the products in MeOD-d_4.

X. Reactions of one SNO-OCT with two competing dipoles or one dipole and one hydrazine.

General procedure 1. This procedure was adopted from a previously published competition methods.[12] Stock solutions (0.02 M in acetonitrile-d_3) of the SNO-OCTs and the dipoles were prepared. One equivalence of SNO-OCTs were combined together in a vial equipped with a stir bar followed by the addition of one equivalence of the dipole solutions. The reaction was allowed to stir under room temperature for one hour for full conversion. All volatiles were removed under reduced pressure. The selectivity was determined based on NMR integrations of the products peaks.



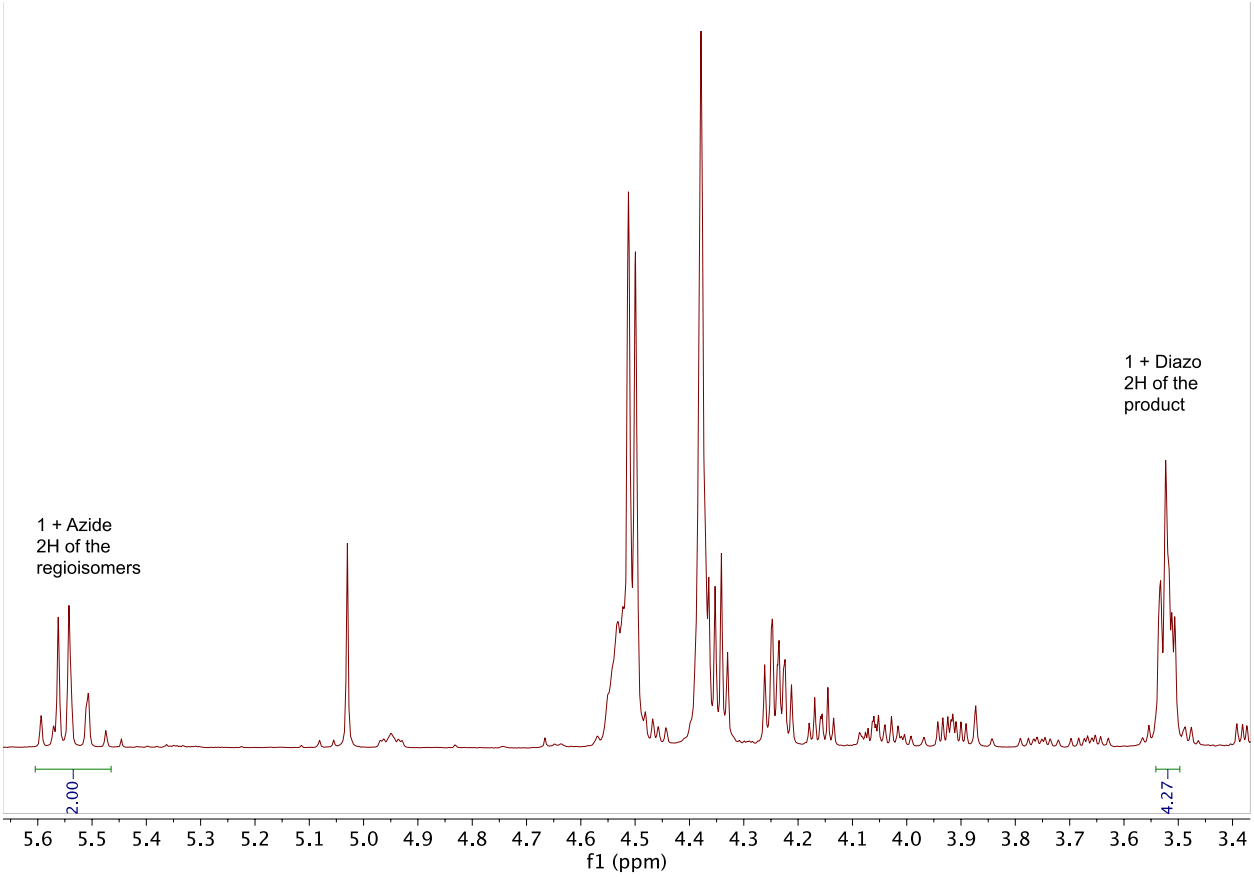


Figure S2-11. In the competition between benzyl diazoacetamide and benzyl azide for **1**, a ratio of the cycloaddition products was calculated based on the product formation ratio between **1** and diazoacetamide. We calculated that there would be 4.27 equiv of **1a** against 2.00 equiv of **1c**. Resulting a ratio of 2 : 1.

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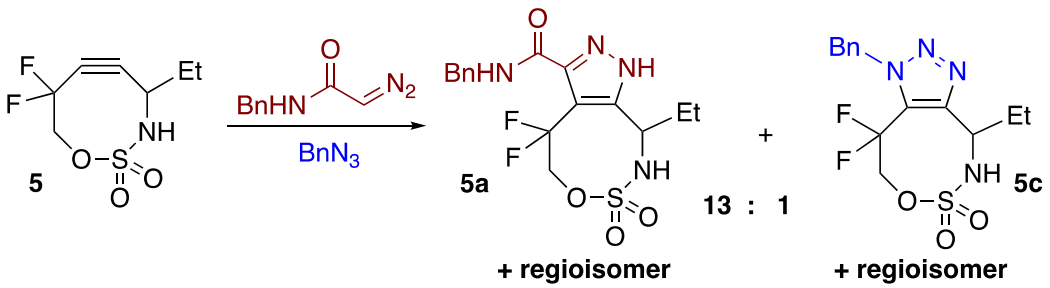

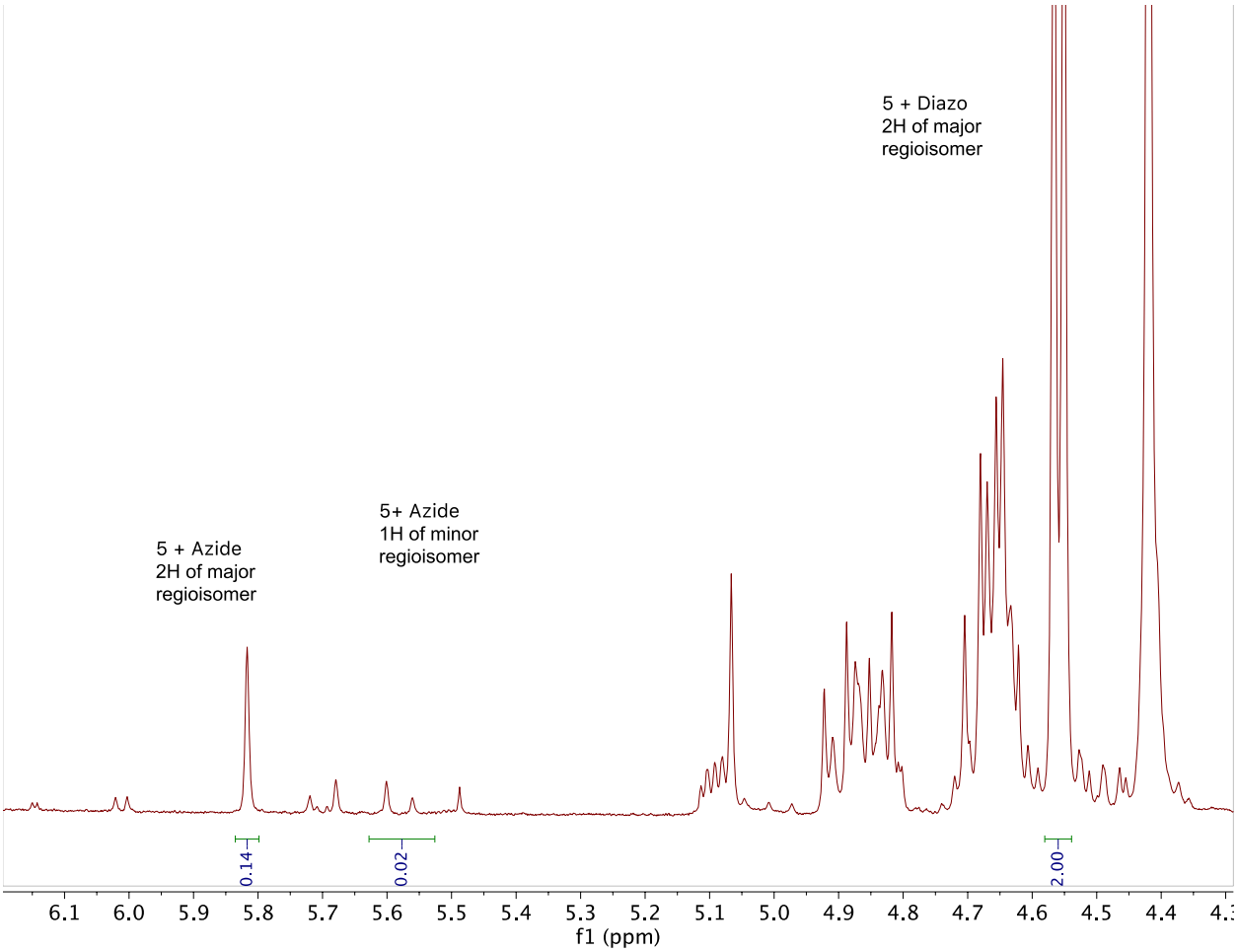

Figure S2-12. In the competition between benzyl diazoacetamide and benzyl azide for **5**, a ratio of the cycloaddition products was calculated based on the product formation ratio between **5** and diazoacetamide. We calculated that there would be 1.16 equiv of **5a** against 0.09 equiv of **5c**. Resulting a ratio of 13 : 1.

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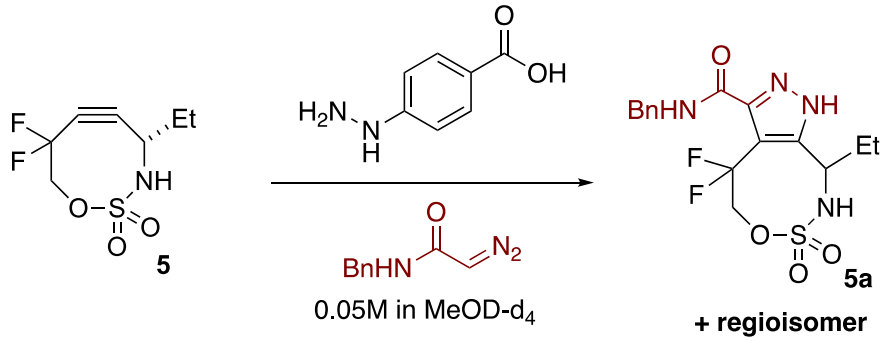
General procedure 2. This procedure was adopted from a previously published competition methods.[12] In a vial equipped with a stir bar, added hydrazine (1 equiv) and benzyl diazoacetamide (1 equiv). The mixture was then dissolved in MeOD-d_4 (0.05M). Then one equivalence of SNO-OCT **5** was added to the mixture and the reaction was stirred under room temperature for 19 hours. The selectivity was determined based on NMR integrations of the products peaks against mesitylene internal standard (1 equiv).



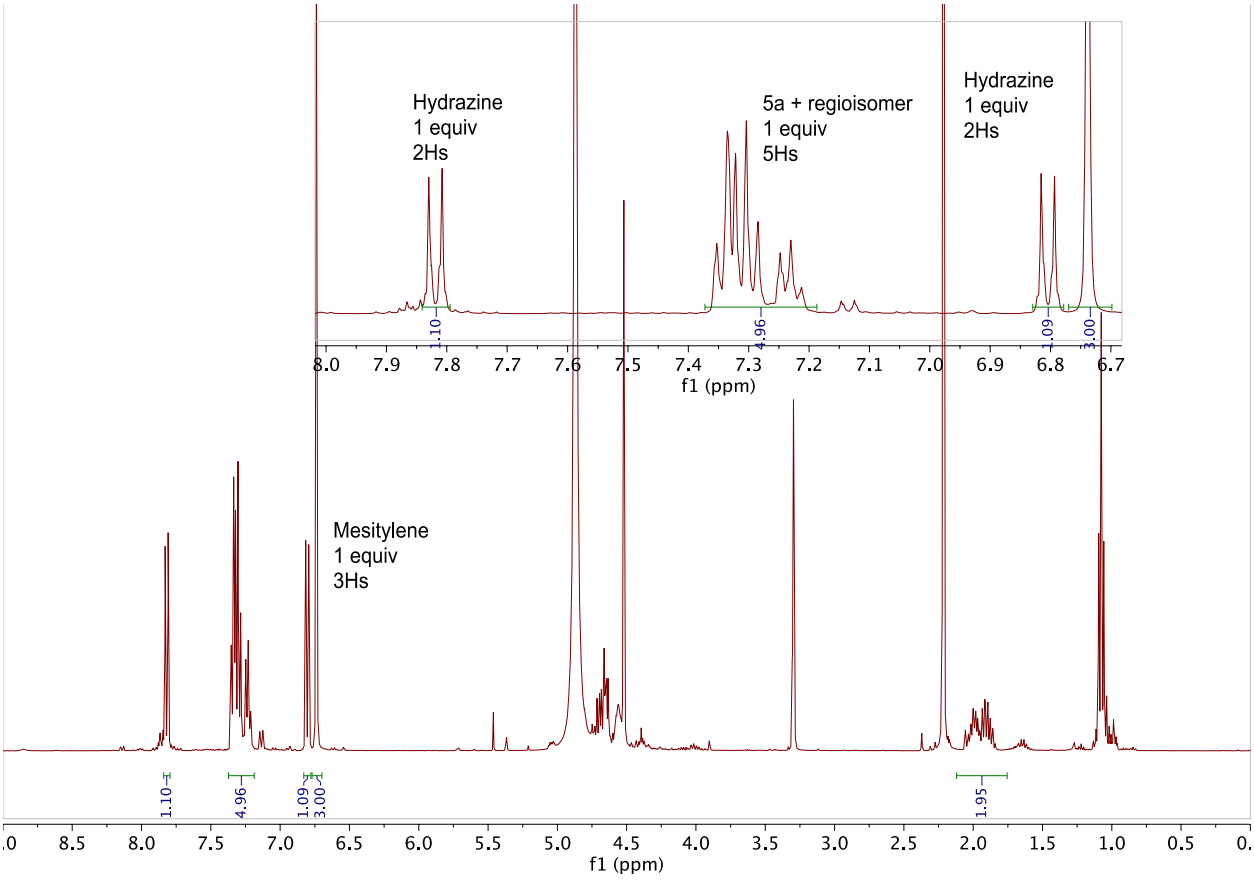


Figure S2-13A. Due to the overlap of major proton signals of **5a** between 5.0 and 4.0 ppm, the selectivity of **5** for benzyl diazoacetamide over hydrazine was determined based on the integration of the remaining hydrazide and **5a** and its regioisomer's aryl protons.

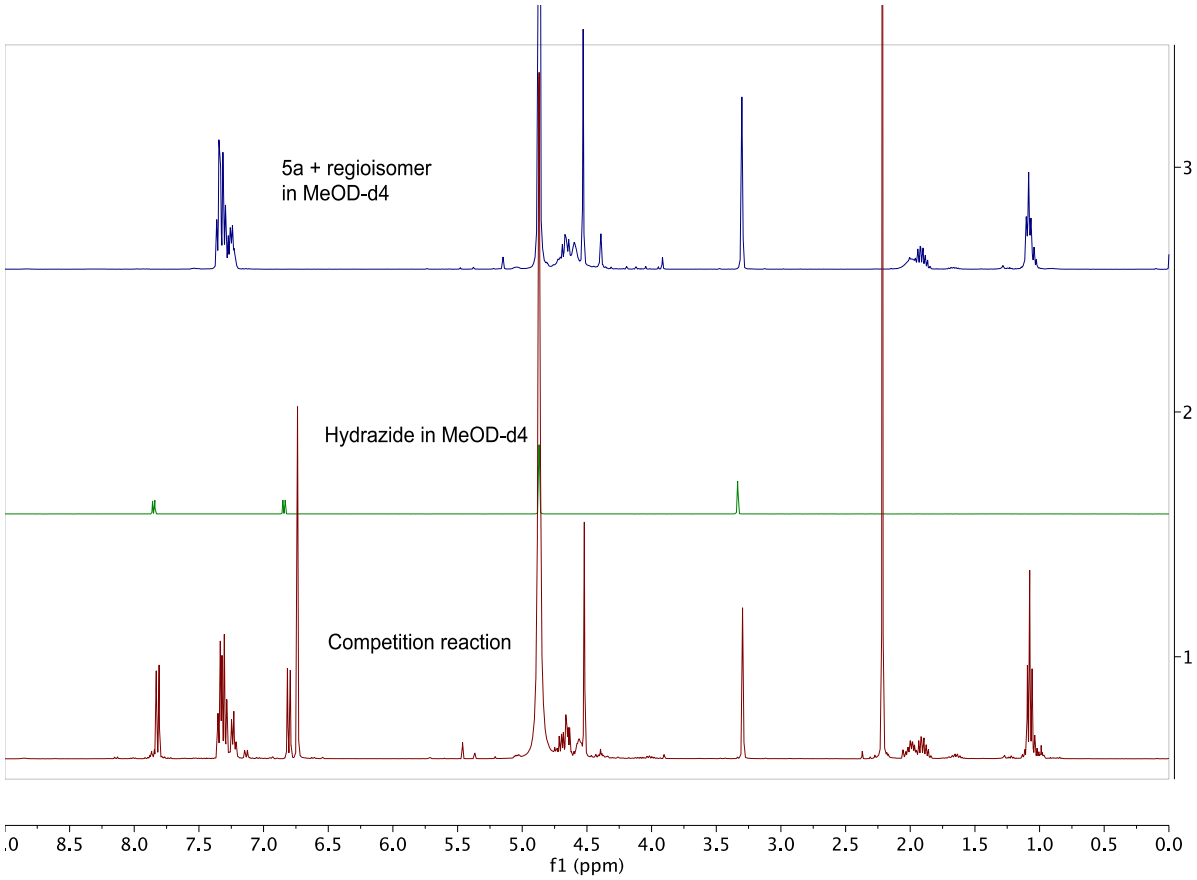
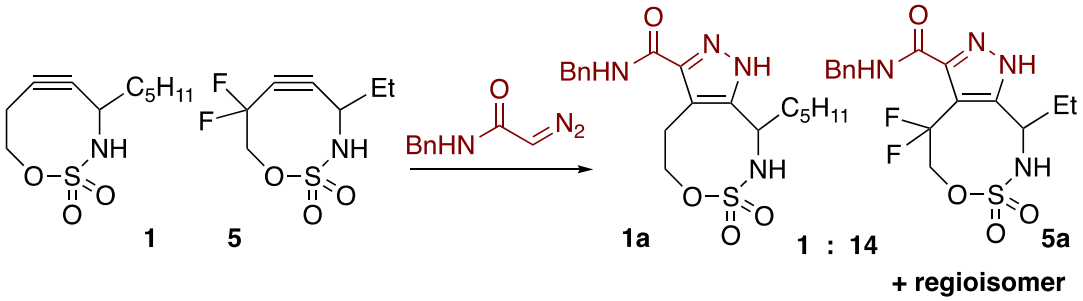


Figure S2-13B. The overlaid spectra show an exclusive formation of the cycloaddition products **5a** (+ regioisomer).

XI. Reactions of two SNO-OCTs for one dipole.

General procedure 1. This procedure was adopted from a previously published competition methods.[12] Stock solutions (0.02 M in acetonitrile-d_3) of the SNO-OCTs and the dipoles were prepared. One equivalence of the SNO-OCTs were combined together in a vial equipped with a stir bar followed by the addition of one equivalence of the dipole solution. The reaction was allowed to stir under room temperature for one hour for full conversion. All volatiles were removed under reduced pressure. The selectivity was determined based on NMR integrations of the products peaks.



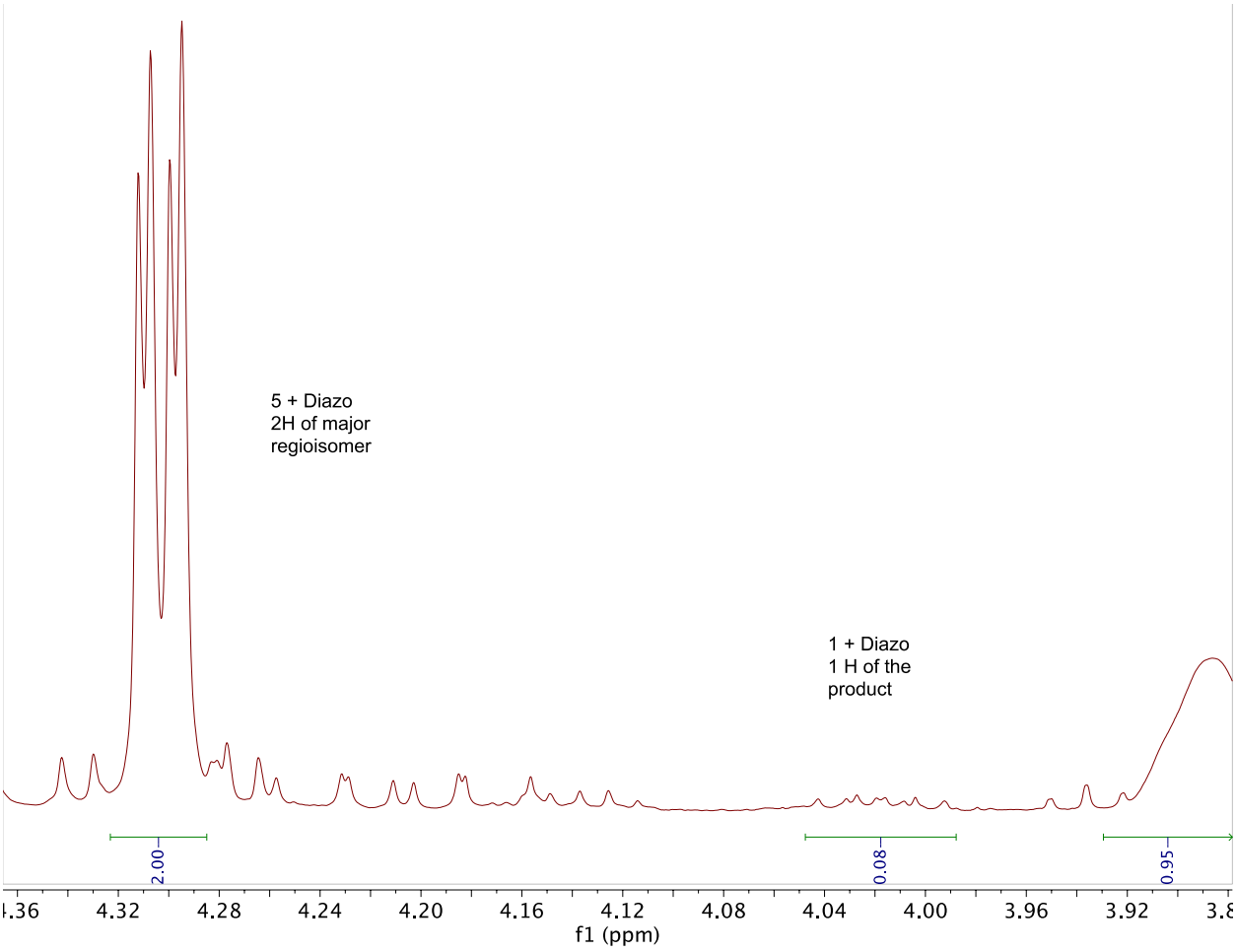


Figure S2-14. In the competition between **1** and **5** for benzyl diazoacetamide, the addition cycloaddition product was calculated based on the product **1a major**'s 2Hs at benzylic position. We calculated that there would be 1.16 equiv of **5a** against 0.08 equiv of **1a**. Resulting a ratio of 14 : 1.

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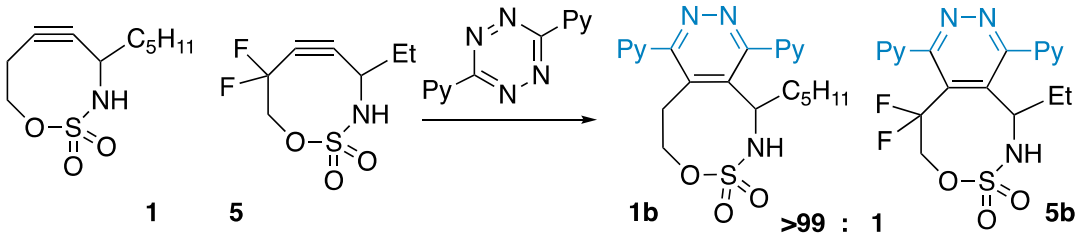

Figure S2-15. In the competition between **1** and **5** for dipyridyl tetrazine, only the product between **1** and dipyridyl tetrazine **1b** was observed. The NMR spectra matches previously reported values.[14]

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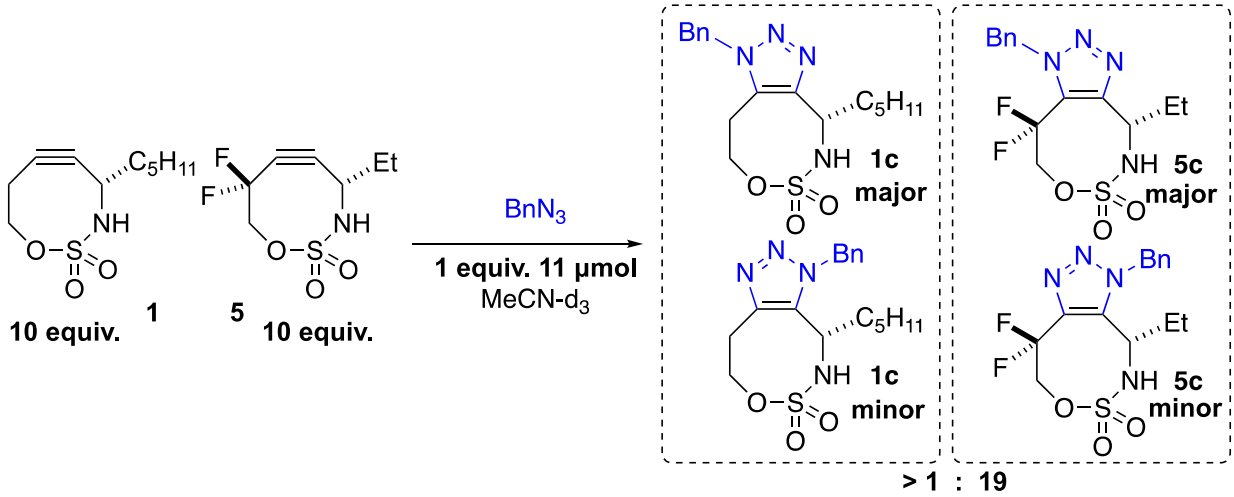
General procedure 2. This procedure was adopted from a previously published competition methods.[13] In a vial equipped with a stir bar, added SNO-OCT **1** and **5** (0.11 mmol, 10 equiv each) and then dissolved both with 0.6 mL MeCN-d_3 or 0.9 mL D_2O/MeCN-d_3 (2:1). One equivalence of the dipole was then added to the stirring solution. The reaction was allowed to stir under room temperature for one hour to achieve completion. The mixture is then transferred directly to an NMR tube for analysis. The selectivity was determined based on the integrations of the products peaks.



Note: Based on experimental measurement, we know the cycloaddition rate k_2 in MeCN-d_3 between **1** and azide is 0.026 $M^{-1}•s^{-1}$, and between **5** and azide is 0.46 $M^{-1}•s^{-1}$. Therefore based on Fox and coworkers' method,[13] we would expect a competition ratio of 1 : 18. Here we have estimated a ratio larger than 1:19

and probably less than 1 : 33, which falls well within an order of magnitude difference. Therefore, we believe this competition experiment is a valid way to provide an estimation for our reaction kinetics.

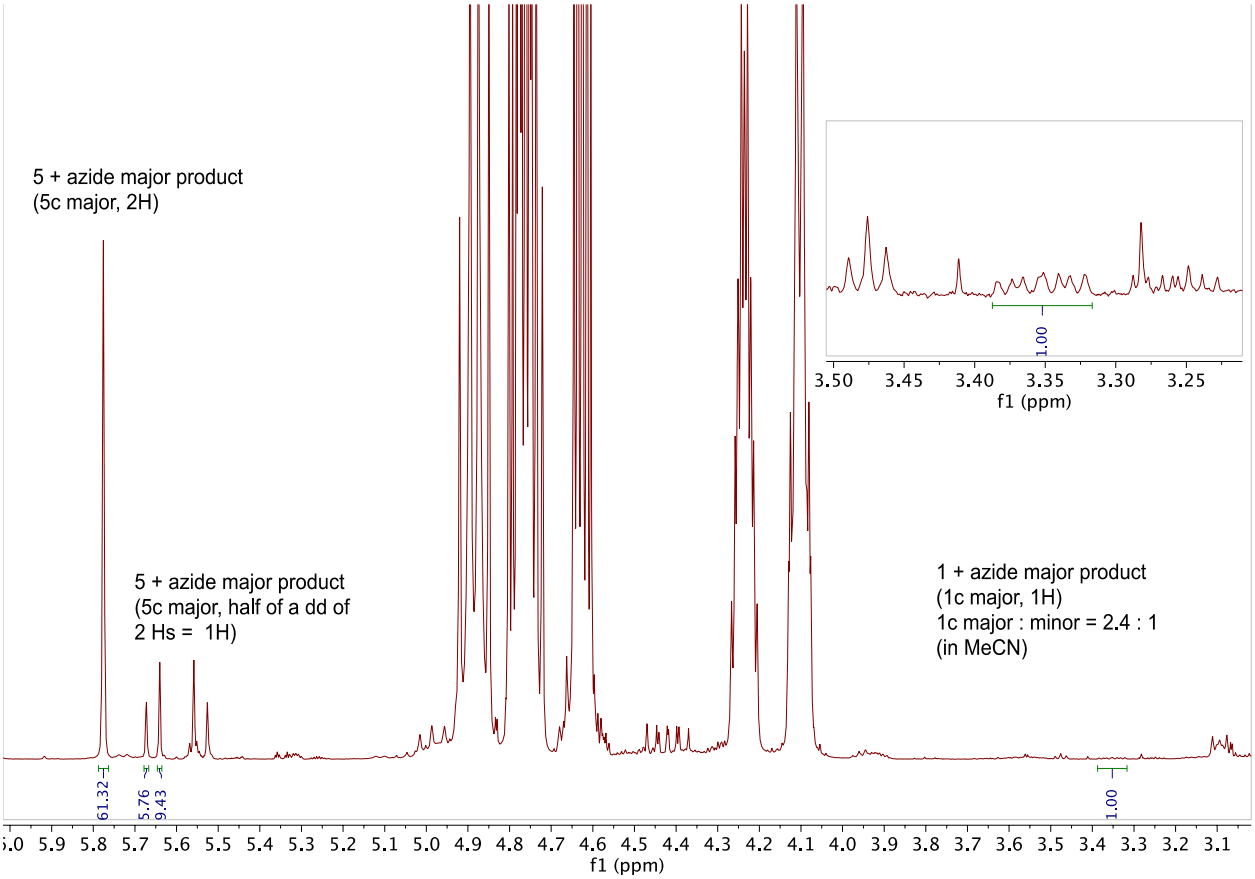


Figure S2-16A. In the competition between **1** (10 equiv) and **5** (10 equiv) for benzylazide (1 equiv), product **1c major** is compared with products **5c** resulting a ratio of **1c : 5c = 1 : 33**. However, considering the detection limit using NMR, we decided the selectivity to be larger than **1 : 19** out of precaution.

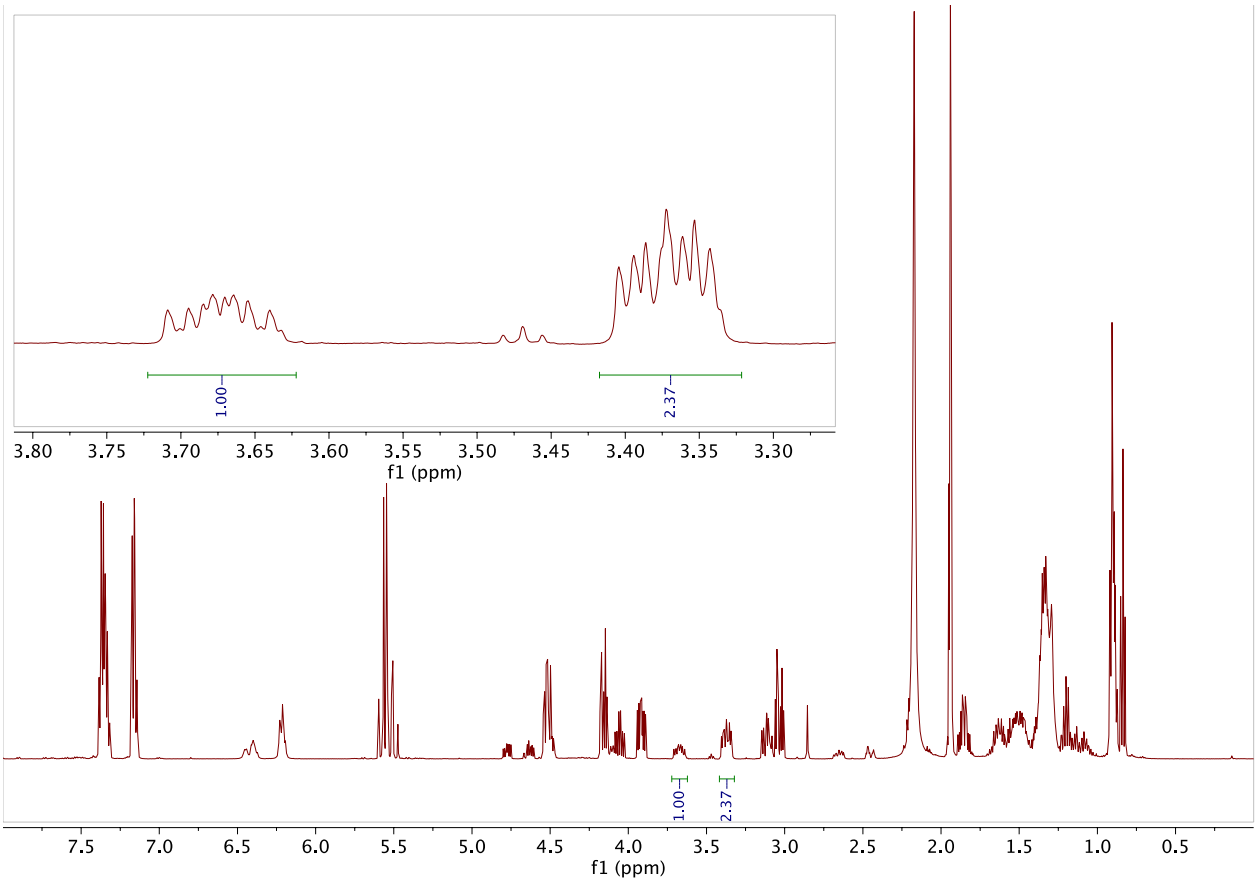
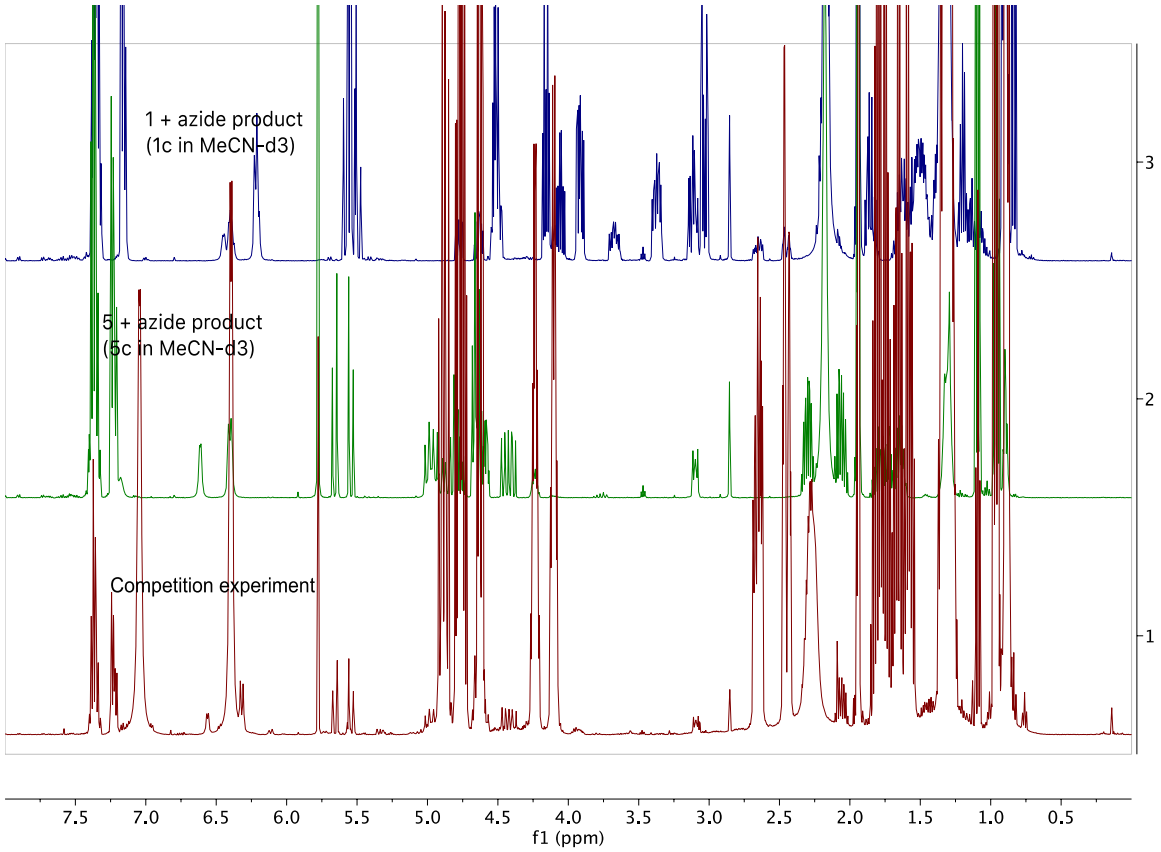


Figure S2-16B. The regioisomer ratio of **1c** was determined by mixing SNO-OCT **1** (1 equiv) and benzylazide (1 equiv) in MeCN-d_3, resulting product resulting **1c major :minor = 2.4 : 1**.


1 + azide product
(1c in MeCN-d3)
5 + azide product
(5c in MeCN-d3)
Competition experiment
3
2
1
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)

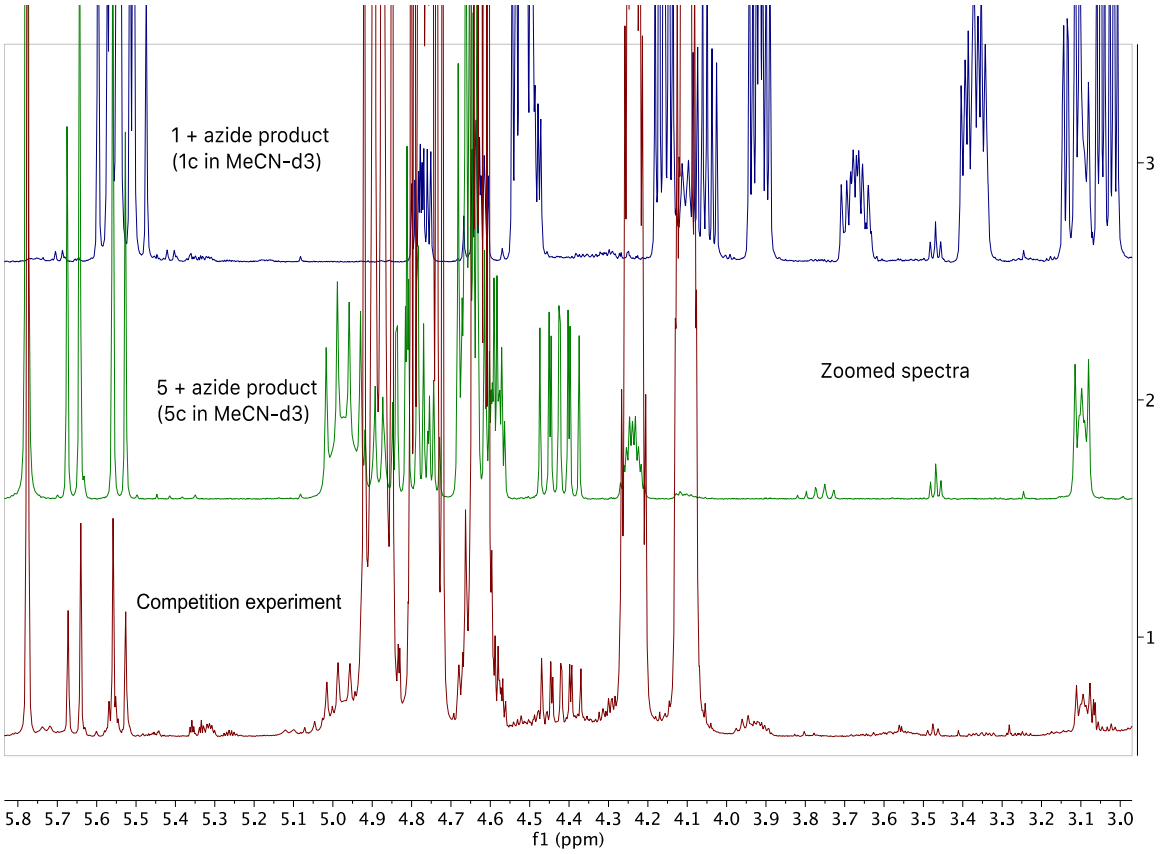

1 + azide product
(1c in MeCN-d3)
5 + azide product
(5c in MeCN-d3)
Competition experiment
Zoomed spectra
3
2
1
5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0
f1 (ppm)

Figure S2-16C. The overlaid spectra show that the competition experiment overwhelmingly favors the reaction between **5** and benzyl azide over **1** and benzyl azide.

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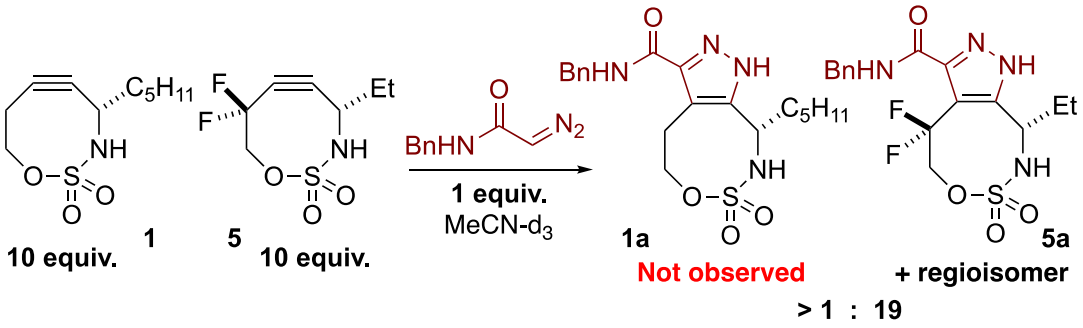
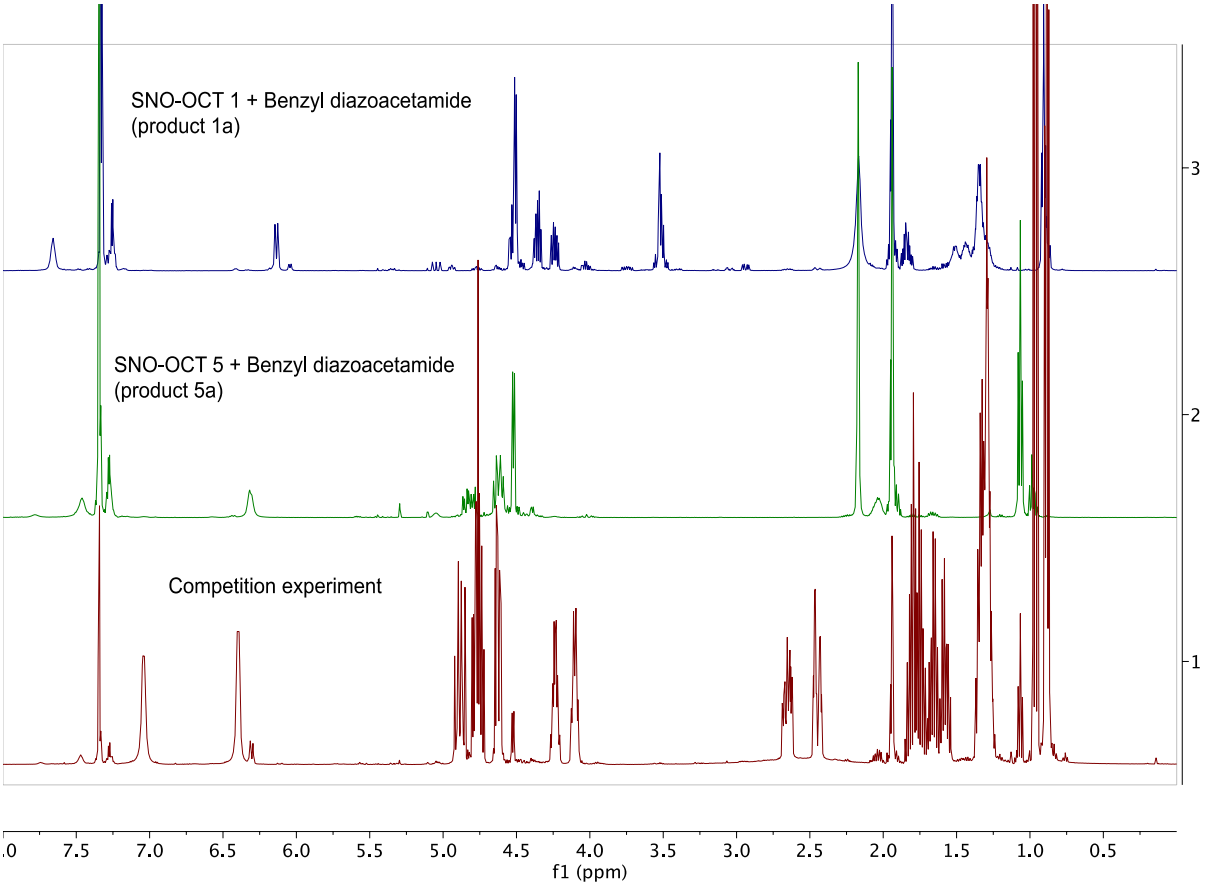


Note: In Figure S2-16, we have concluded the validity of estimating kinetics using competition experiment. Herein, based on the overlaid spectra, we observed an even larger degree of selectivity since we could not even observe any formation of **1a**. Therefore, comparing with the competition reaction using benzyl azide (Figure S2-16), we believe that this reaction has an even higher degree of selectivity. The kinetics rate between **1** and diazoacetamide was measured to be 0.270 $M^{-1}•s^{-1}$, so the estimated rate between **5** and diazoacetamide is estimated to be > 5.13 $M^{-1}•s^{-1}$.



