Cell Chemical Biology, Volume *23*

Supplemental Information

Fluorescent Visualization of Cellular Proton Fluxes

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Supplemental Figures

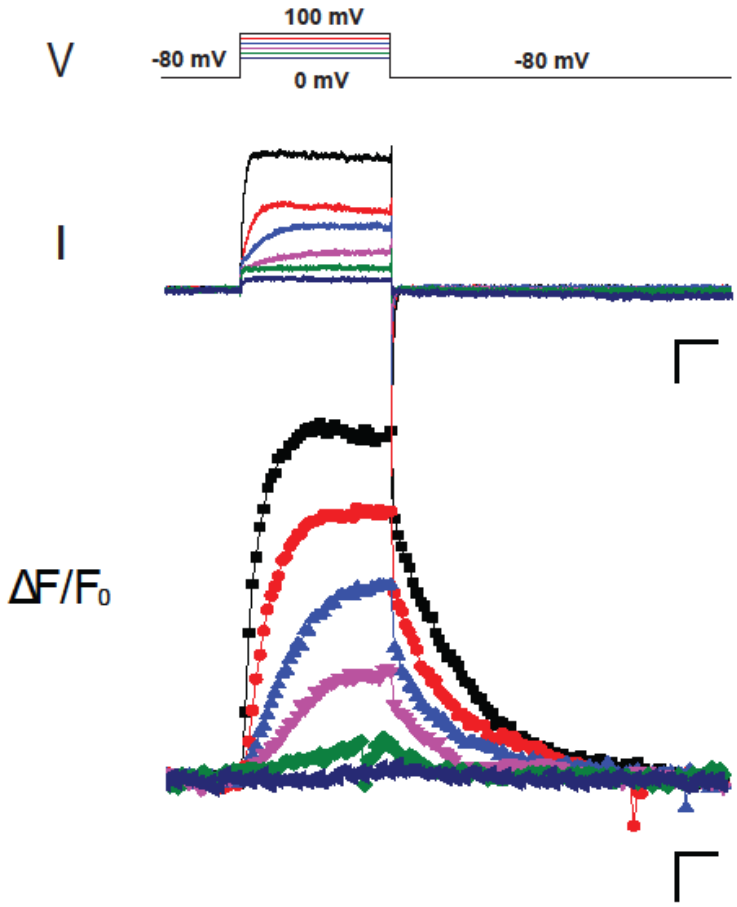


Figure S1, related to Figure 2. Voltage-clamp fluorometry of Hv-1 expressing HEK293T cells. The cell was held at – 80 mV, and currents and pH-DIBO fluorescence were elicited from 4-s command voltages from 0 to 100 mV in 20-mV increments. Scale bars represent 100 pA, 2% and 1 s; pH_o/pH_i = 7.5/6.0 (0.1 mM HEPES).

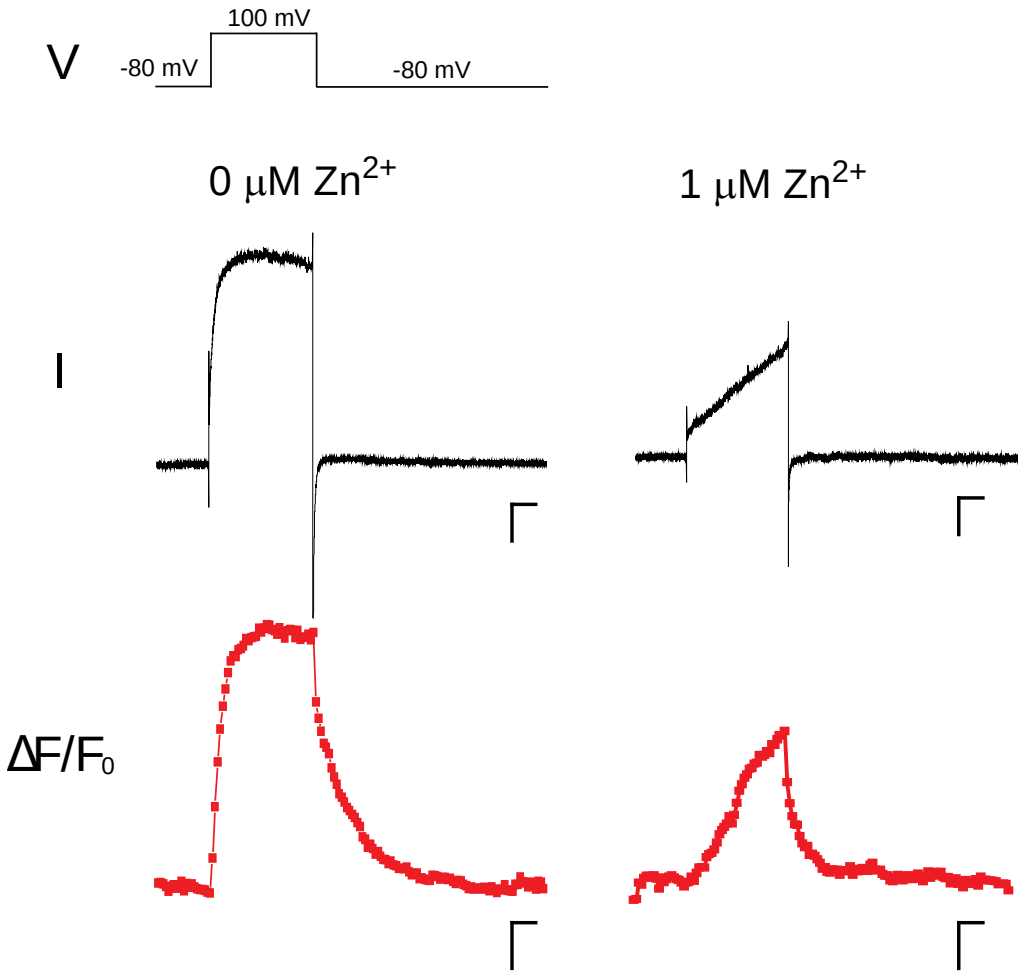


Figure S2, related to Figure 2A. Voltage-clamp fluorometry current and pH-DIBO fluorescent traces of Hv-1 expressing CHO cells in the presence or absence of Zn^{2+}. Scale bars represent 50 pA, 2% and 1 s; pH_o/pH_i = 7.5/6.0 (0.1 mM HEPES).

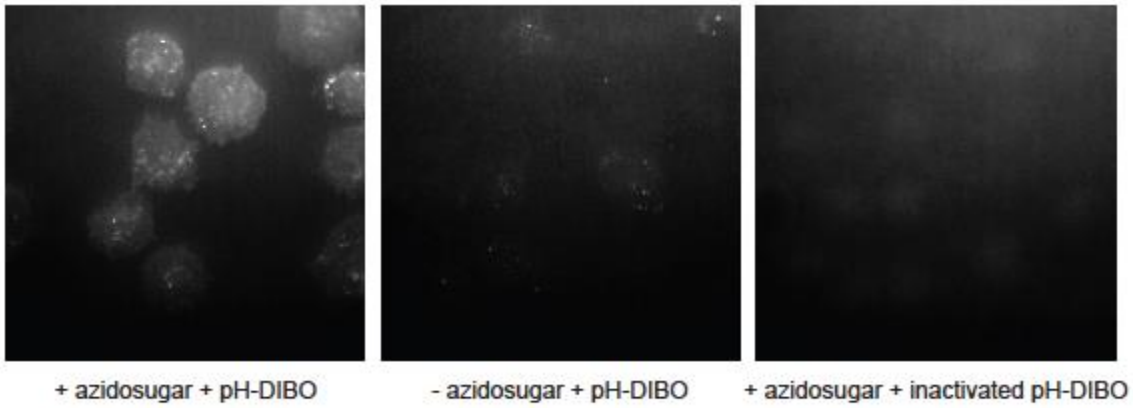


Figure S3, related to Figure 2F. TIRF images of cells incubated with or without azidosugar for 48 h and treated with either pH-DIBO or inactivated pH-DIBO (50 μM, 30 min). pH-DIBO was inactivated by 3-azido-1-propanol (100 eq, 24 h). Images were acquired with an Olympus IX71 microscope with an 60× 1.49 Olympus objective and a 1.6× optivar with TIRF illumination. Exposure was set to 100ms and the excitation light was provided by a Cobolt Jive 561 laser set to 20mW. Emitted light was first passed through a dual dichroic (525/50nm, 645/140nm) and then a 525/50nm band-pass. An Andor iXon EM+ 885i CCD (1004x1002 with 8μm2 pixels) was used to collect the light.

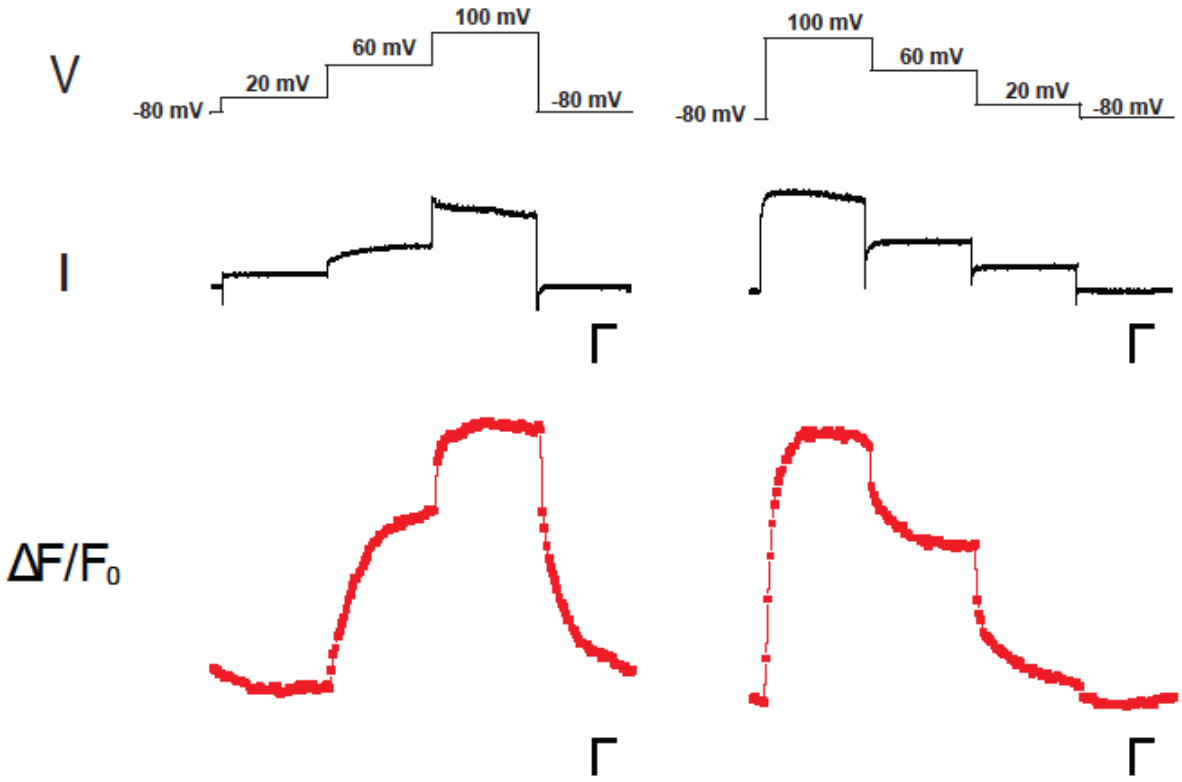


Figure S4, related to Figure 2A. Voltage-clamp fluorometry current and pH-DIBO fluorescent traces of Hv-1 expressing CHO cells for a series of voltage step-ups (*left*) or step-downs (*right*). Scale bars represent 100 pA, 2%, and 1 s; pH_o/pH_i = 7.5/6.0 (0.1 mM HEPES).