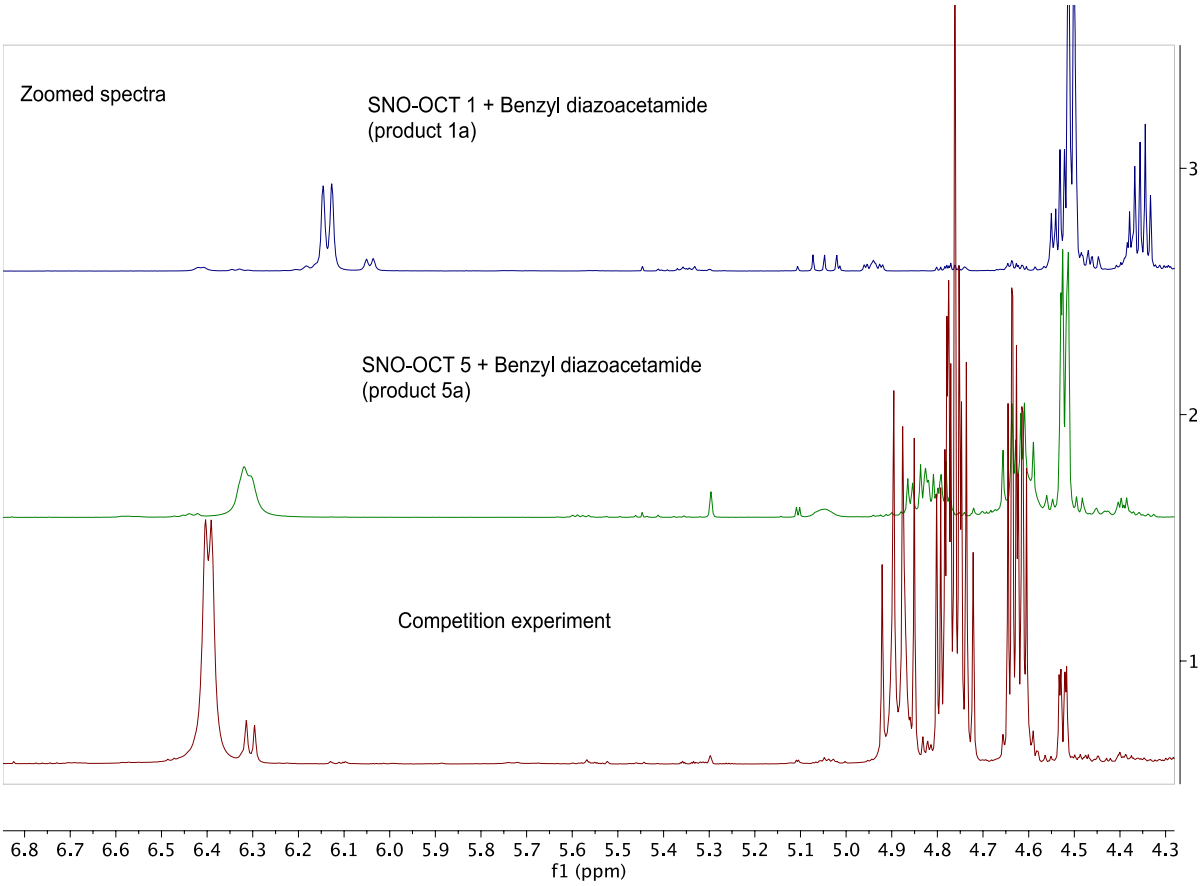


Figure S2-17. Overlaid spectra show that the competition experiment overwhelmingly favors the reaction between **5** and benzyl diazoacetamide over **1** and benzyl diazoacetamide. Based on the overlay, no **1a** was observed. We cautiously determined that the selectivity is larger than **1 : 19**.

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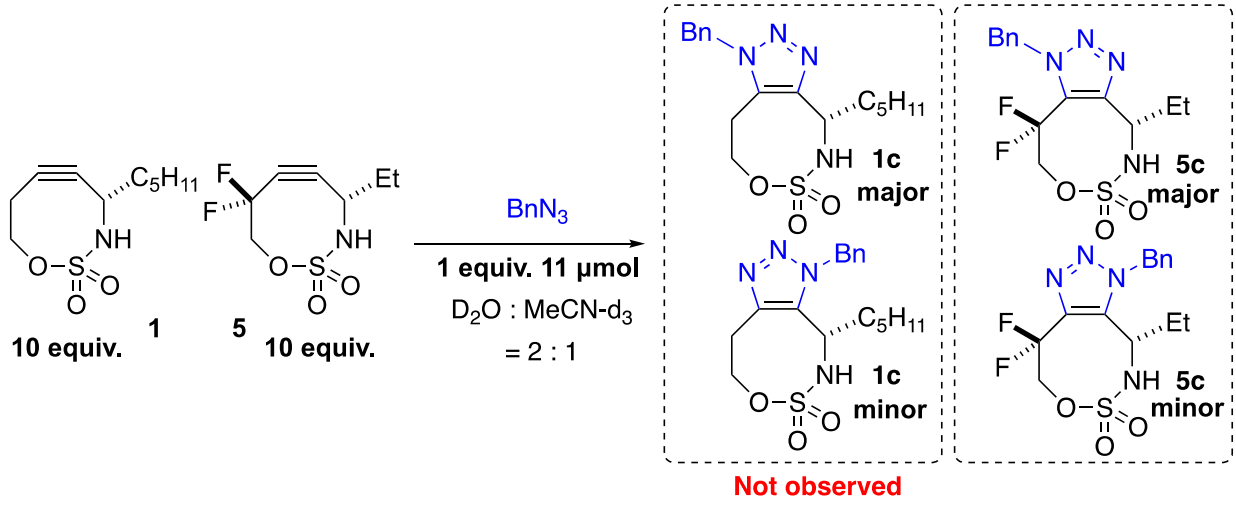
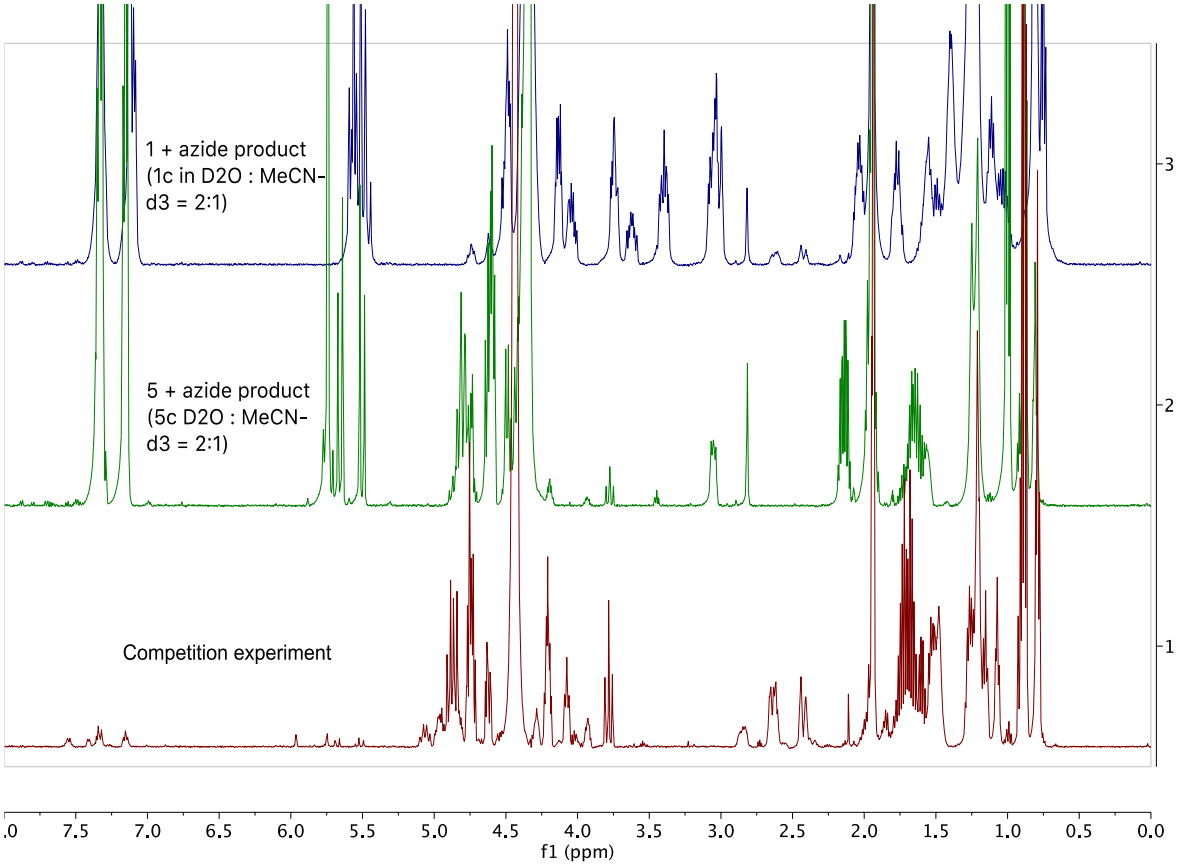


Note: Due to the poor solubility of the SNO-OCTs under the aqueous condition and the usage of an excess amount of SNO-OCTs in this competition experiment, we observed a layer of organics on top the aqueous

mixture despite the usage of sonicator. Additionally, the poor solubility require us to prepare a more dilute NMR sample solution. The solubility issue also resulted a difficult rate measurement, when we attempted to measure the rate between **1** and benzyl azide. As a result, we could not provide an estimation for the rate between **5** and azide, but we can conclude a high chemoselectivity for **5** over**1** even under an aqueous reaction condition.



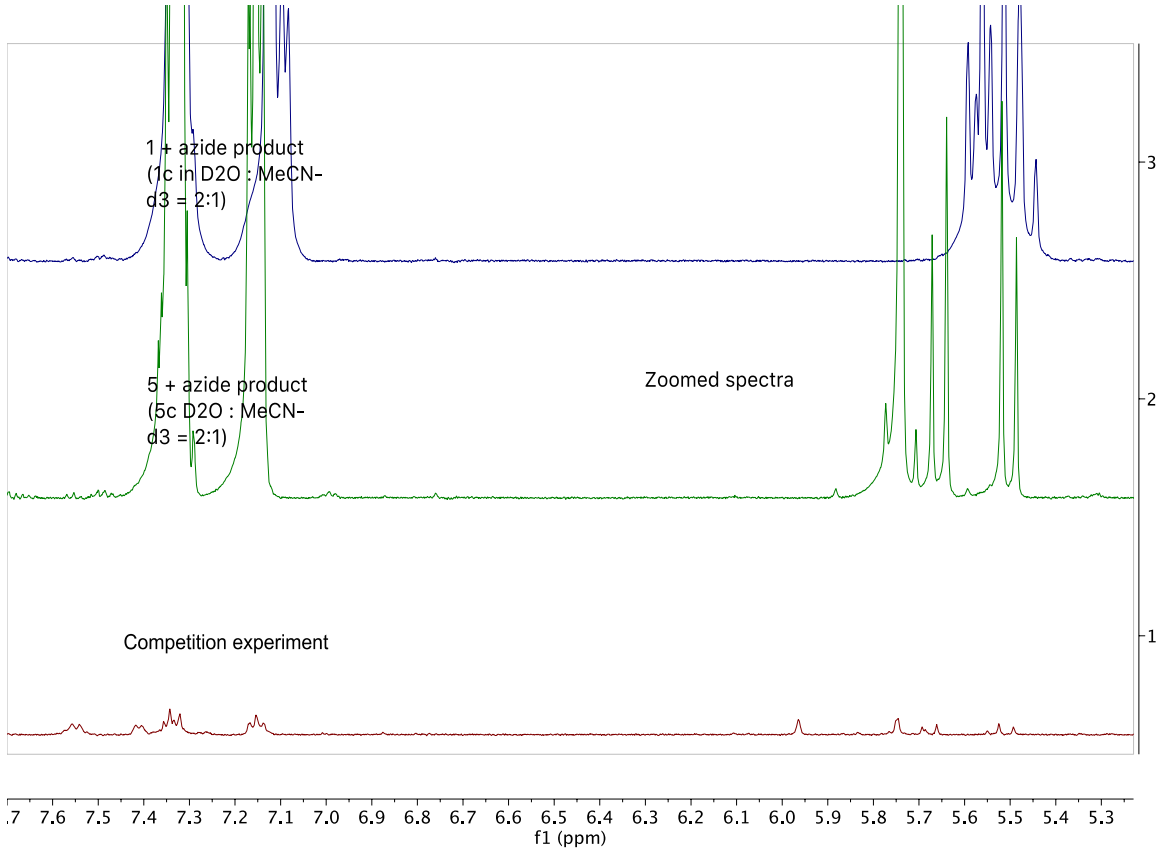


Figure S2-18. Overlaid spectra show that the competition experiment overwhelmingly favors the reaction between **5** and benzyl azide over **1** and benzyl azide in D_2O : MeCN-d_3 = 2 : 1. No peaks from **1c** was observed. However, due to a baseline much worse than the competition reactions performed in MeCN-d_3 (see Figure S2-13 and S2-14), we decided not to estimate the ratio.

XII. Reactions of two SNO-OCTs competing for two dipoles or for one dipole and one hydrazine.

General procedure 1. This procedure was adopted from a previously published competition methods.[12] Stock solutions (0.1 M in methanol) of the SNO-OCTs and the dipoles were prepared. One equivalence of dipoles were combined together in a vial equipped with a stir bar followed by simultaneous addition of two SNO-OCT solutions (one equivalence for each SNO-OCT). The reaction was allowed to stir under room temperature for one hour for full conversion. All volatiles were removed under reduced pressure. The orthogonality was analyzed using either NMR or UPLC/LC-SM calibration curve.

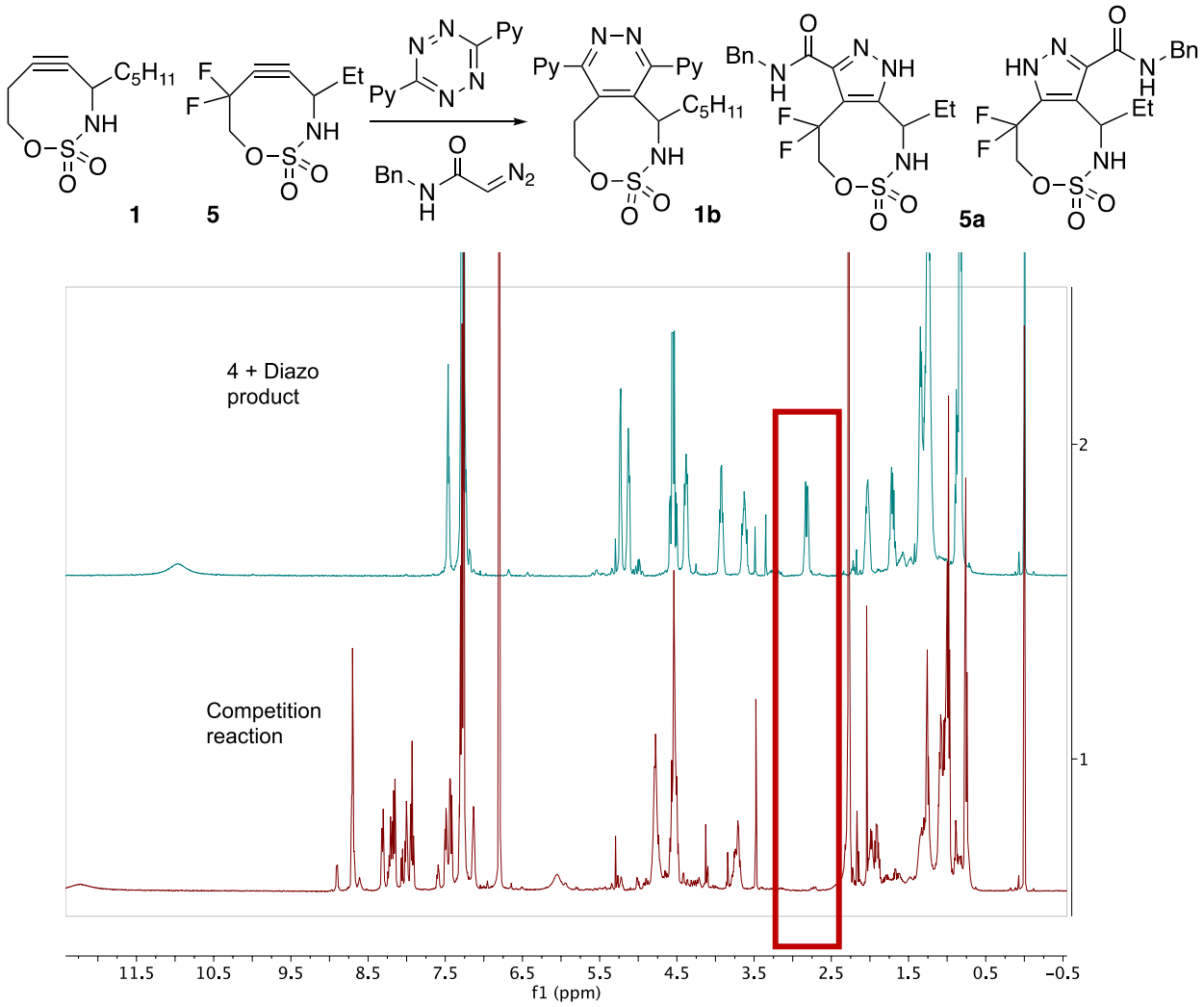
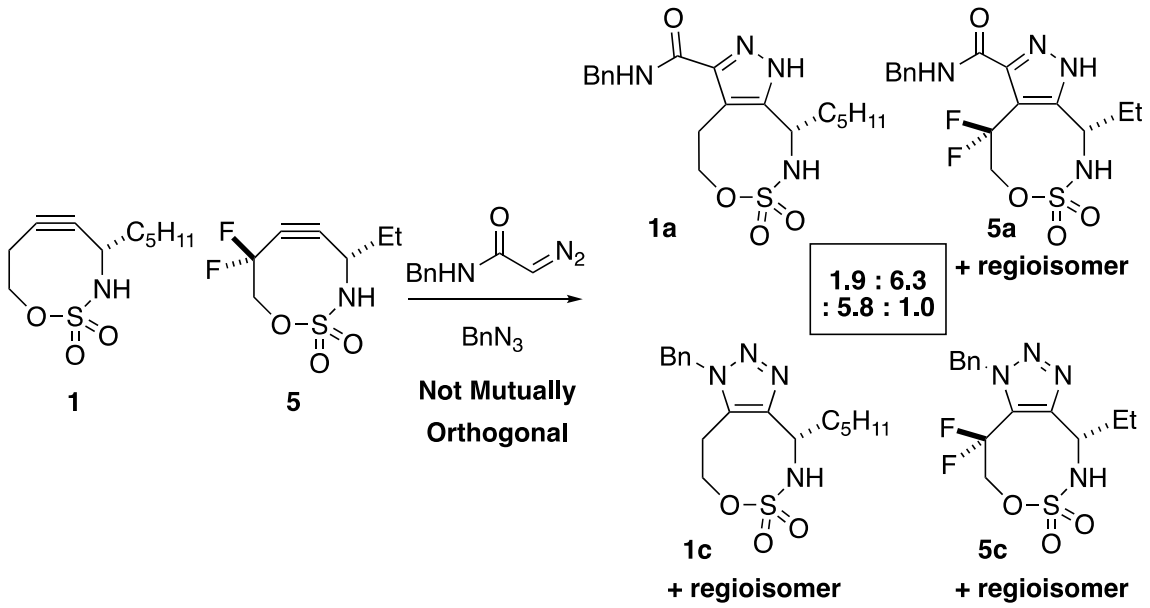
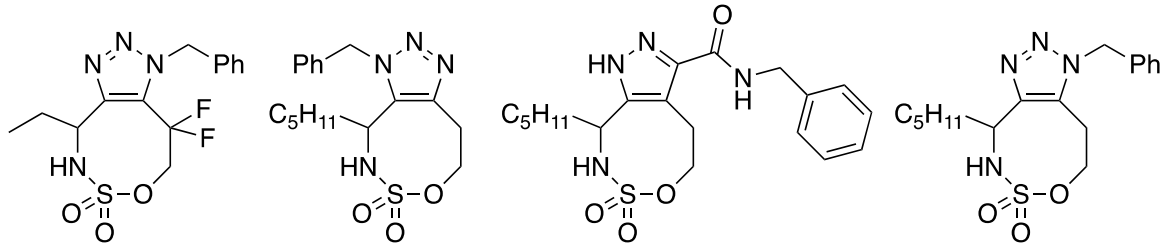


Figure S2-19. In the competition between **1** and **5** for dipyridyl tetrazine and benzyl diazoacetamide, only product **1b** and **5a** (two regioisomers) were observed. The NMR spectra matches previously reported values.[5,14]

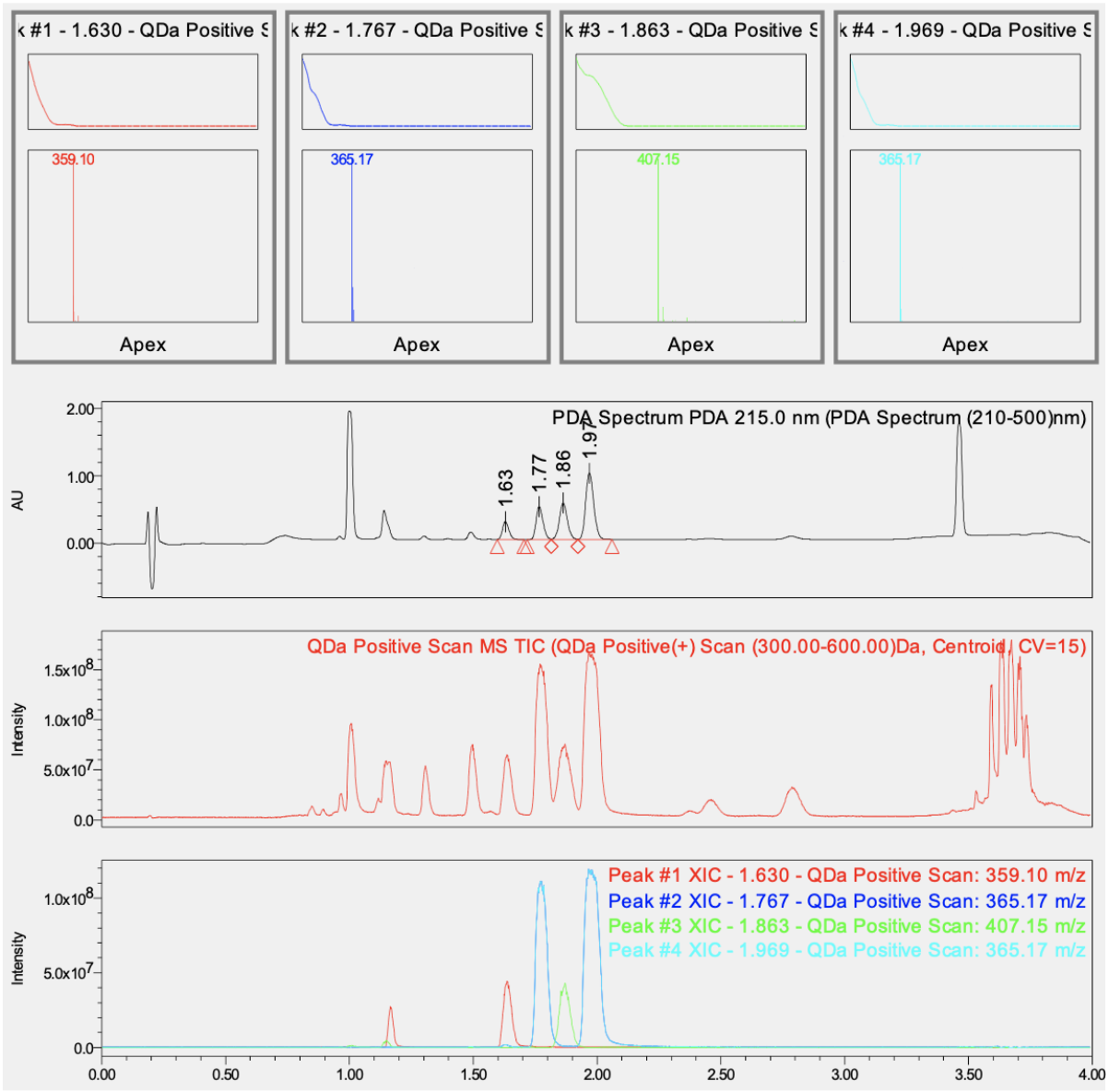


Method 1	RT (mins)	Area (V•sec)*
Peak 1 **5c maj**	1.63066667	0.48869333
Peak 2 **1c min**	1.76766667	0.969846
Peak 3 **1a maj**	1.86366667	1.25377233
Peak 4 **1c maj**	1.96966667	2.307522

Method 1

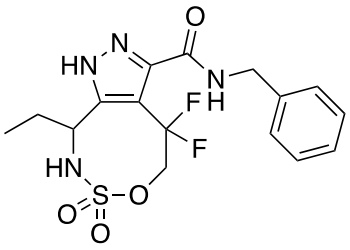


5c Major Peak 1 | **1c Minor Peak 2** | **1a Major Peak 3** | **1c Major Peak 4**

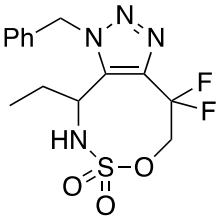


Method 2	RT (mins)	Area (V•sec)*
Peak 1 **5a maj**	2.086	4.89384233
Peak 2 **5c min**	2.607	0.15620833
Peak 3 **5a min**	2.751	0.48344133

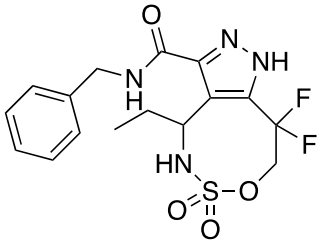
Method 2



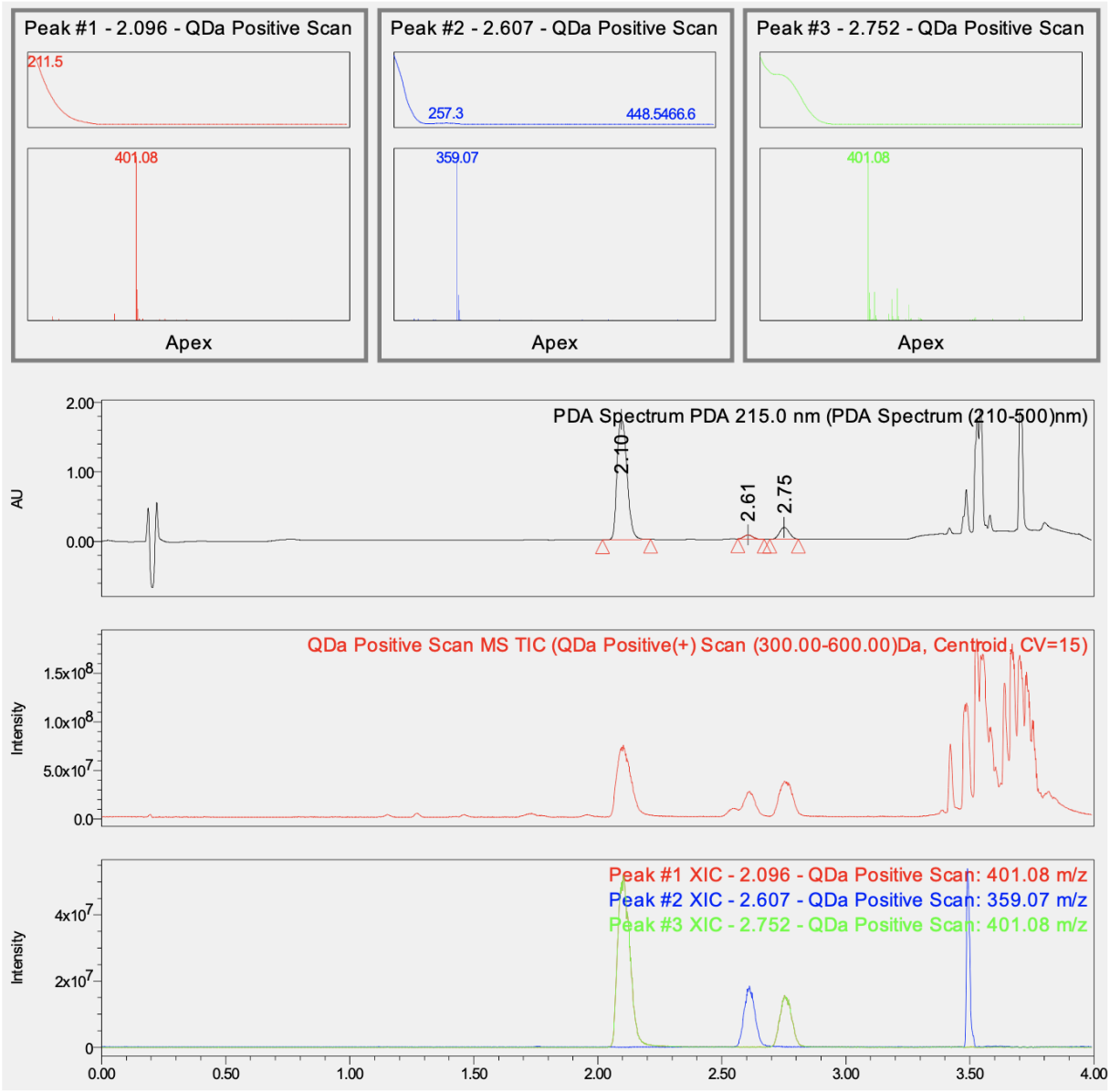
5a Major
Peak 1



5c Minor
Peak 2



5a Minor
Peak 3



Ratio Calculation		
	[Conc.]	Ratio

1a	1.39554881	1.9
1c	4.29578295	5.8
5a	4.60929659	6.3
5c	0.73729236	1.0

Figure S2-20. In the competition between **1** and **5** for benzyl diazoacetamide and benzyl azide, all possible products were observed. Due to the difficulties of analyzing the mixture using NMR, we employed LC-MS to assess ratio between each products. Two LC-MS method were used to analyze the this mixture. The compound concentration was calculated based on the calibration curve in Section XIV.

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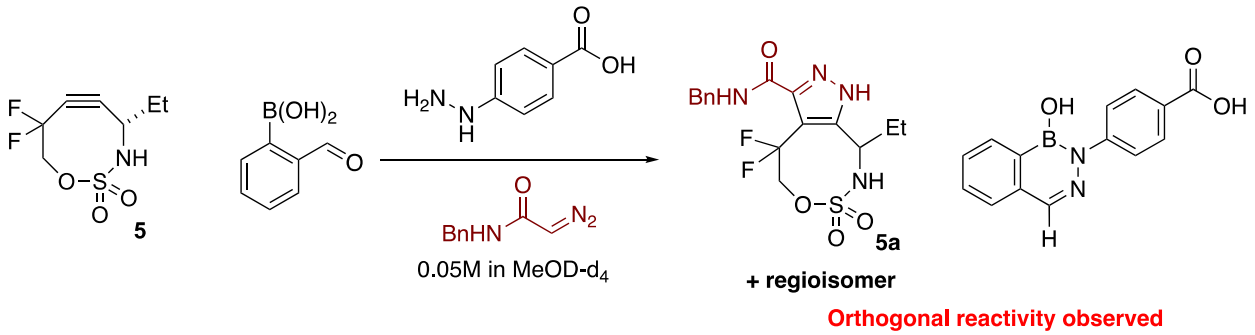
General procedure 2. This procedure was adopted from a previously published competition methods.[12] In a vial equipped with a stir bar, added hydrazine (1 equiv) and benzyl diazoacetamide (1 equiv). The mixture was then dissolved in MeOD-d_4 (0.05M). Then one equivalence of SNO-OCT **5** and one equivalence of boronic acid were added as solids at once to the mixture and the reaction was stirred under room temperature for 19 hours. One equivalence of mesitylene internal standard was also added. The selectivity was determined based on overlaid NMR spectra.



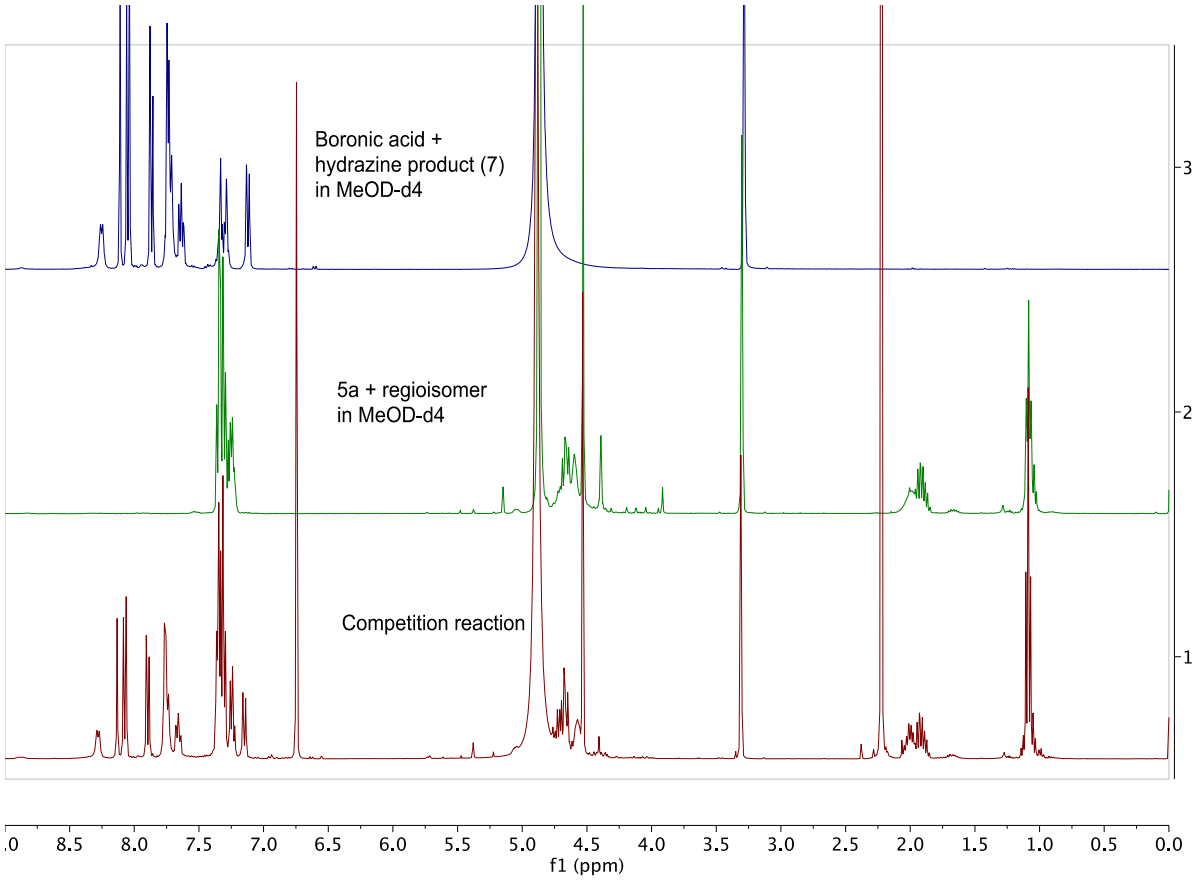
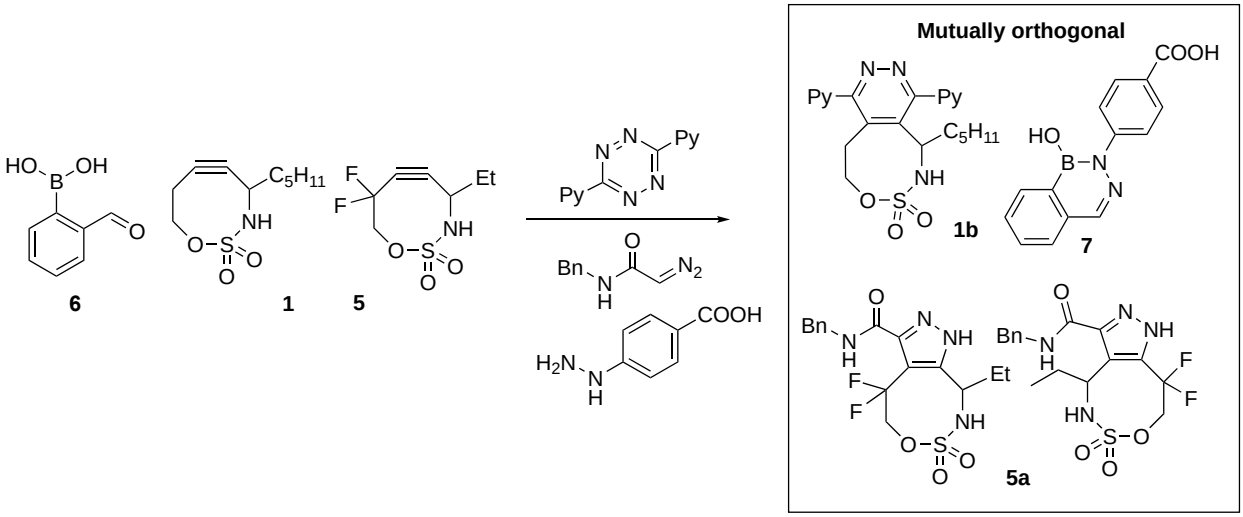
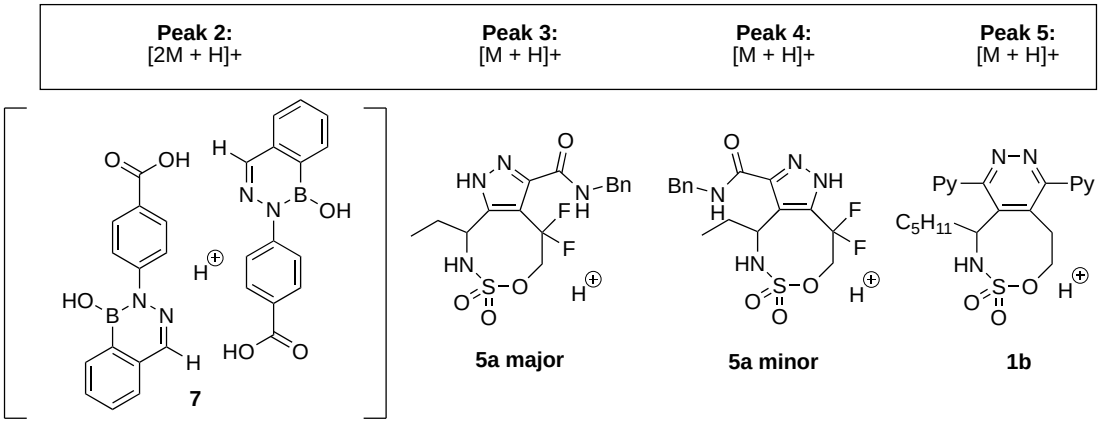


Figure S2-21. Despite the reactivity between **5** and hydrazine, overlaid spectra show two parallel bioorthogonal reactions with orthogonality.

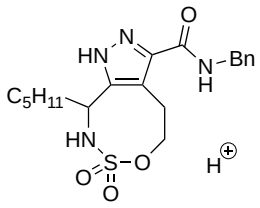
XIII. Triple Ligation Results.

General procedure. Stock solutions (0.025 M in methanol) of the SNO-OCTs and the dipoles were prepared, while the stock solutions for boronic acid and hydrazide were prepared at 0.0125 M, due to their slight lower solubility. One mole equivalence (12.5 μmol each) of tetrazine, diazoacetamide, and hydrazine solutions were combined together in a vial equipped with a stir bar followed by the addition of one equivalence of the SNO-OCT solutions and boronic acid solution all at once. The reaction was allowed to stir under room temperature for one hour for full conversion. All volatiles were removed under reduced pressure. The selectivity was dissolved in 1.5 mL methanol and analyzed based on UPLC/LC-MS analysis using method 1.



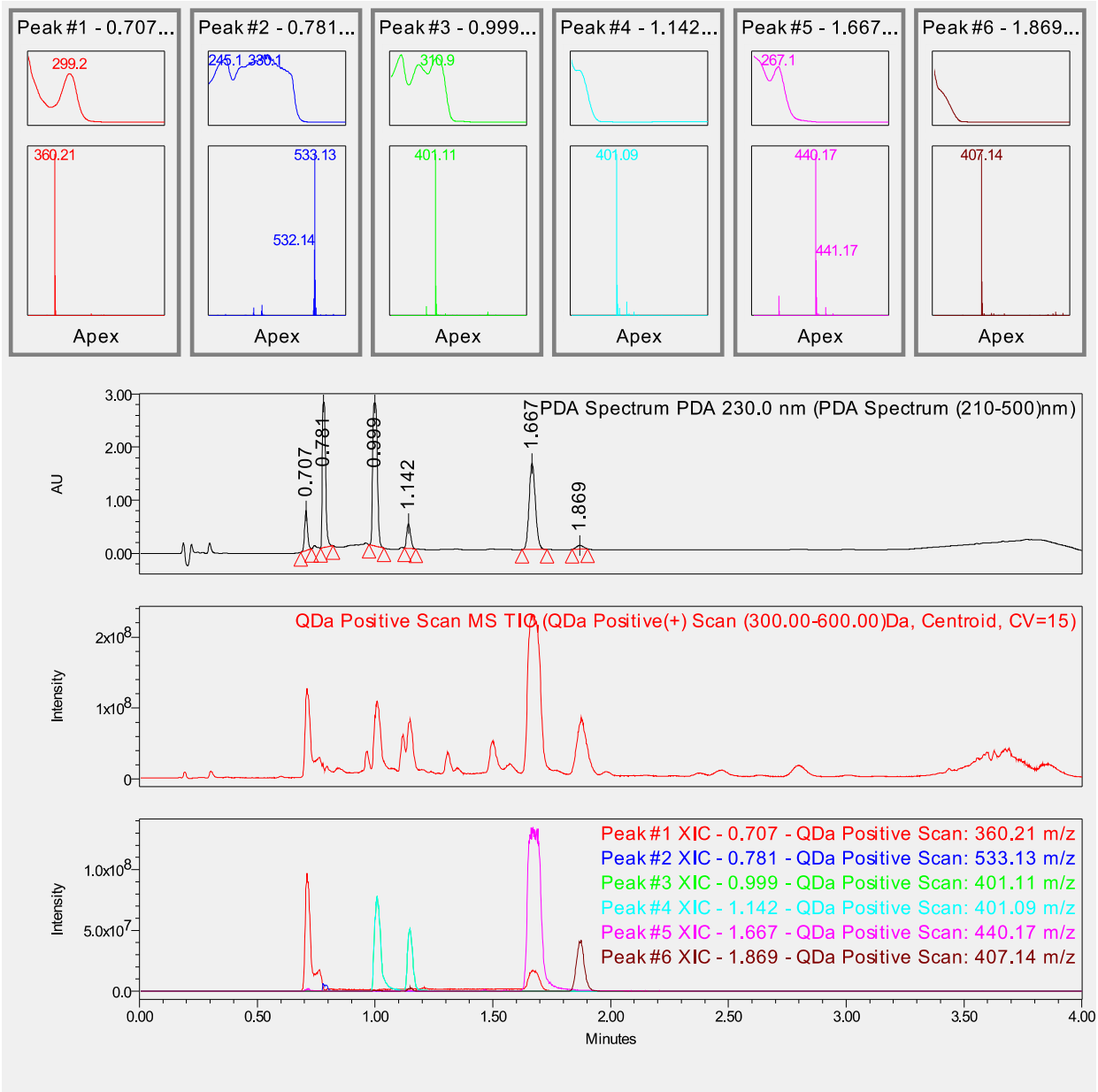


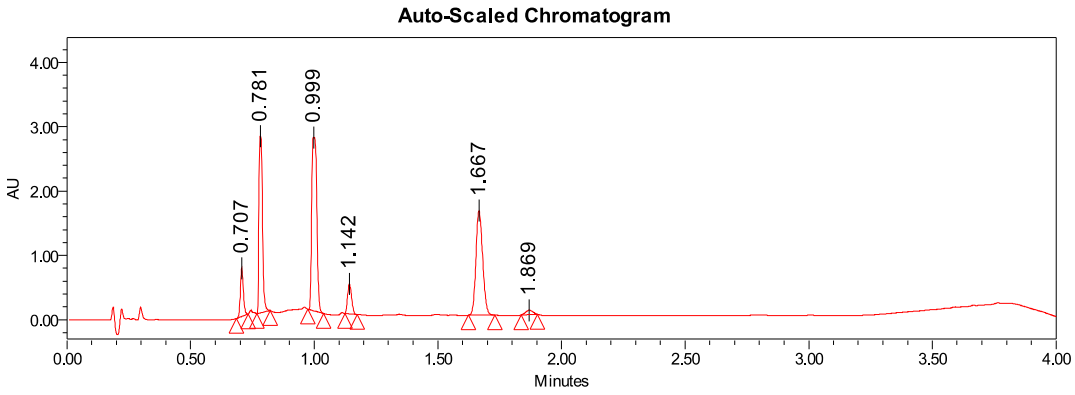
Peak 1: Unknown (from **4 + tetrazine**)	**Peak 6:** [M + H]+



1a

Note: Peak 1 is a unknown we have observed from the cycloaddition between **1** and dipyridyl tetrazine (see Figure S2-22B). We suspect this might be a by-product from the loss of one of the pyridine moiety. Peak 6 is an undesired cross product. We did not calculate the cross product ratio to the desired product, due to the cross product exceeds below the calibration while the desired products exceeds the detection limit on the other end of the calibration curve. We have observed similar minor cross reactivity in our dual ligation system when the mixture was injected into UPLC/LC-MS, yet the cross product is not observable on NMR, thus the selectivity is > 19:1.

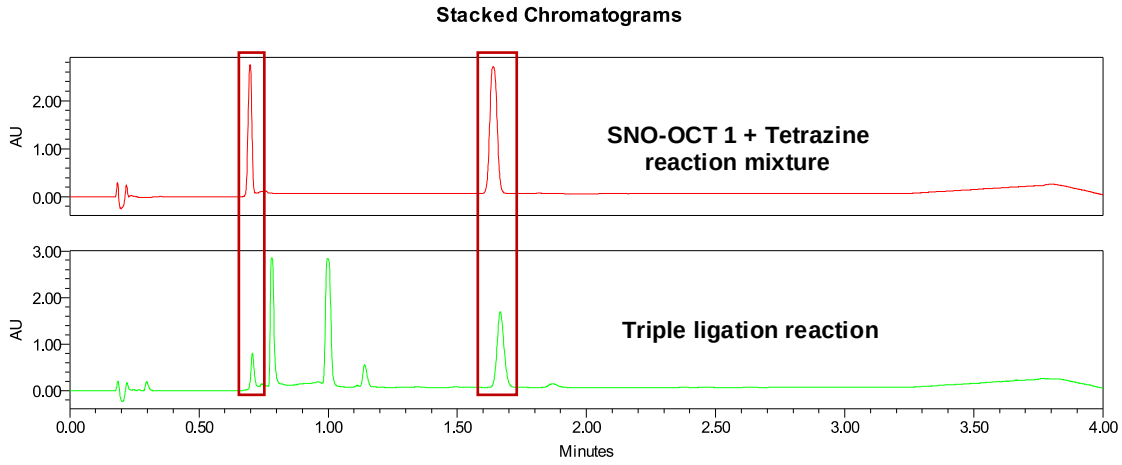

Peak #1 - 0.707...
299.2
360.21
Apex
Peak #2 - 0.781...
245.1 330.1
533.13
532.14
Apex
Peak #3 - 0.999...
316.9
401.11
Apex
Peak #4 - 1.142...
401.09
Apex
Peak #5 - 1.667...
267.1
440.17
441.17
Apex
Peak #6 - 1.869...
407.14
Apex
PDA Spectrum PDA 230.0 nm (PDA Spectrum (210-500)nm)
AU
0.707
0.781
0.999
1.142
1.667
1.869
QDa Positive Scan MS TIC (QDa Positive(+) Scan (300.00-600.00)Da, Centroid, CV=15)
Intensity
Peak #1 XIC - 0.707 - QDa Positive Scan: 360.21 m/z
Peak #2 XIC - 0.781 - QDa Positive Scan: 533.13 m/z
Peak #3 XIC - 0.999 - QDa Positive Scan: 401.11 m/z
Peak #4 XIC - 1.142 - QDa Positive Scan: 401.09 m/z
Peak #5 XIC - 1.667 - QDa Positive Scan: 440.17 m/z
Peak #6 XIC - 1.869 - QDa Positive Scan: 407.14 m/z
Minutes



Peak Results

	RT	Area	Height
1	0.707	644721	751404
2	0.781	2721388	2752541
3	0.999	3748532	2695284
4	1.142	523325	466101
5	1.667	3041547	1624144
6	1.869	142268	69868

Figure S2-22A. The triple ligation of three pairs of bioorthogonal reagents. Based on the analysis of UPLC/LC-MS analysis, mutual orthogonality was observed. Peak 3 and Peak 4 are regioisomers of **5a**, and they are unfortunately overlapped with each other in the QDa mass analysis. Additional spectra, see Figure S2-22C.



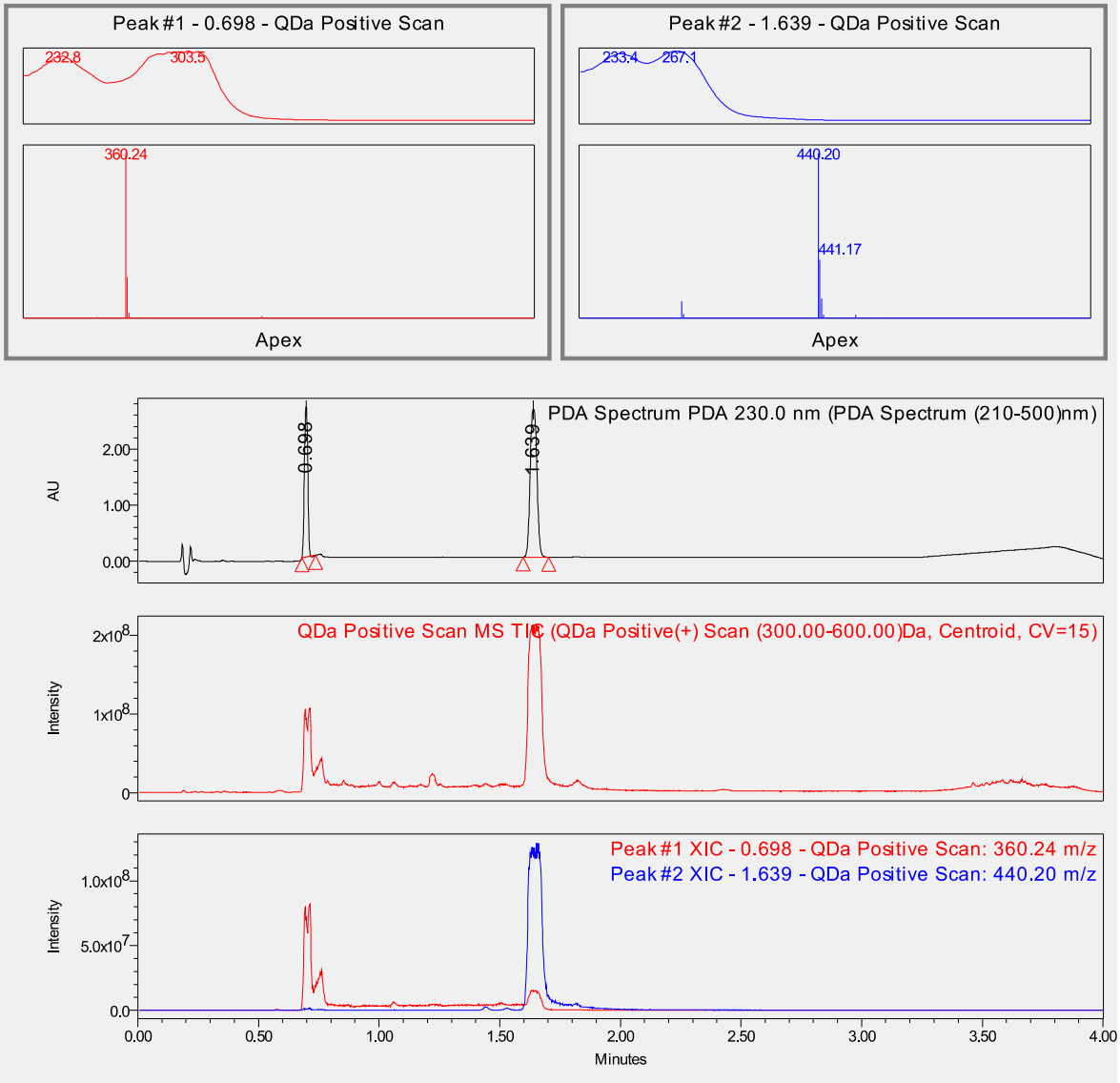


Figure S2-22B. Two stacked spectra showed the reaction mixture between SNO-OCT **1** and tetrazine resulting product **1b** (**[M + H]** measured 440.20) and a byproduct with m/z = 360.24. This reaffirmed that the unknown compound during the triple ligation experiment comes from the reaction between **1** and dipyridyl tetrazine. Unfortunately, we were not able to isolate this compound.

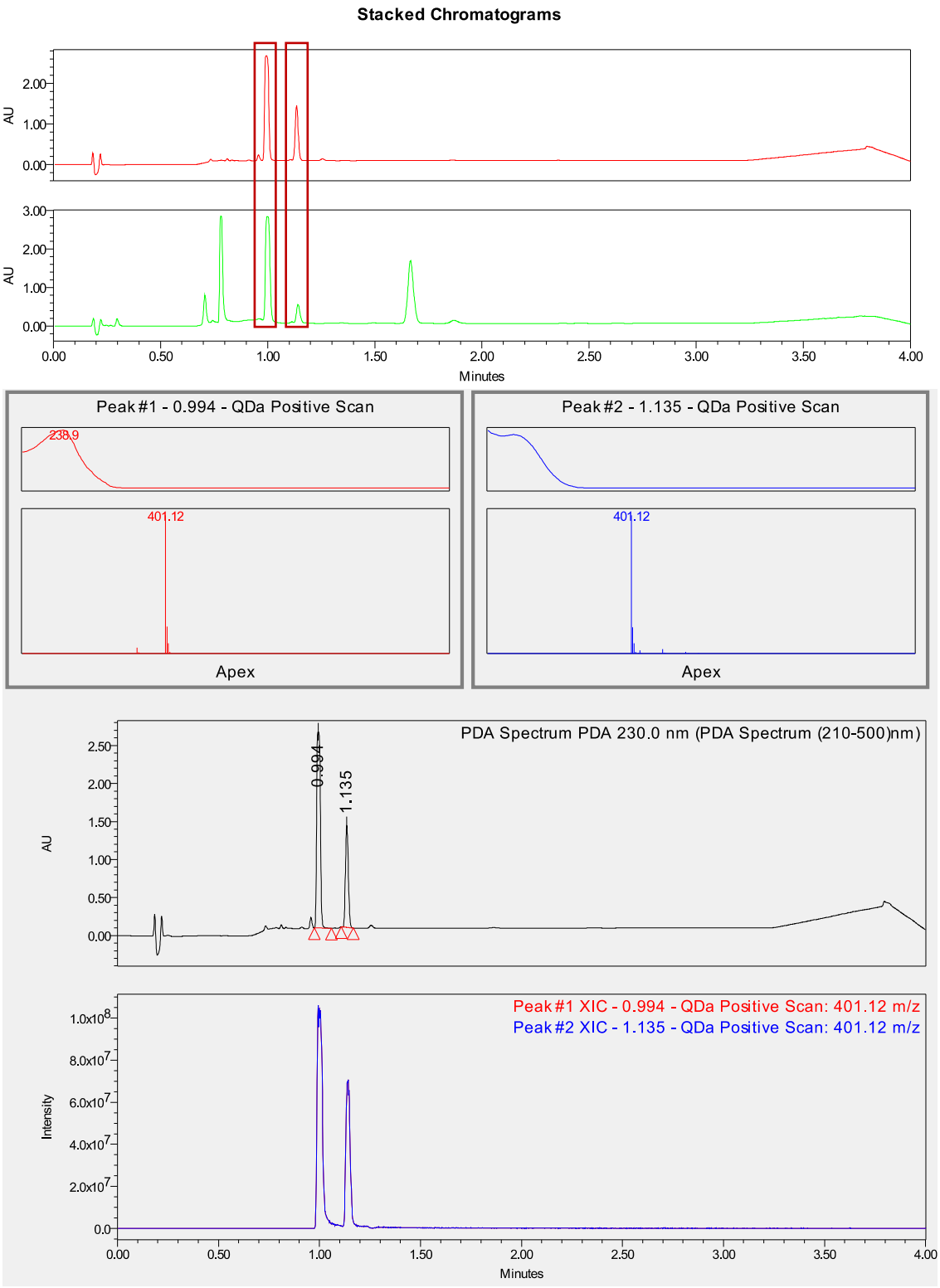


Figure S2-22B. Two stacked spectra showed the reaction mixture between SNO-OCT **5** and benzyl diazoacetamide resulting two regioisomers. Both stacked trace and QDa mass analysis reaffirmed that the two peaks are from the cycloaddition reaction. We, however, were unable to completely differentiate the color in the QDa analysis spectrum.

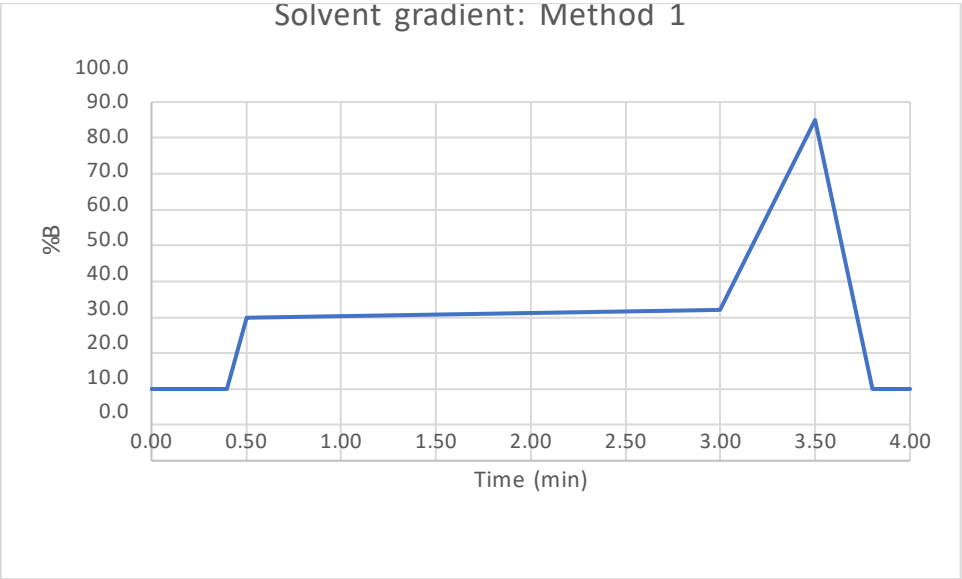
XIV. UPLC-MS methods and calibration curves.

General procedure for calibration. Response factor curves were created for each compound, which were synthesized and purified based on the method above or the previously reported method.[5] Serial dilutions were performed in a 96-welled plate to create a series of concentrations (5.0 mM – 0.4 mM). The UV absorption at 215 nm were measured based on 1 μL injection of samples with varied concentrations. The calibration curves were generated based on the peak area of the corresponding compound against its concentrations. Each curve was generated in triplicates to create the following calibration curves. Error bars were also incorporated into each averaged point.

Note: Due to the difficulties in separating all components in the competition mixture using a single method, two methods were developed to achieve optimal separation and efficiency.

UPLC-MS methods:

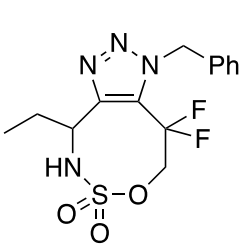
Method 1



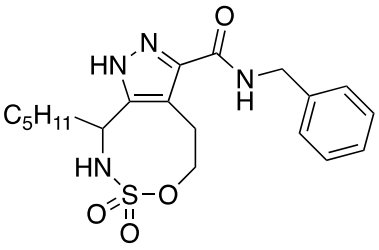
Solvents	A2:	H2O + 0.1% formic acid	B2:	MeCN + 0.1% formic acid
1 μL per injection	Time (mins)	Flow speed (mL/min)	%A	%B
1	0.00	0.700	80.0	20.0
2	0.40	0.700	80.0	20.0
3	0.50	0.700	60.0	40.0
4	3.00	0.700	58.0	42.0
5	3.50	0.700	5.0	95.0

6	3.80	0.700	80.0	20.0
7	4.00	0.700	80.0	20.0

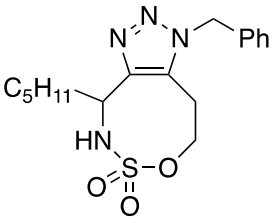
The following compounds were calibrated using **method 1:**



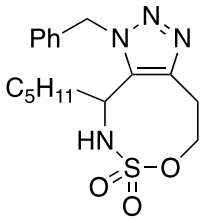
5c Major



1a Major

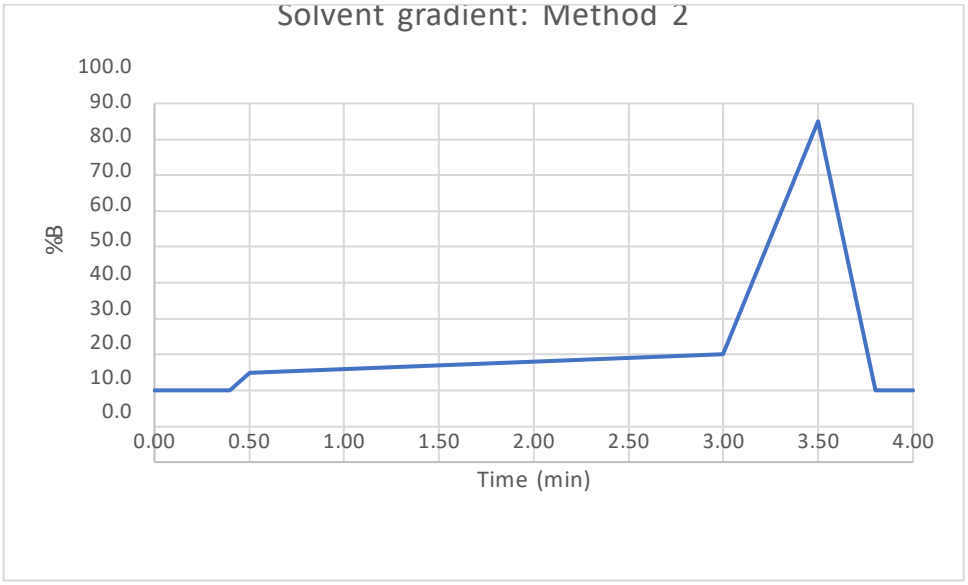


1c Major



1c Minor

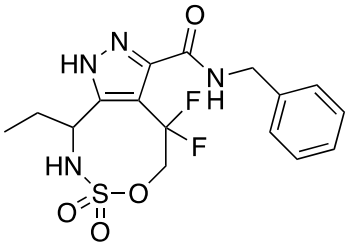
Method 2



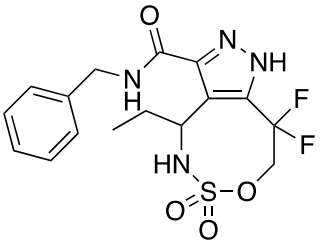
Solvents	A2:	H2O + 0.1% formic acid	B2:	MeCN + 0.1% formic acid
1 µL per injection	Time (mins)	Flow speed (mL/min)	%A	%B
1	0.00	0.700	80.0	20.0
2	0.40	0.700	80.0	20.0
3	0.50	0.700	75.0	25.0
4	3.00	0.700	70.0	30.0

5	3.50	0.700	5.0	95.0
6	3.80	0.700	80.0	20.0
7	4.00	0.700	80.0	20.0

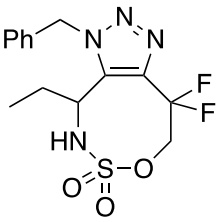
The following compounds were calibrated using **method 2:**



5a Major

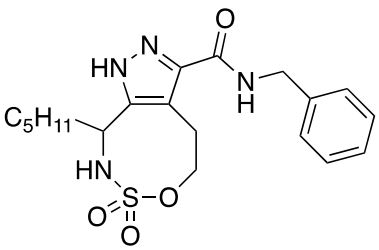


5a Minor

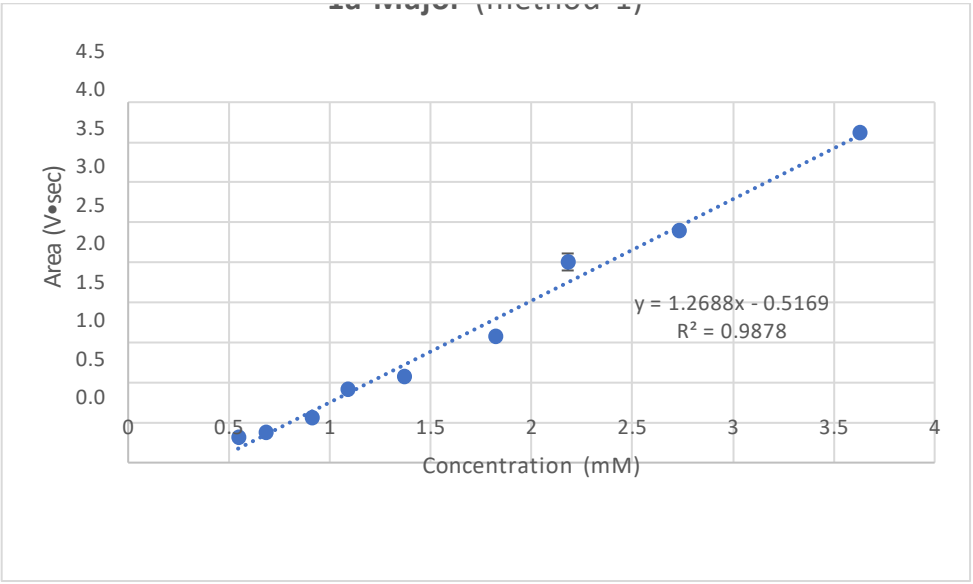


5c Minor

Compound 4a major calibration curve.



1a Major



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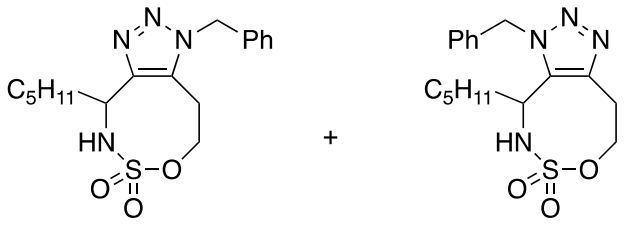
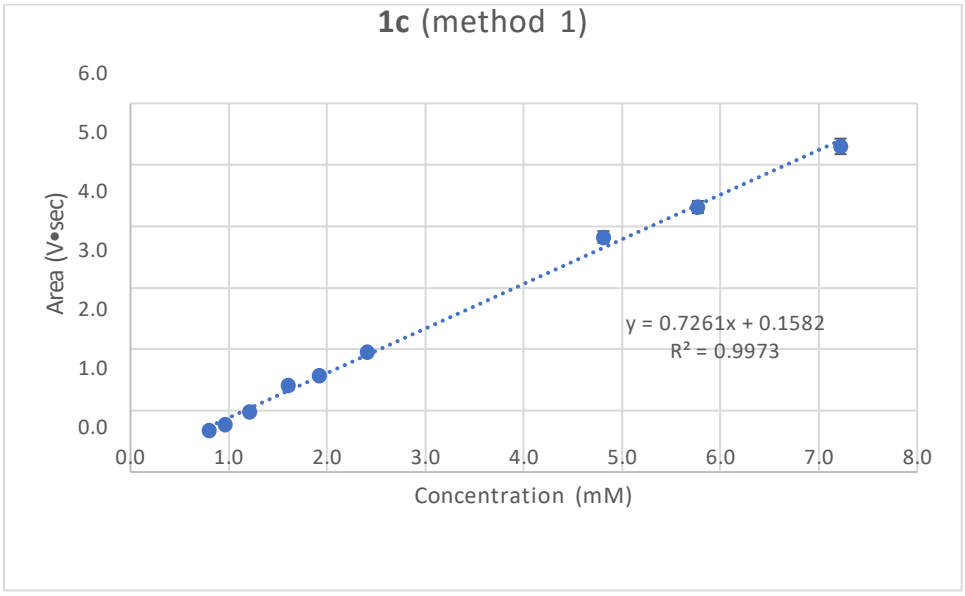
Compound 1c major and 1c minor calibration curve.



1c Major **1c Minor**

Note: Due to the difficulties in purification, the compound was calibrated as a mixture. The area was determined by the total combined integrated value.



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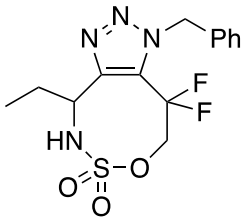
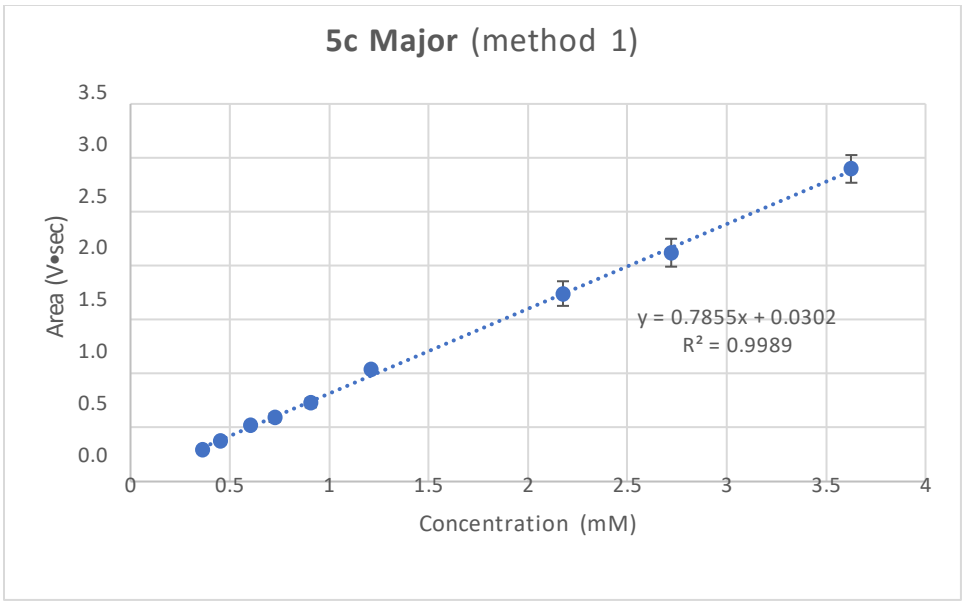
Compound 5c major calibration curve.



5c Major



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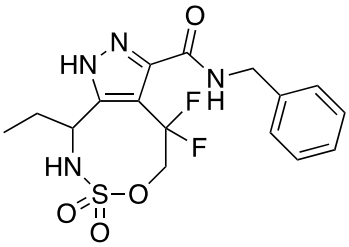
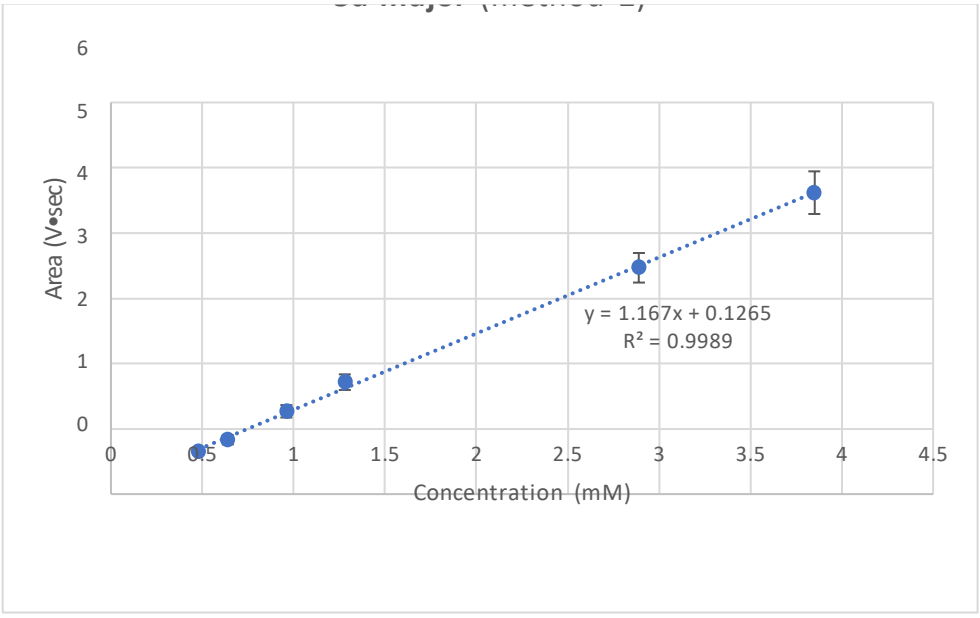
Compound 5a major calibration curve.



5a Major



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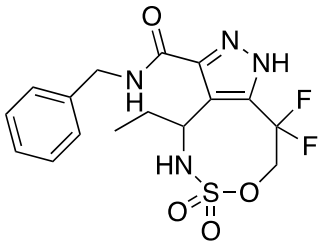
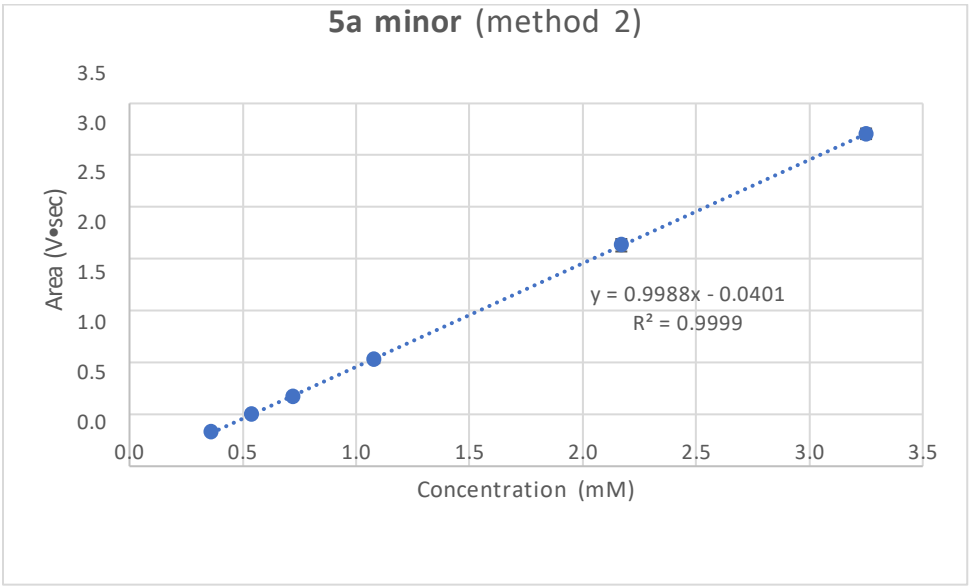
Compound 5a minor calibration curve.



5a Minor



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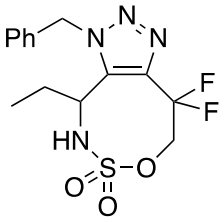
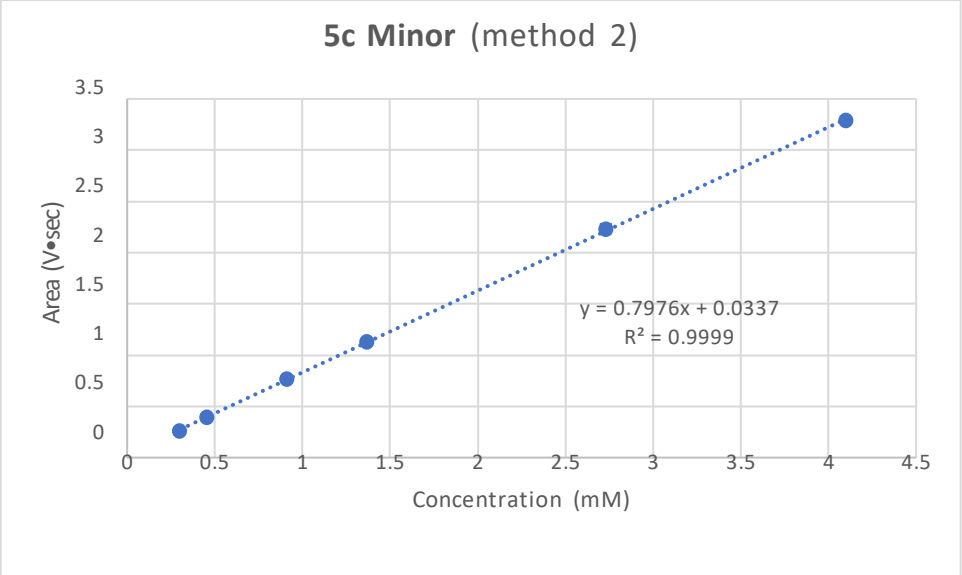
Compound 5c minor calibration curve.



5c Minor


5c Minor (method 2)
Area (V•sec)
Concentration (mM)
y = 0.7976x + 0.0337
R² = 0.9999
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5

XV. *In vitro* assays.

Mammalian Tissue Culture

Chinese Hamster Ovary cells (CHO-K1, American Tissue Culture Catalog CCL-61™) were cultured in sterile T-75 flasks with 20mL F12-K media supplemented with 10% Fetal Bovine Serum (FBS) 100 units/mL penicillin, 100 µg/mL streptomycin, and 2mM glutamine. Unless otherwise stated, supplemented F12-K media contains 10% Fetal Bovine Serum (FBS) 100 units/mL penicillin, 100 µg/mL streptomycin, and 2mM glutamine. Cells were cultured at 37°C with 5% v/v CO_2 in a cell culture incubator. Cells were counted to determine seeding density using a Countess™ II Automated Cell Counter, employing trypan blue and disposable countess chamber slides according to manufacturer recommendation.

Microscopy:

Chinese hamster ovary cells (CHO-K1) were plated on at 50,000-75,000 cells/well in an 8-well glass bottom microscopy dish (µ-Slide 8 well) in supplemented F-12K media. These cells were incubated overnight at 37 °C. Prior to treatment cells were washed twice with 200 µL of Dulbecco's Phosphate-Buffered Saline supplemented with calcium and magnesium (DPBS, 0.9mM $CaCl_2$, 0.49 $MgCl_2 \cdot 6H_2O$, 2.67mM KCl, 1.467mM KH_2PO_4, 138mM NaCl, 8.059 mM $Na_2HPO_4 \cdot 7H_2O$), followed by the addition of 200 µL of supplemented F-12K media. Probe solutions were prepared in supplemented F-12K were added to achieve the appropriate concentration of the molecule and allowed to incubate for 2 hours at 37°C. CHO-K1 cells were then washed twice with 200 µL DPBS, and 160-200 µL DPBS was added to each well prior to counterstaining or 200 µL of Invitrogen Live-Cell Imaging solution (140 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20mM HEPES, pH 7.4). Cells when counterstained were treated with Hoechst 33342 at 2 µg/mL, or Invitrogen Wheat-Germ Agglutinin-AlexaFluor-647™ at 5 µg/mL for 15 minutes on ice, then washed twice with 200 µL of Invitrogen Live-Cell Imaging Solution before a final 200 µL of Invitrogen Live-Cell Imaging solution was added to each well.

Images were recorded on an Andor Spinning Disk Confocal Microscope using a 1.4 NA, 60x Plan Apo objective. Sample excitation was performed with a 50 mW 405 nm, 50 mW 488nm, 50 mW 561nm, or 100 mW 640nm wavelength laser and recorded upon either an Andor Zyla sCMOS camera or Andor iXion+ EMCCD camera. Images were recorded with the MetaMorph acquisition software. Further background subtraction and analysis was performed using Fiji. Inspection of these images abated concerns of bleed over from **SNO-C343** into the 488 nm channel used to observe **DCF** as bright, high-intensity objects found in **SNO-C343** micrographs are not observed in the 488 nm channel.

Metabolic labeling

A sterile-stock solution of Ac_4ManDiaz was prepared in absolute ethanol at a concentration of 10mM. One-hour prior to seeding CHO-K1 cells, 0-15 µL of the 10mM Ac_4ManDiaz stock solution was added to either a sterile 8 well Ibidi glass bottom slide or a sterile 12 well plate and allowed to evaporate prior to seeding. CHO-K1 cells were seeded at 50,000 cells/well at a volume of 200 or 500 µL in supplemented F12-K and allowed to incubate at 37°C with 5% v/v

CO_2 in a tissue culture cabinet for 24 hours. Prior to labeling, cells were washed twice with DPBS followed by the addition of 200 or 500 µL of fresh supplemented F-12k to each well. Cells were then treated with Rho-DIFO-SNO at 1 µM for 2 hours, before being washed twice and with DPBS and analyzed with flow cytometry or confocal microscopy.

Flow Cytometry

Cells were released from 12 well culture dishes by incubation with 200 µL of 0.25% v/v Trypsin-EDTA mix for 5 minutes 37°C with 5% v/v CO_2 in a tissue culture cabinet, trypsinolysis was then quenched with 500 µL supplemented F-12K media. Cells were then subject to centrifugation at □200g for 5 minutes and resuspended in 1mL DPBS supplemented with 1% w/v bovine serum albumin (BSA). To these cells 1 µL of 30 µM SYTOX® Green for a staining concentration of 30 nM, they were then lightly vortexed and allowed to incubate for 20 minutes. Cells centrifuged again at □200g for 5 minutes, resuspended in 1mL DPBS supplemented with 1% w/v BSA, this process was repeated one more time to remove excess dye before a final resuspension in 1mL DPBS supplemented with 1% w/v BSA. Cells were then analyzed with an Attune NxT Flow Cytometer and fluorescence intensity was quantified on 20,000 live, single cell events at the experimental conditions. Data was analyzed using FlowJo version 10.7.

Protein Labeling

Preparation of BA-RNase A, Tz-RNase A, and Diaz-CytoC.

Solutions 10mg/mL of bovine seminal Ribonuclease (RNase A) and Horse heart Cytochrome c (Cyto c) were prepared in DPBS. Solutions of 10 mM tetrazine-NHS, Diaz-NHS, and BE-NHS in DMSO were prepared immediately before use and stored at −20 °C. In a typical reaction 1 equivalent of tetrazine-NHS, or BE-NHS was added to a 1mg (100 µL) solution of RNase A on ice, then gently mixed for two hours. In an equivalent transformation 1 mg of Cyto c was functionalized with 1 equivalent of Diaz-NHS on ice, then gently mixed for two hours. Protein conjugates were then diluted to a volume of 250 µL with DPBS, eluted from a PD-25 column equilibrated with DPBS elution was monitored by absorption measurements at A_{280}. Functionalized proteins were then concentrated with a 3.5 kDa molecular weight cut-off spin concentrator at □10,000g for 10 minutes. Mass of protein conjugates were measured using matrix assisted laser desorption ionization mass spectrometry (MALDI), and carried forward.

Assessing Mutual Orthogonality

Protein-conjugates and small molecule mixtures were prepared to assay the extent of orthogonality among reactive species. The corresponding lane number presented in Figure 4A and Figure S2-23A-D imaged with *in situ* gel fluorescence is indicated in parenthetical bold, e.g. (**x**). To demonstrate the mutual orthogonality of reactive pairs an aliquot of cyto*c*-AcDiaz (**7**), BA-RNase A (**6**), or Tz-RNase A (**5**) was mixed with 5 equivalents of SNO-DF-Rho, SNO-C343, and Hyd-TR and allowed to react shielded from light at room temperature overnight with gentle mixing. In a similar experiment 1 equivalent of each cyto*c*-AcDiaz, BA-RNase A, and Tz-RNase A were mixed with 5 equivalents of SNO-DF-Rho (**4**), SNO-C343 (**2**), and Hyd-

TR (**3**) allowed to react shielded from light at room temperature overnight with gentle mixing. Finally, the mutual orthogonality of all three pairs was demonstrated by mixture of 0.34 equivalents of cyto*c*-AcDiaz, BA-RNase A, and Tz-Rnase A was mixed with 1.5 equivalents of SNO-DF-Rho, SNO-C343, and HYD-TR (**8**) and allowed to react shielded from light at room temperature overnight with gentle mixing. A 20 µL was then drawn from each reaction, and diluted 10-fold, to 200 µL. From the diluted stock, a 10 µL portion was added to 10 µL of 5% β-mercaptoethanol in Laemmli sample buffer and heated to 95 °C for 20 minutes. Samples were then cooled and separated by gel electrophoresis. Fluorescent imaging of the gel was recorded with an Amersham Imager 680 blot and gel imager. Some bleed over between blue and green channels is apparent. Excitation of with 460nm, 520nm, and 630nm with corresponding filter sets 525/20 nm, 605/40 nm and 705/40 nm intrinsically lead to bleed through between SNO-DF-Rho and Hyd-TR conjugates which excitation maxima at 570 nm and 600 nm respectively.

Figure S2-23. *in situ* gel fluorescence validation of tripartite biorthogonal set with protein labeling with a GE Amersham Imager 680. (A) 460 excitation 525/20 nm filter set (B) 520 nm excitation, 605/40 nm filter set. (C) 630 nm excitation and 705/40 nm filter set. (D) Combination of images in Figure S2-23A-C with false-color.

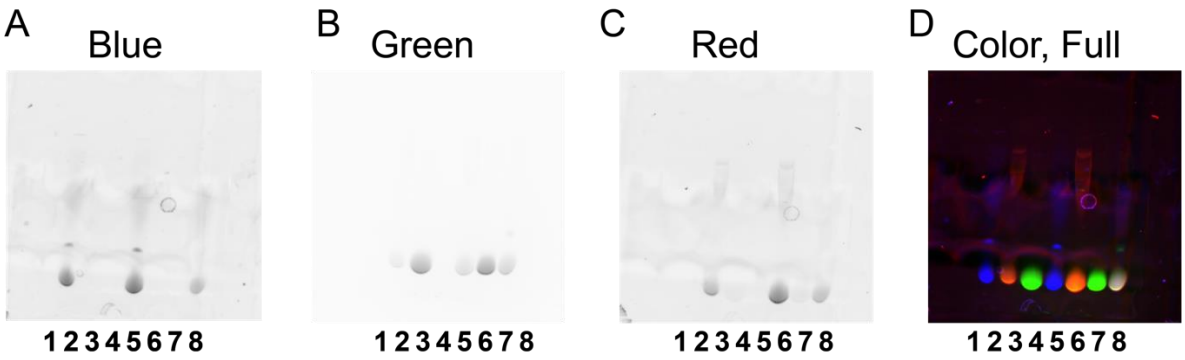


Figure S2-24. Uptake of SNO-DF-Rho, 60% Laser power, 200ms exposure time SCMOS camera. Scale Bar: 10 µm.

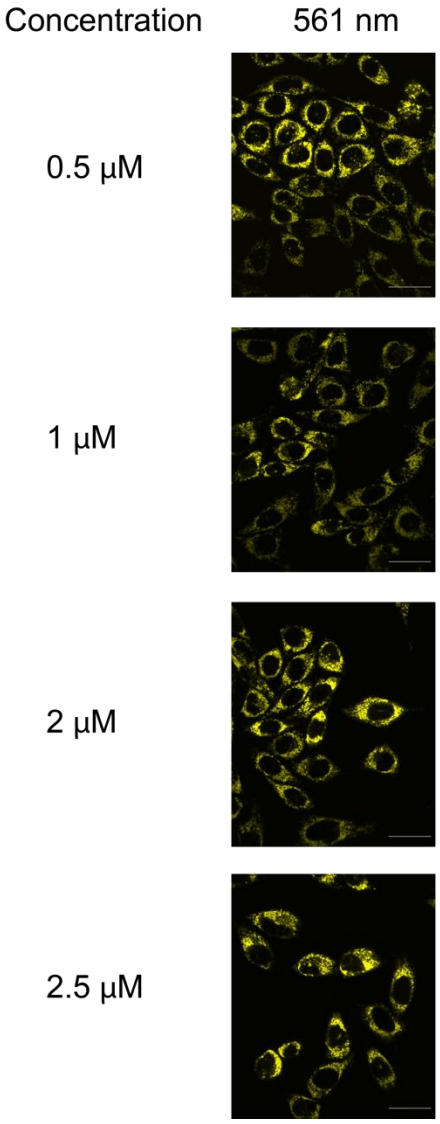


Figure S2-25. Uptake of SNO-C343, 45% Laser power, 150ms exposure time SCMOS camera. Scale Bar: 10 µm.

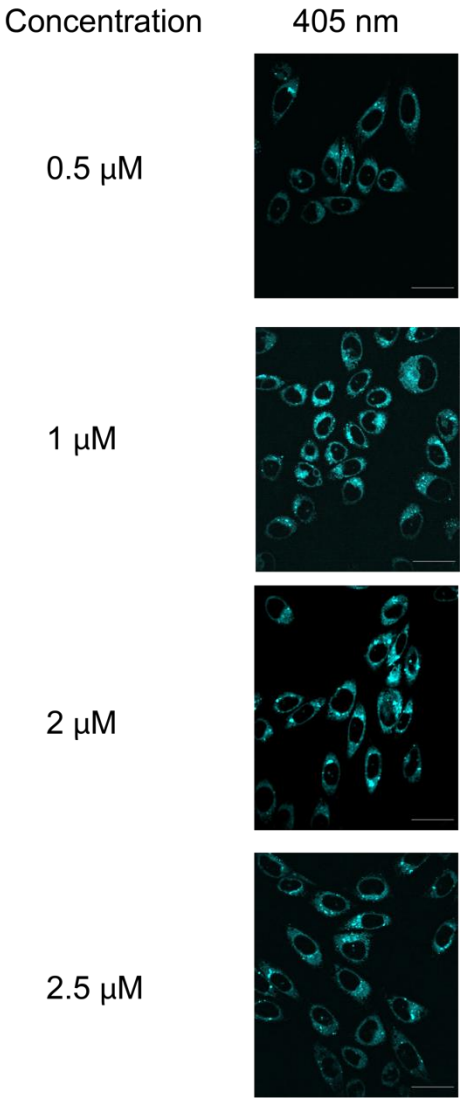


Figure S2-26. Mitochondrial labeling colocalization experiments of SNO-DF-Rho. (A) comparison of CHO-K1 cells treated with and without MitoTracker green™ and SNO-DF-Rho. (B) 2-D intensity histogram of SNO-DF-Rho (561 nm channel) and MitoTracker green™ (488 nm channel). The Pearsons R coefficient for colocalization of MitoTracker green™ and SNO-DF-Rho, R = 0.90 ± 0.01 (*N* = 3).

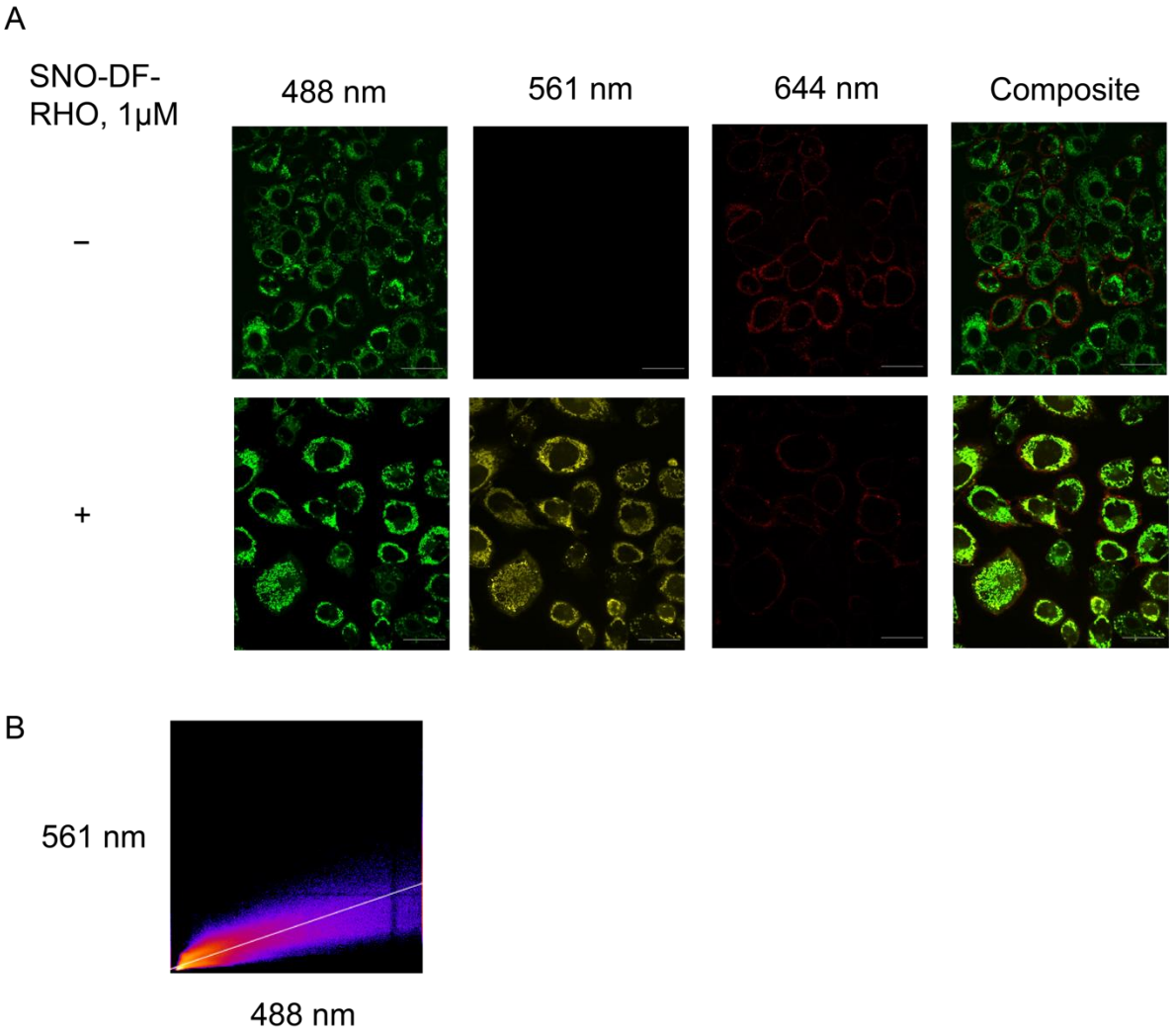


Figure S2-27. Uptake of DBCO-PEG4-Rho, 60% Laser power, 200ms exposure time SCMOS camera. Scale Bar: 7.5 µm.