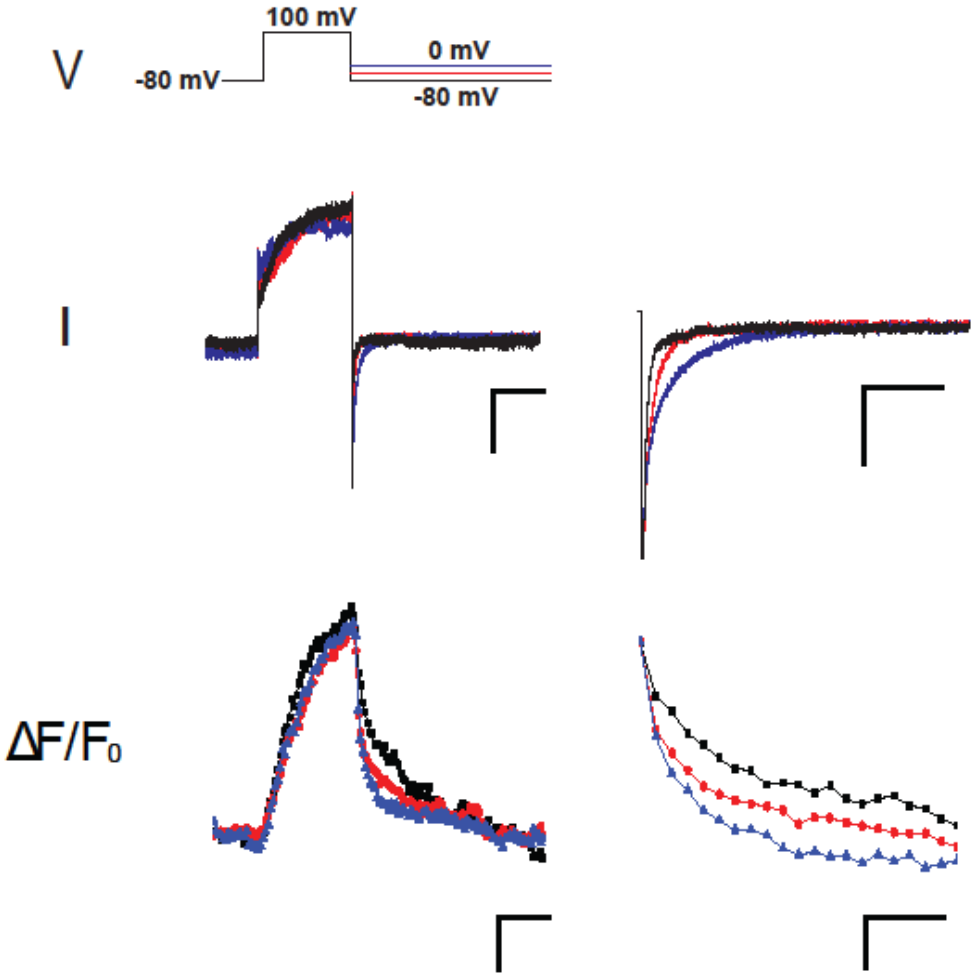


Figure S5, related to Figure 2A, 2C, 3C and 4. Tail current and pH-DIBO fluorescent decay kinetics at different closing voltages. Cells were held at – 80 mV, depolarized to 100 mV, and tail currents were elicited at 0 mV (blue), – 40 mV (red), and – 80 mV (black). Current and fluorescence traces of the entire voltage protocol are shown on the *left*. Scale bars represent 50 pA, 2%, and 2 s. Enlargement of the tail region are shown on the *right*. Scale bars represent 50 pA, 2%, and 0.5 s. No pH gradient was used: pH_o/pH_i = 7.0/7.0 (0.1 mM HEPES).

Supplementary movie legends

Supplementary movie 1, related to Figure 2A. ΔF movie of a representative CHO cell expressing Hv-1, the cell was held at – 80 mV, and depolarized at 100mV for 4 s. The F_0 was the fluorescence of the first frame. The frame rate is 10 fps.

Supplementary movie 2, related to Figure 2A. Raw fluorescence images of movie S1. The frame rate is 30 fps.

Supplementary movie 3, related to Figure 4A. ΔF movie of a representative CHO cell expressing Shaker R362H, the cell was held at 30 mV, and hyperpolarized at – 120mV for 4 s. The F_0 was the fluorescence of the last frame at the – 120 mV command voltage. The frame rate is 10 fps.

Supplementary movie 4, related to Figure 4A. Raw fluorescence images of movie S3. The frame rate is 30 fps.

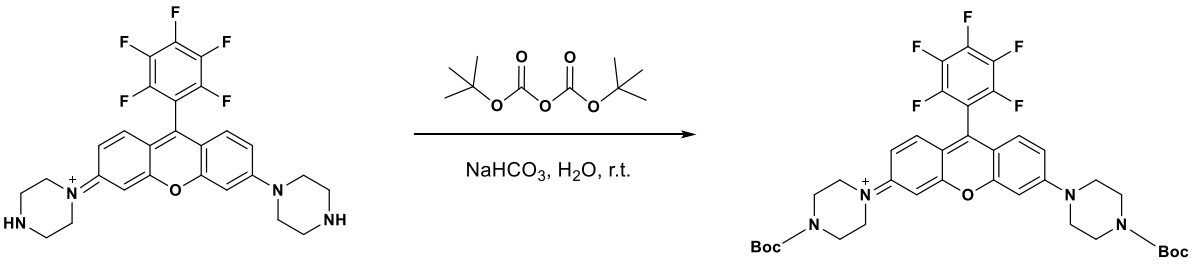
Supplementary movie 5, related to Figure 4B. ΔF movie of a representative CHO cell expressing Hv-1 and its neighboring cells, the cell in the center was held at – 80 mV and depolarized at 100mV for 4 s. The F_0 was the fluorescence at the first frame. The frame rate is 10 fps.

Supplementary movie 6, related to Figure 4B. Raw fluorescence images of movie S5. The frame rate is 30 fps.

Supplemental Experimental Procedures, related to Figure 1B

General experimental procedures: Unless otherwise stated, all reactions were run under an inert environment of argon (Ar) from which water and oxygen were rigorously excluded. Pentafluorobenzaldehyde and methanesulfonic acid were purchased from Acros. 3-(1-Piperazinyl) phenol was from Alfa Aesar. All other reagents and solvents were obtained from Sigma-Aldrich. Deuterated solvents were purchased from CIL. Thin layer chromatography was used to monitor the progress of reactions with EM Science silica gel 60 F_{254} plates or neutral aluminum oxide F_{254} plates from EMD Chemicals. Flash chromatography was performed using silica gel 60 (40-63 μm) from BDH. The final compound was purified by HPLC using a Higgins Analytical PROTO 300 C-18 column (10 μm), 250 × 10 mm (RS-2510-W181) on a Hewlett Packard Agilent 1100 HPLC instrument equipped with G1315A DAD absorbance detector. NMR spectra were recorded in $CDCl_3$, CD_3OD on a Varian 400 MHz spectrometer; ^{1}H NMR and ^{13}C NMR signals are reported in chemical shift relative to the NMR solvent peak; 19^{F} NMR are reported in chemical shift relative to an internal triflouroacetic acid (TFA) standard. Coupling constants are reported as J values in Hz. NMR splitting patterns are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Coupling constants (J) are reported in Hz. High resolution mass spectra (HRMS) were obtained on a Waters Q-TOF Premier Mass Spectrometer at the University of Massachusetts Medical School Proteomics and Mass Spectrometry Laboratory. Fluorescence spectroscopic measurements were performed on an F4500 (Hitachi).

Compound 2 (Boc-pH)

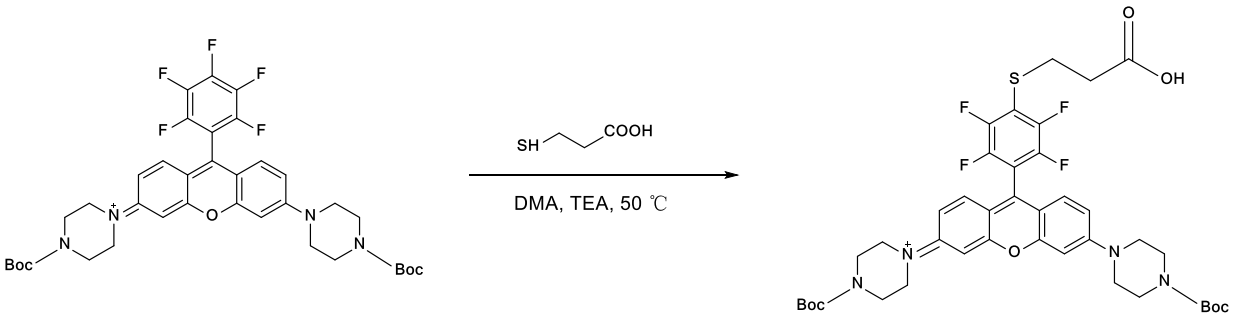


1 **2**

Crude 1 was synthesized following procedure described in the literature without further purification. Di-tert-butyl dicarbonate (4g, 18 mmol) was added to a stirred solution of crude 1 (4 g) and $NaHCO_3$ (2.7 g, 32 mmol) in H_2O (100 mL). After being stirred at r.t. overnight, the reaction mixture was extracted with CH_2Cl_2 (3 x 100 mL). The combined organic layers were washed with H_2O (3 x 100 mL), dried (Na_2SO_4), filtered and the filtrate was concentrated under reduced pressure. The crude product was purified by chromatography over silica gel (50:1, v/v, CH_2Cl_2/Methanol) to give compound 2 (1.54 g, two steps yield 28%). ^{1}H-NMR (400 MHz, CD_3OD) δ = 7.53 (d, J = 9.6 Hz, 2H), 7.36 (dd, J = 9.6, 2.4 Hz, 2H), 7.25 (d, J = 2.4 Hz, 2H), 3.93 – 3.84 (m, 8H), 3.68 (s, 8H), 1.50 (s, 18H). ^{19}F-NMR (376 MHz, CD_3OD) δ = -138.80 (d, J = 17.6 Hz, 2F), -150.66 (t, J = 20.3 Hz, 1F), -160.47 (dt, J = 20.3, 5.6 Hz, 2F). ^{13}C-NMR (101 MHz, CD_3OD) δ = 159.58,

158.91, 156.17, 131.93, 117.14, 115.59, 98.82, 81.91, 48.06, 28.60. HRMS (ESI): m/z calculated for $C_{37}H_{40}F_5N_4O_5$ (M^+): 715.2913; found: 715.2890.

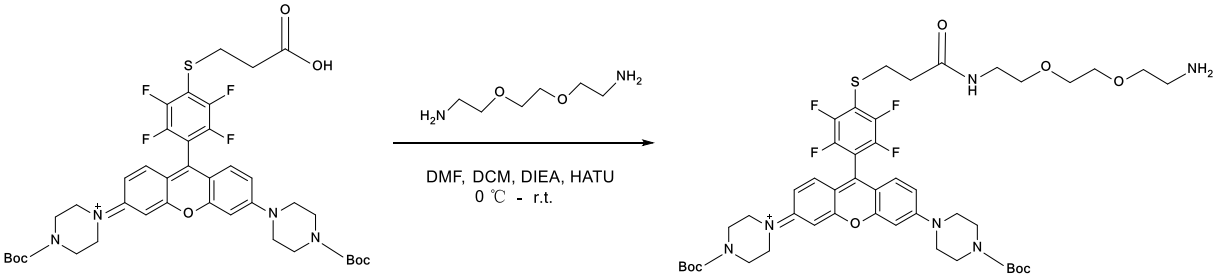
Compound 3 (Boc-pH-COOH)



2 **3**

Compound 2 (580 mg, 0.8 mmol), N,N-dimethylacetamide (25 mL) and trimethylamine (720 uL) were heated to 50 ℃ and 3-mercaptopropionic acid (100 uL) was added dropwise. After being stirred at 50 for 3h, the solvents were evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (100 mL) and the resulting solution was washed with H_2O (3 x 50 mL), which was back extracted with CH_2Cl_2 (3 x 30 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude was purified by chromatography over silica gel (50:1 -> 10:1, v/v, CH_2Cl_2/Methanol) to give compound 3 (0.3 g, 46%). ^{1}H NMR (400 MHz, CD_3OD) δ = 7.54 (d, J = 9.5 Hz, 2H), 7.36 (dd, J = 9.5, 2.4 Hz, 2H), 7.26 (d, J = 2.4 Hz, 2H), 3.95 – 3.81 (m, 8H), 3.68 (s, 8H), 3.39 – 3.33 (m, 2H), 2.73 – 2.64 (m, 2H), 1.50 (s, 18H). ^{19}F-NMR (376 MHz, CD_3OD) δ = -131.87 (2F, dd, J = 25.8, 12.4 Hz, 2F), -138.96 (dd, J = 26.8, 14.5 Hz, 2F). ^{13}C NMR (101 MHz, CD_3OD) δ = 159.52, 158.85, 156.16, 132.04, 117.14, 115.38, 98.84, 81.86, 48.06, 31.25, 28.60. HRMS (ESI): m/z calculated for $C_{40}H_{45}F_4N_4O_7S$ (M^+): 801.2940; found: 801.2933.

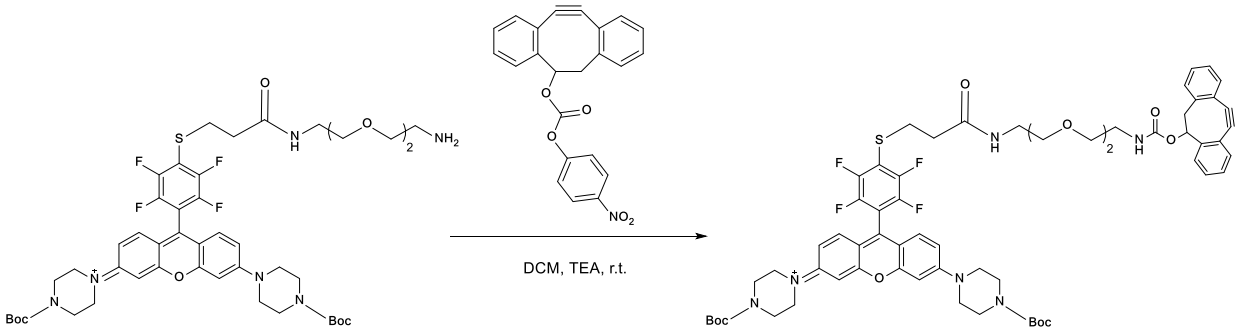
Compound 4 (Boc-pH-NH2)



3 **4**

N,N-Diisopropylethylamine (90 mg, 0.7 mmol) and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (171 mg, 0.45 mmol) was added slowly to a stirred solution of compound 3 (300 mg, 0.37 mmol), 2, 2'-(Ethylenedioxy) bis(ethylamine) (555 mg, 3.7 mmol), DMF (5mL) and CH_2Cl_2 (5 mL) at 0 ℃. After being stirred at r.t. for 2 h, the solvents were evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (50 mL) and the resulting solution was washed with brine (3 x 50 mL), H_2O (3 x 50 mL) which was back extracted with CH_2Cl_2 (3 x 30 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated under reduced pressure. The crude was purified by chromatography over silica gel (50:1 -> 10:1, v/v, CH_2Cl_2/Methanol) to give compound 4 (0.2 g, 57%). ^{1}H NMR (400 MHz, CD_3OD) δ = 7.58 (d, J = 9.6 Hz, 2H), 7.38 (dd, J = 9.6, 2.4 Hz, 2H), 7.26 (d, J = 2.4 Hz, 2H), 3.93 – 3.82 (m, 8H), 3.72 – 3.64 (m, 12H), 3.58 (t, J = 5.7 Hz, 4H), 3.37 (dd, J = 9.7, 4.1 Hz, 4H), 3.11 – 3.05 (m, 2H), 2.67 (t, J = 6.8 Hz, 2H), 1.50 (s, 18H). ^{19}F NMR (376 MHz, CD_3OD) δ = -131.63 (dd, J = 25.1, 12.6 Hz, 2F), -138.89 (dd, J = 24.5, 11.9 Hz, 2F). ^{13}C NMR (101 MHz, CD_3OD) δ = 173.21, 159.57, 158.90, 156.18, 117.11, 115.41, 98.82, 81.92, 71.35, 70.55, 67.93, 48.06, 40.67, 40.31, 37.46, 31.52, 28.60. HRMS (ESI): m/z calculated for $C_{46}H_{59}F_4N_6O_8S$ (M^+): 931.4046; found: 931.4034.

Compound 5 (Boc-pH-DIBO)

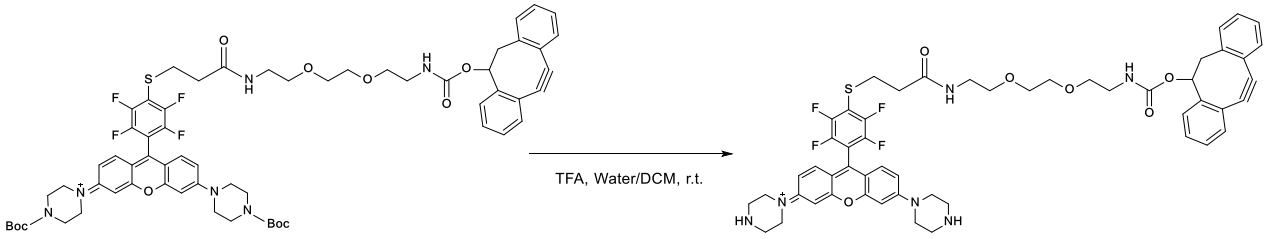


4 **5**

DIBO-4-nitrophenyl ester (196 mg, 0.51 mmol) was added to a stirred solution of compound 4 (240 mg, 0.26 mmol) and triethylamine (36 uL, 0.26 mmol) in CH_2Cl_2 (30 mL). After being stirred at r.t. for 16 h, the solvent were evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (50 mL) and the resulting solution was washed with H_2O (3 x 50 mL), which was back extracted with CH_2Cl_2 (3 x 30 mL). The combined organic layers were dried (Na_2SO_4, filtered and concentrated under reduced pressure. The crude was purified by chromatography over silica gel (50:1 -> 20: 1, v/v, CH_2Cl_2/Methanol) to give compound 5 (110 mg, 36%). ^{1}H NMR (400 MHz, CD_3OD) δ = 7.48 (dd, J = 12.3, 8.7 Hz, 4H), 7.35 – 7.21 (m, 8H), 7.14 (d, J = 2.1 Hz, 2H), 5.29 (s, 1H), 3.89 – 3.77 (m, 8H), 3.63 (d, J = 6.2 Hz, 12H), 3.54 (t, J = 5.1 Hz, 4H), 3.37 – 3.33 (m, 4H), 3.26 (t, J = 5.2 Hz, 2H), 3.12 (dd, J = 15.1, 2.1 Hz, 1H), 2.68 (dd, J = 15.1, 3.9 Hz, 1H), 2.60 (t, J = 6.7 Hz, 2H), 1.51 (s, 18H). ^{19}F NMR (376 MHz, CD_3OD) δ = -131.27 (dd, J = 27.3, 14.7 Hz, 2F), -138.41 (dd, J = 26.8, 14.2 Hz, 2F). ^{13}C NMR (101 MHz, CDCl3) δ = 171.40, 158.10, 157.33, 155.94, 154.44, 152.42, 151.37, 132.65,

130.24, 128.17, 128.14, 126.97, 126.94, 126.02, 125.84, 124.36, 123.78, 121.09, 116.32, 114.74, 112.90, 110.12, 97.45, 81.07, 70.46, 70.34, 70.22, 70.05, 47.19, 46.24, 40.99, 39.22, 36.61, 30.43, 28.48. HRMS (ESI): m/z calculated for $C_{63}H_{69}F_4N_6O_{10}S$ (M^+): 1177.4727; found: 1177.4729.