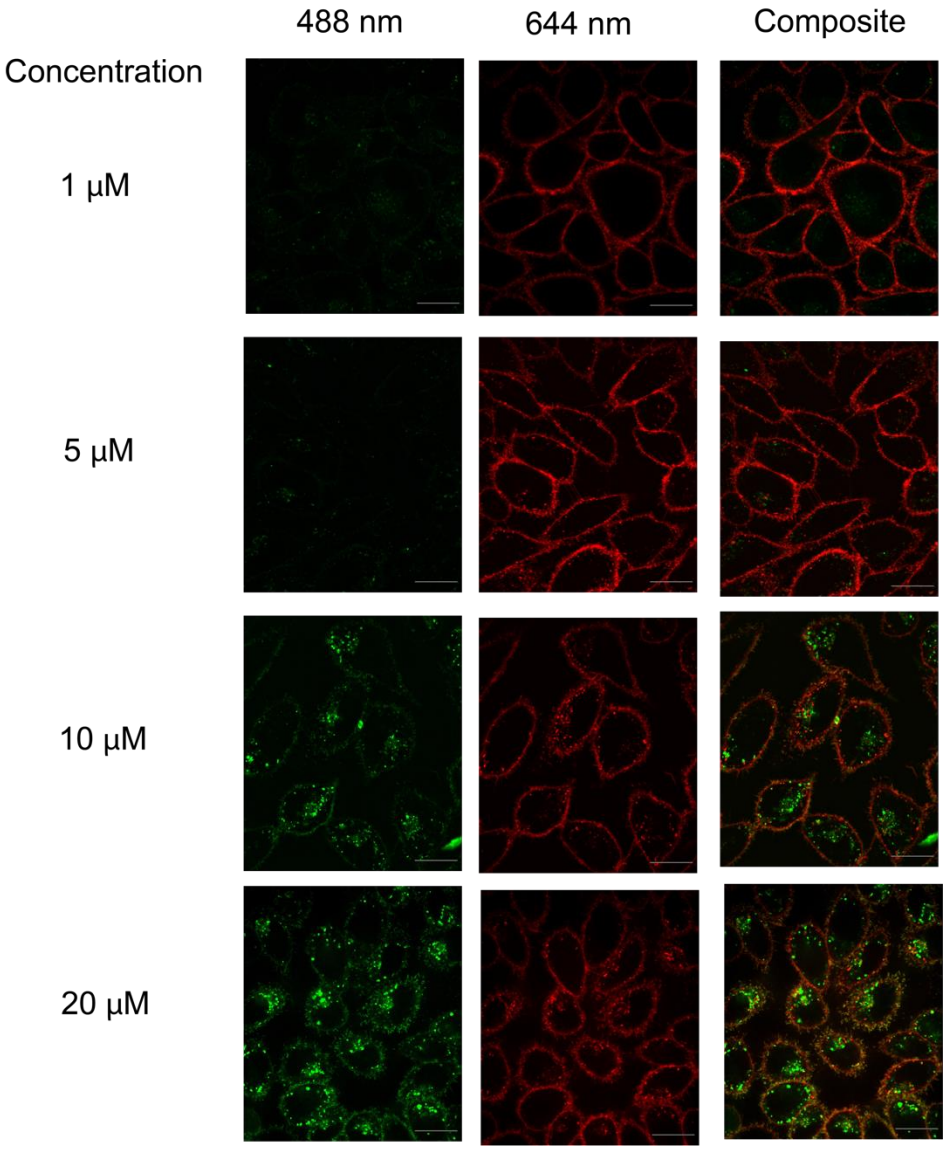


Figure S2-28. Uptake of DBCO-C343, 45% Laser power, 200ms exposure time SCMOS camera. Scale Bar: 7.5 µm.

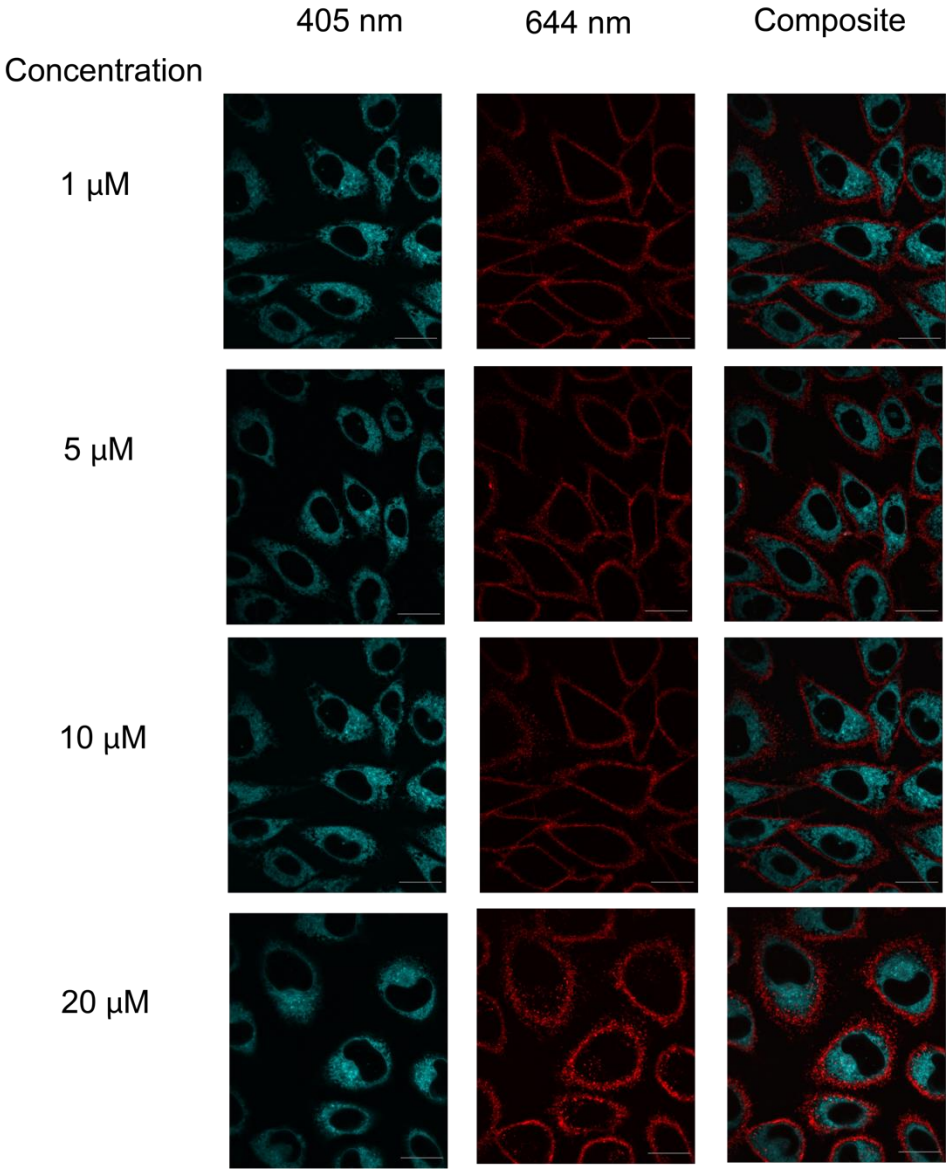


Figure S2-29. Titration of Ac_4ManDiaz concentration on CHO-K1 cells labeled with SNO-DF-Rho (1 µM), imaged at 561 nm, gain 10, exposure time 100 ms. Scale Bar: 10 µm.

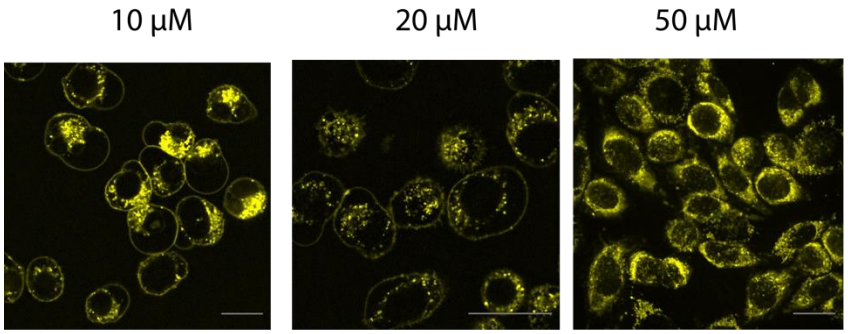
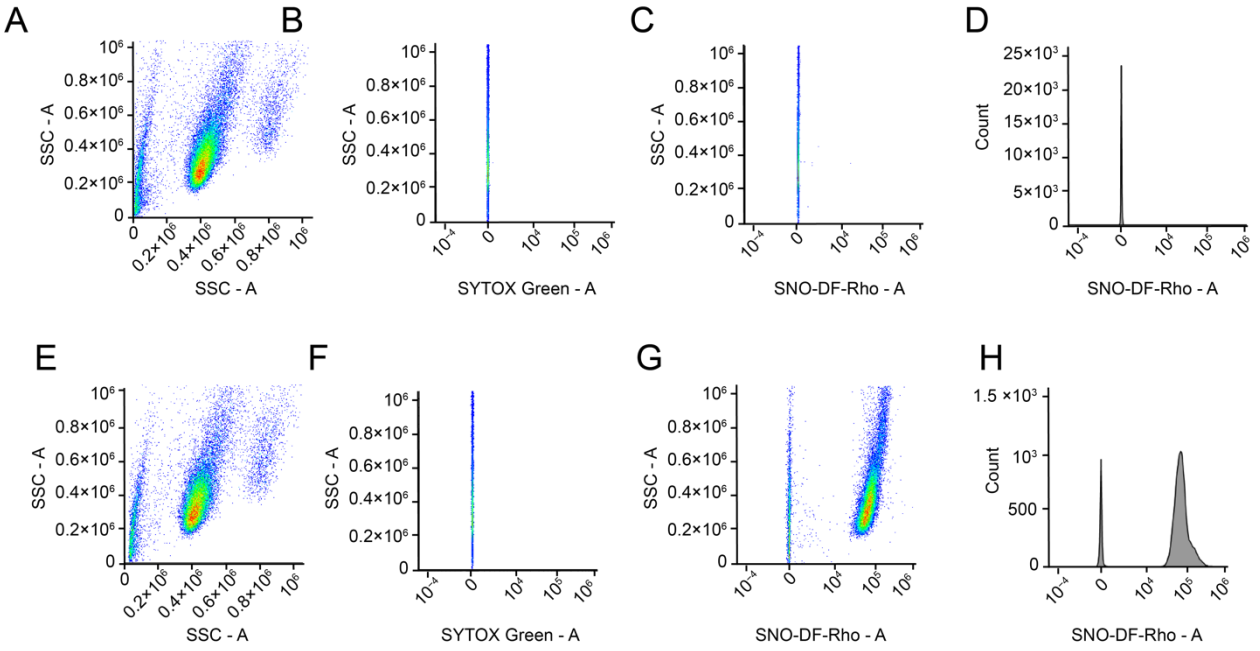


Figure S2-30. Analysis of CHO-K1 Cells metabolically labeled with Ac_4ManDiaz (20 µM) after incubation with SNO-DF-Rho (1 µM) for one hour, cells were the incubated with SYTOX green for 15 minutes (*see in vitro methods for more information*). (A) Scatter plot of metabolically labeled CHO-K1 cells, (B) Scatter plot of SYTOX green treated CHO-K1 cells, 488 nm excitation (C) Scatter plot of metabolically labeled CHO-K1 cells, 561 nm excitation. (D) Fluorescence intensity histogram of live-metabolically labeled CHO-K1 cells at 561 nm excitation. (E) Scatter plot of metabolically labeled CHO-K1 cells incubated with 1 µM SNO-DF-Rho. (F) Scatter plot of CHO-K1 cells incubated with 1 µM SNO-DF-Rho, excitation 488 nm. (G) Scatter plot of CHO-K1 cells incubated with 1 µM SNO-DF-Rho, excitation 561 nm. (H) Fluorescence intensity histogram of live-metabolically labeled cells treated with 1 µM SNO-DF-Rho.



XVI. Reference.

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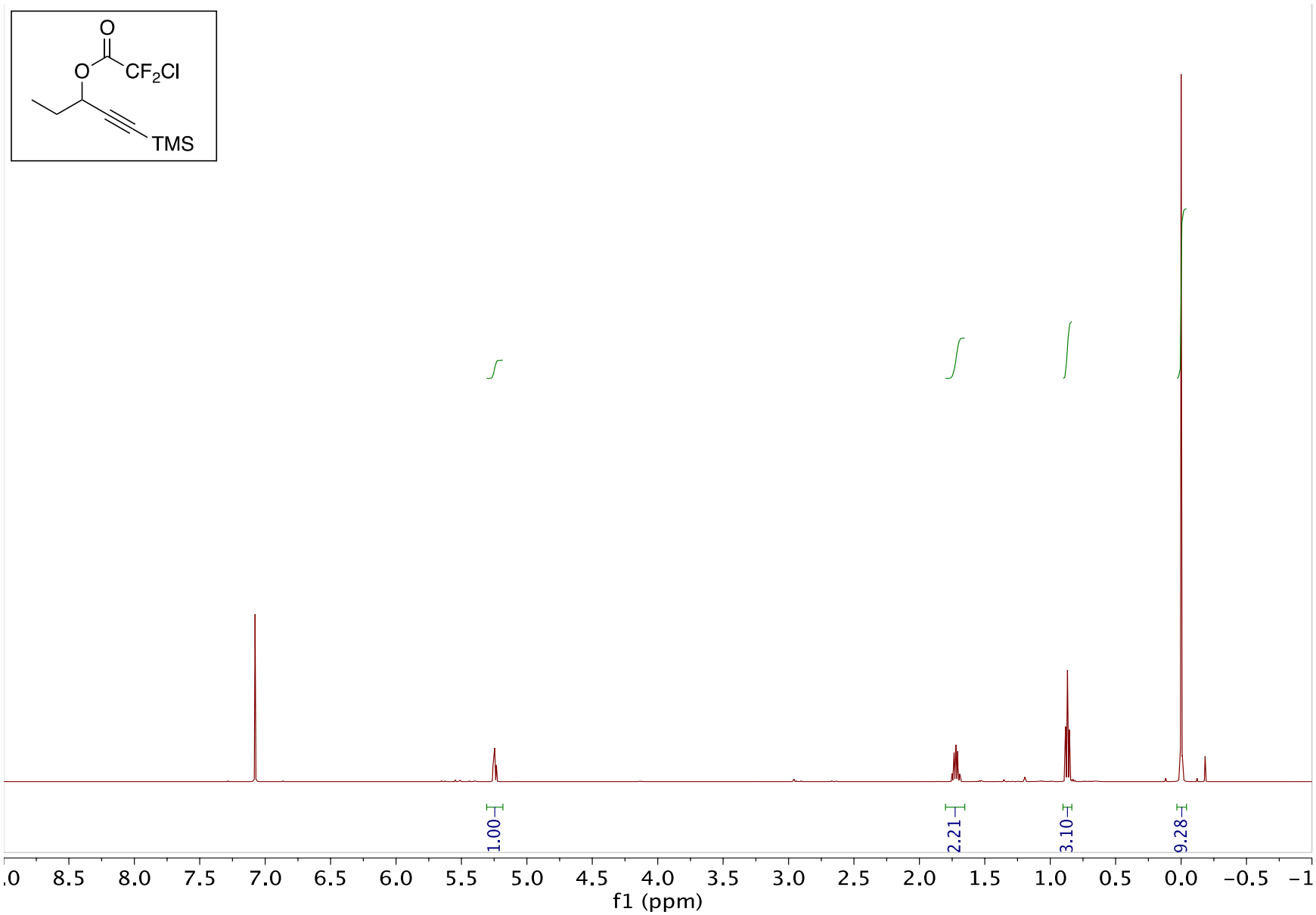
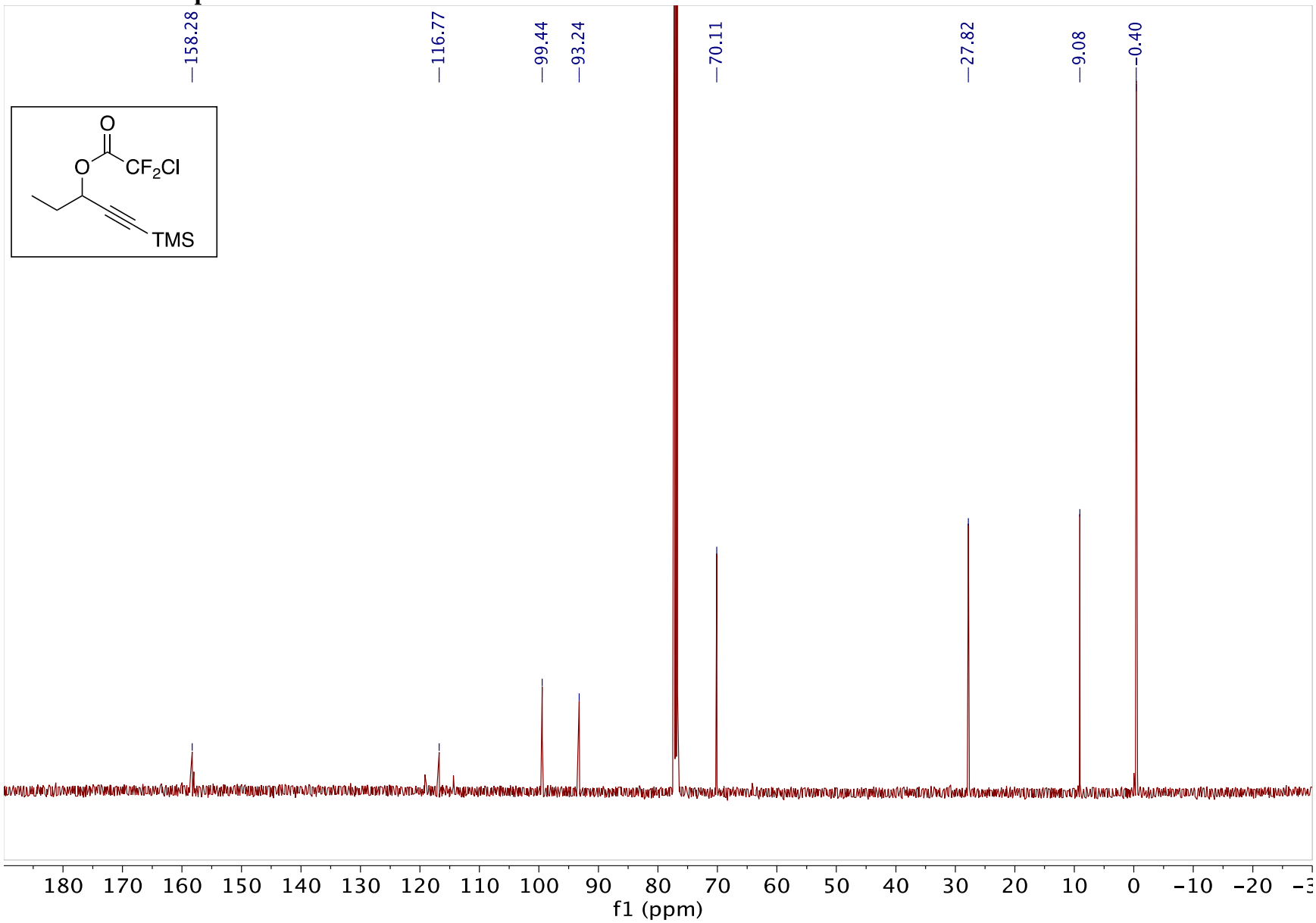
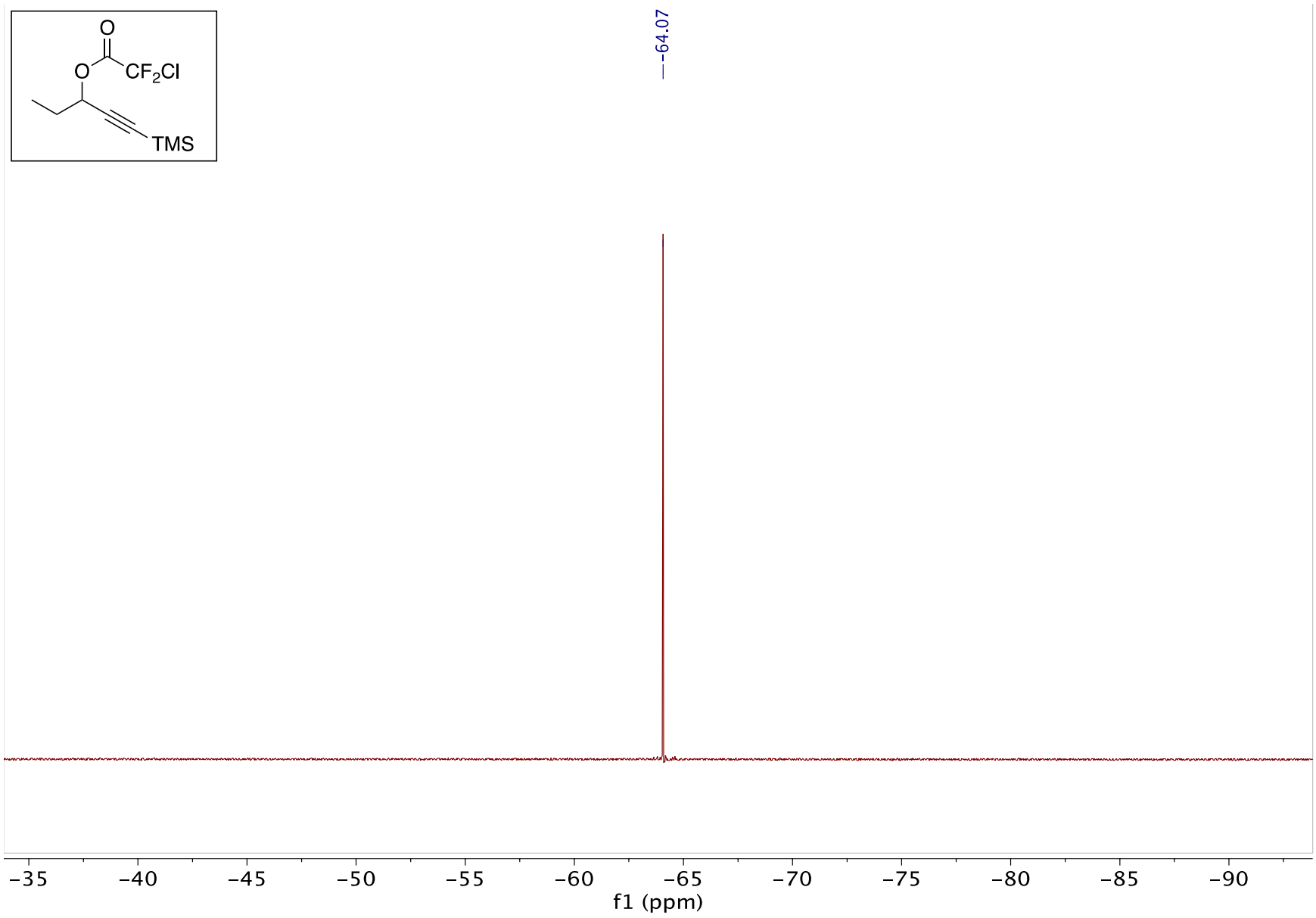
^{1}H NMR for Compound S2.



^{13}C NMR for Compound S2.



^{19}F NMR for Compound S2.



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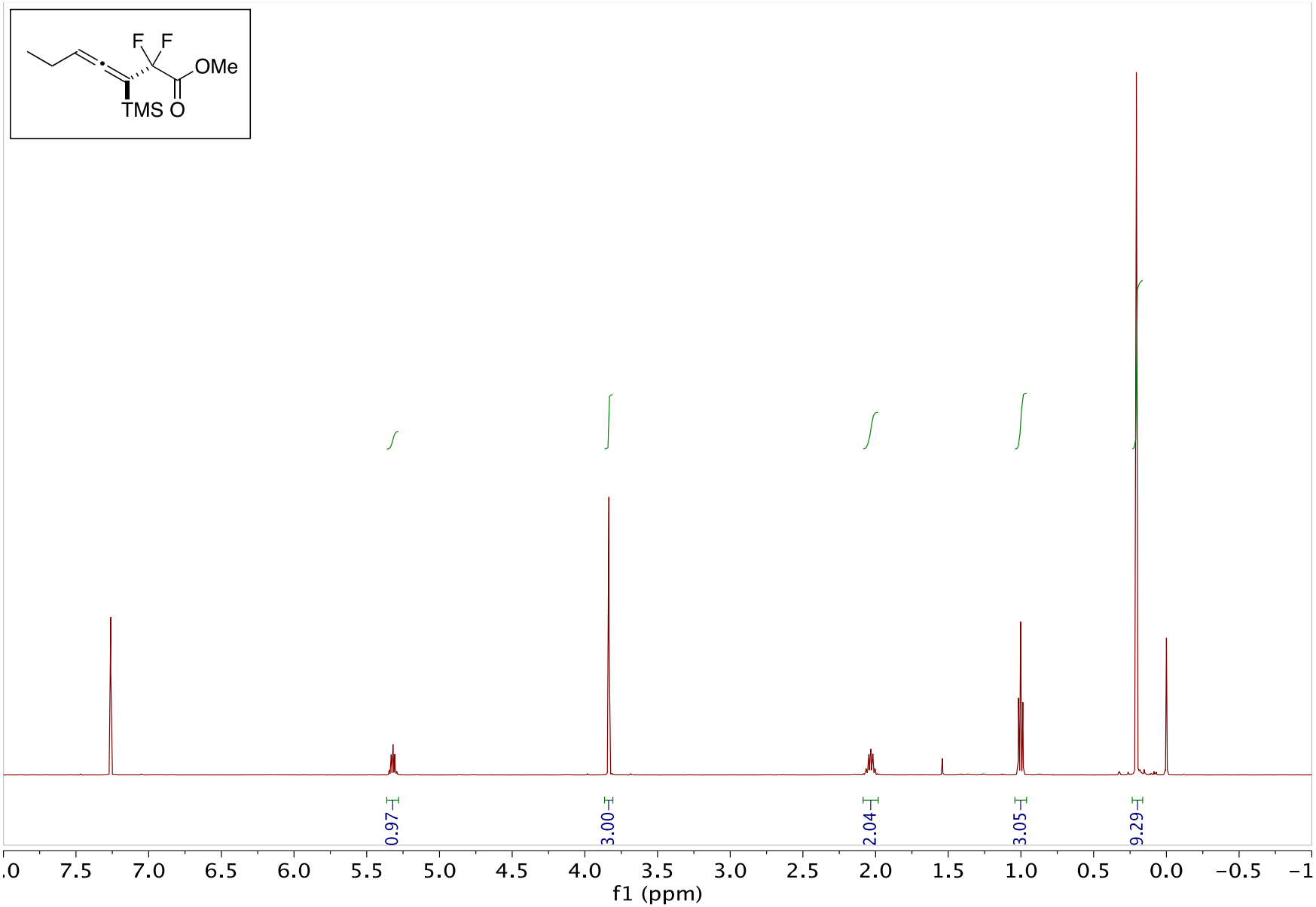

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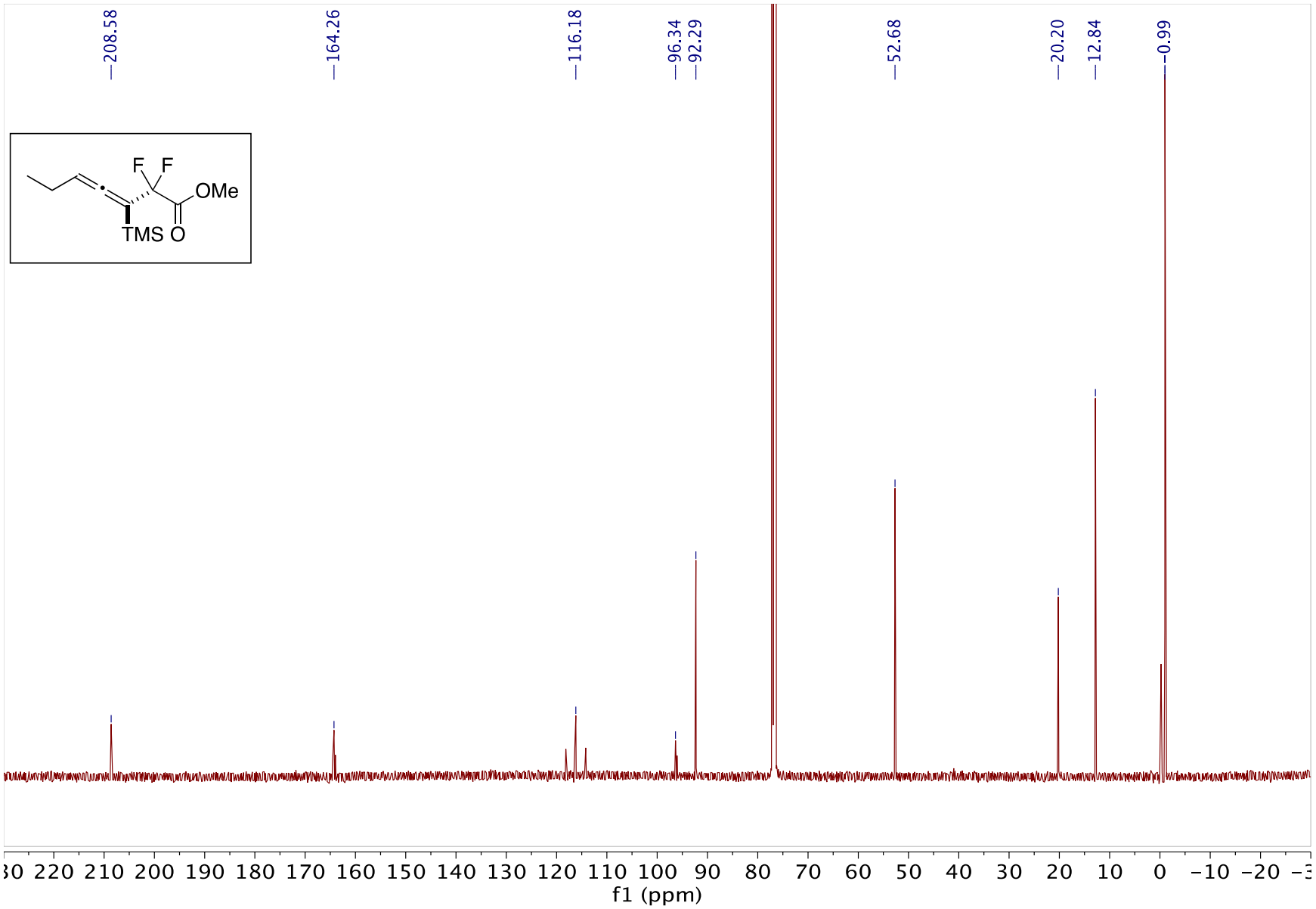

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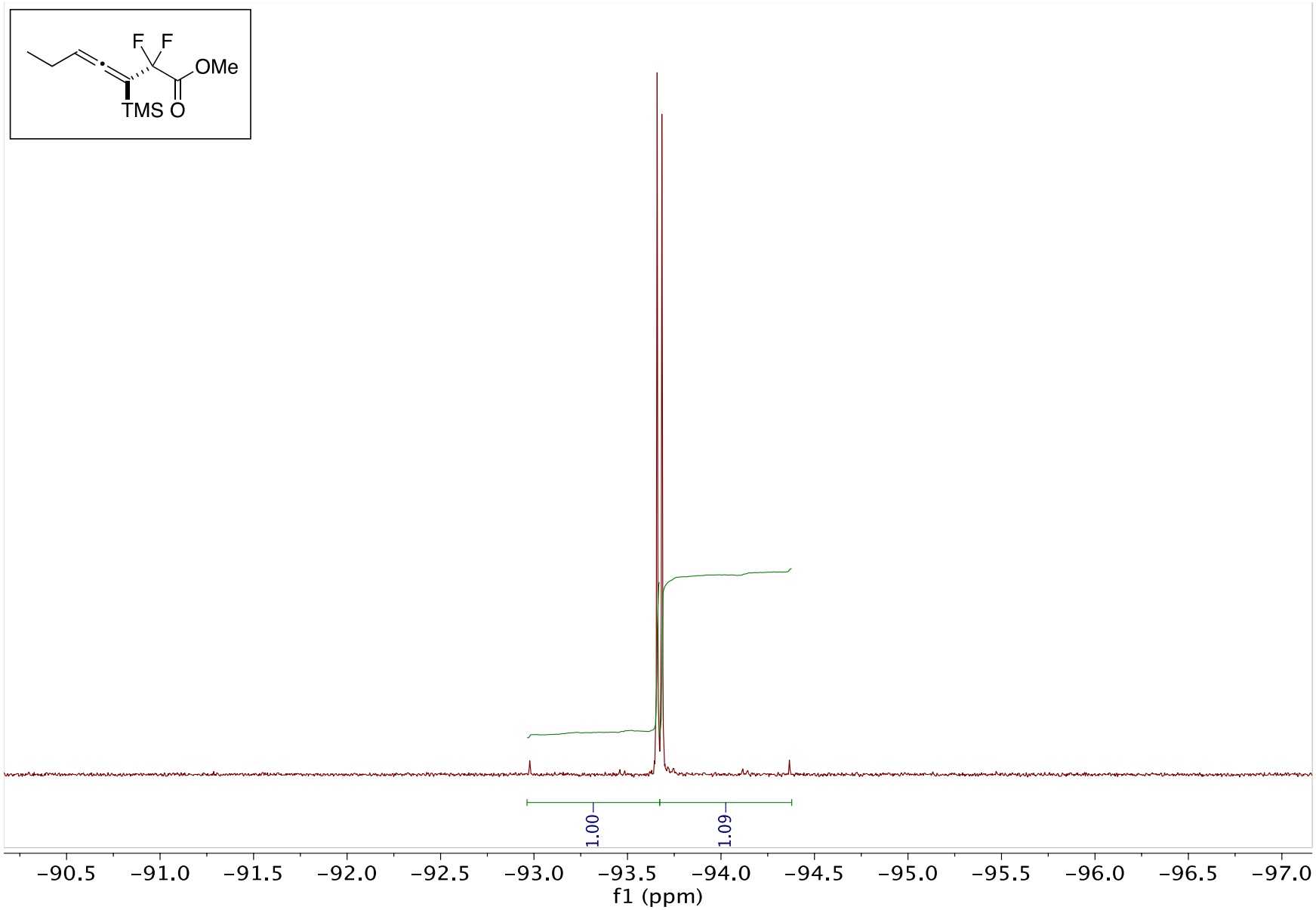
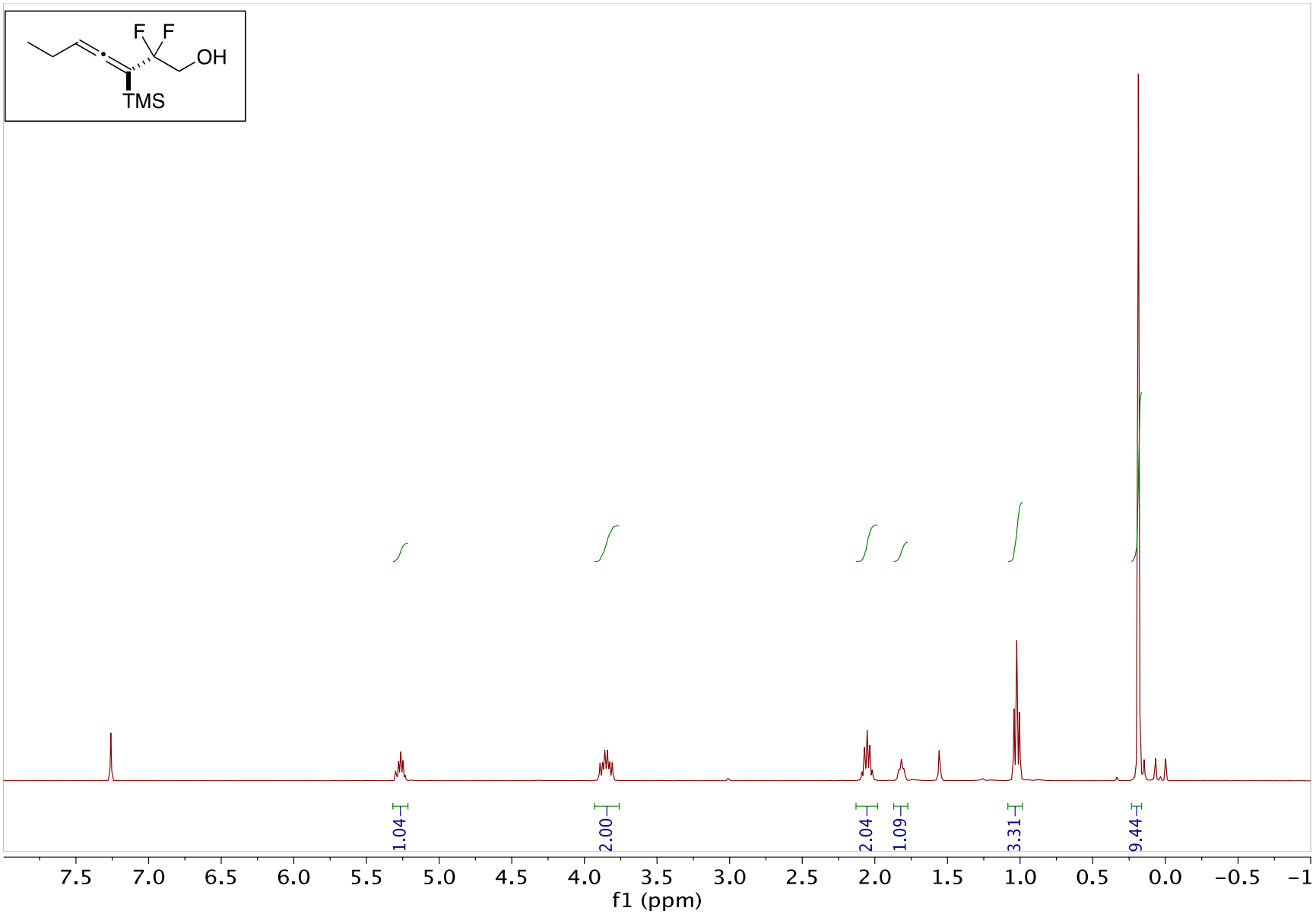


^{1}H NMR for Compound S4.



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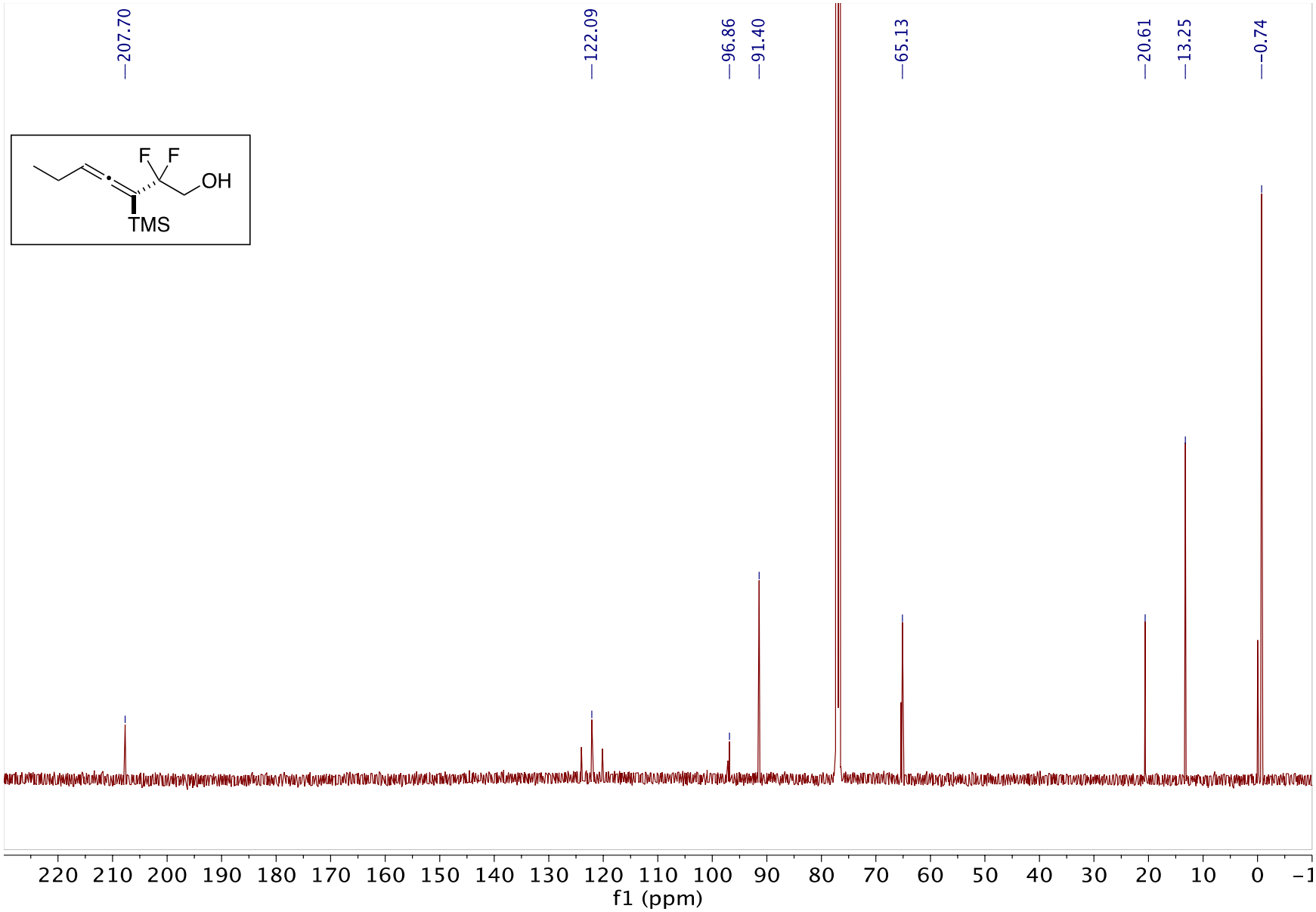