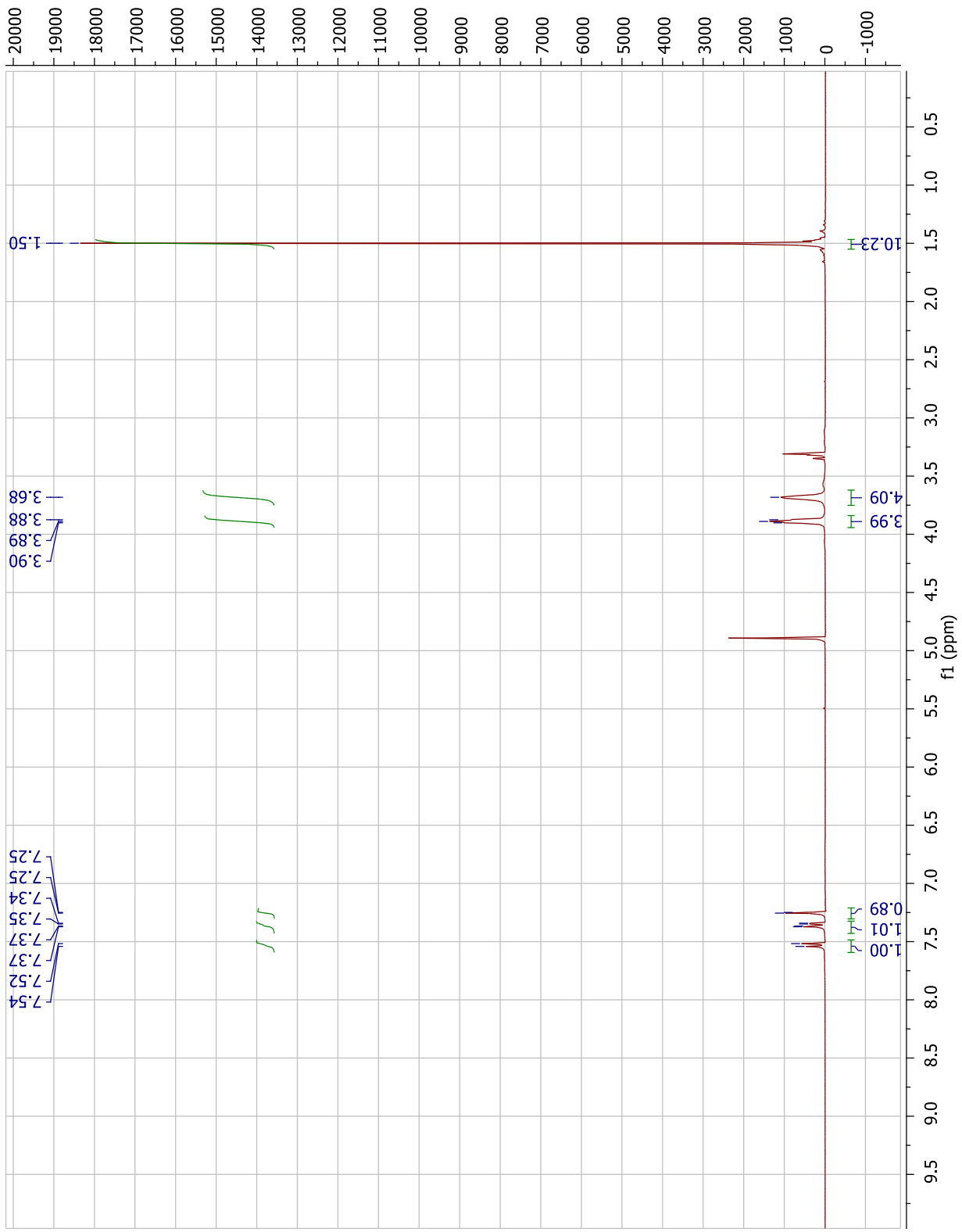
Compound 6 (pH-DIBO)



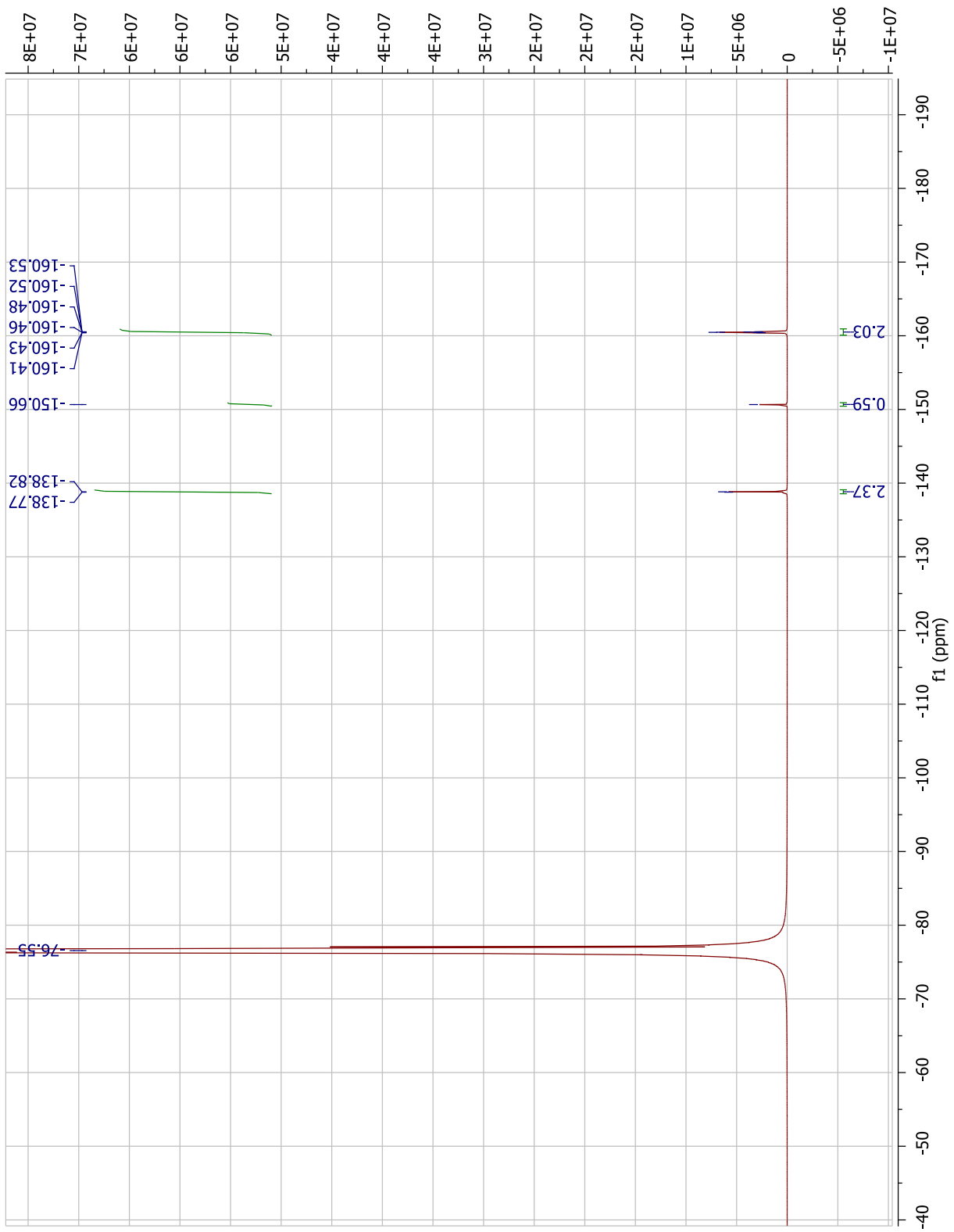
5 **6**

Trifluoroacetic acid (5mL) was added dropwise to a stirred solution of compound 5 (100 mg, 0.085 mmol) in CH_2Cl_2 (5 mL), H_2O (1 mL). After being stirred at r.t. for 1.5 h, the solvents were evaporated under reduced pressure. The residue was dissolved in H_2O (25 mL) and the resulting solution was washed with CH_2Cl_2 (3 x 20 mL). The aqueous layers were concentrated under reduced pressure and purified by HPLC. 25 mg crude yielded 15 mg pure compound 6. If up-scaled, 60 mg (0.051 mmol, 72%). ^{1}H NMR (400 MHz, CD_3OD) δ = 7.59 (d, J = 9.5 Hz, 2H), 7.51 (d, J = 7.9 Hz, 1H), 7.38 (d, J = 9.6 Hz, 3H), 7.33 (s, 4H), 7.30 – 7.24 (m, 4H), 5.31 (s, 1H), 4.07 (d, J = 4.6 Hz, 8H), 3.62 (s, 4H), 3.54 (d, J = 2.6 Hz, 4H), 3.44 (d, J = 4.7 Hz, 8H), 3.35 (m, 4H), 3.27 (t, J = 5.5 Hz, 2H), 3.15 (dd, J = 15.1 Hz, 1H), 2.75 – 2.67 (m, 1H), 2.61 (t, J = 6.7 Hz, 2H). ^{19}F NMR (376 MHz, CD3OD) δ = -132.77 (dd, J = 27.2, 14.4 Hz, 2F), -139.95 (dd, J = 27.1, 14.4 Hz, 2F). ^{13}C NMR (126 MHz, CD3OD) δ = 173.04, 159.86, 158.97, 157.95, 153.54, 152.46, 145.26, 132.40, 131.09, 129.32, 129.26, 128.29, 128.26, 127.19, 126.91, 124.99, 124.88, 122.26, 117.57, 116.14, 113.78, 110.97, 99.66, 77.87, 71.37, 71.30, 70.94, 70.51, 47.19, 45.34, 44.04, 41.76, 40.43, 37.62, 31.41. HRMS (ESI): m/z calculated for $C_{53}H_{53}F_4N_6O_6S$ (M^+): 977.3678; found: 977.3669.

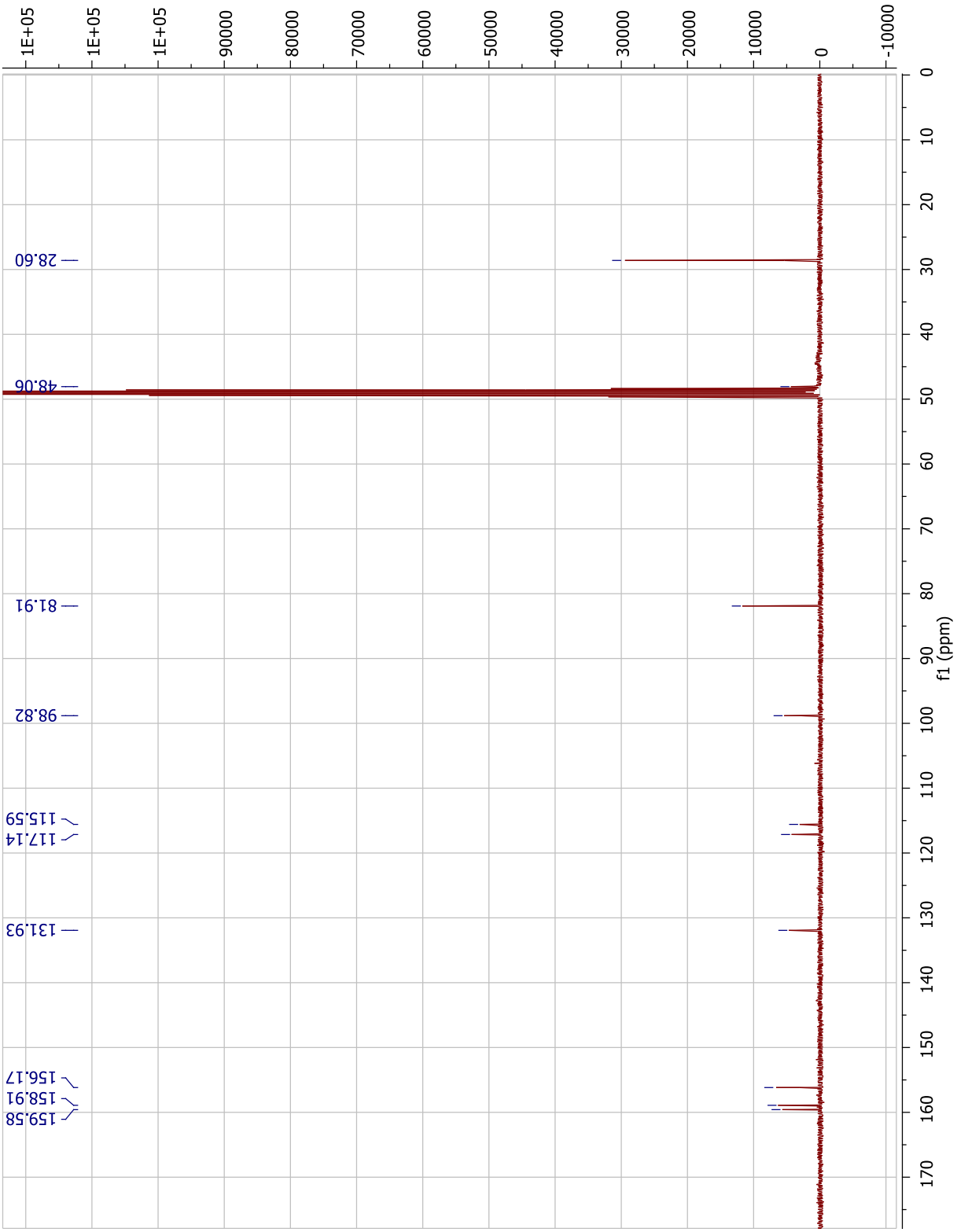
Compound 2 (Boc-pH) ^{1}H-NMR in MeOD



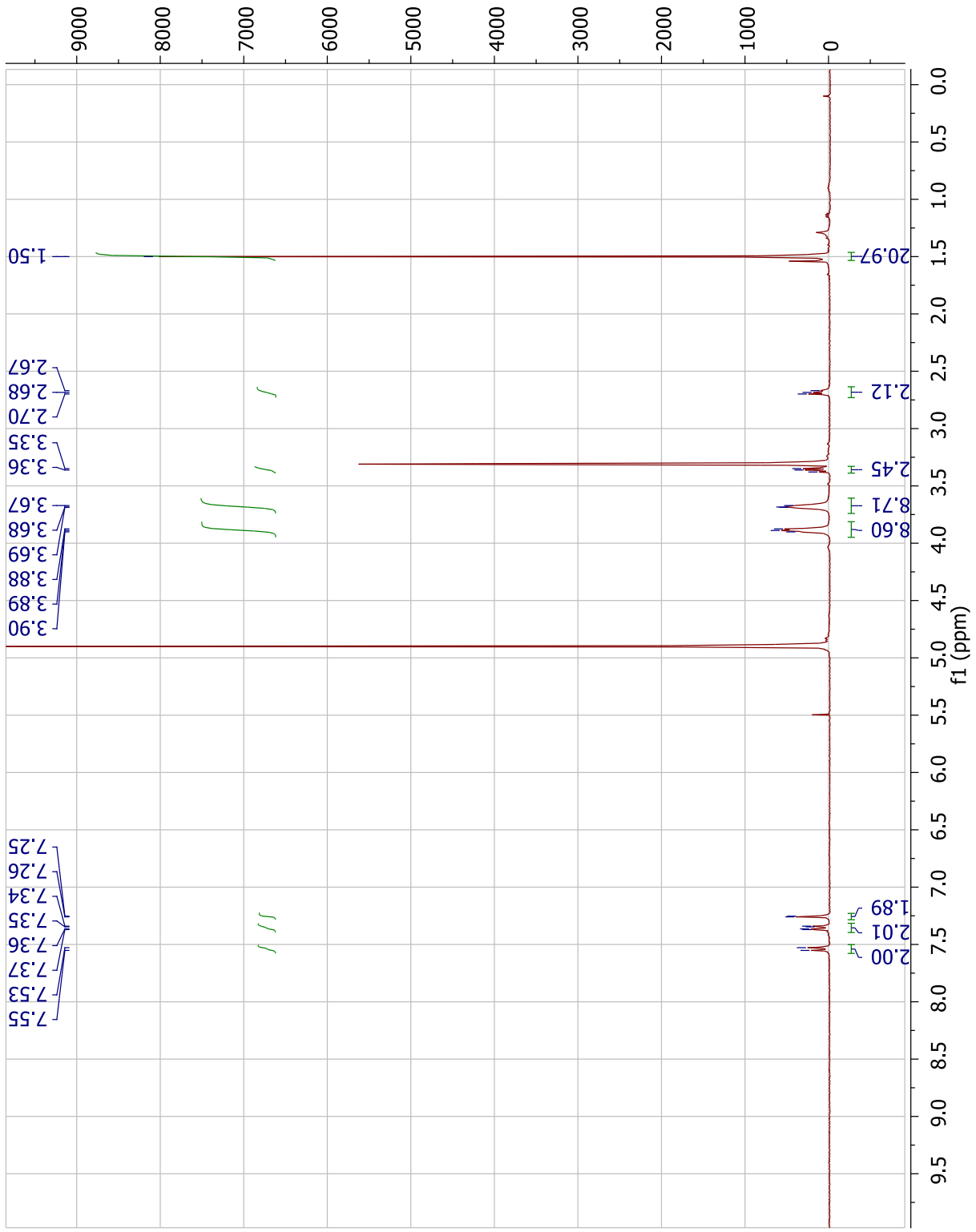
Compound 2 (Boc-pH) ^{19}F-NMR in MeOD



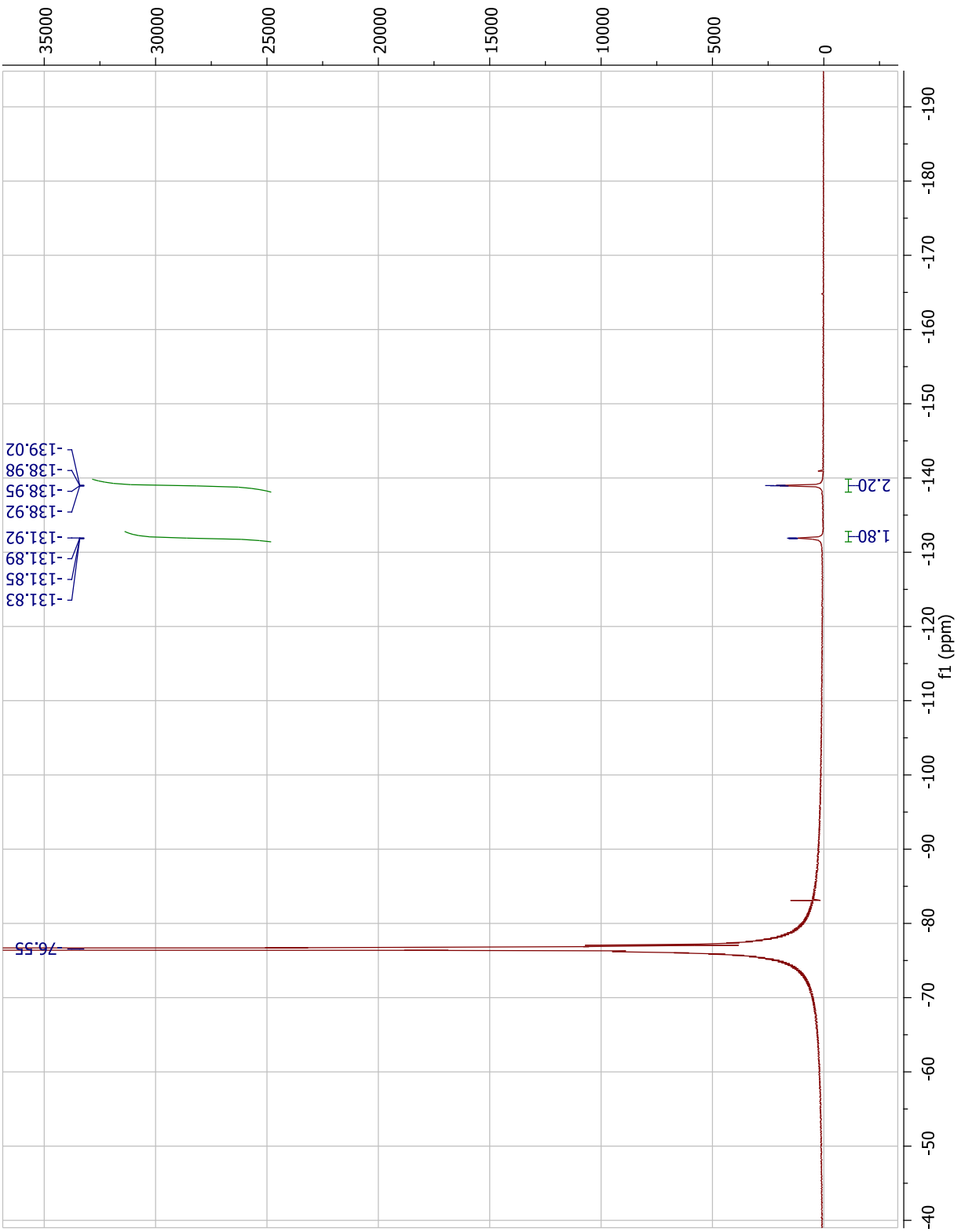
Compound 2 (Boc-pH) ^{13}C-NMR in MeOD



Compound 3(Boc-pH-COOH) ^{1}H-NMR in MeOD