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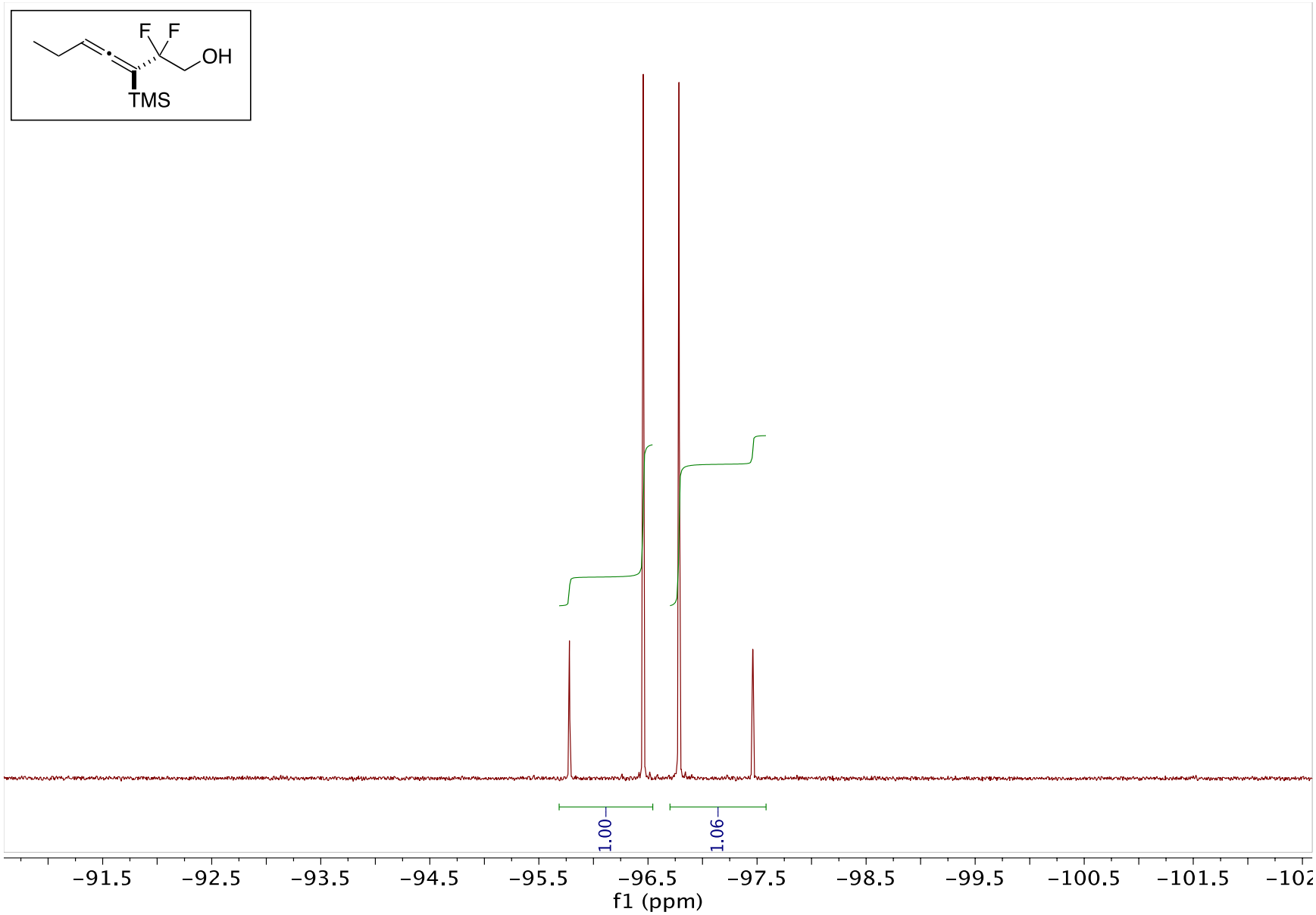
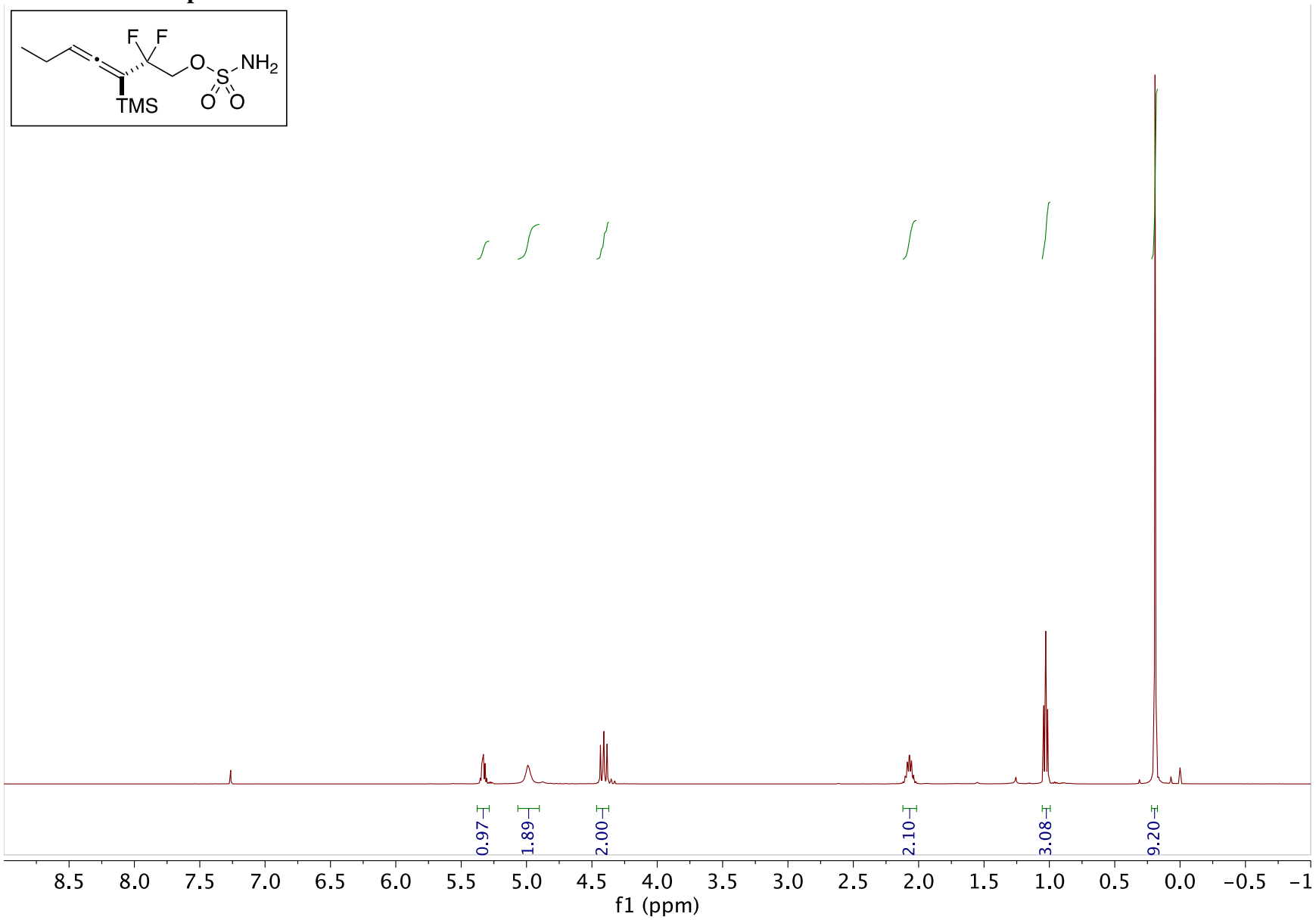
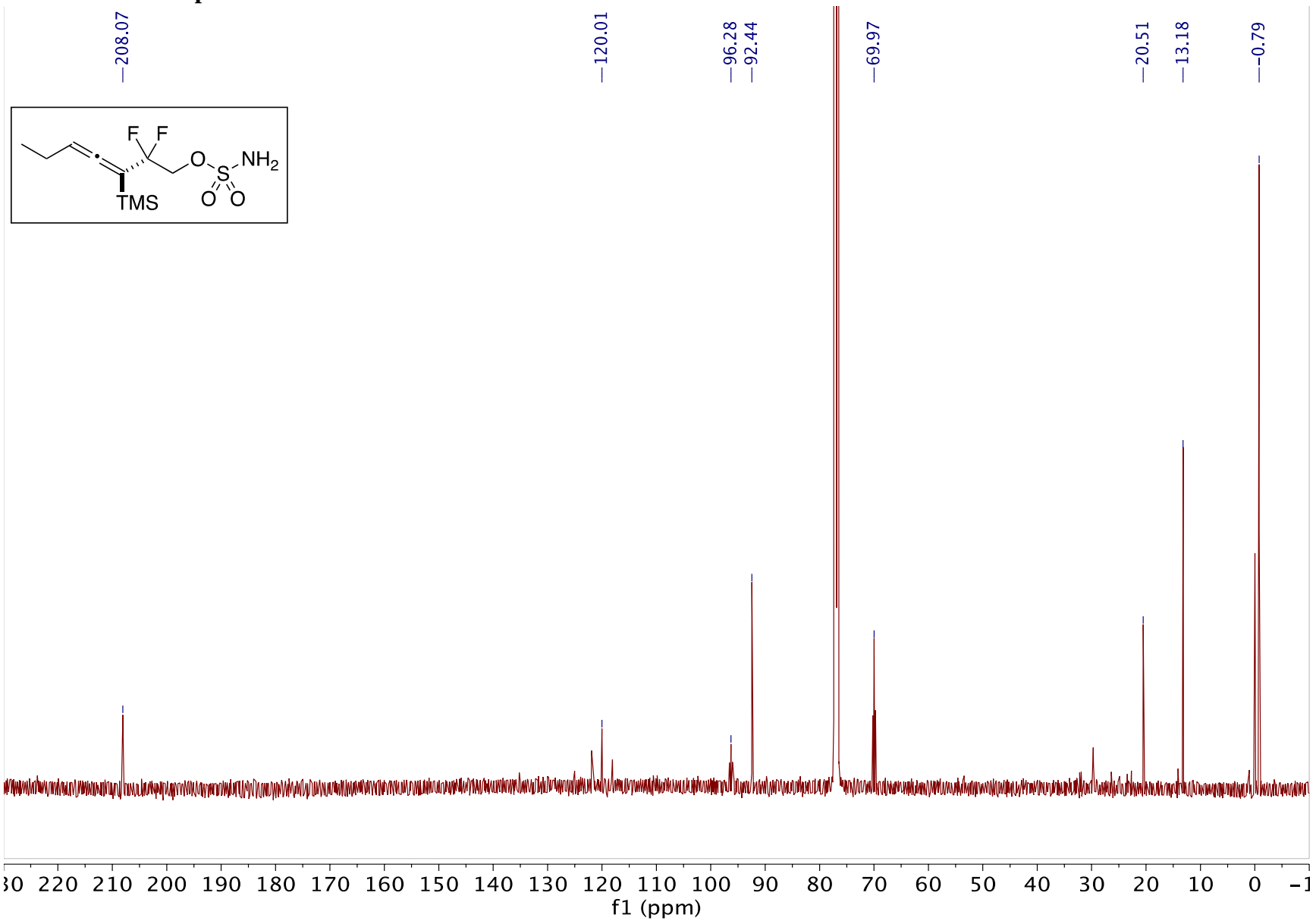


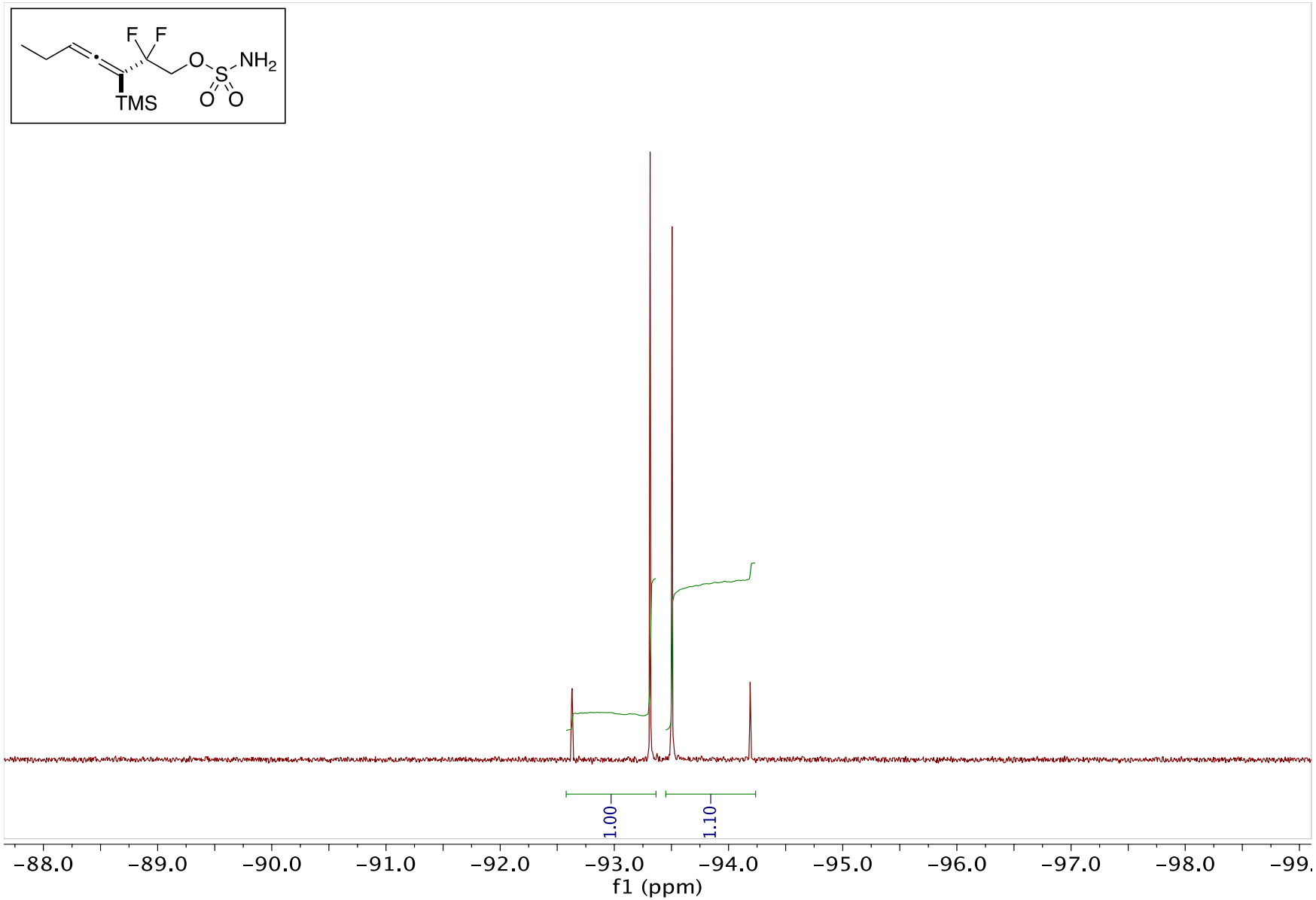
^{1}H NMR for Compound S5.



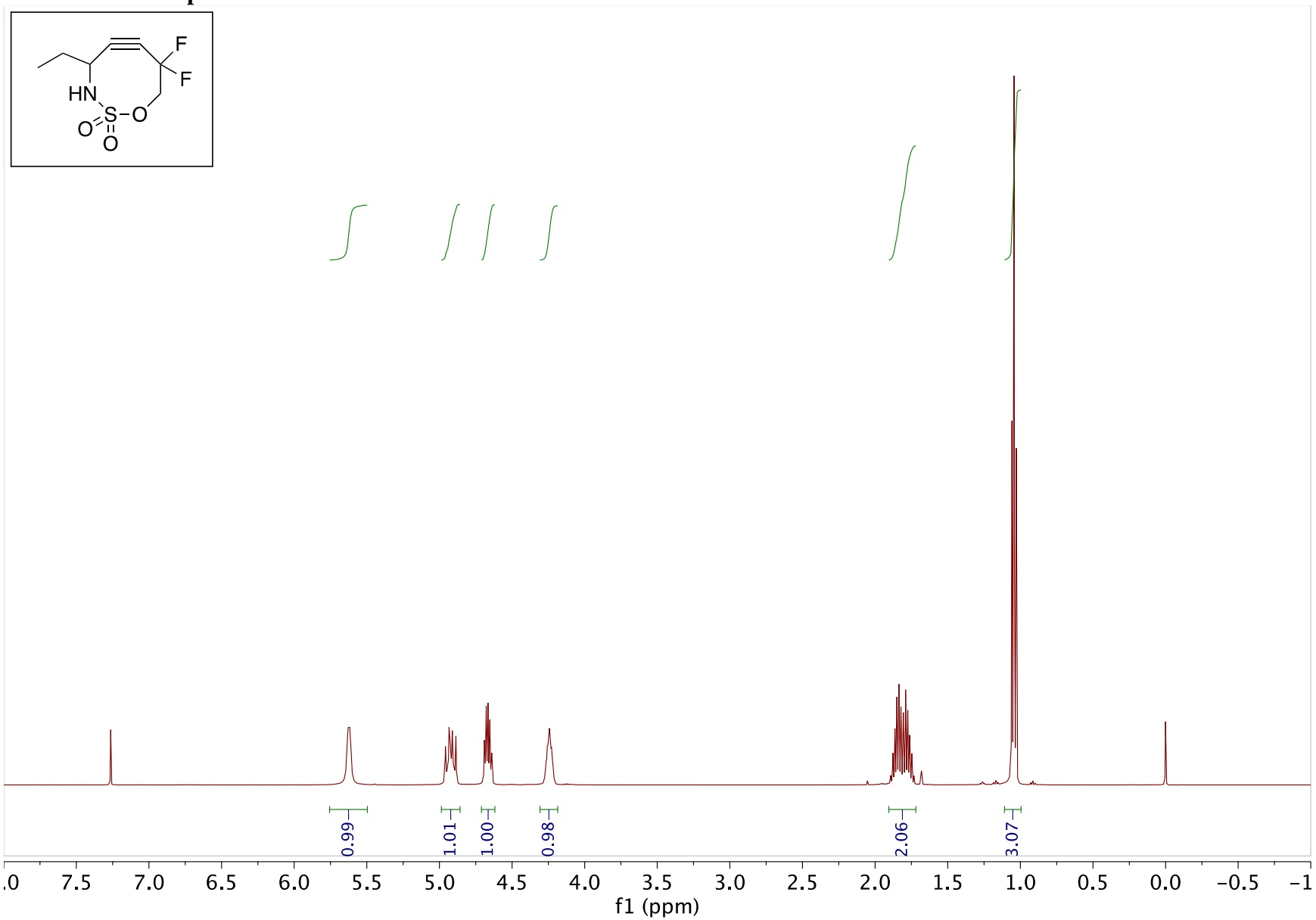
^{13}C NMR for Compound S5.



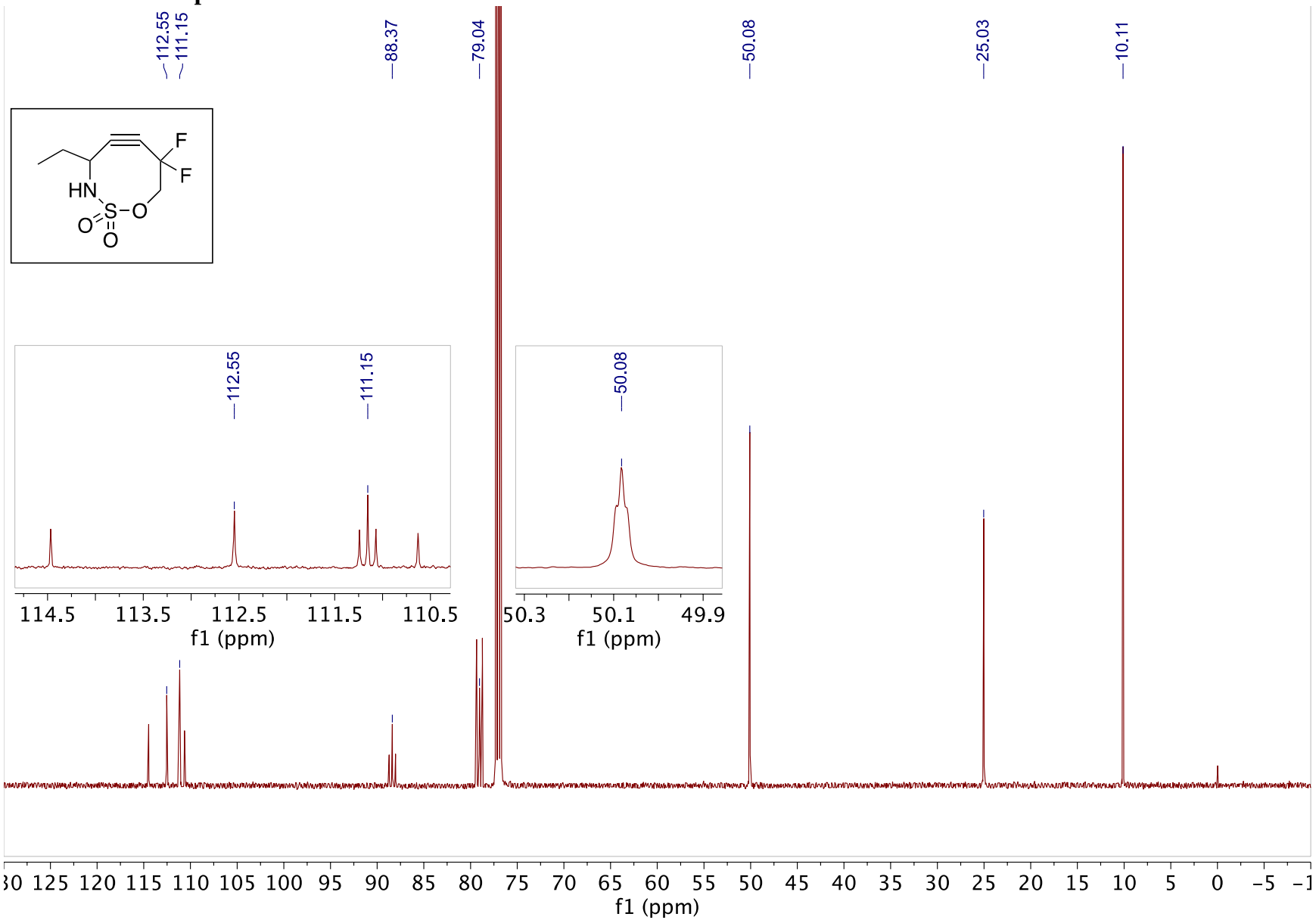
^{19}F NMR for Compound S5.



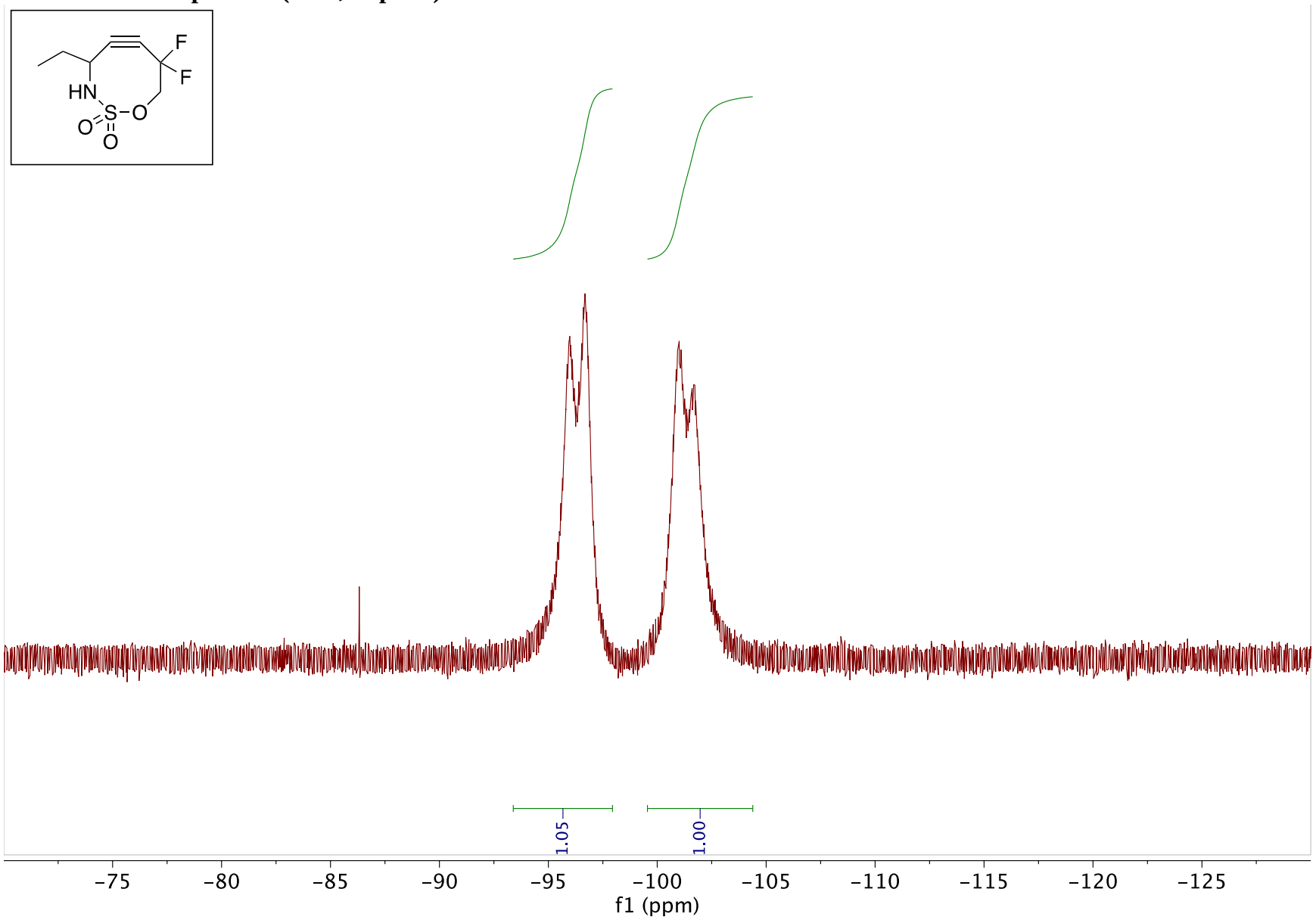
^{1}H NMR for Compound 5.



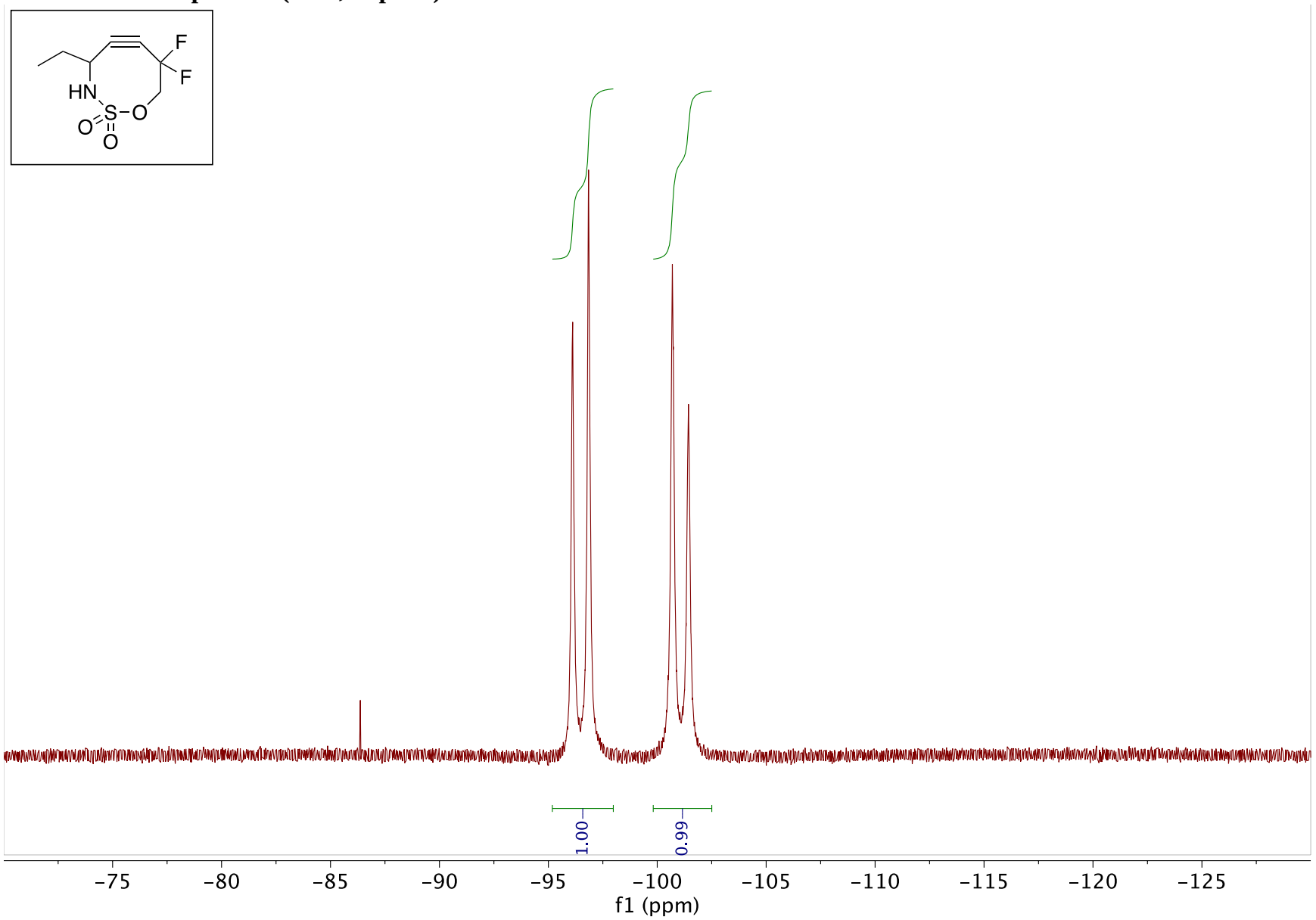
^{13}C NMR for Compound 5.



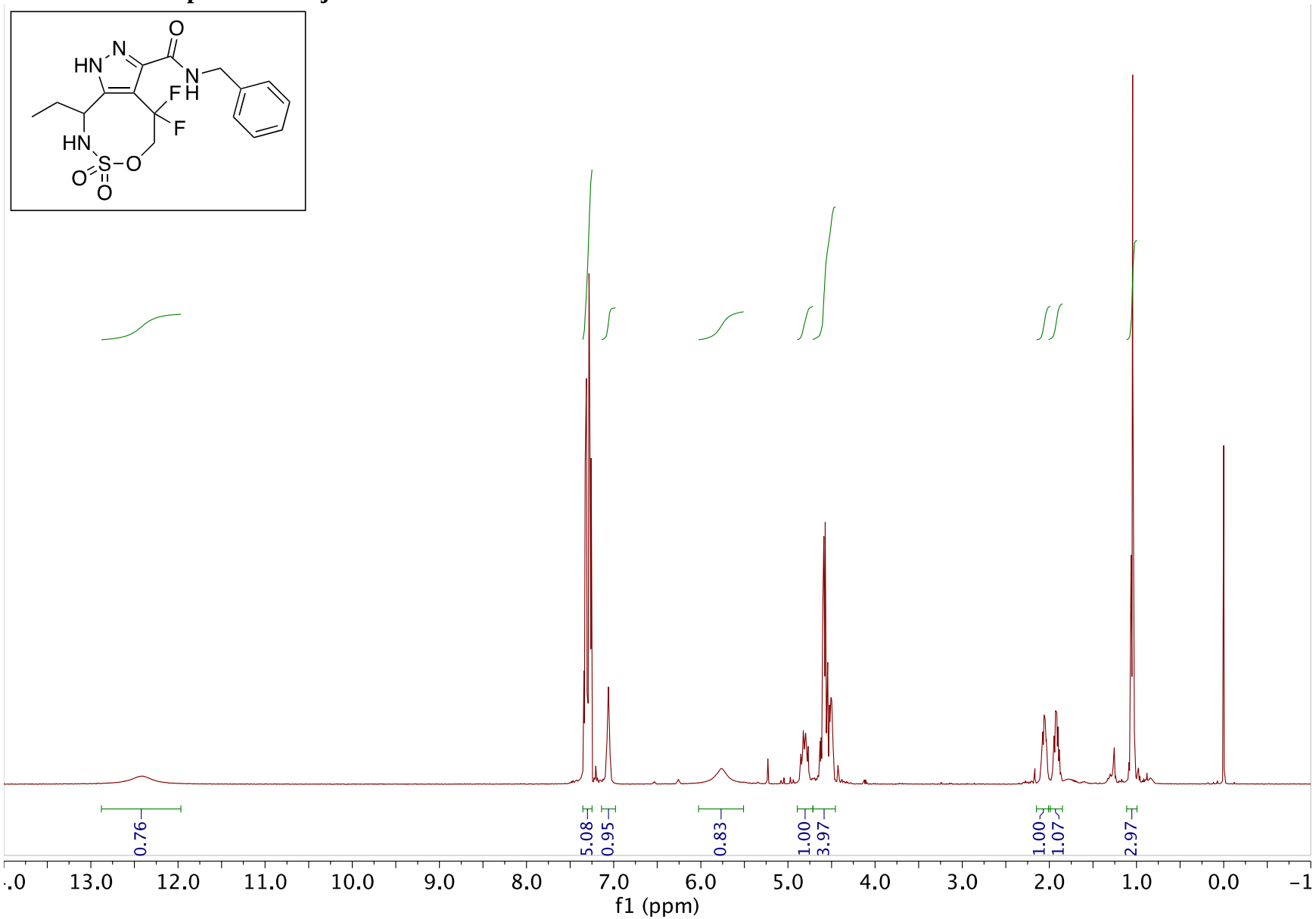
^{19}F NMR for Compound 5 (24°C, setpoint).



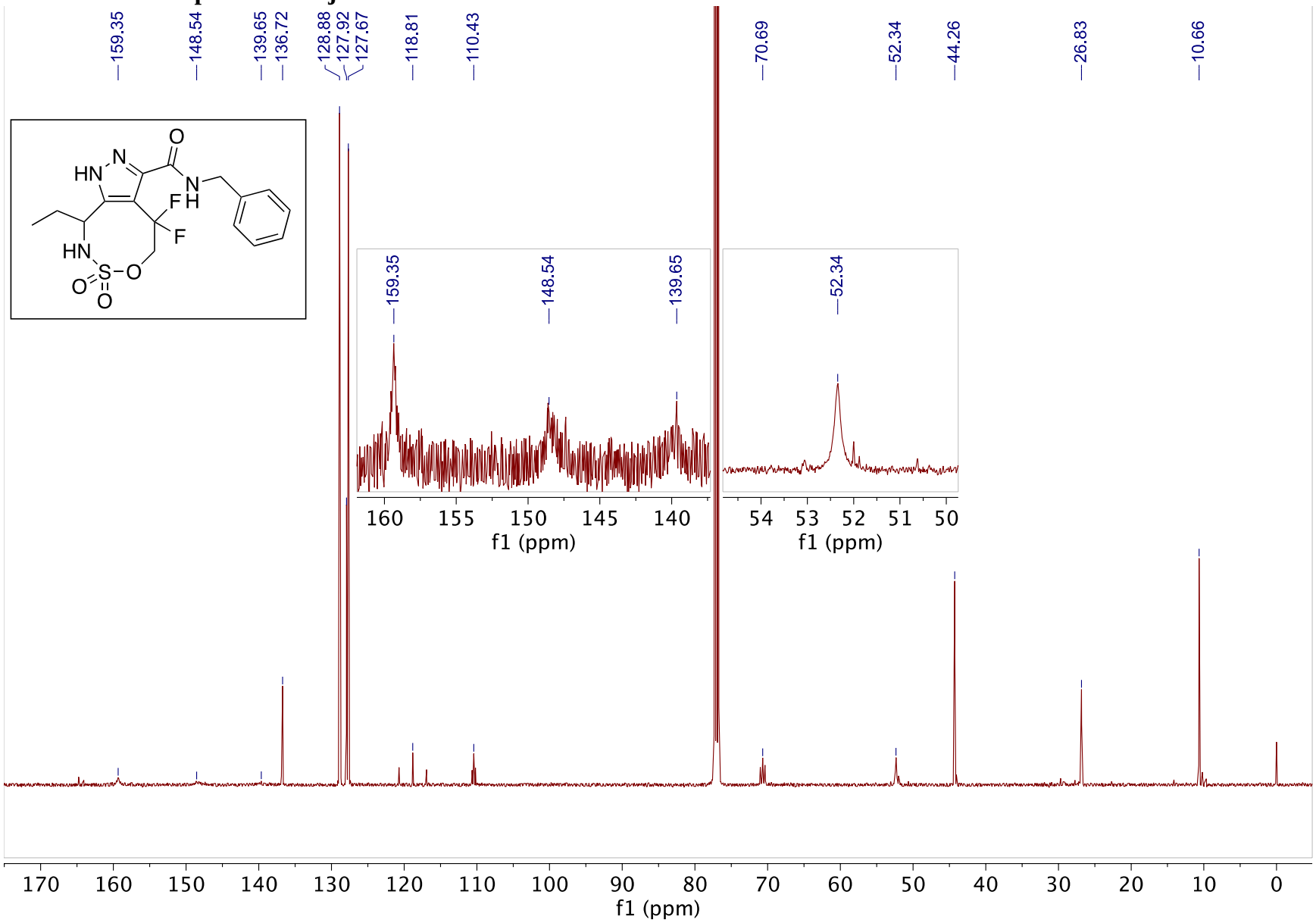
^{19}F NMR for Compound 5 (55°C, setpoint).



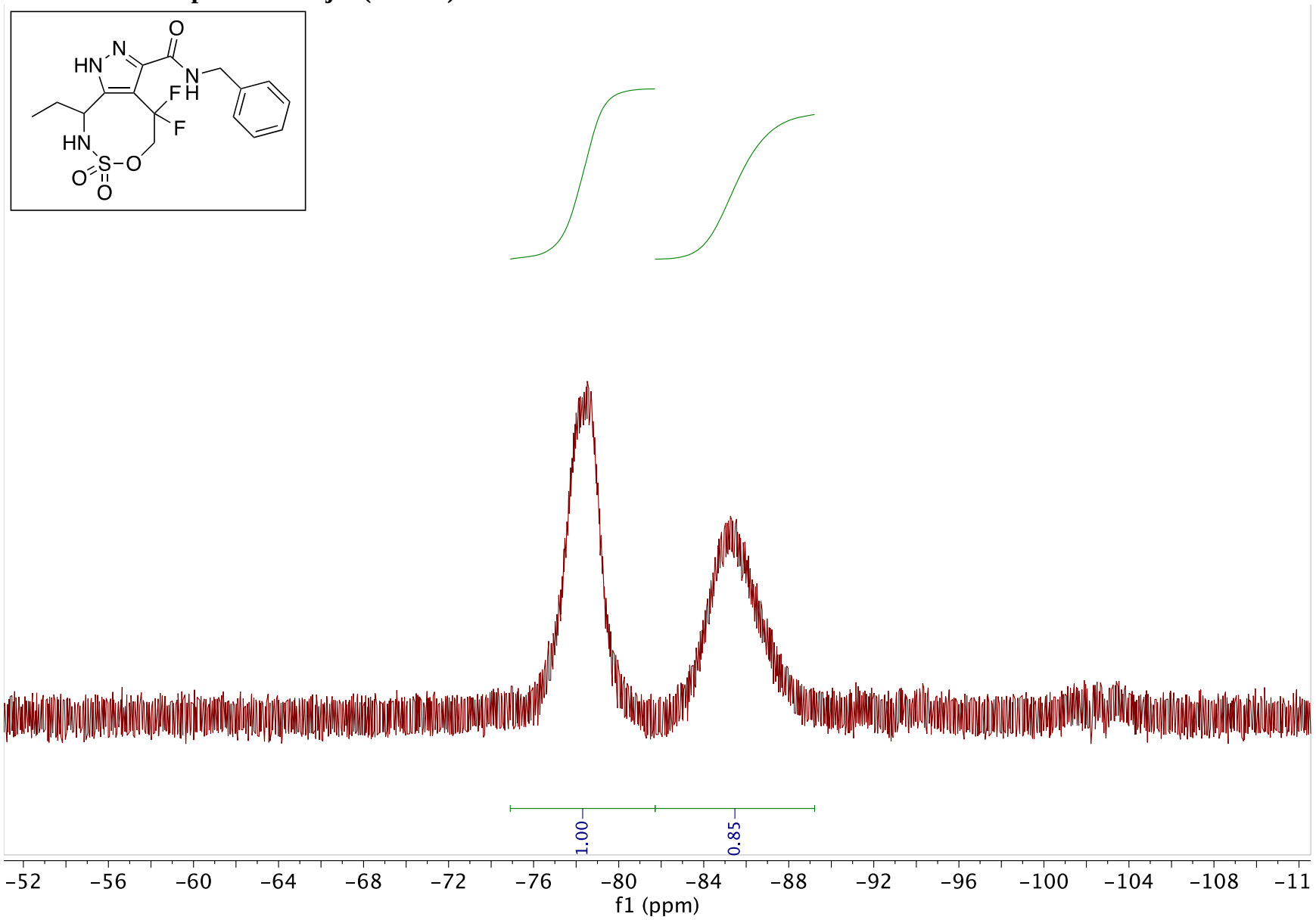
^{1}H NMR for Compound 5a major.



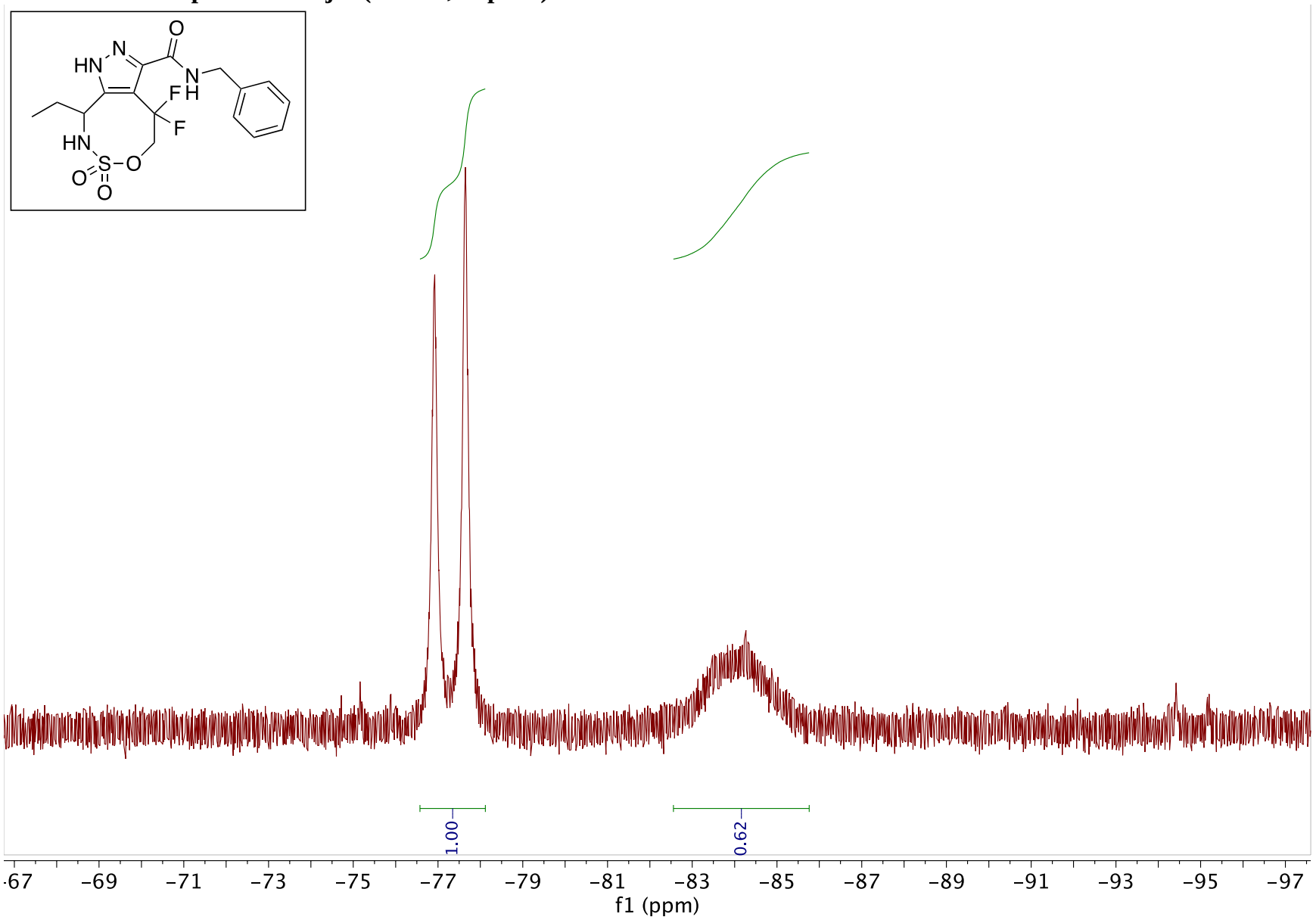
^{13}C NMR for Compound 5a major.



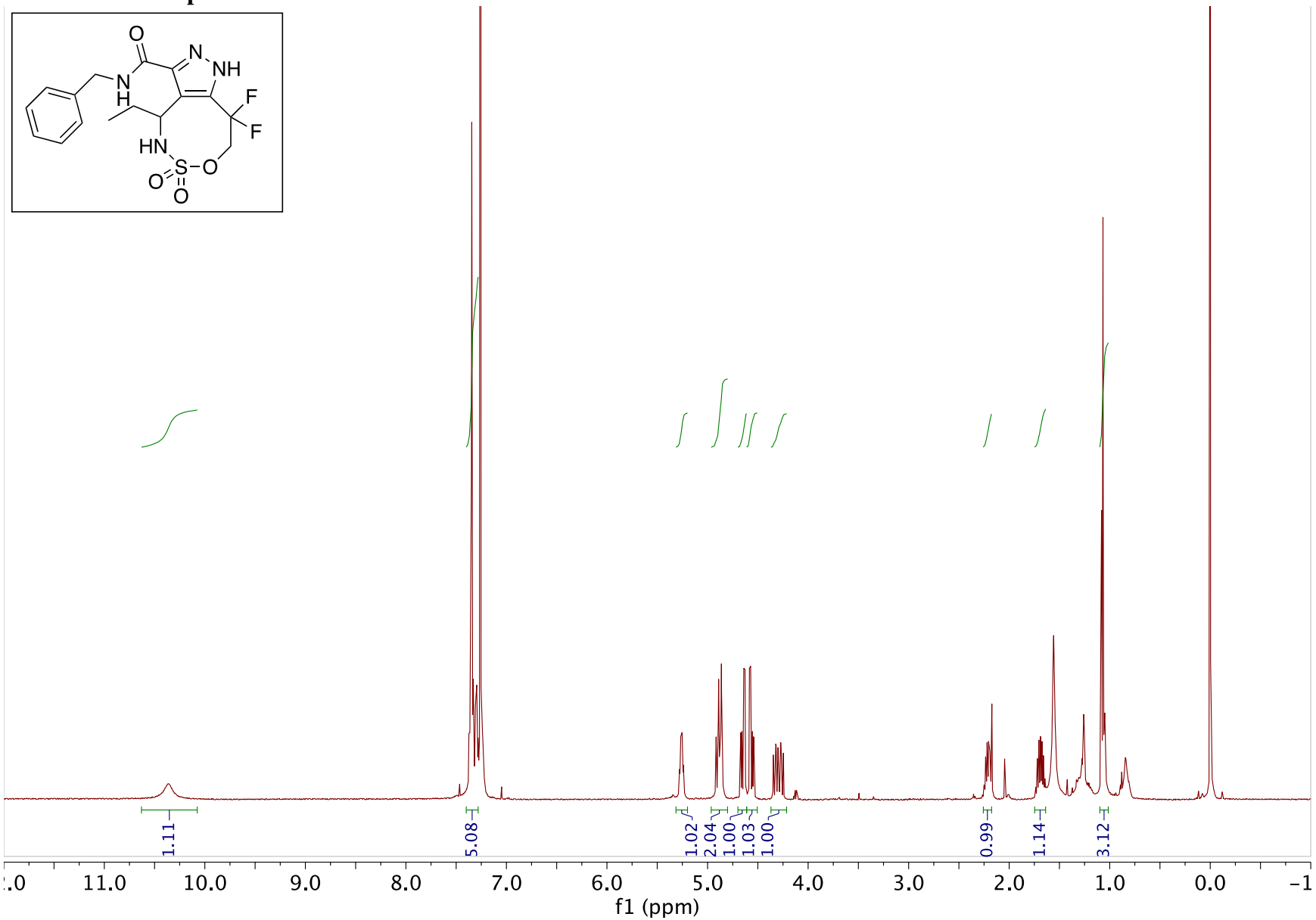
^{19}F NMR for Compound 5a major (at 24ºC).



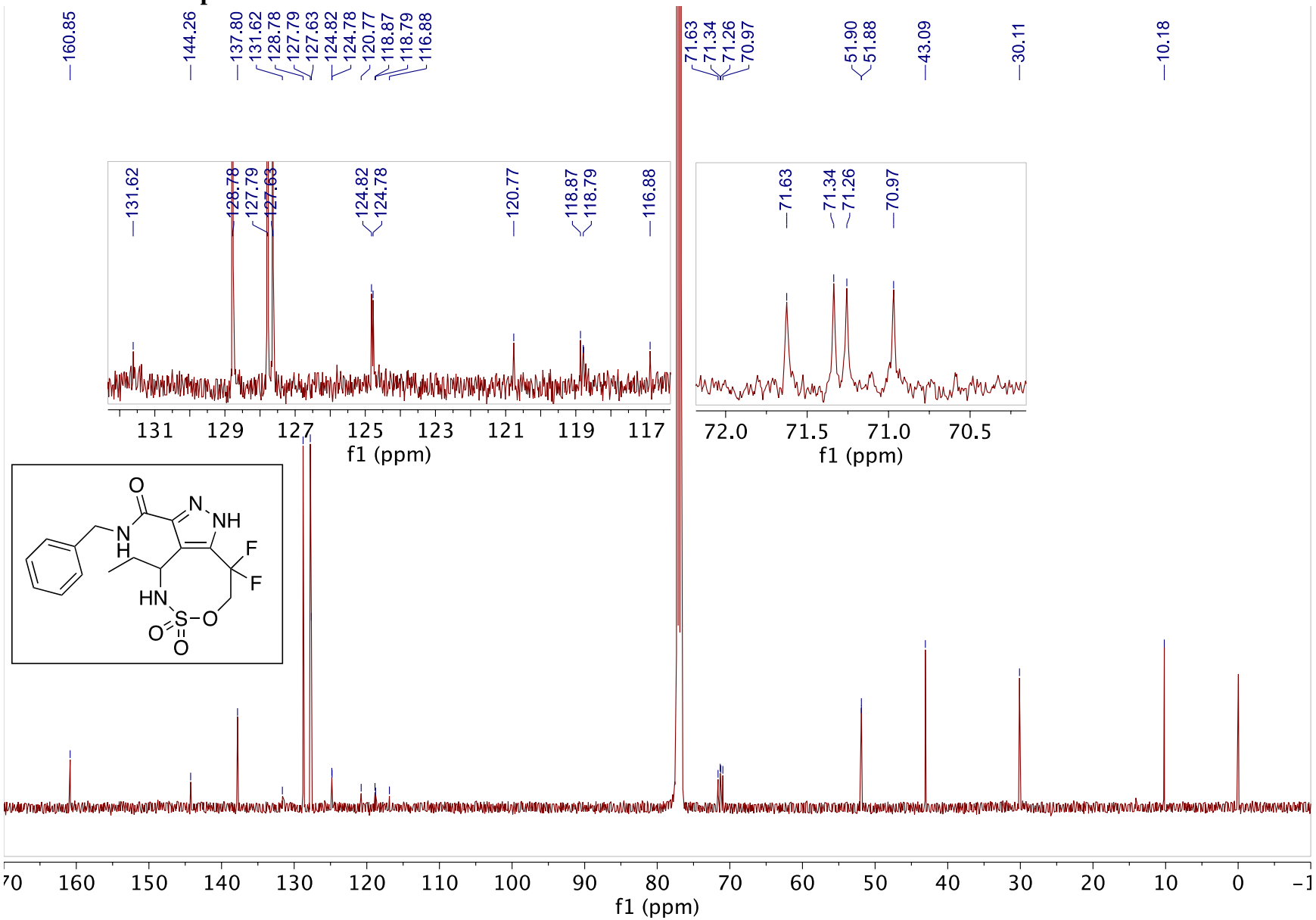
^{19}F NMR for Compound 5a major (at 55°C, setpoint).