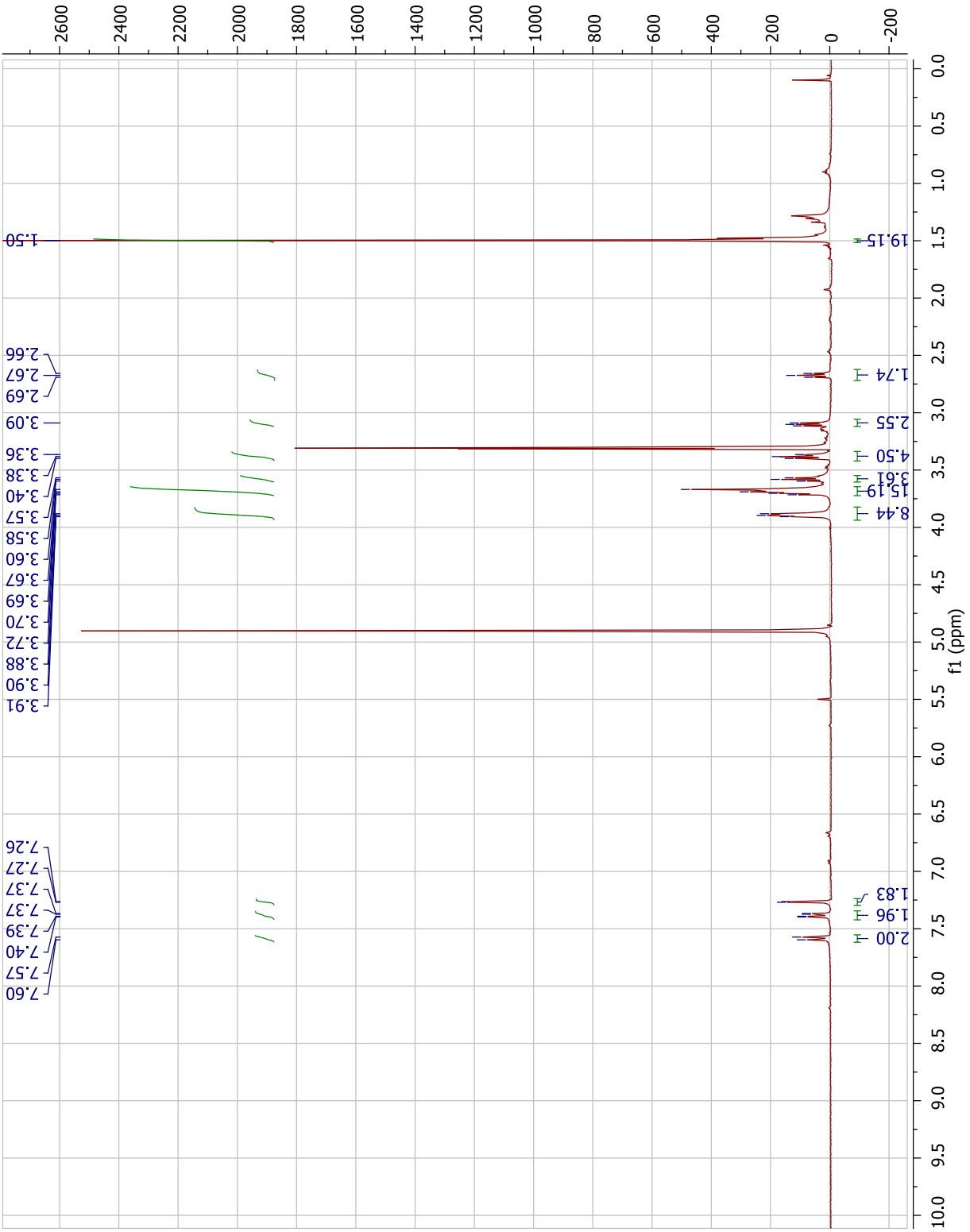


Compound 3 (Boc-pH-COOH) ^{9}F-NMR in MeOD

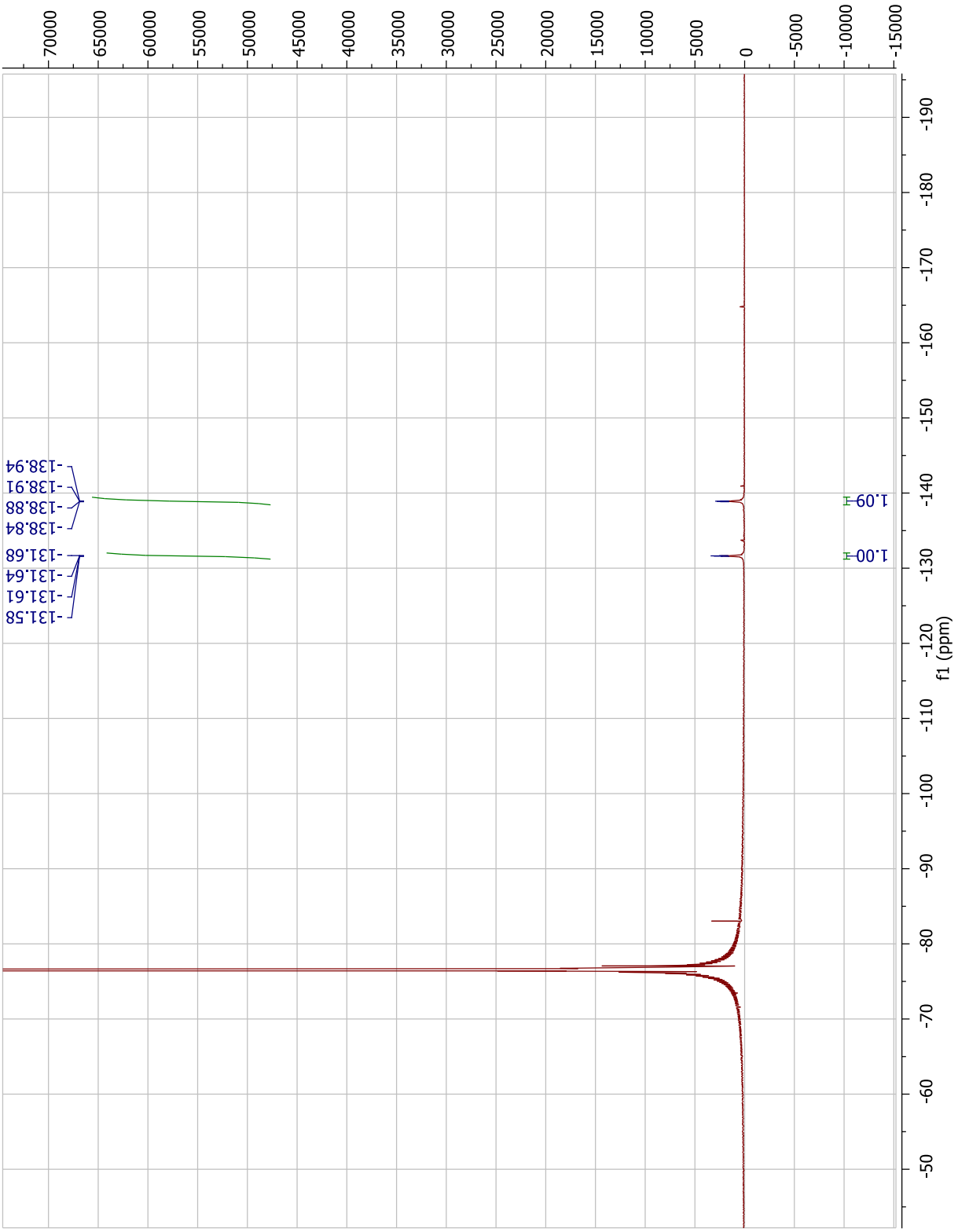


Compound 3 (Boc-pH-COOH) ^{13}C-NMR in MeOD

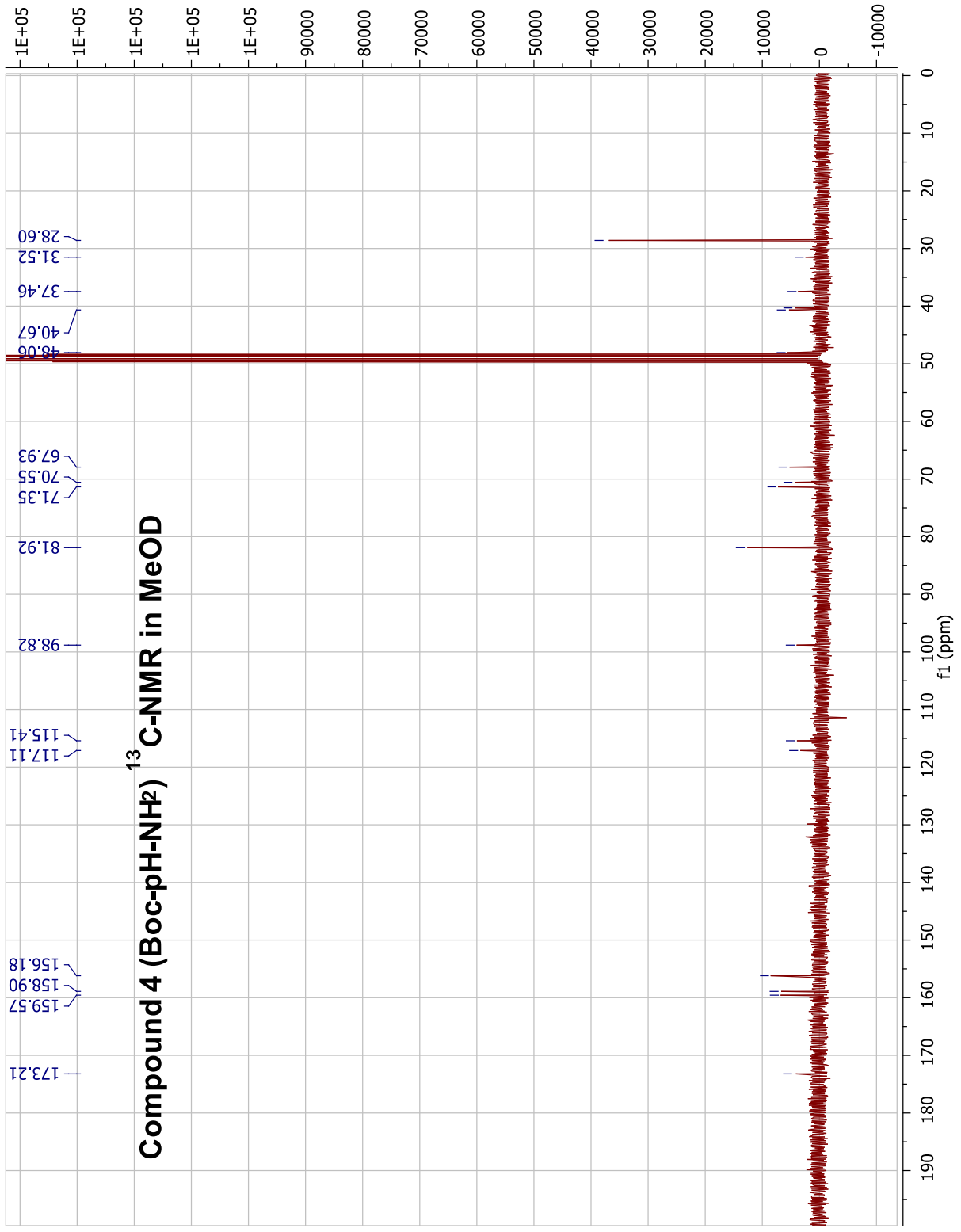
Compound 4(Boc-pH-NH2) ^{1}H-NMR in MeOD