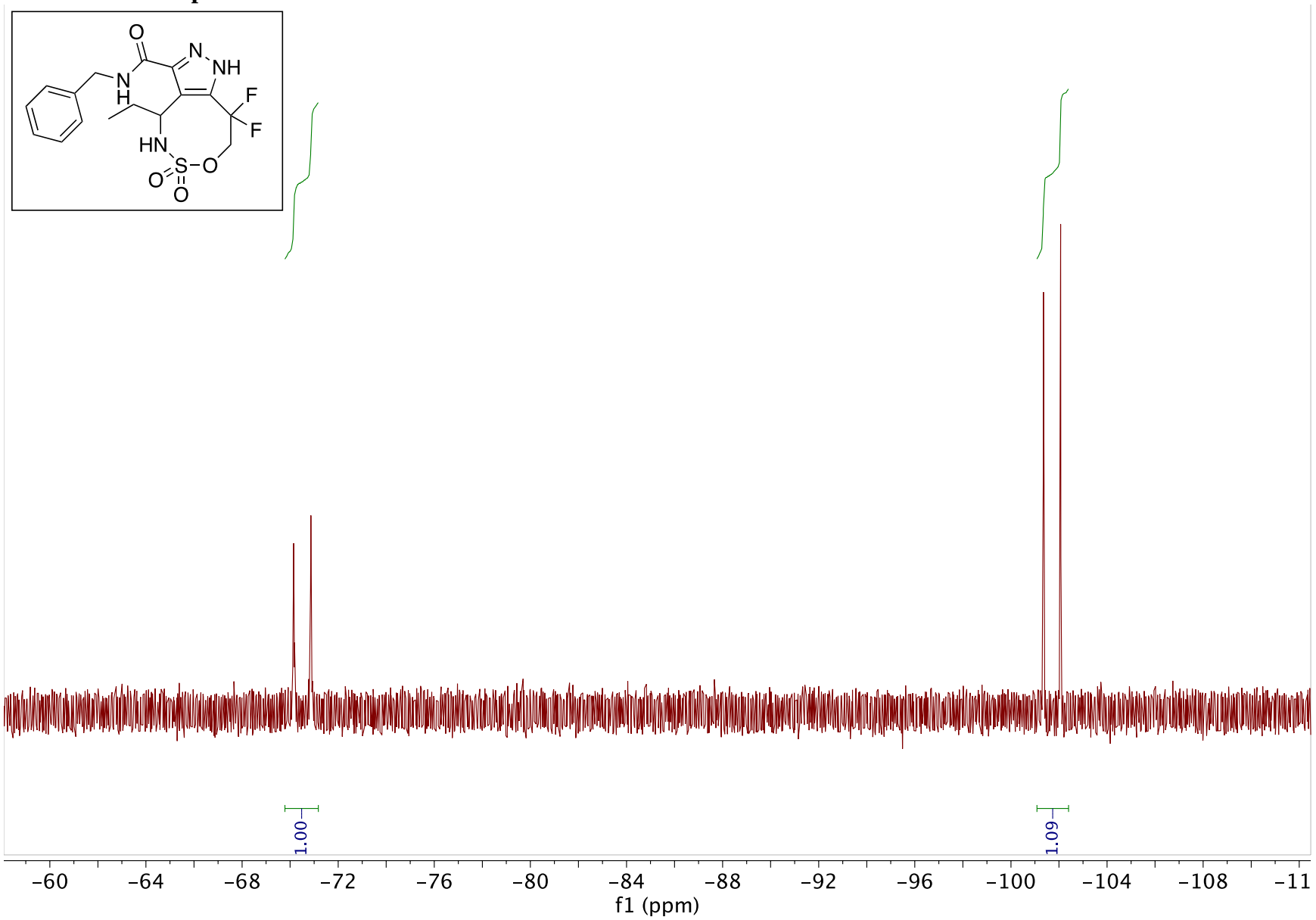
^{1}H NMR for Compound 5a minor.



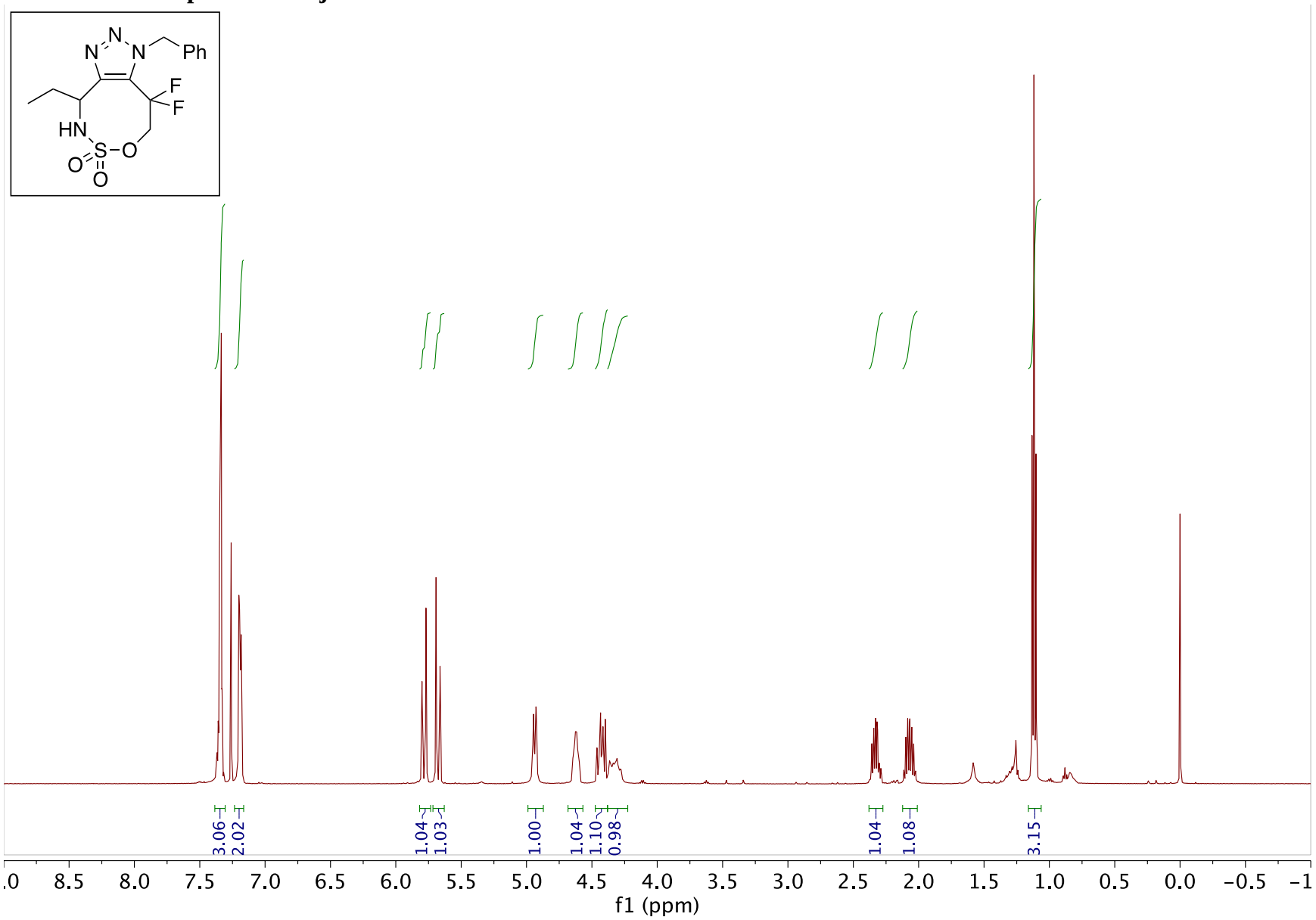
^{13}C NMR for Compound 5a minor.



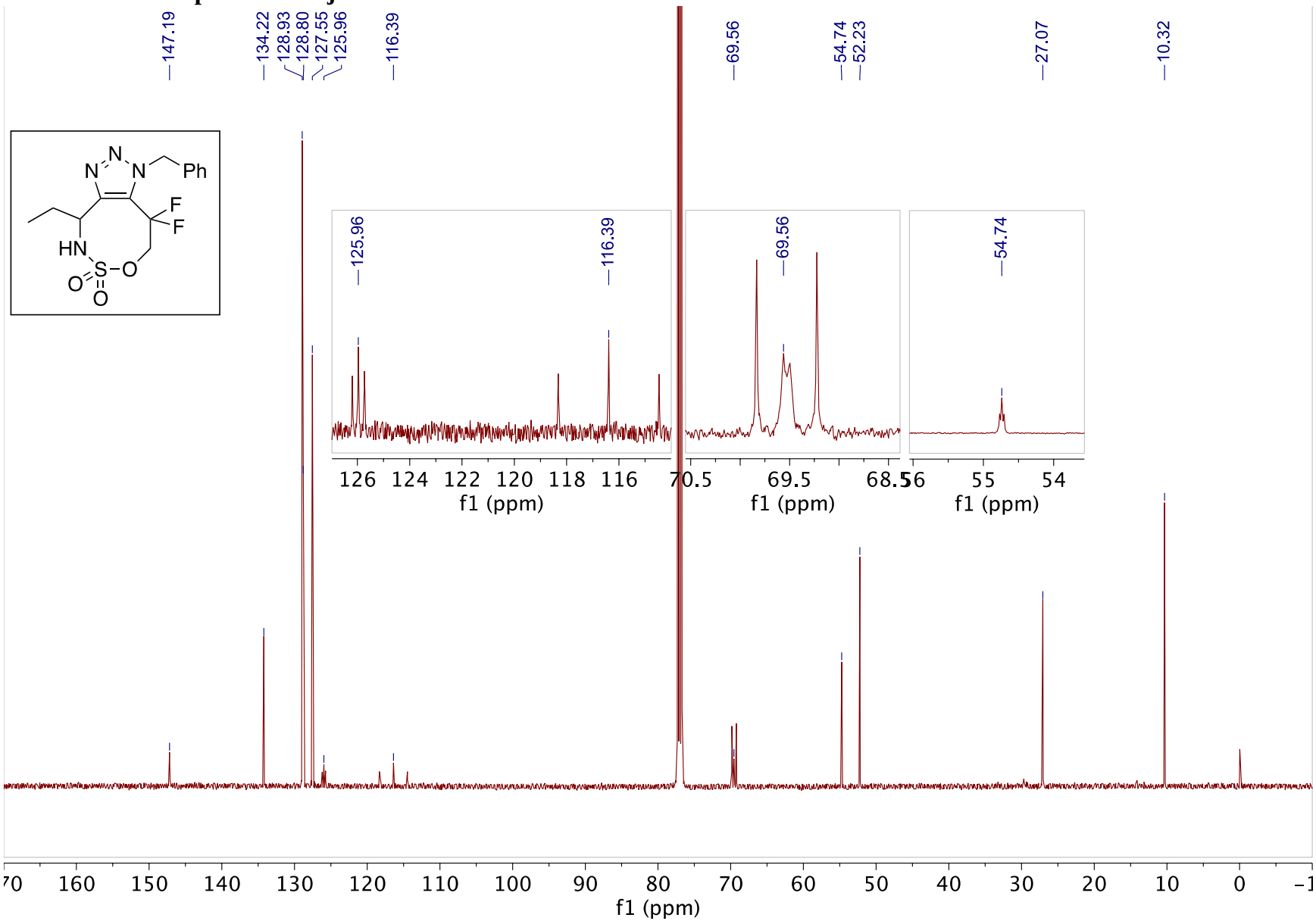
^{19}F NMR for Compound 5a minor.



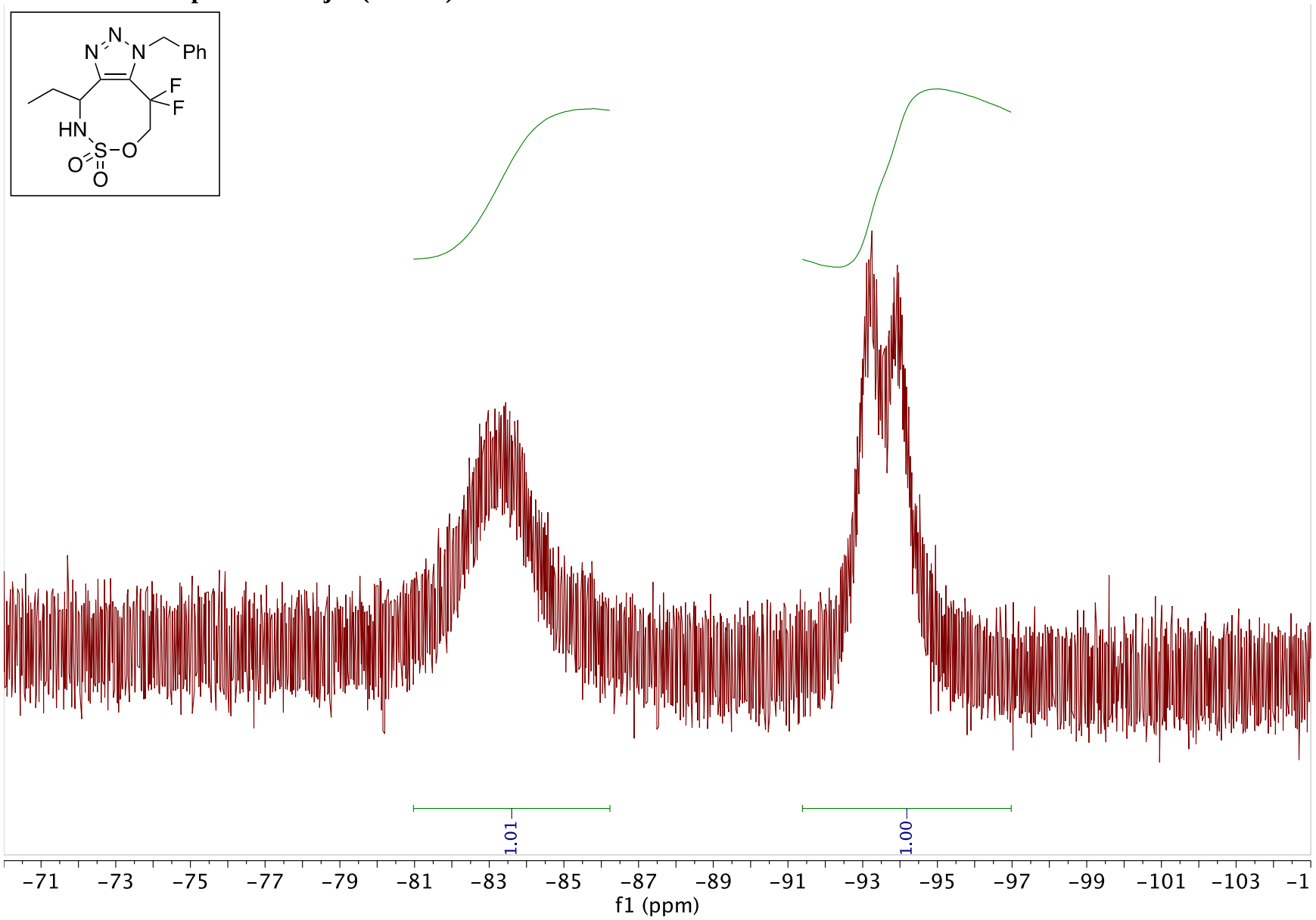
^{1}H NMR for Compound 5c major.



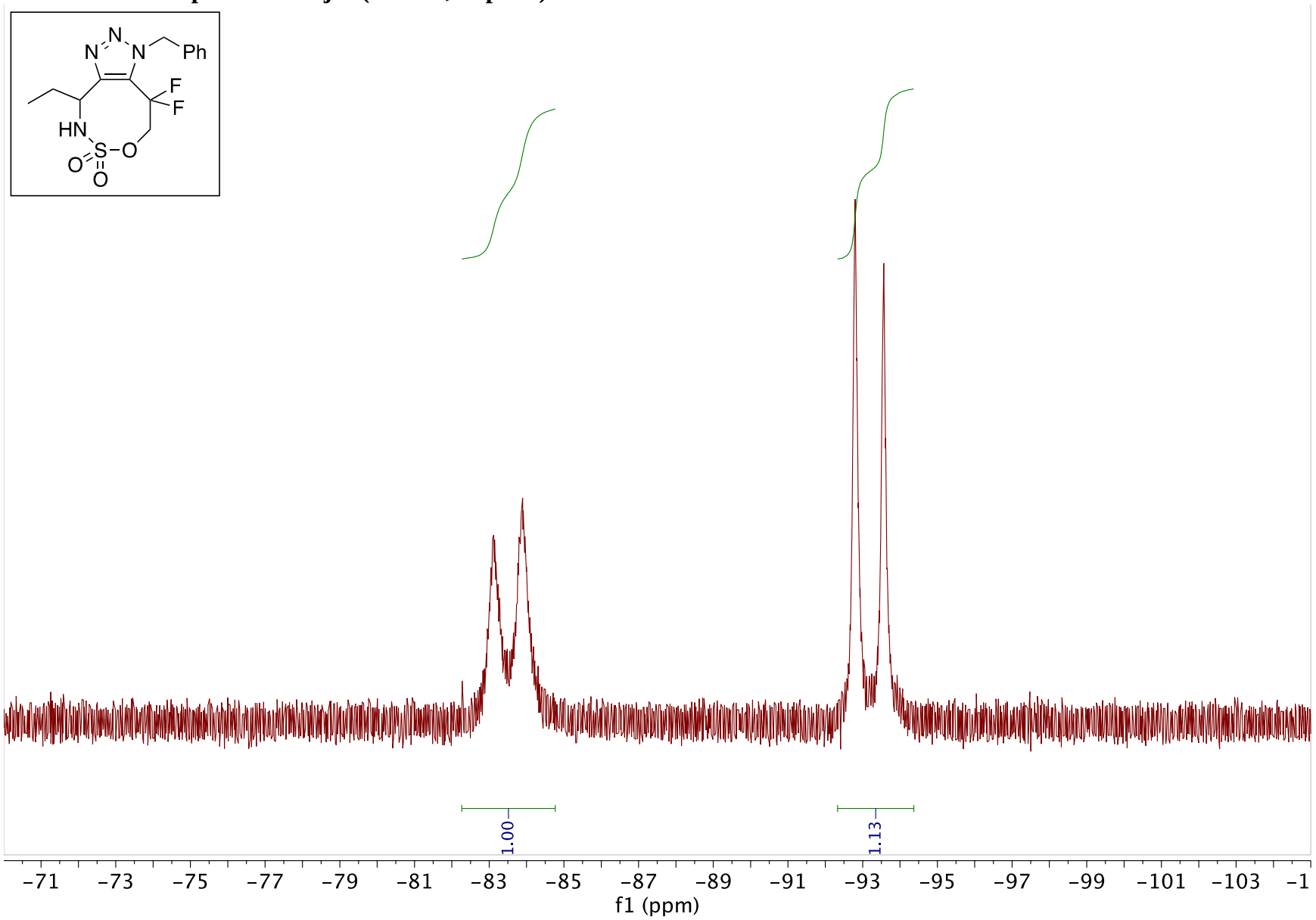
^{13}C NMR for Compound 5c major.



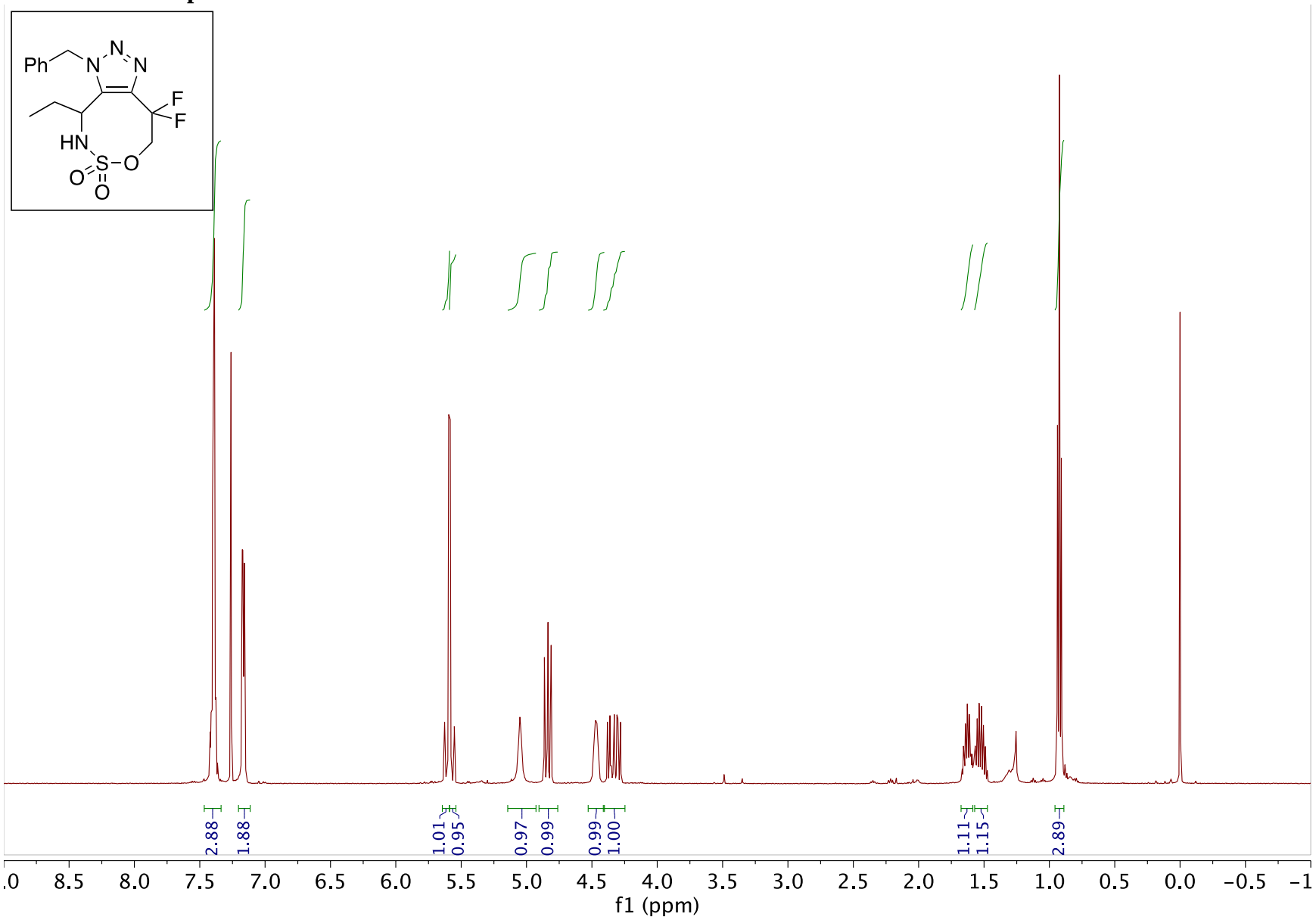
^{19}F NMR for Compound 5c major (at 24°C).



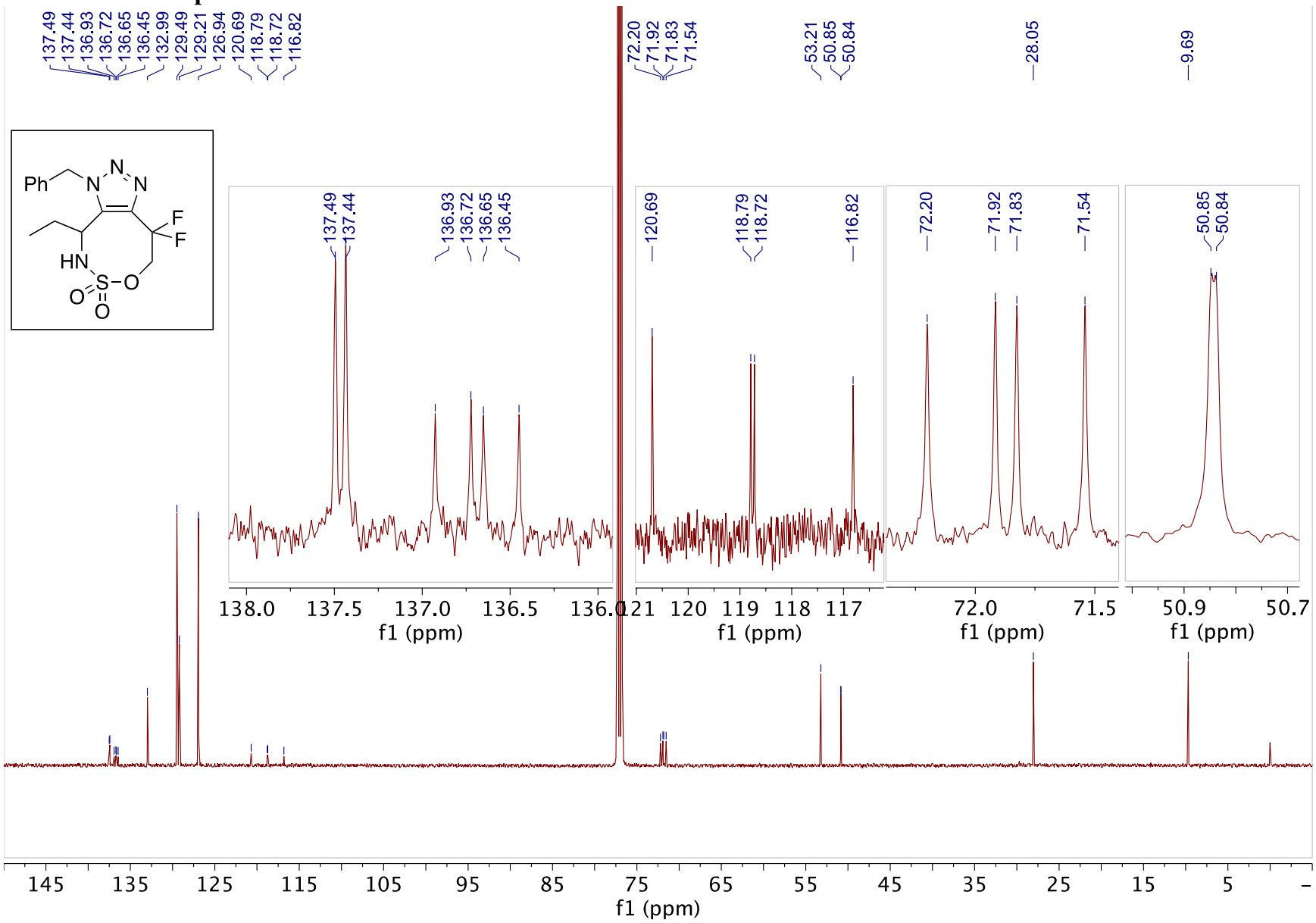
^{19}F NMR for Compound 5c major (at 55°C, setpoint).



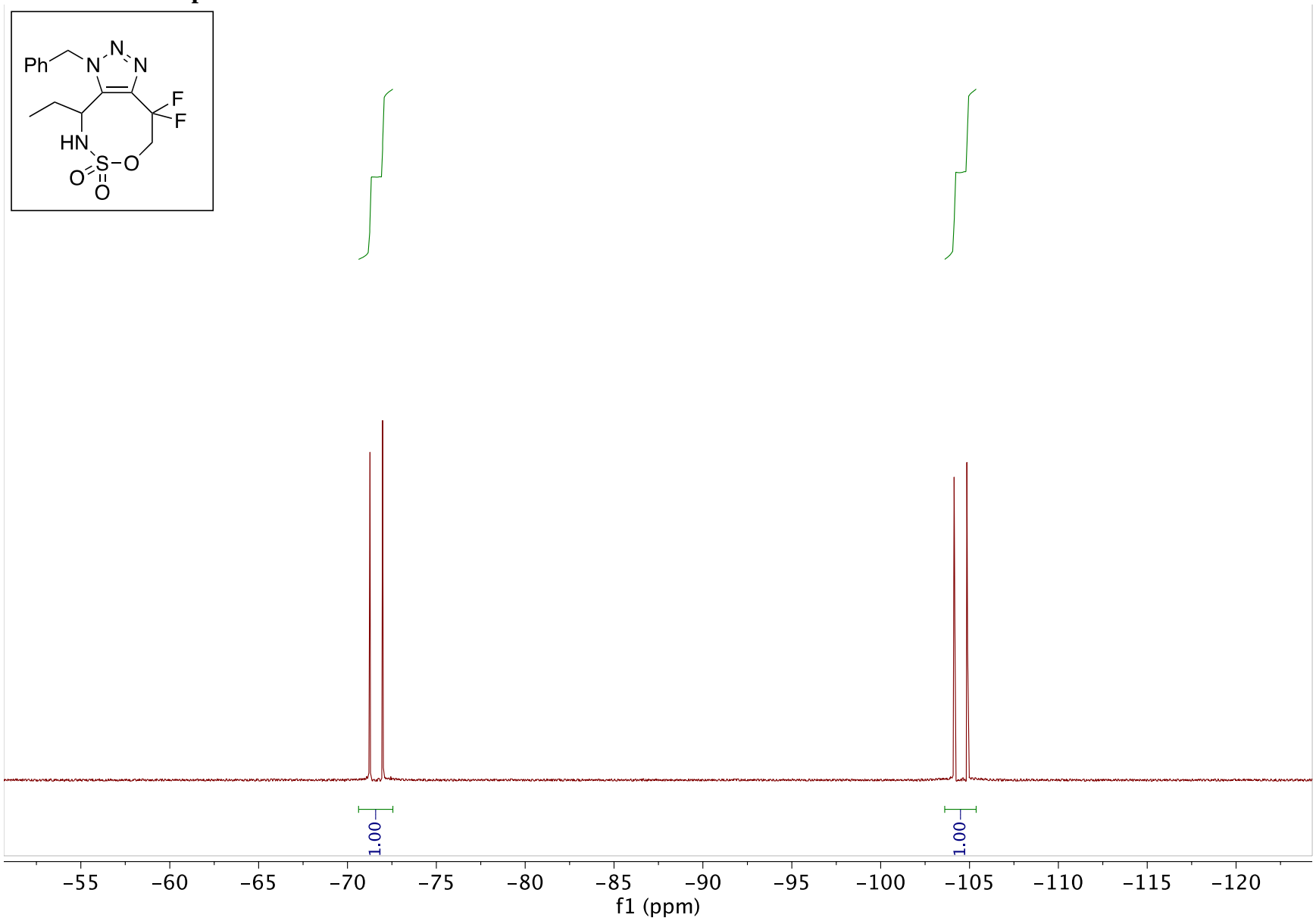
^{1}H NMR for Compound 5c minor.



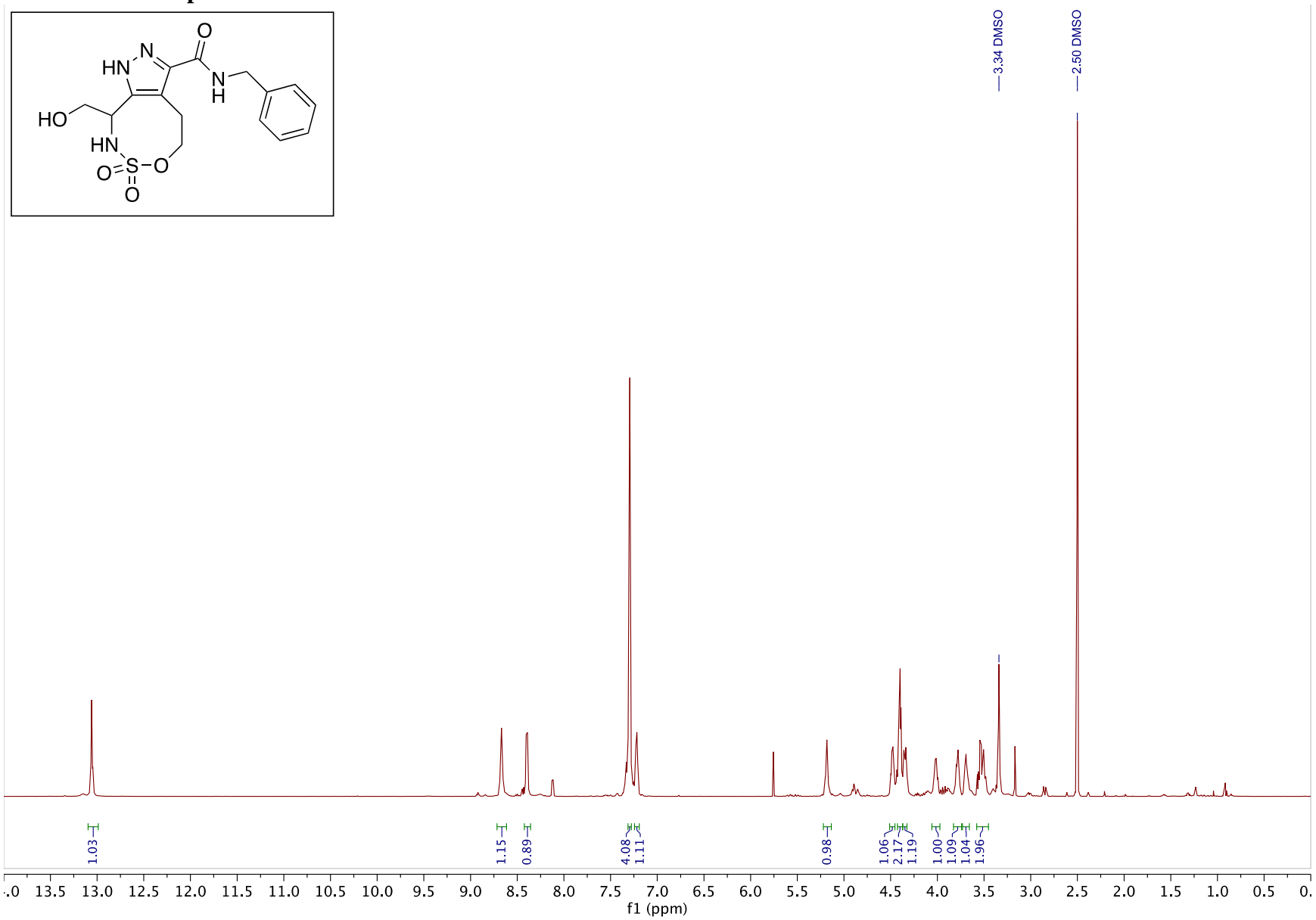
^{13}C NMR for Compound 5c minor.



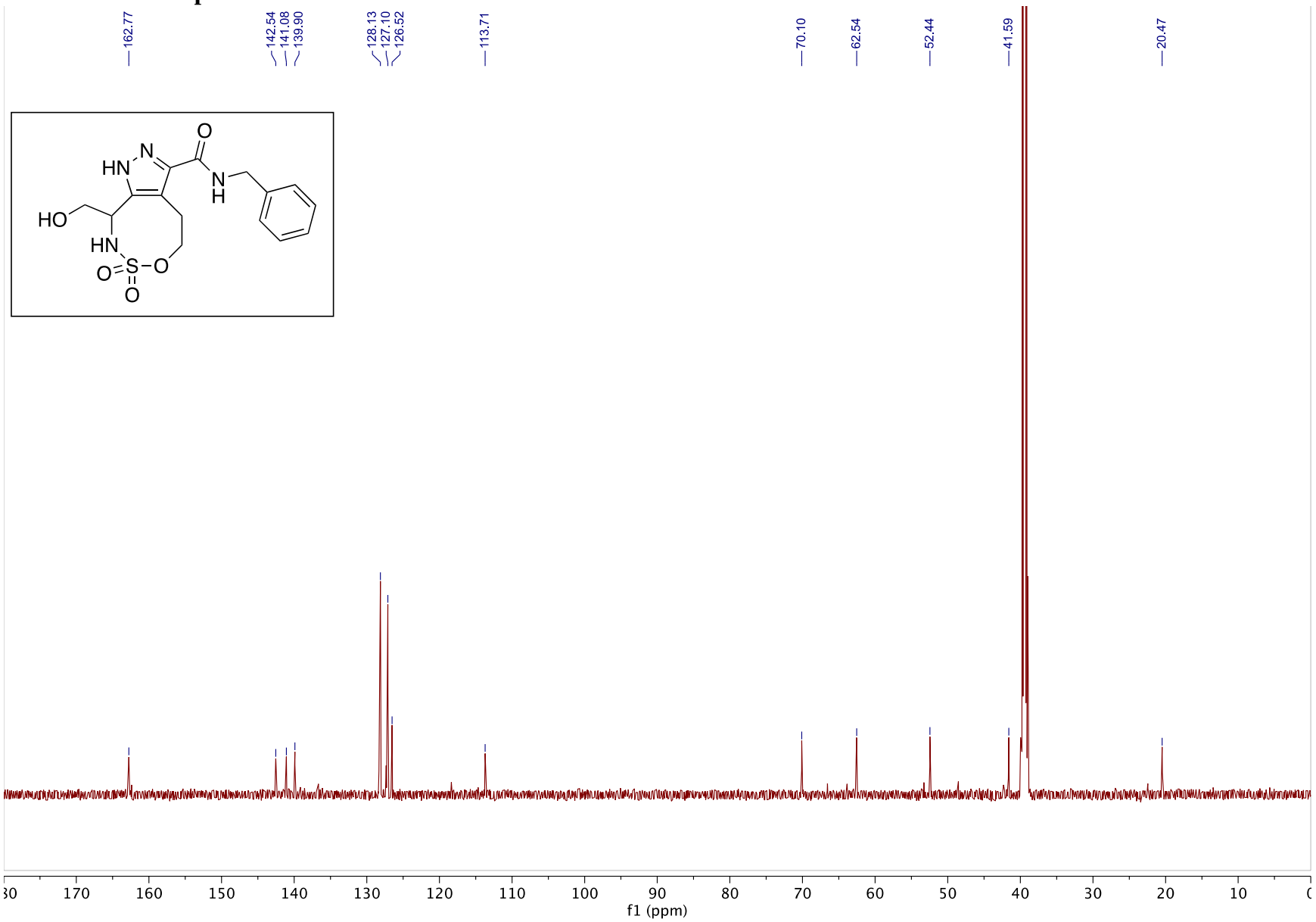
^{19}F NMR for Compound 5c minor.



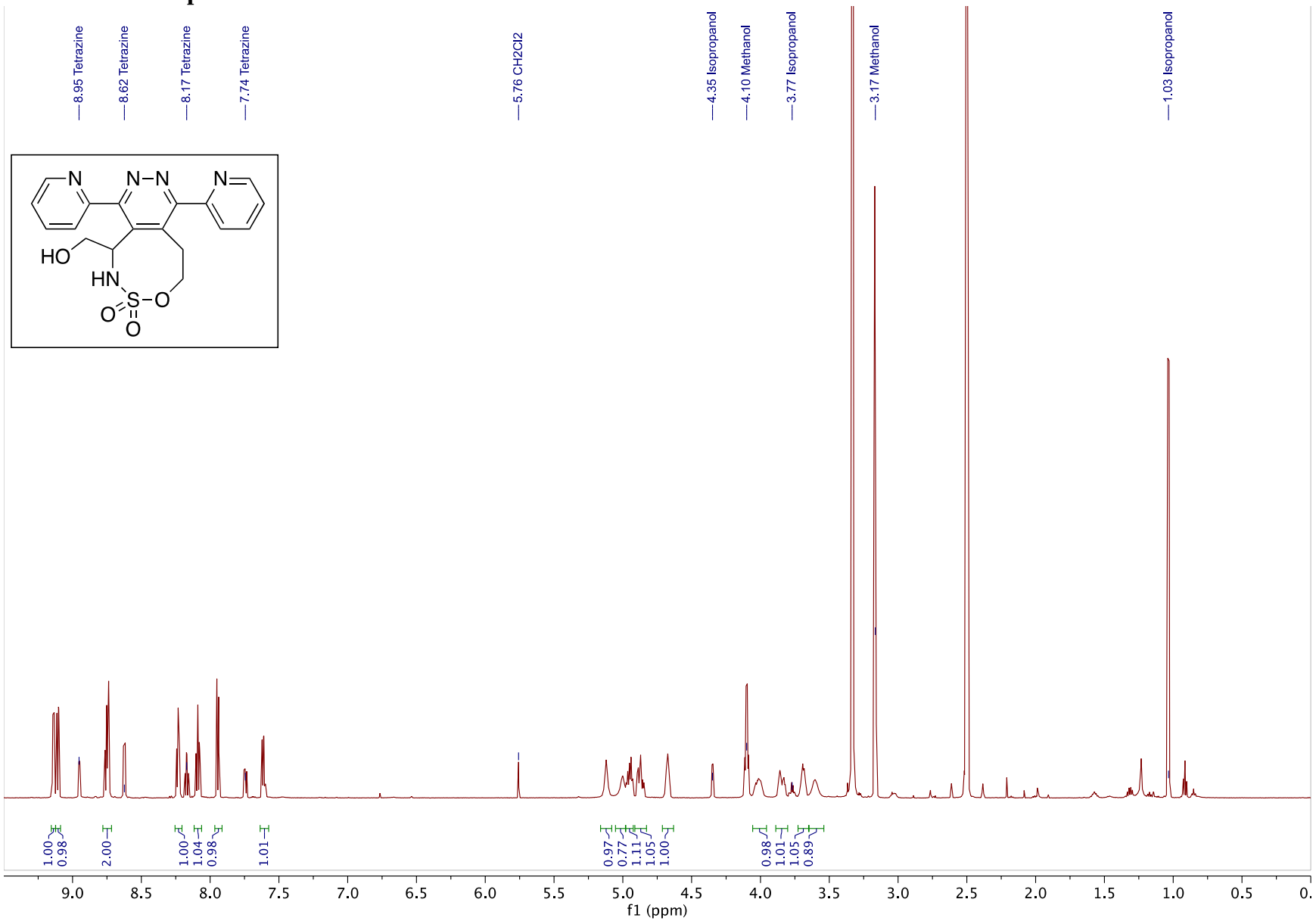
^{1}H NMR for Compound 2a.



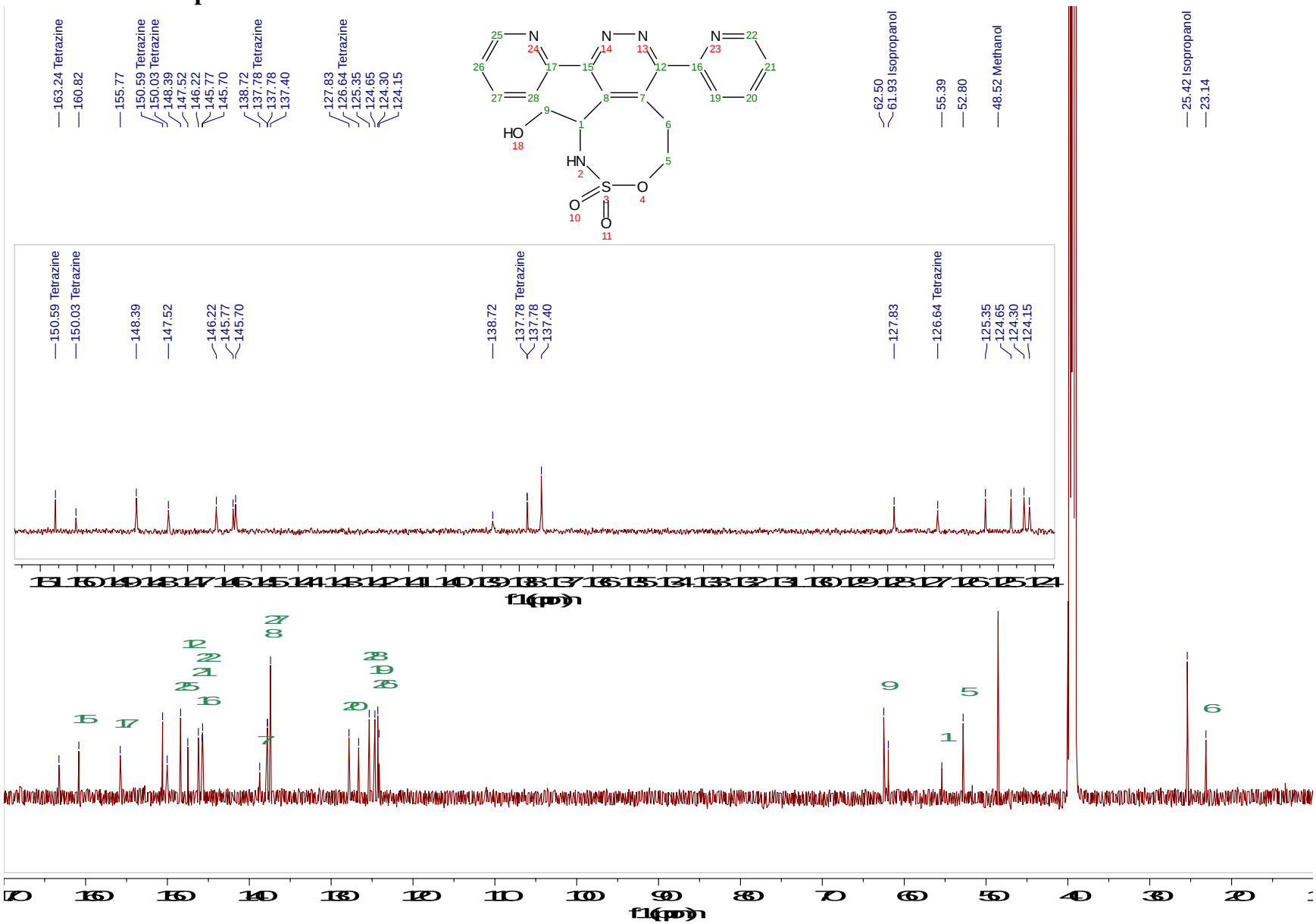
^{13}C NMR for Compound 2a.



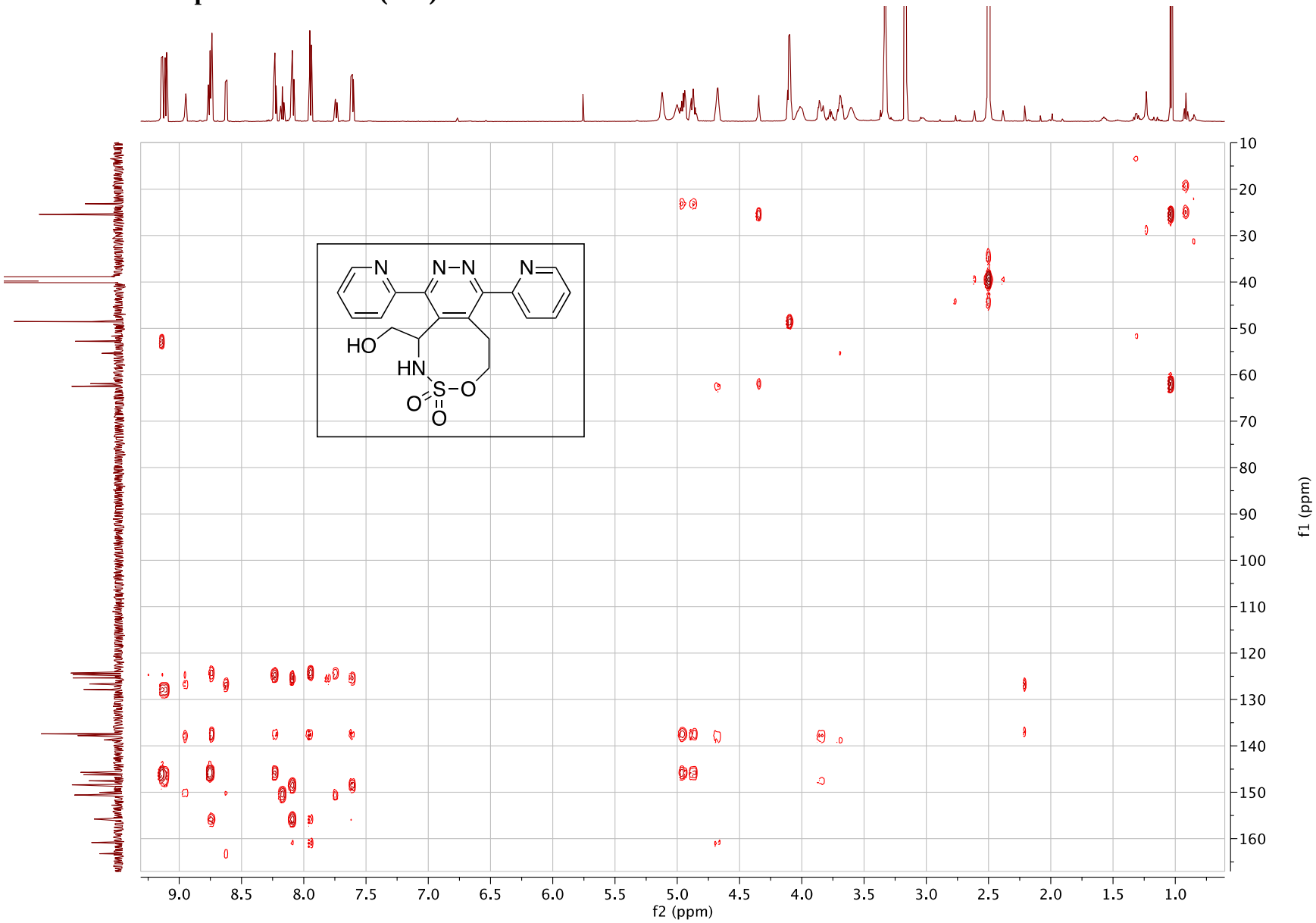
^{1}H NMR for Compound 2b.



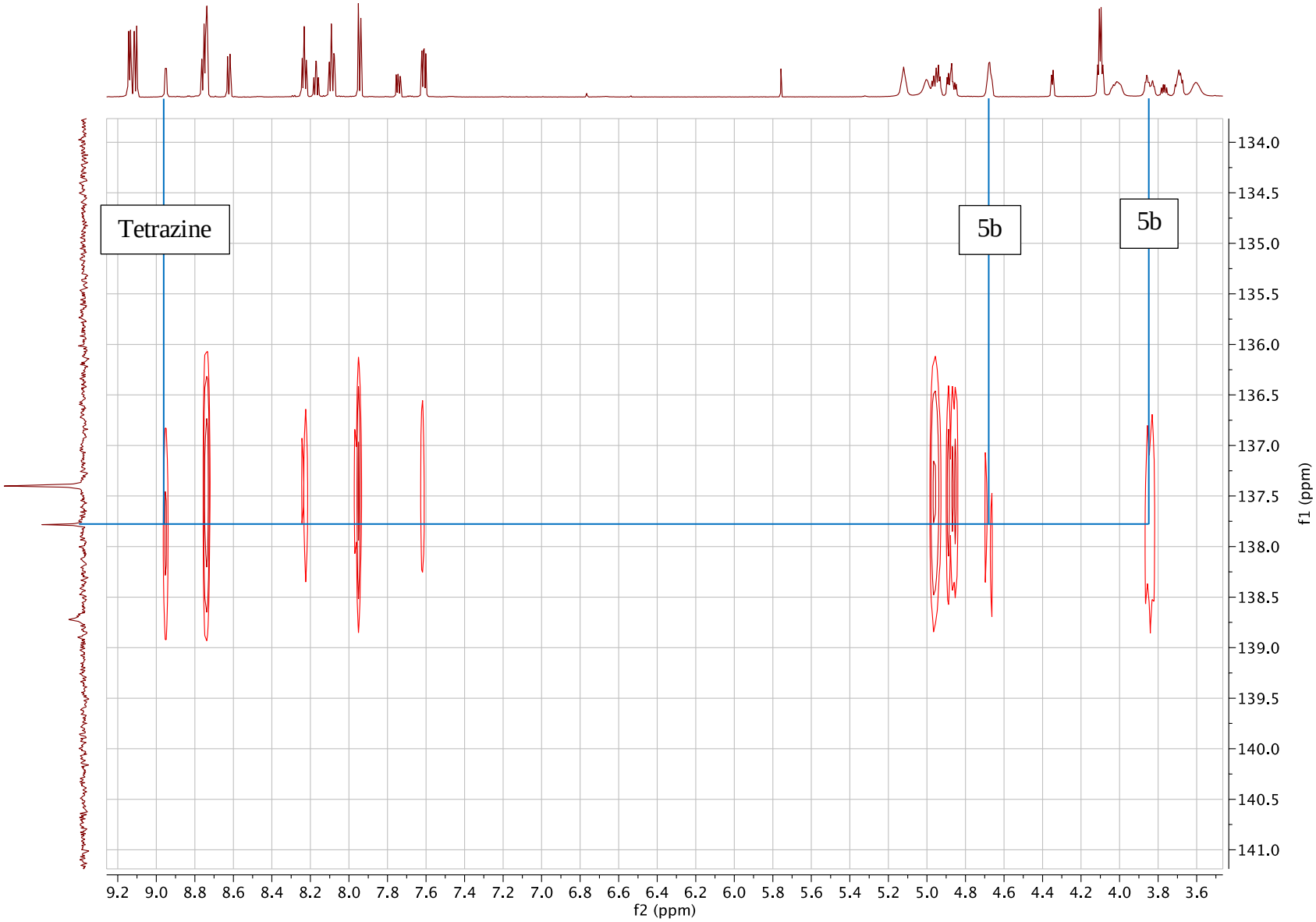
^{13}C NMR for Compound 2b.



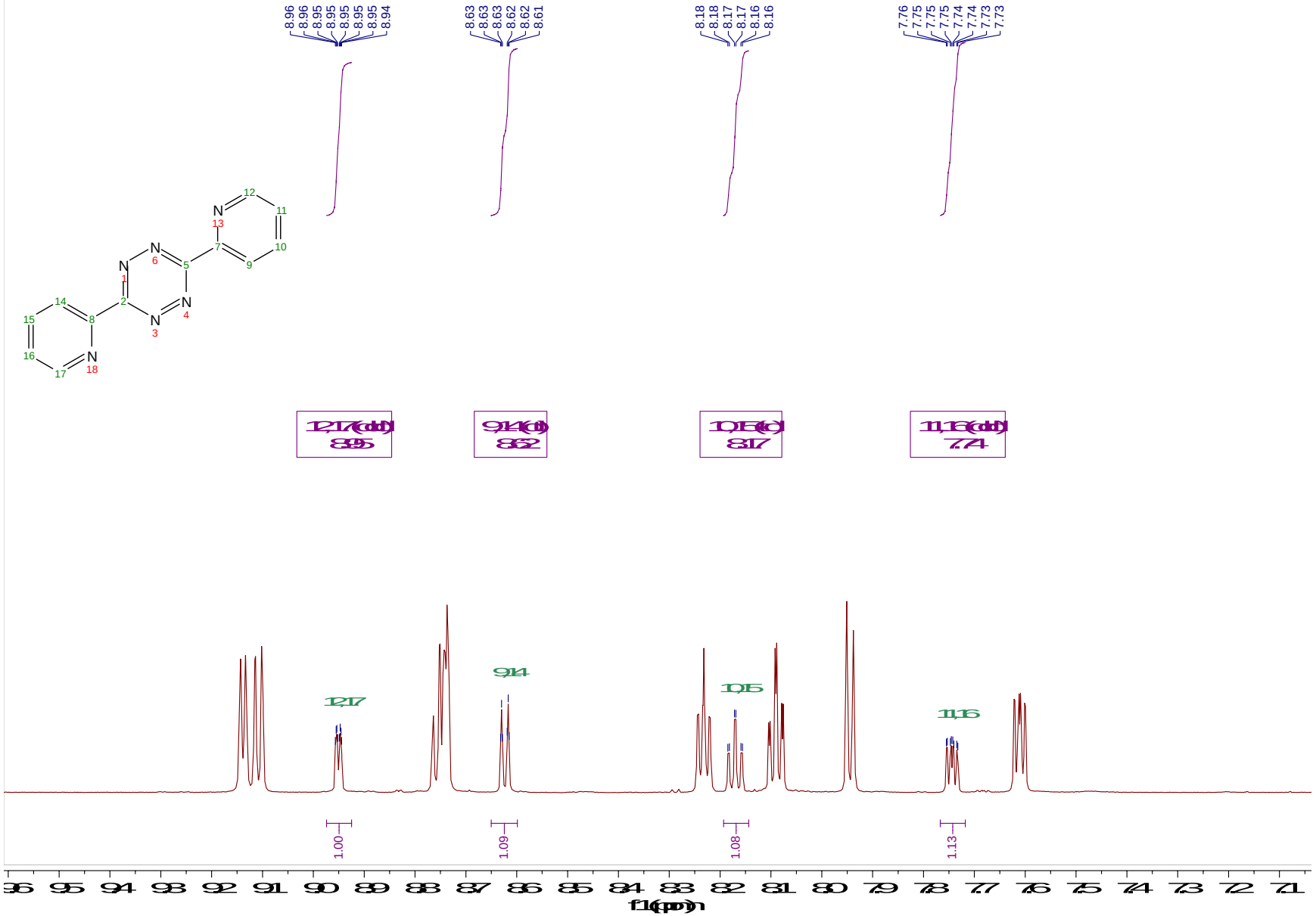
^{13}C NMR for Compound 2b HMBC (Full).



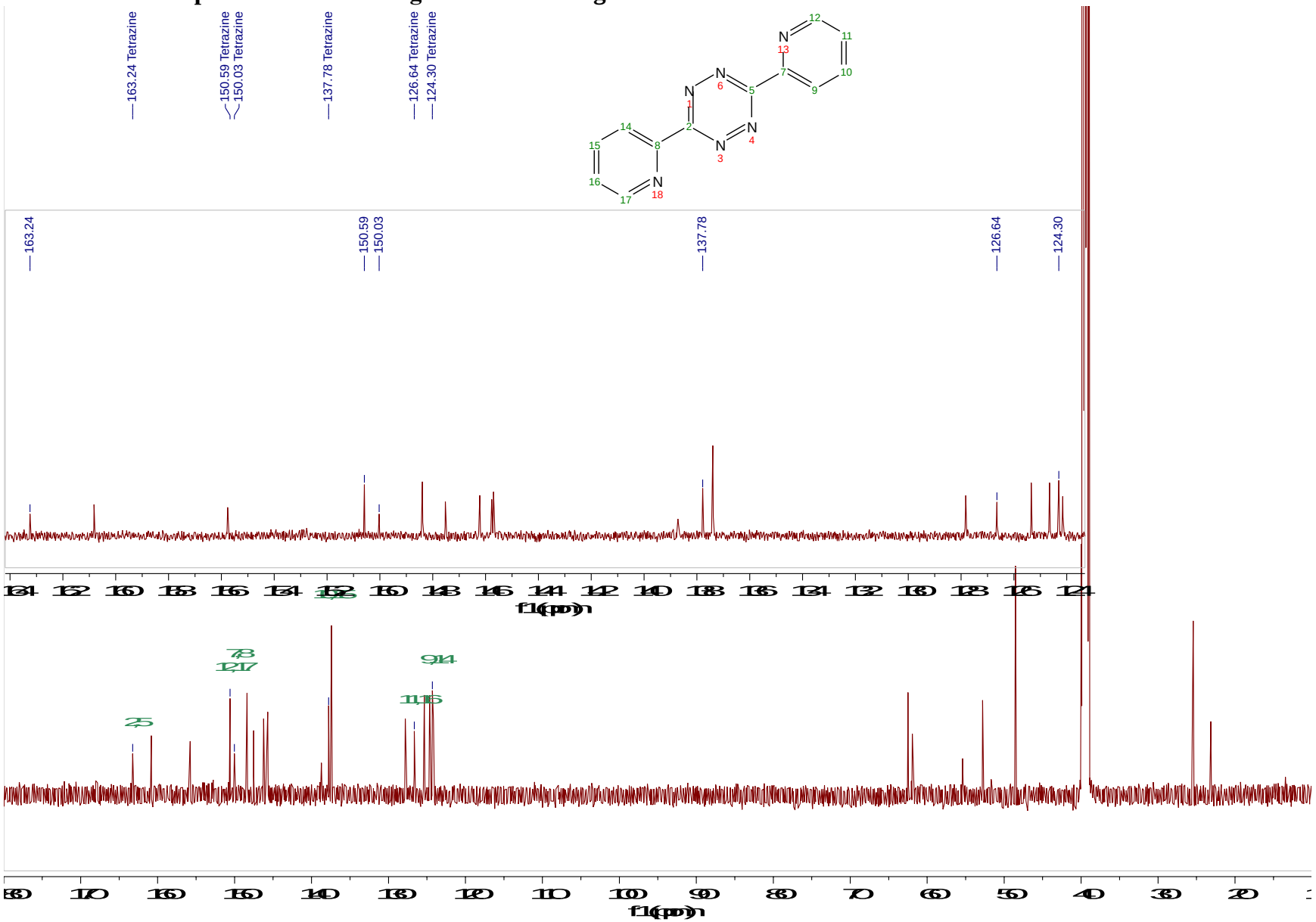
^{13}C NMR for Compound 2b HMBC (Zoom: Tetrazine and 2b overlap).



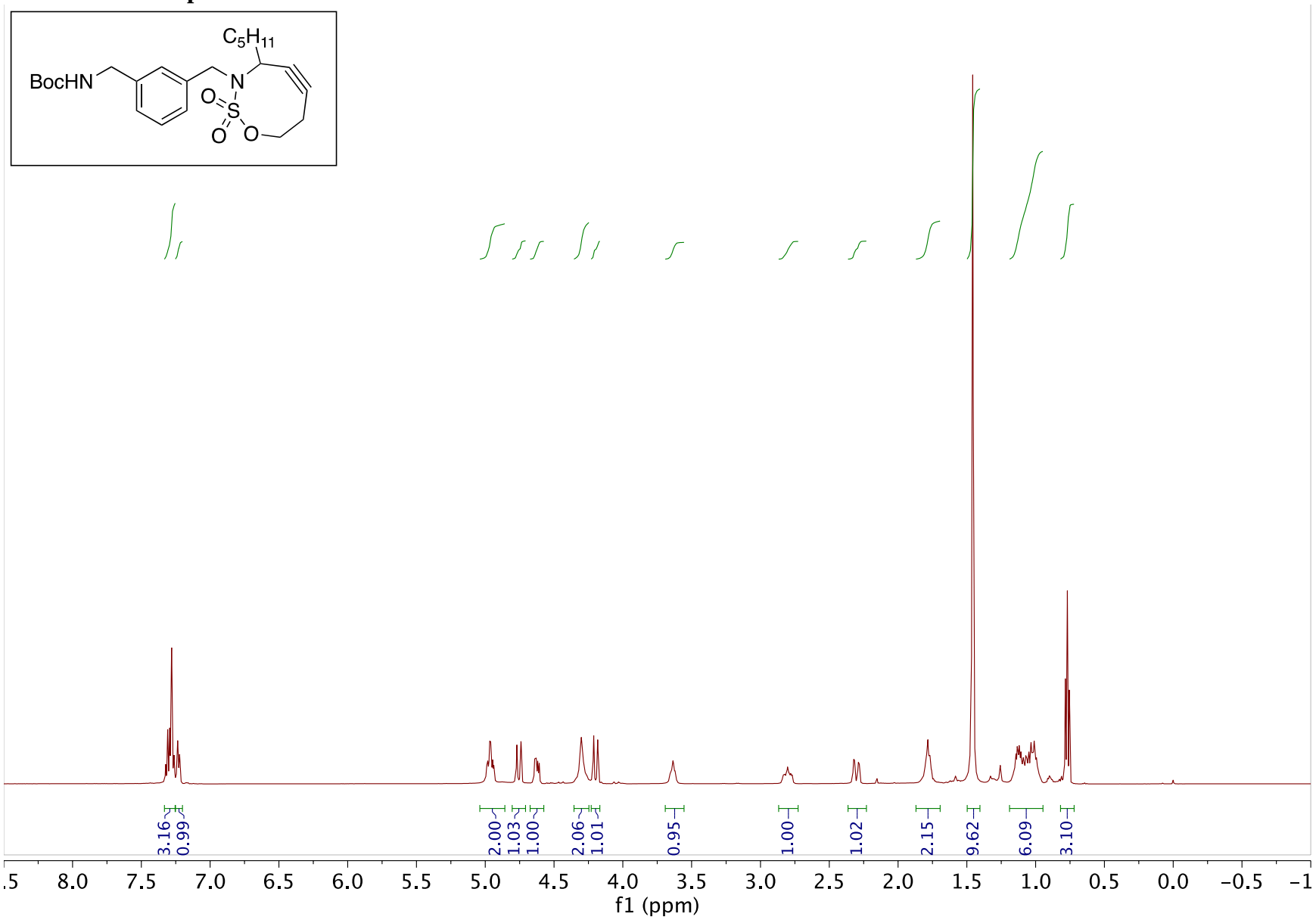
^{1}H NMR for Compound 2b's remaining tetrazine starting material.



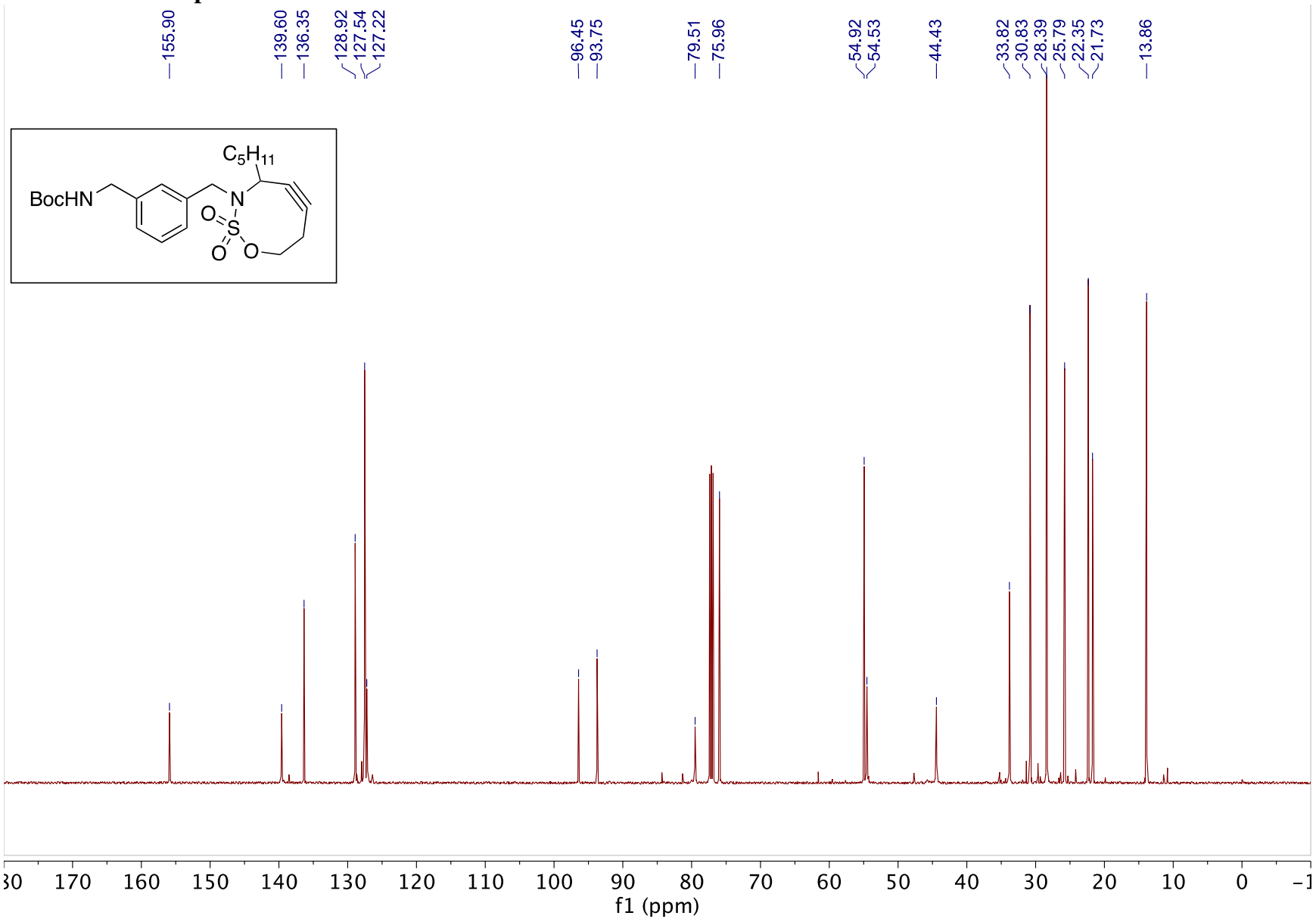
^{13}C NMR for Compound 2b's remaining tetrazine starting material.



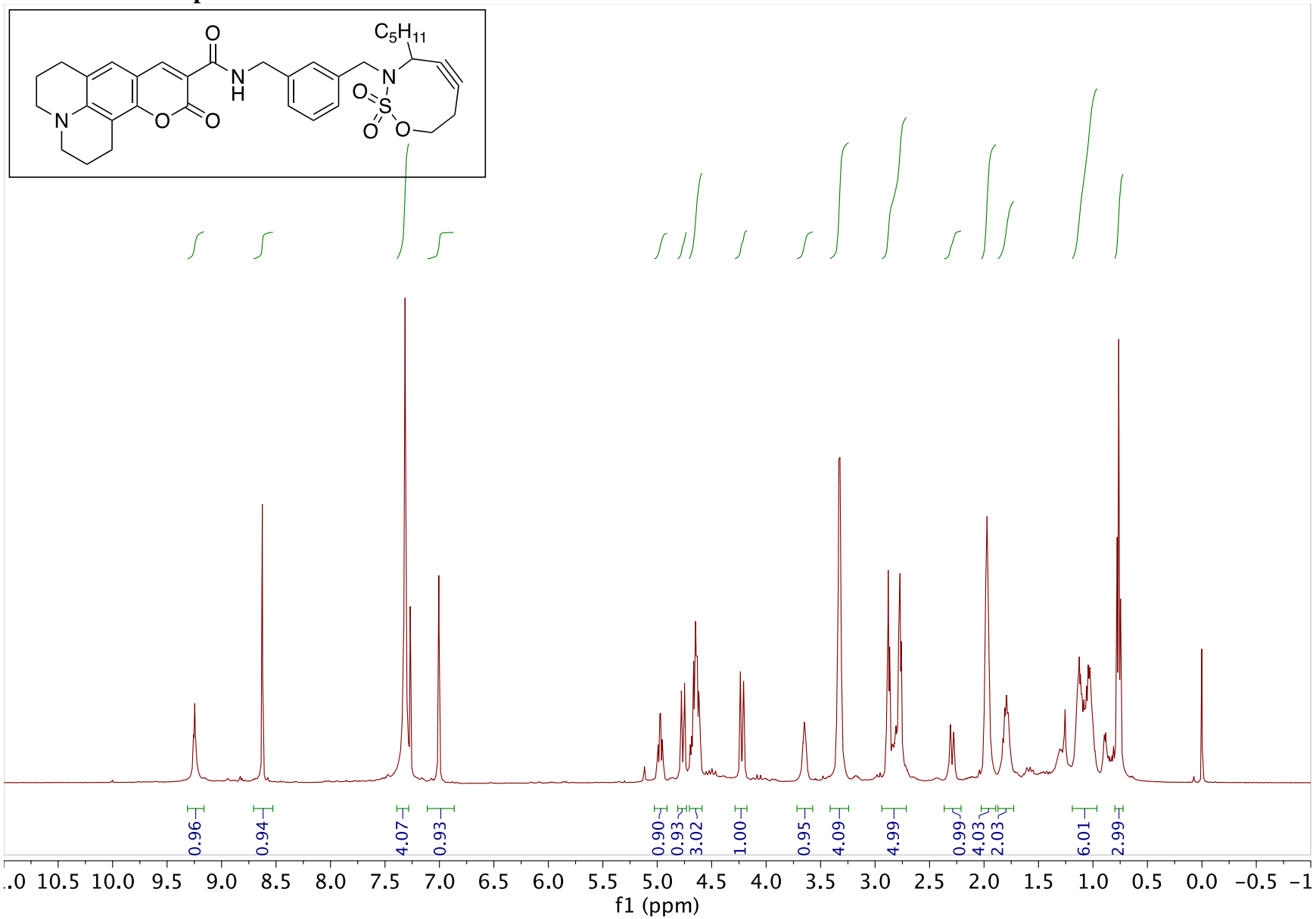
^{1}H NMR for Compound S6.



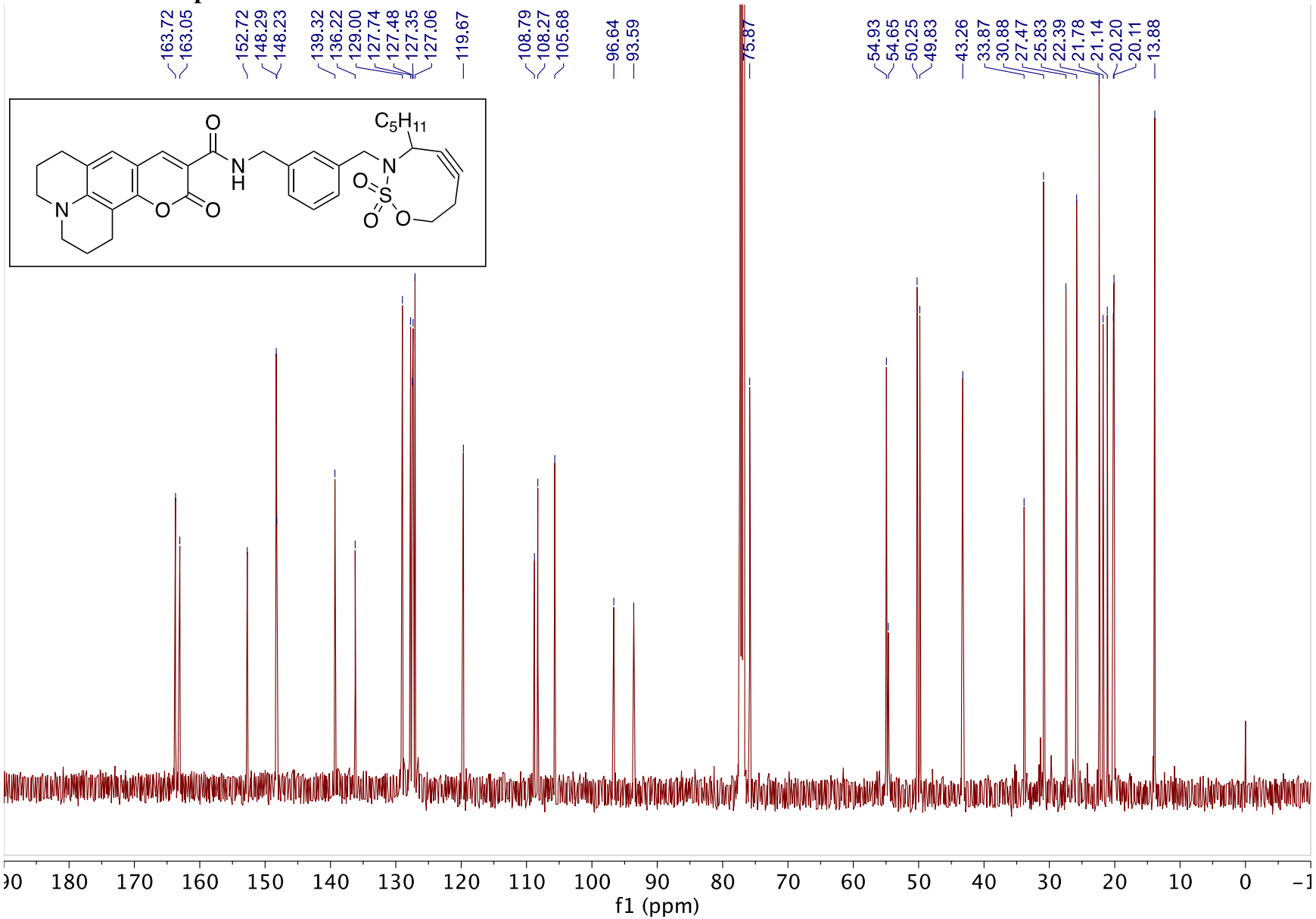
^{13}C NMR for Compound S6.



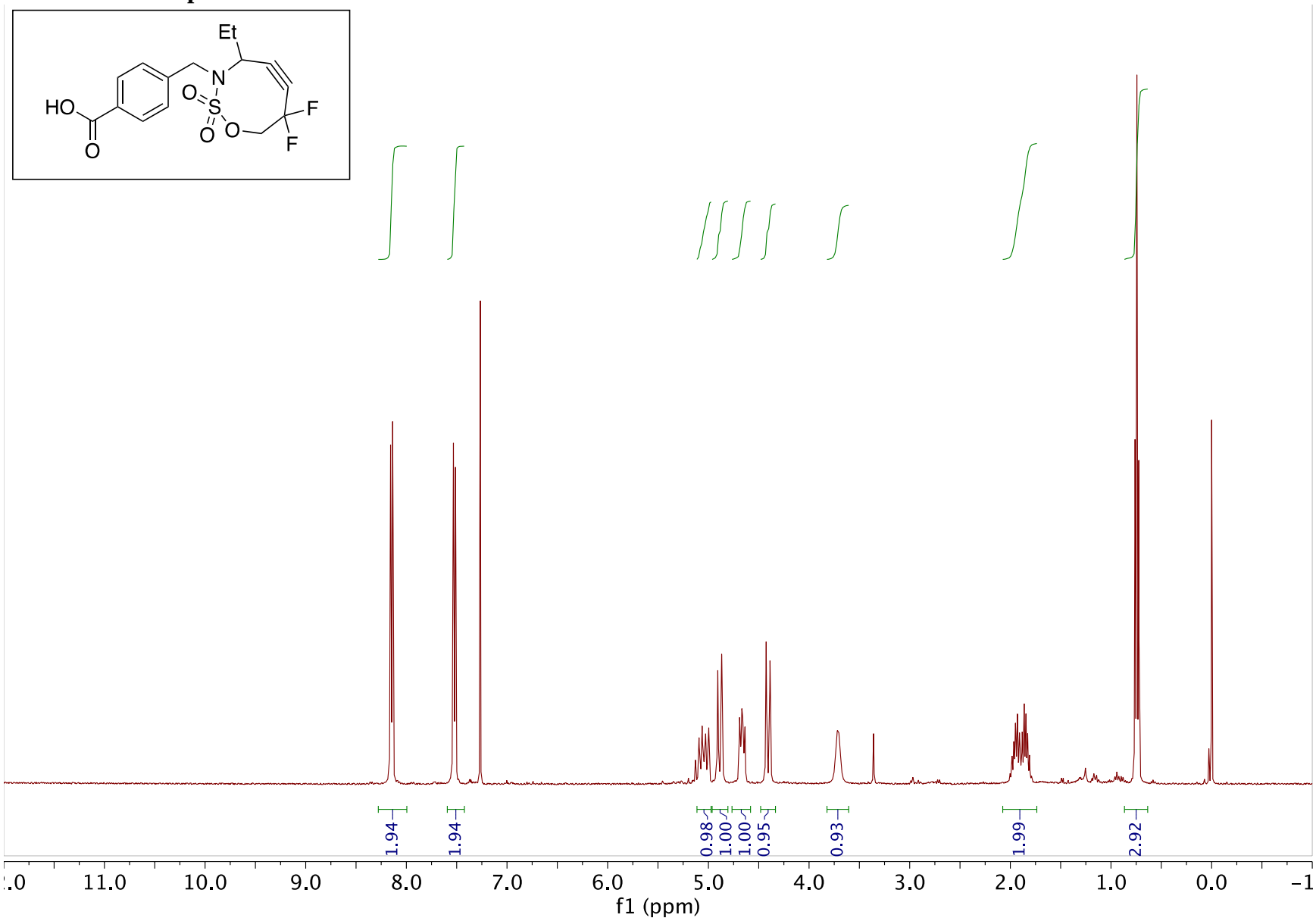
^{1}H NMR for Compound SNO-C343.



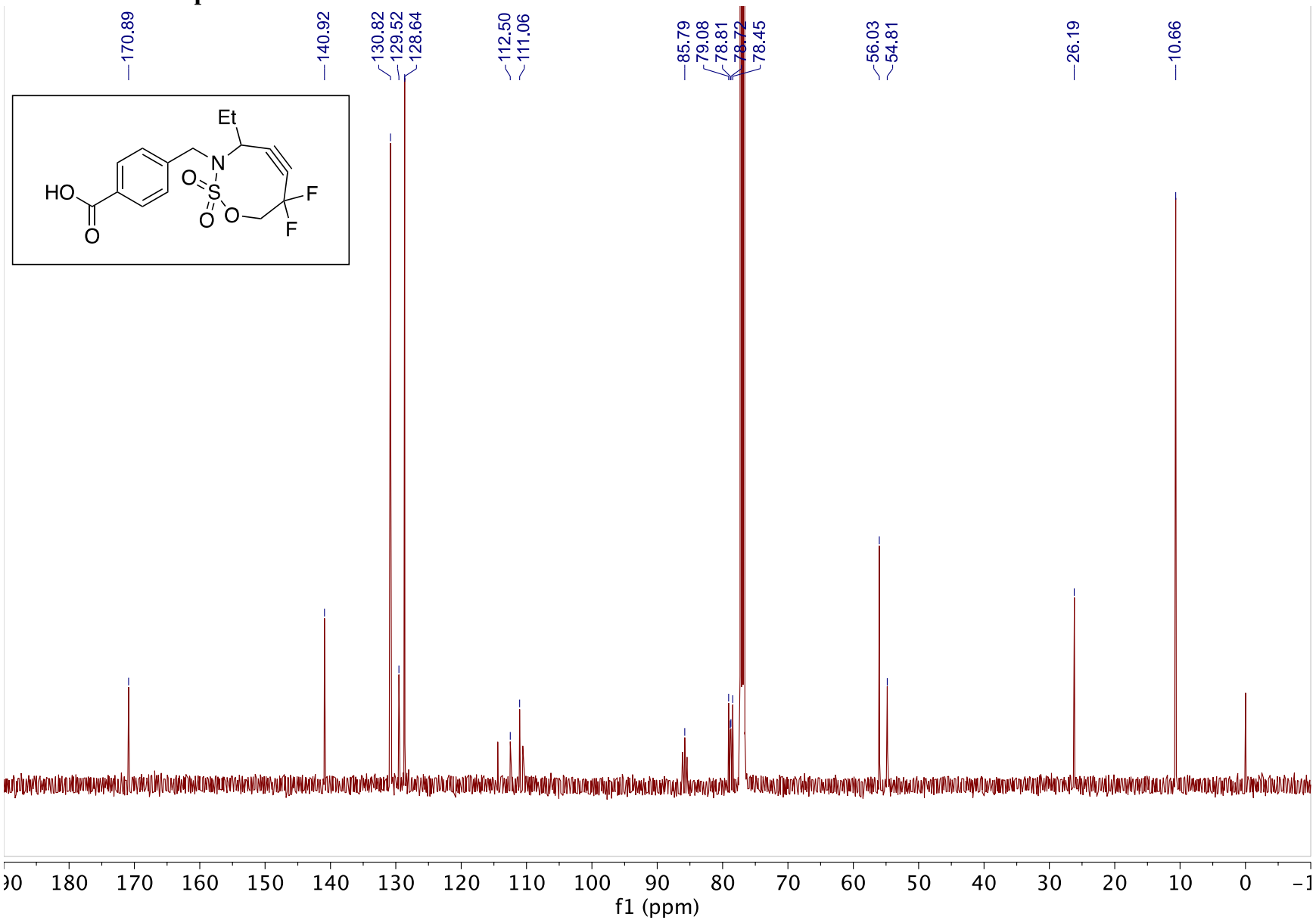
^{13}C NMR for Compound SNO-C343.



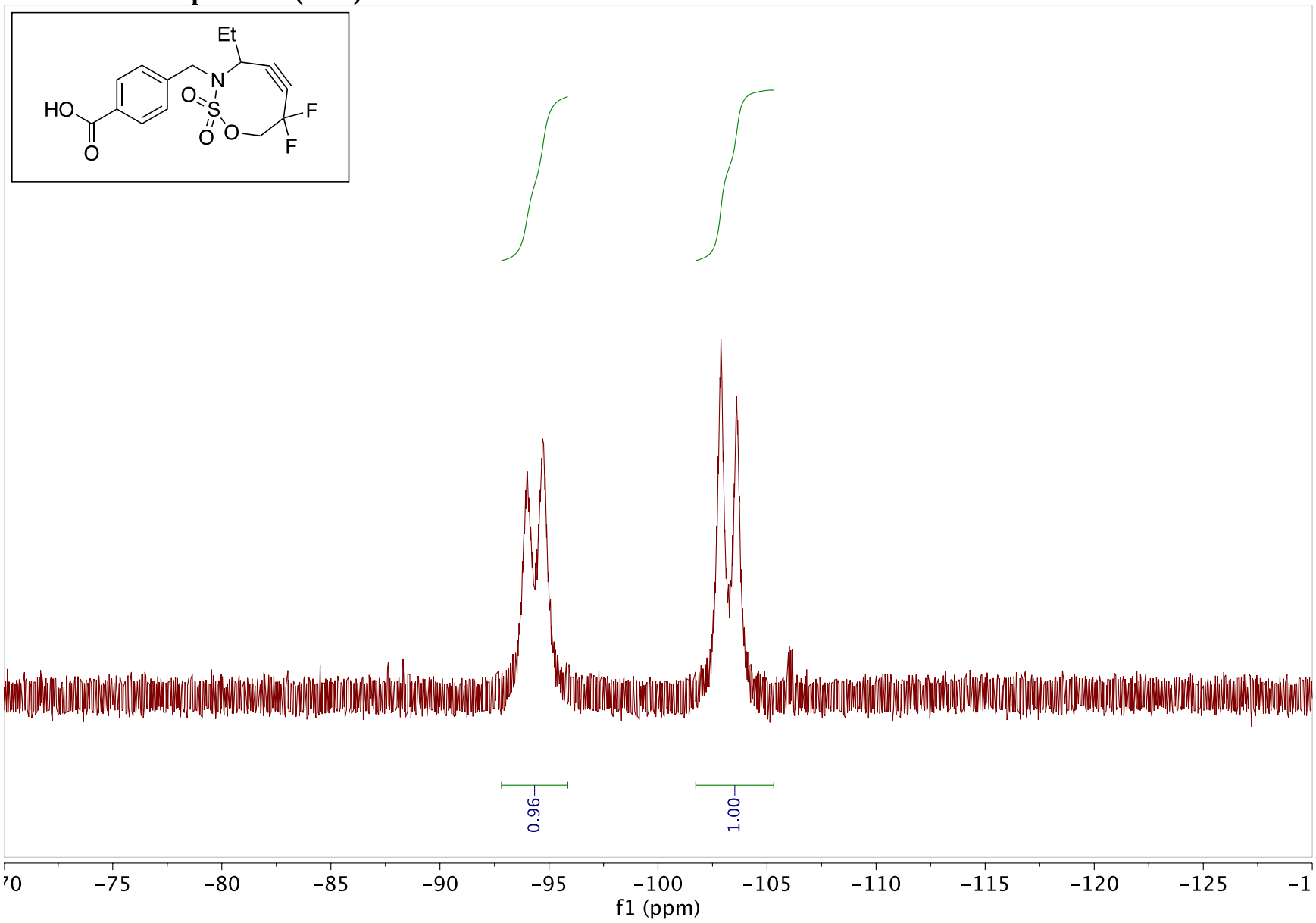
^{1}H NMR for Compound S7



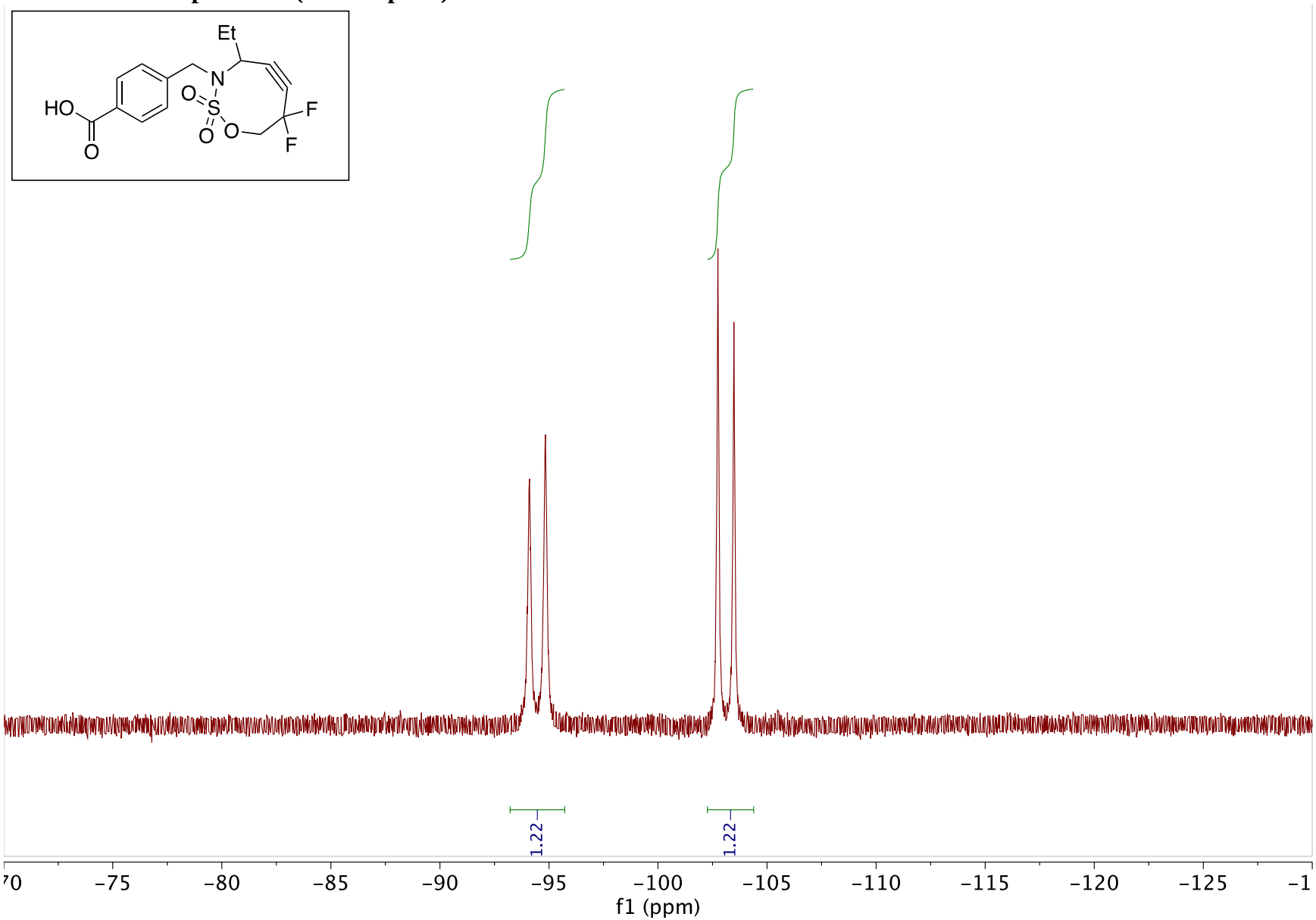
^{13}C NMR for Compound S7



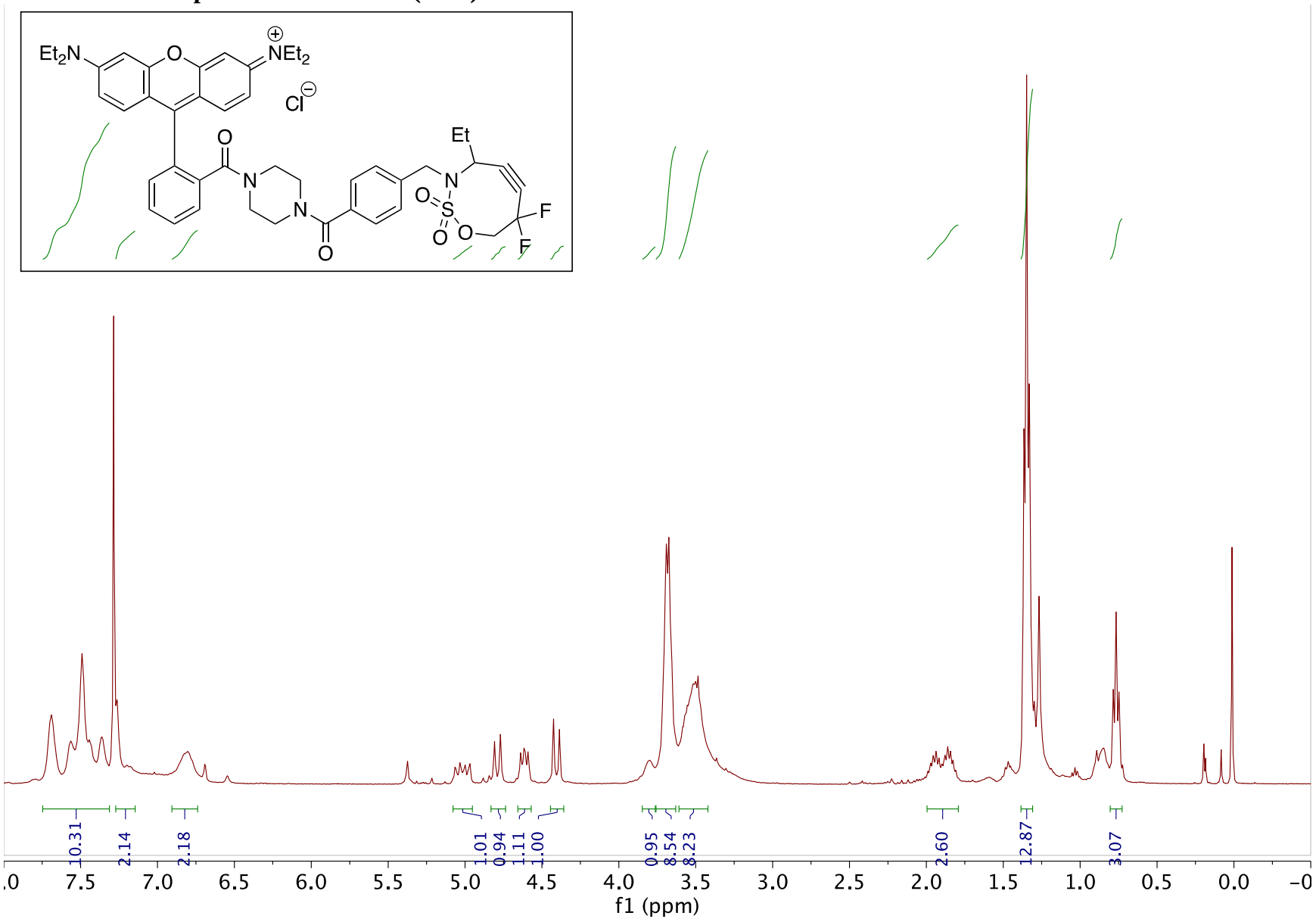
^{19}F NMR for Compound S7 (24ºC)