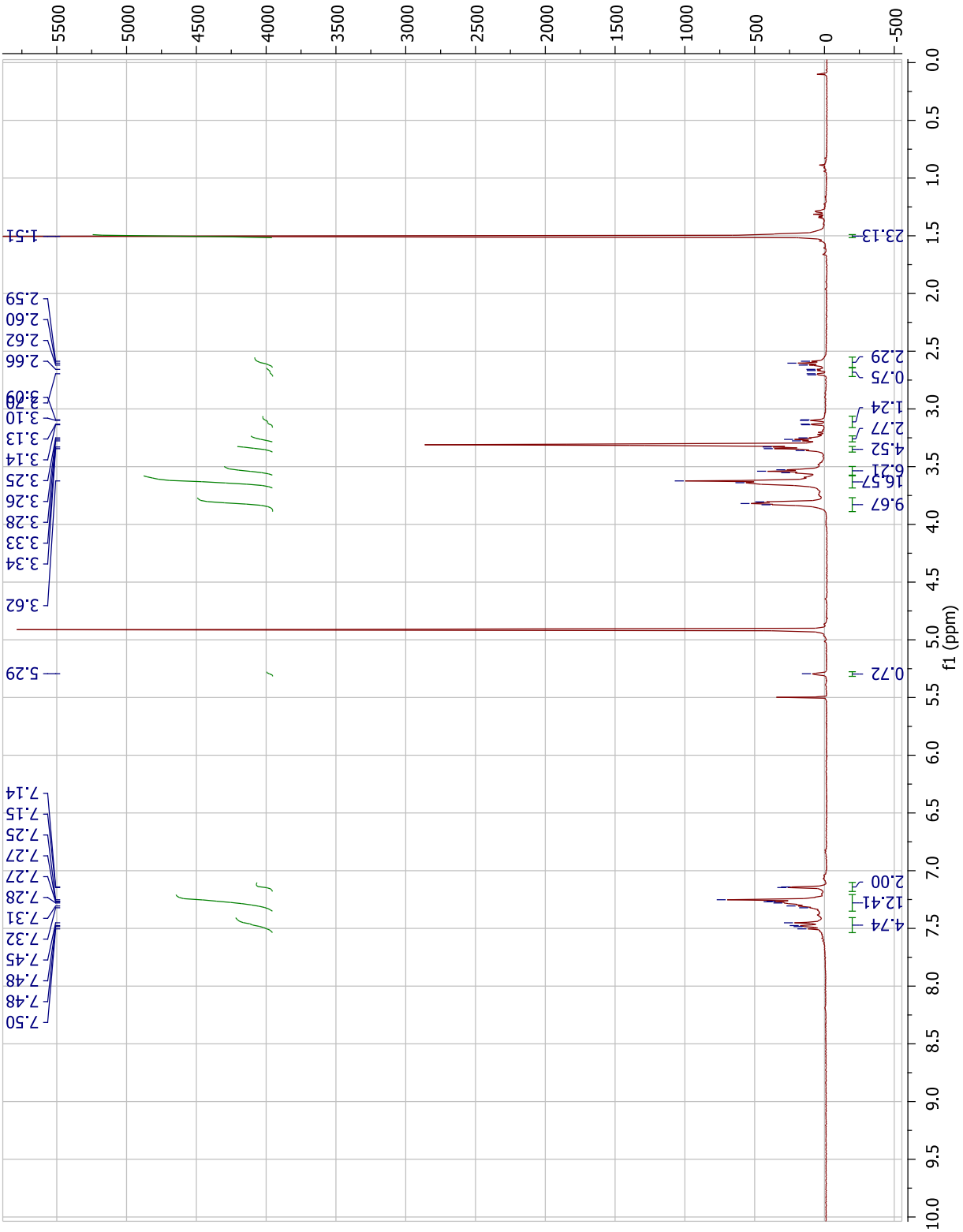
Compound 4(Boc-pH-NH2) ^{9}F-NMR in MeOD



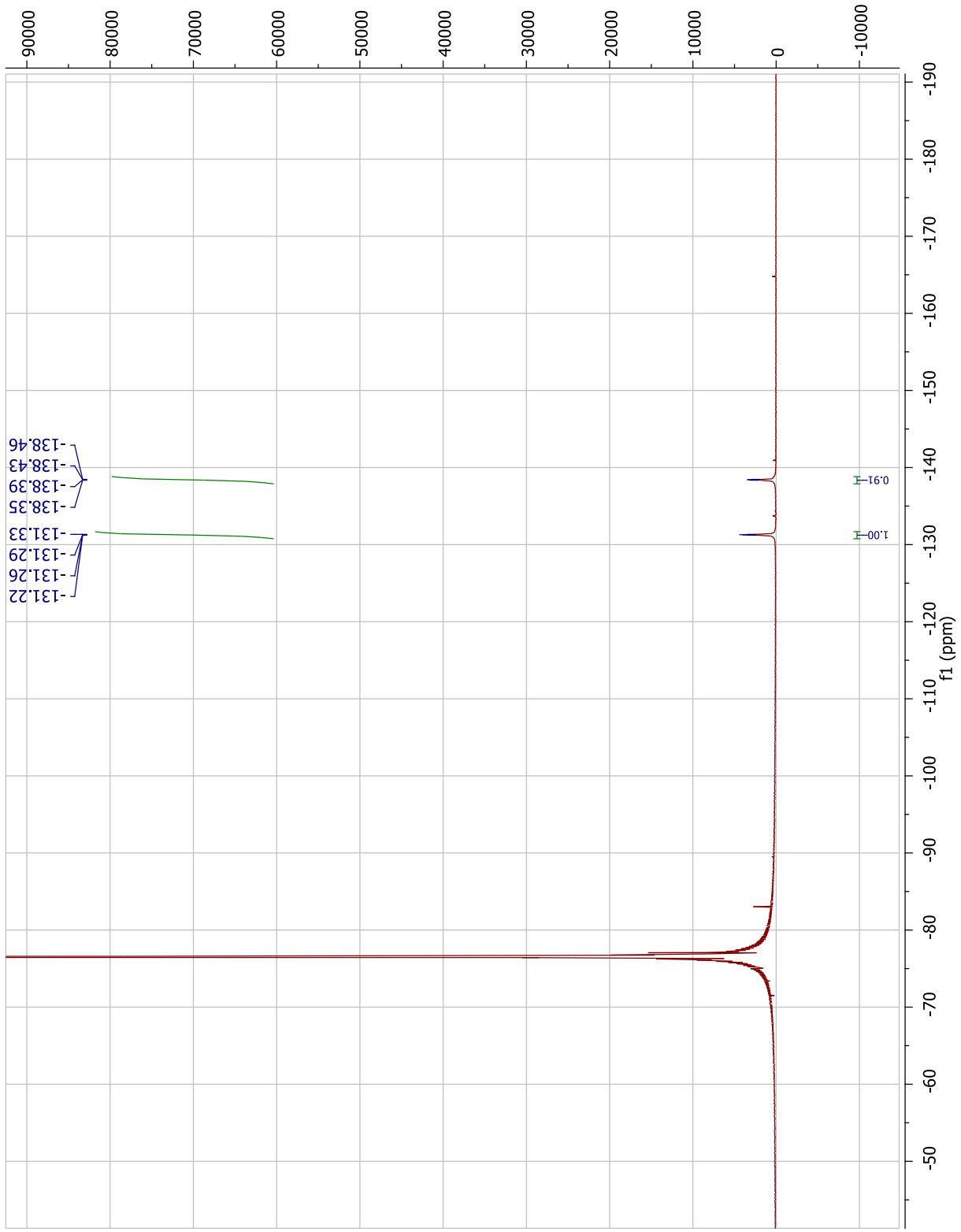
Compound 4(Boc-pH-NH2) ^{13}C-NMR in MeOD



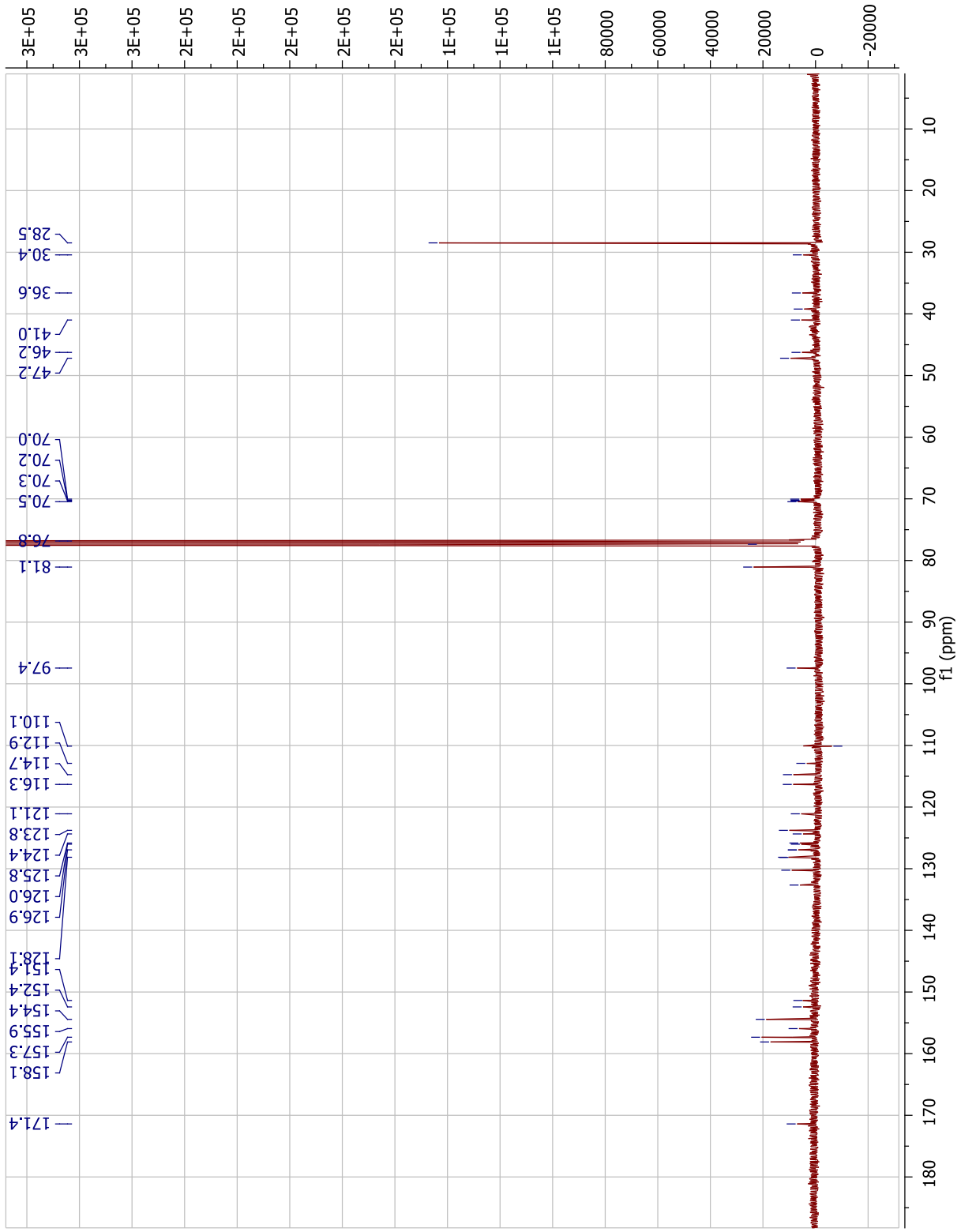
Compound 5(Boc-pH-DIBO) ^{1}H-NMR in MeOD



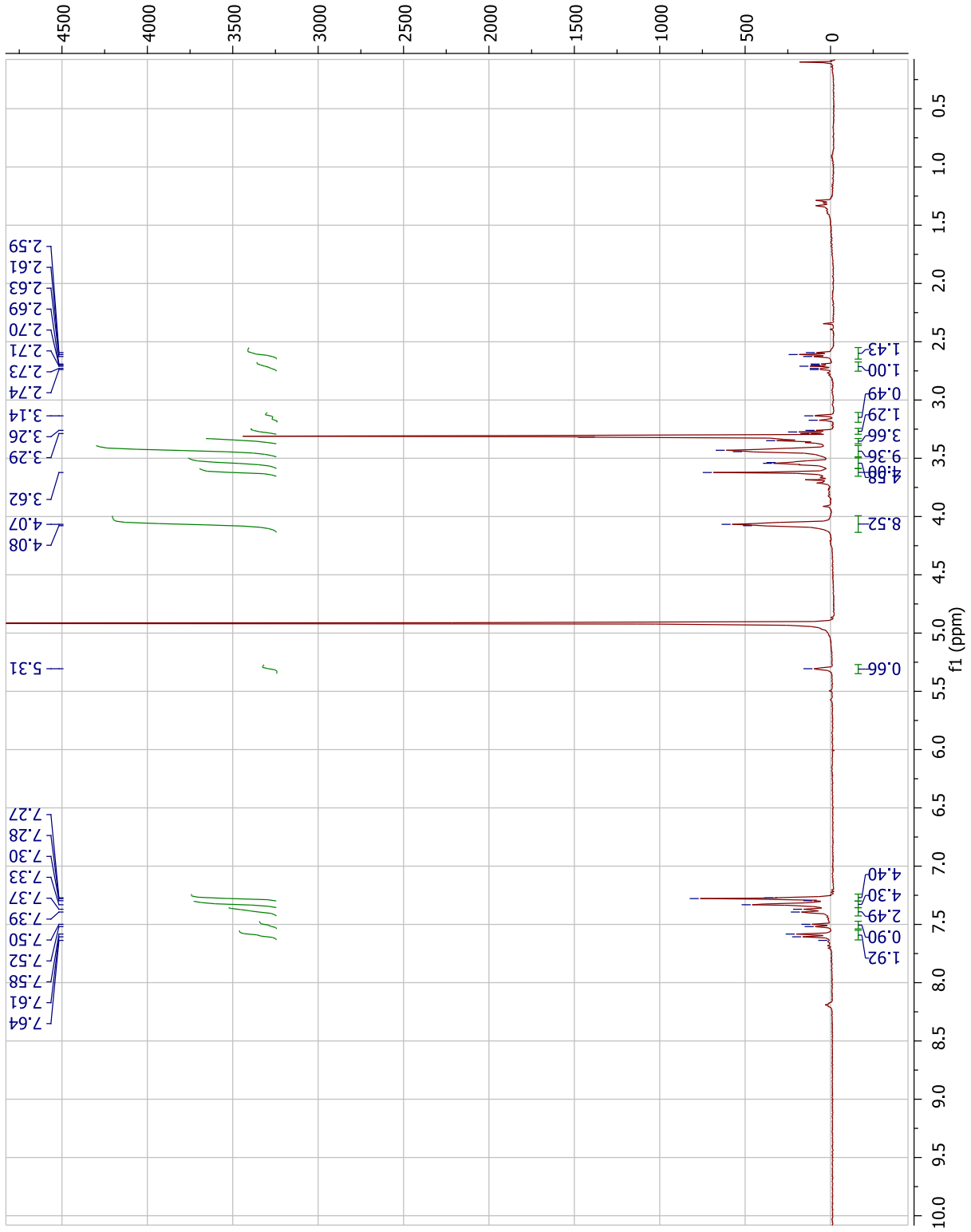
Compound 5(Boc-pH-DIBO) ^{9}F-NMR in MeOD



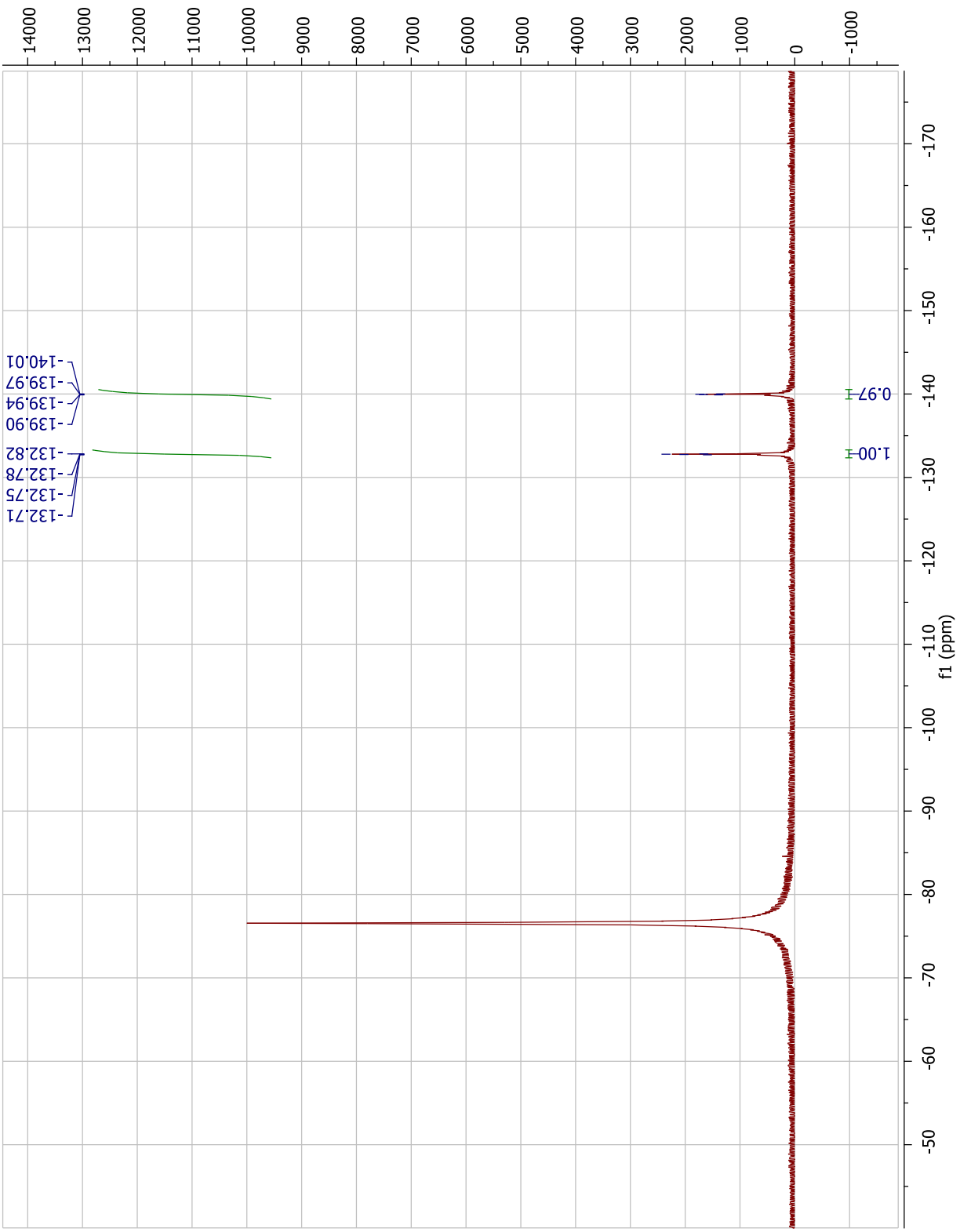
Compound 5(Boc-pH-DIBO) ^{13}C-NMR in CDCl3



Compound 6(pH-DIBO) ^{1}H-NMR in MeOD



Compound 6(pH-DIBO) ^{9}F-NMR in MeOD



Compound 6(pH-DIBO) ^{13}C-NMR in MeOD