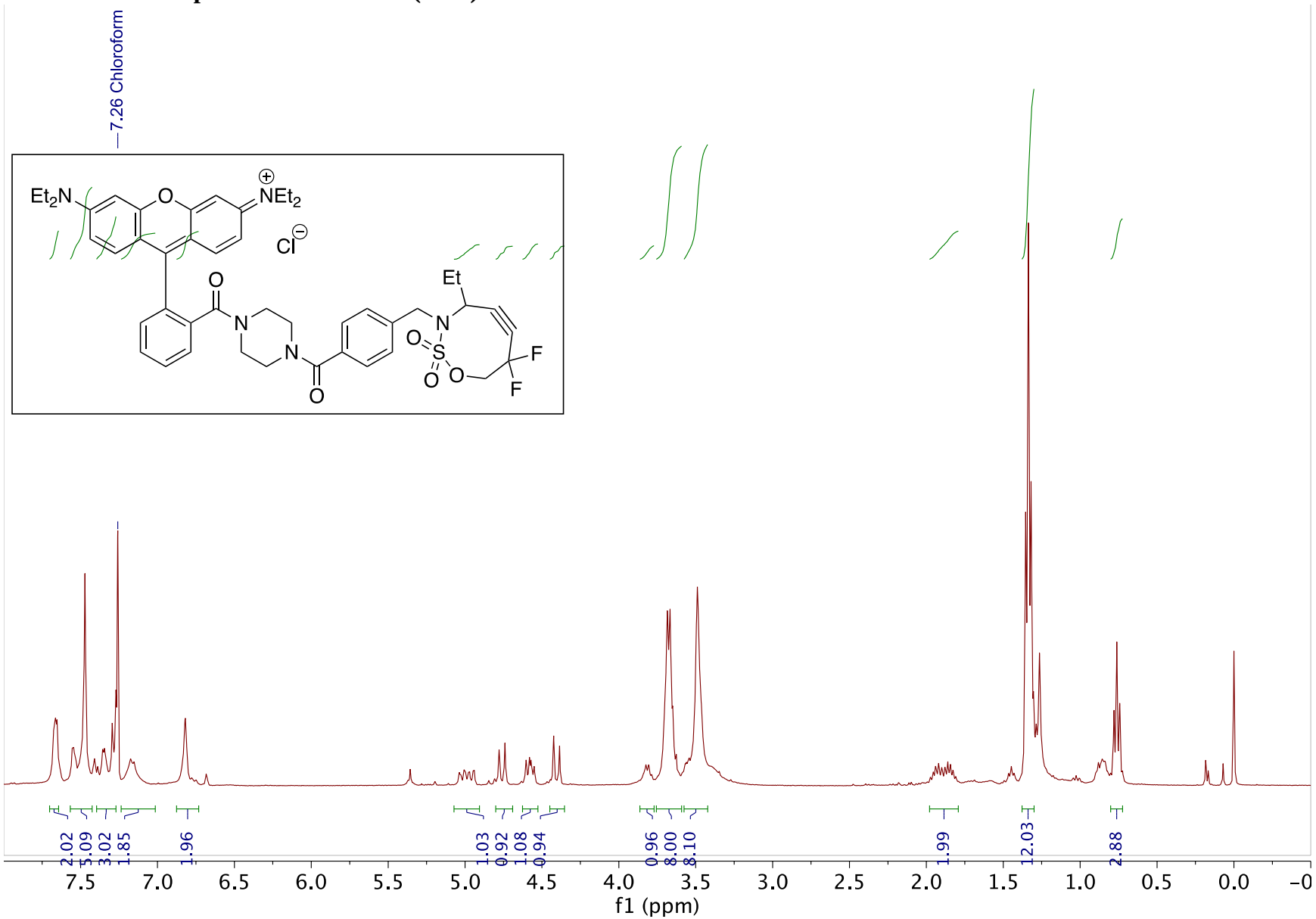
^{19}F NMR for Compound S7 (50°C setpoint)



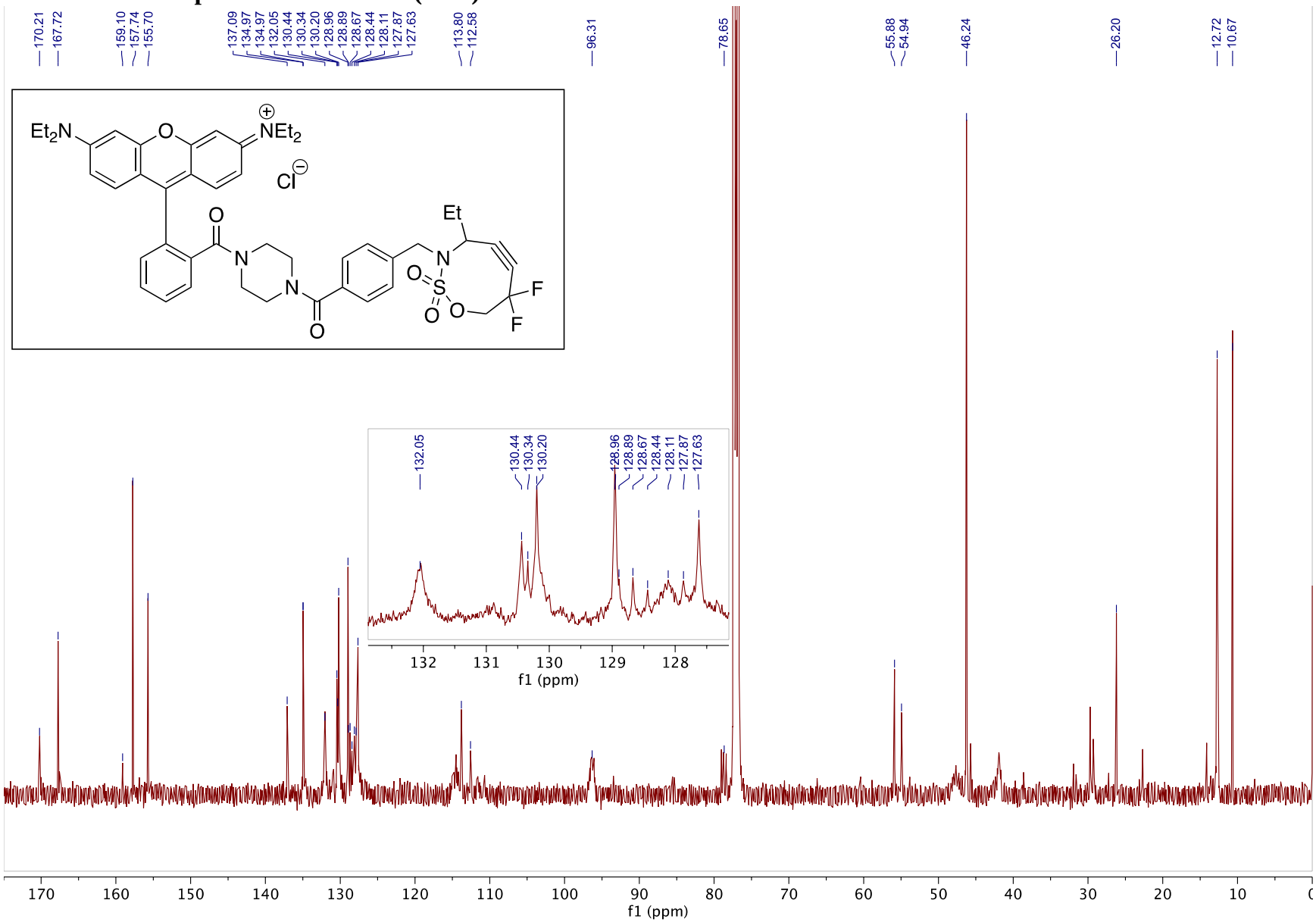
^{1}H NMR for Compound SNO-DF-Rho (24°C).



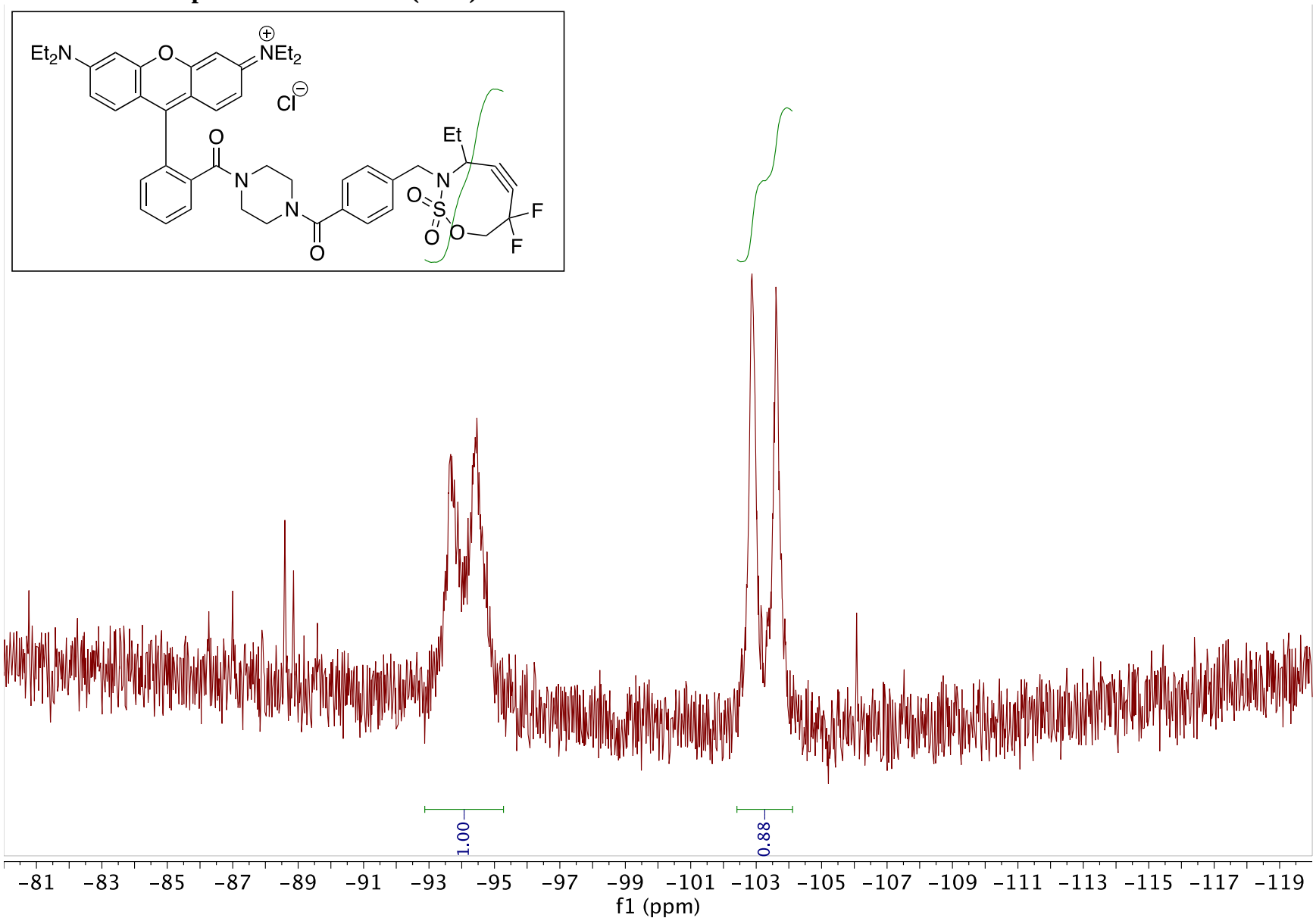
^{1}H NMR for Compound SNO-DF-Rho (55°C)



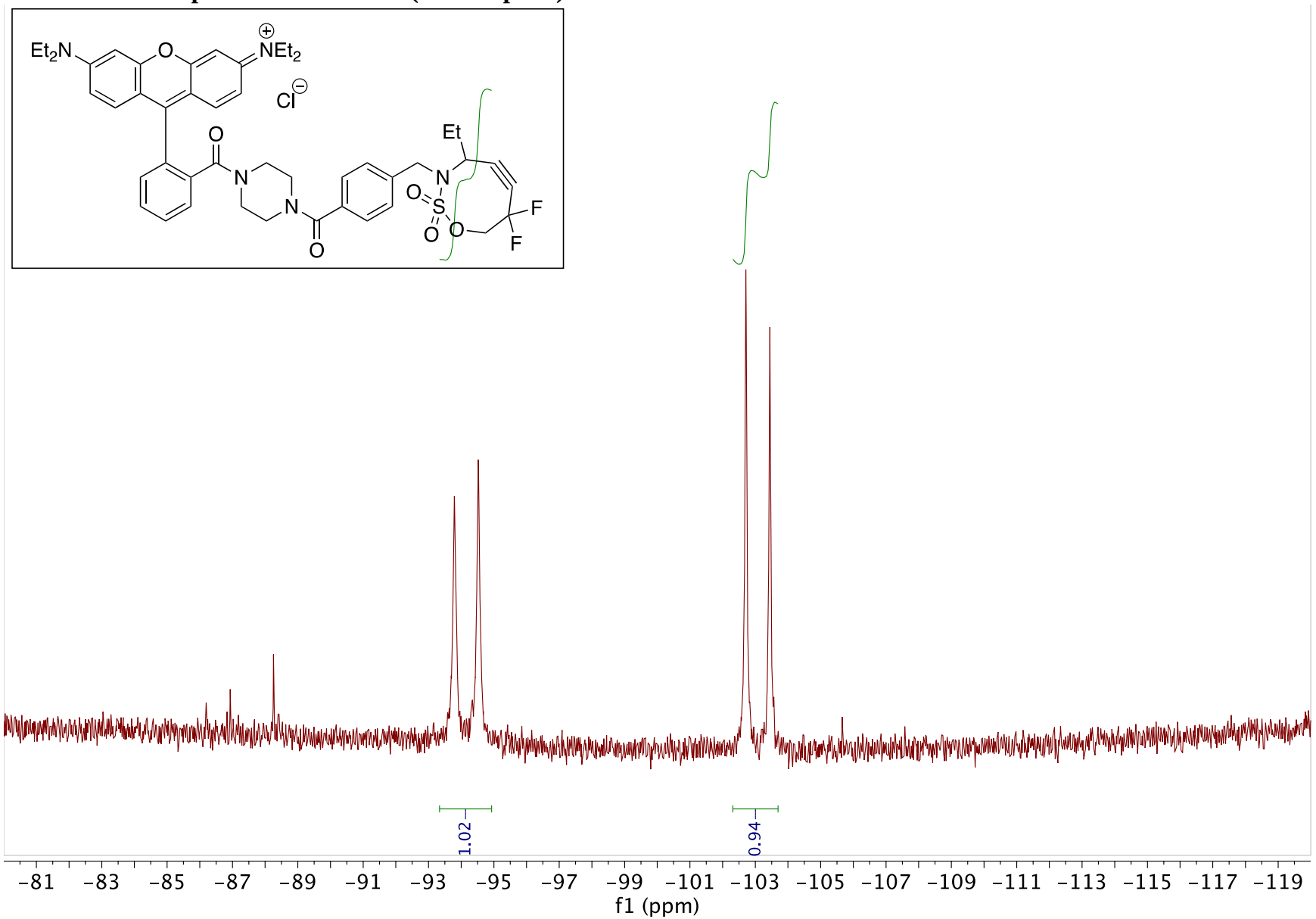
^{13}C NMR for Compound SNO-DF-Rho (24°C).



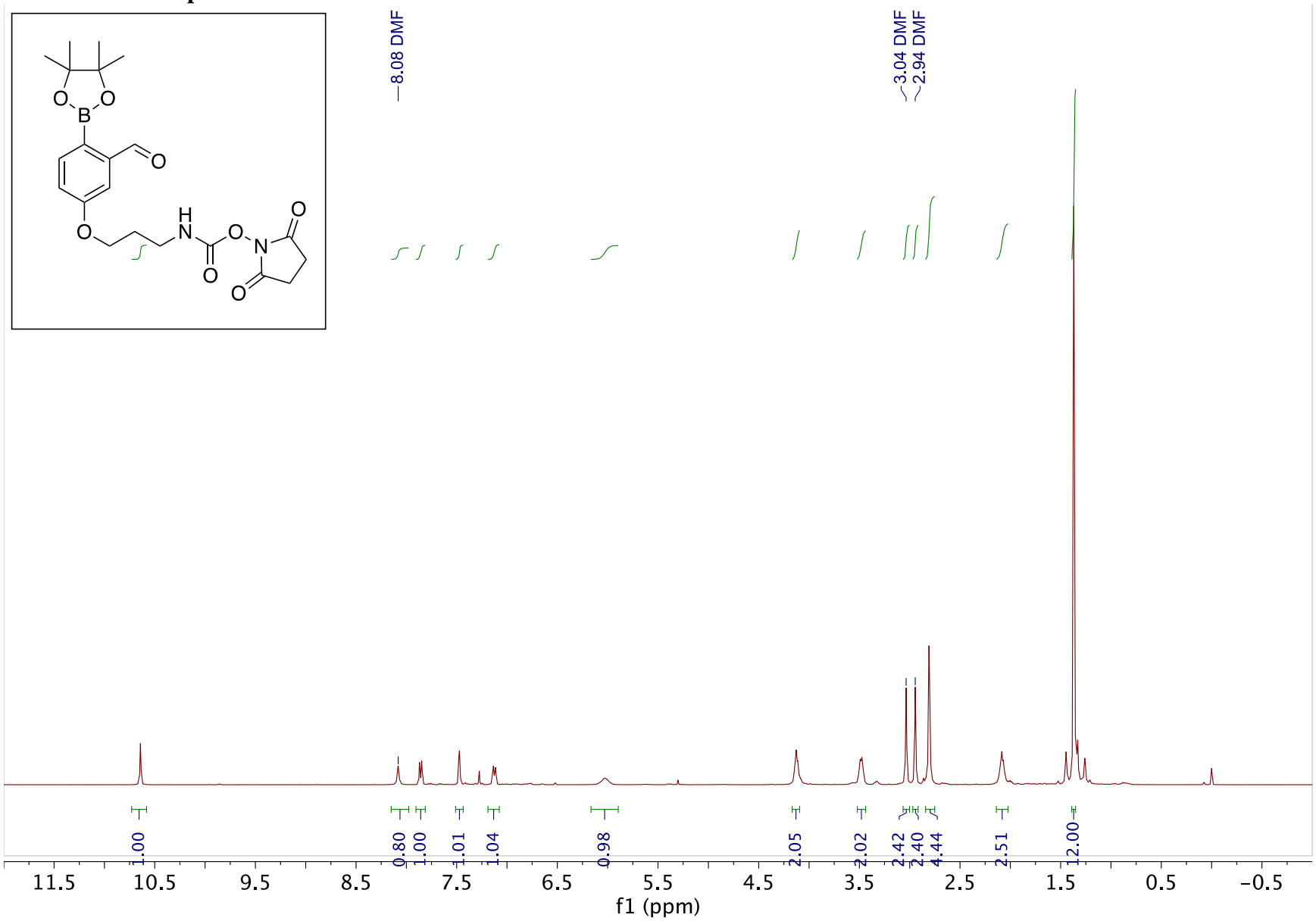
^{19}F NMR for Compound SNO-DF-Rho (24ºC)



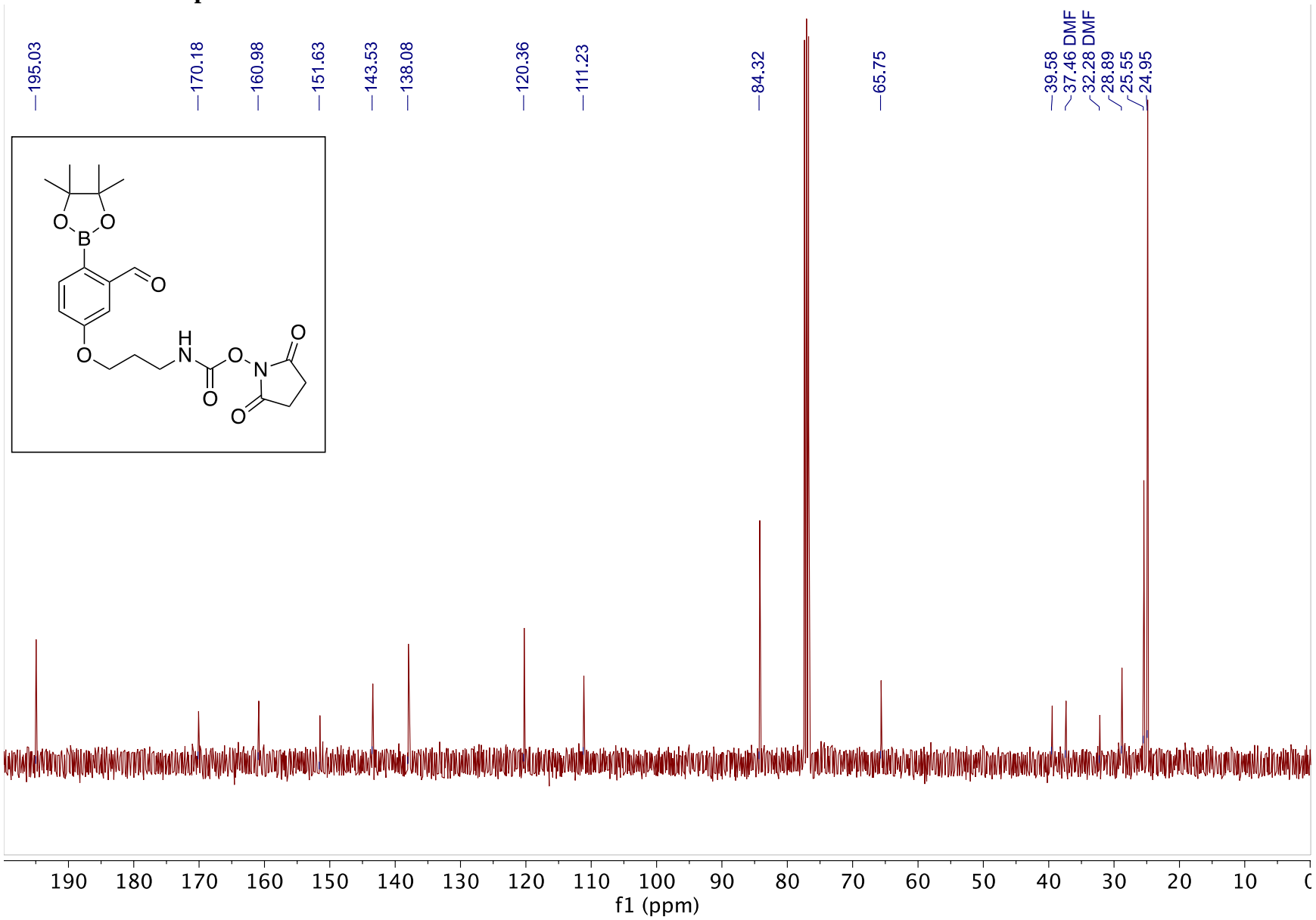
^{19}F NMR for Compound SNO-DF-Rho (55ºC setpoint)



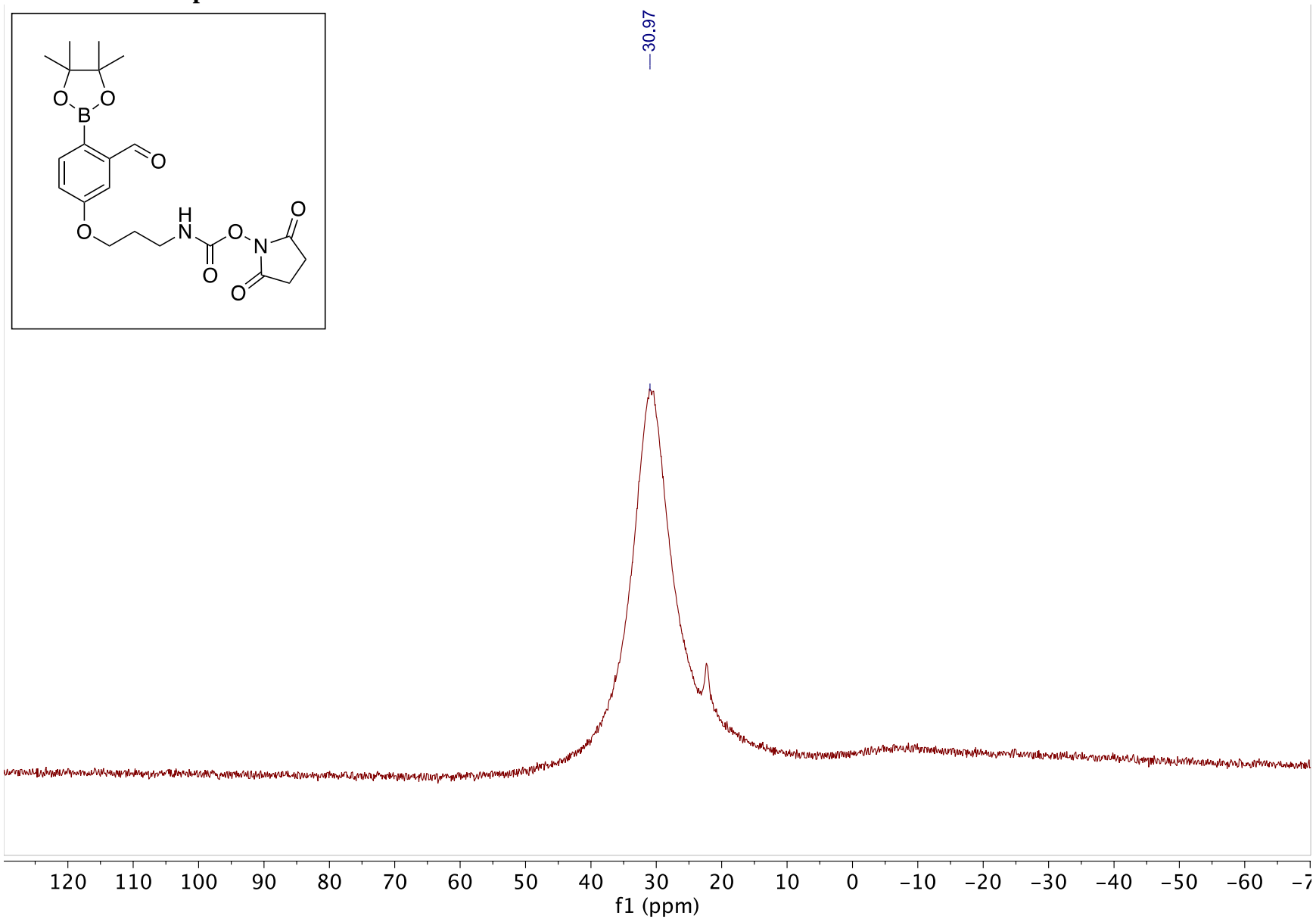
^{1}H NMR for Compound BE-NHS



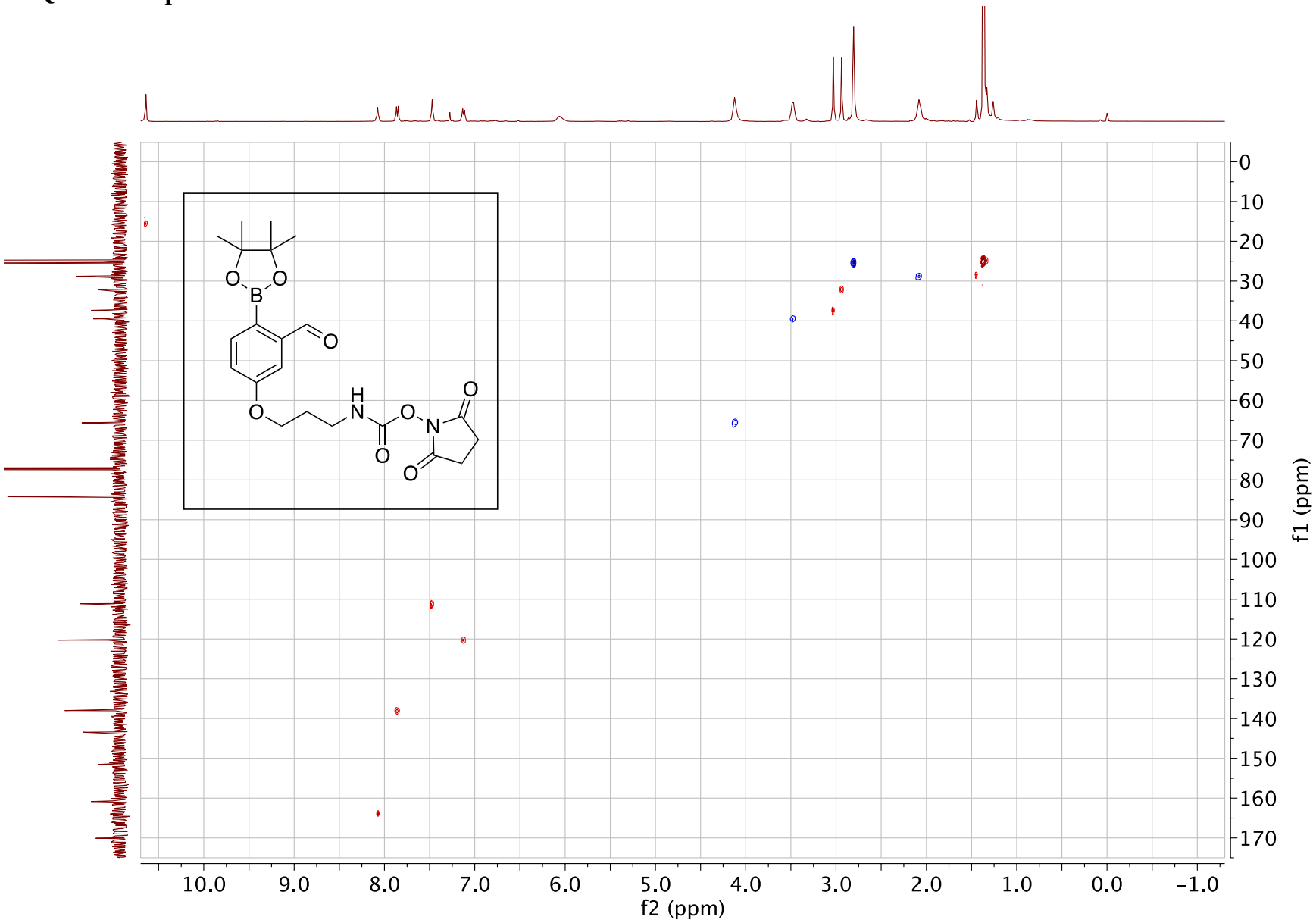
^{13}C NMR for Compound BE-NHS



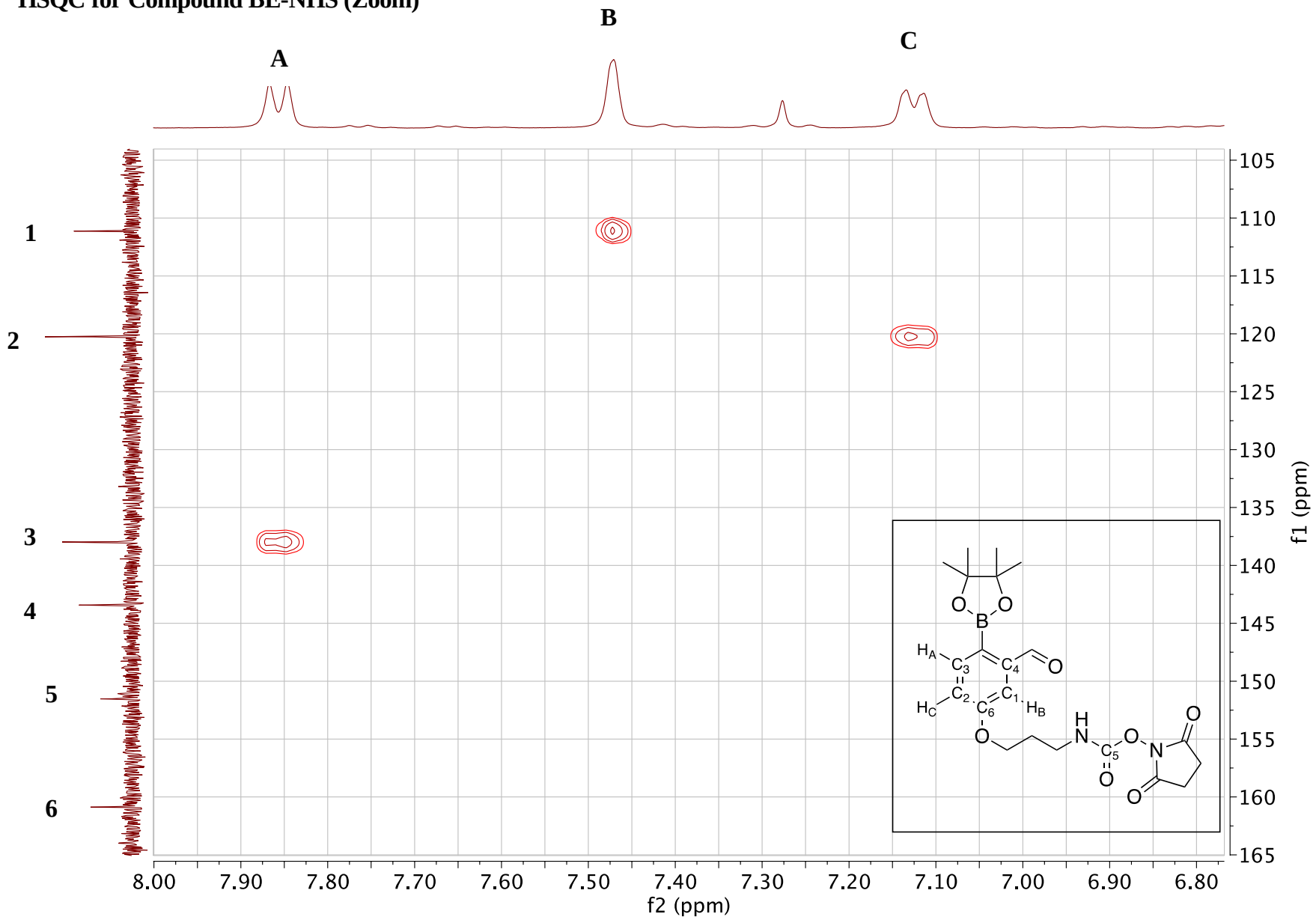
^{11}B NMR for Compound BE-NHS



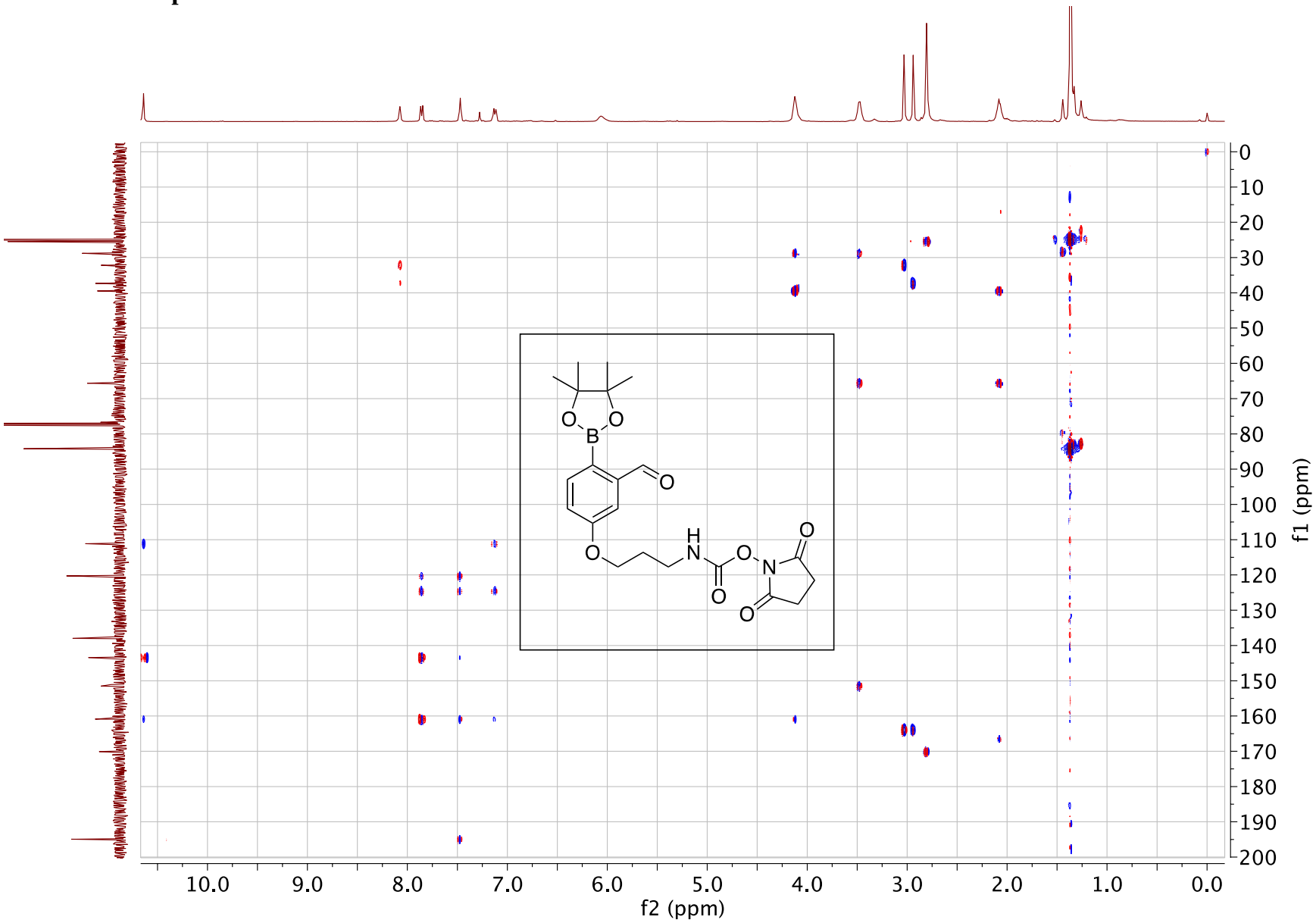
HSQC for Compound BE-NHS



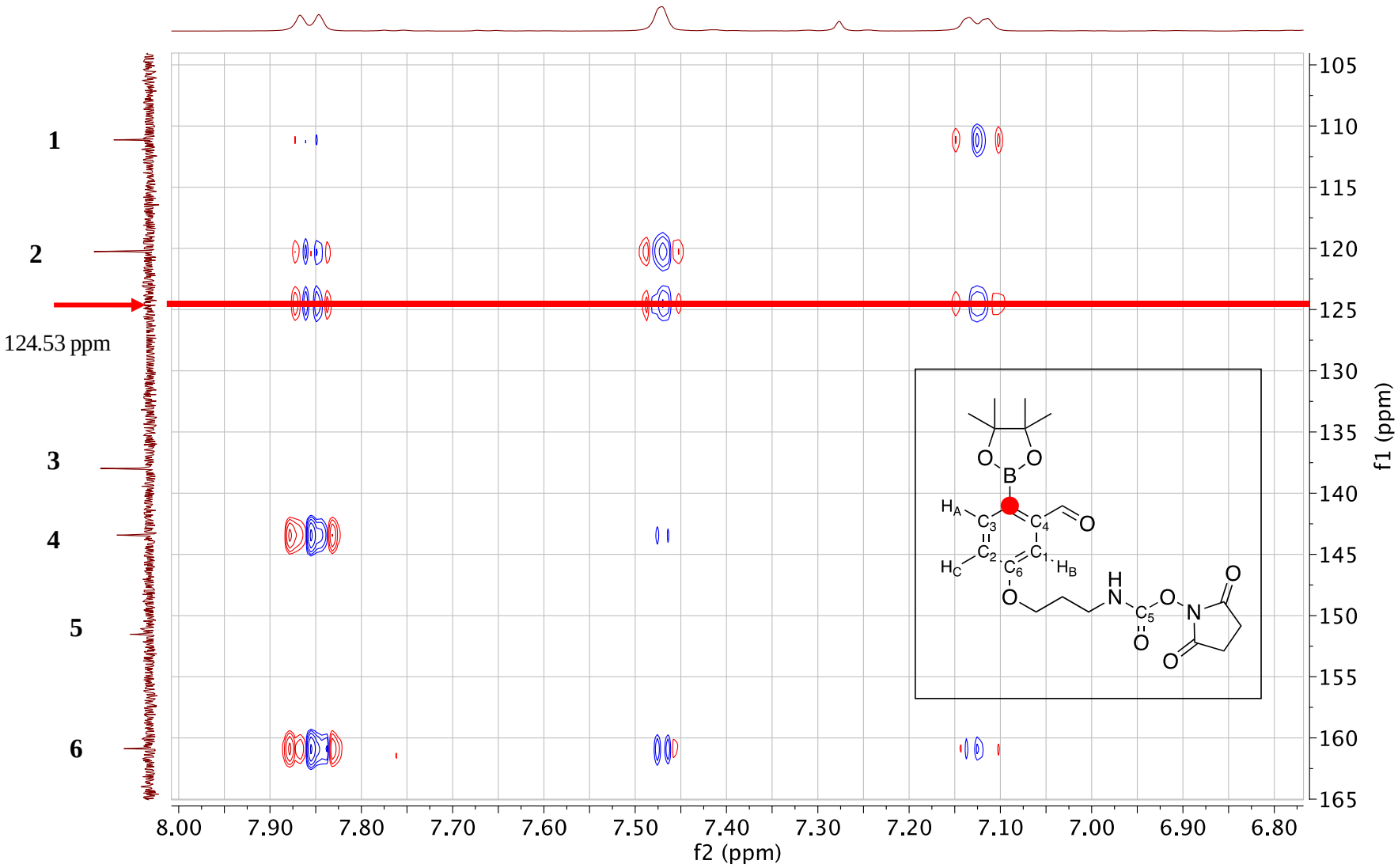
HSQC for Compound BE-NHS (Zoom)



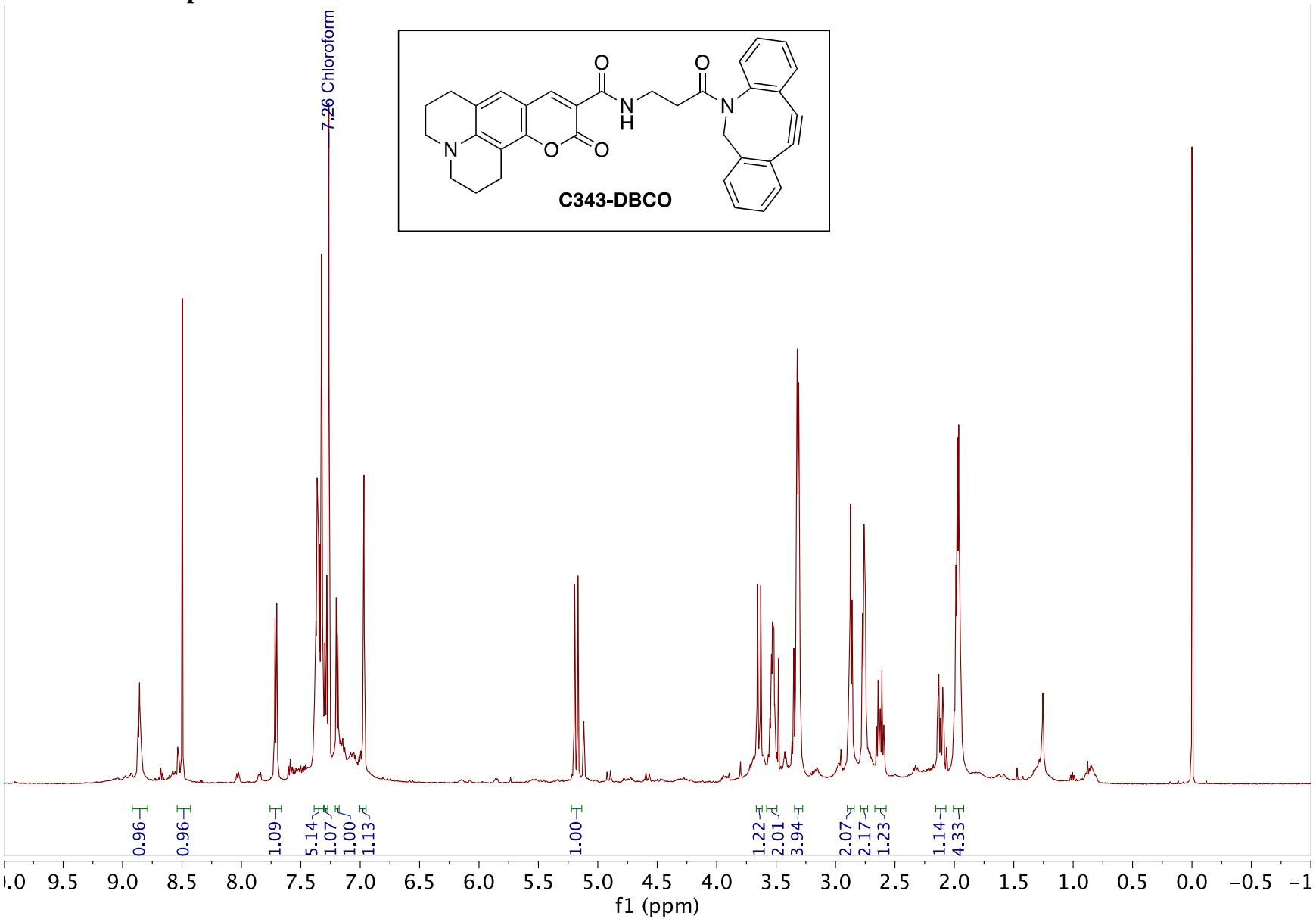
HMBC for Compound BE-NHS



HMBC for Compound BE-NHS (Zoom)



^{1}H NMR for Compound C343-DBCO



^{13}C NMR for Compound C343-DBCO