Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

Design, Synthesis and Evaluation of Halogenated Phenazine Antibacterial Prodrugs Targeting Nitroreductase Enzymes for Activation

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Supporting Information

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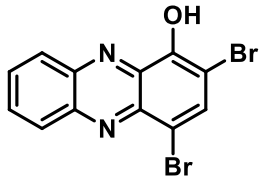
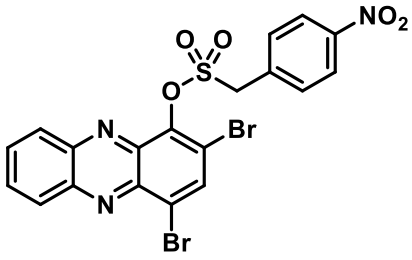
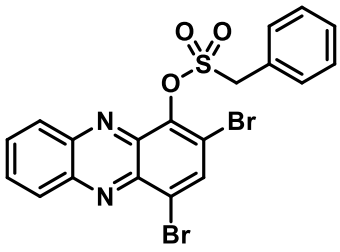
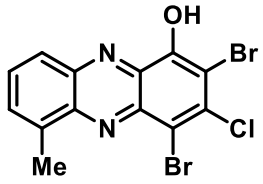
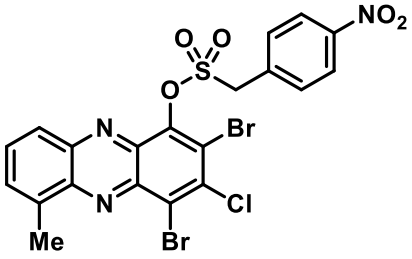
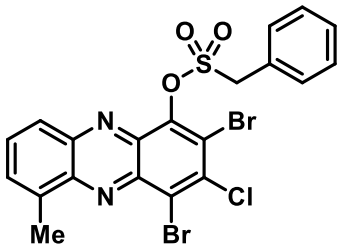
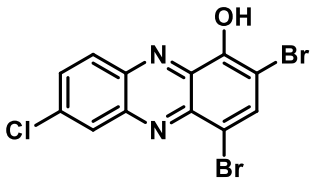
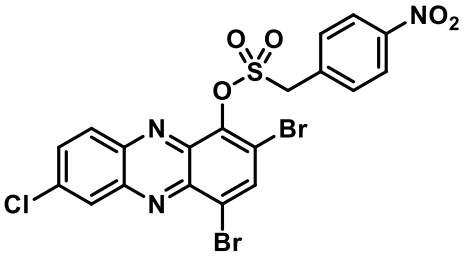
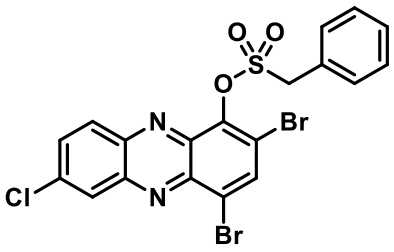
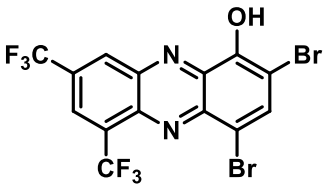
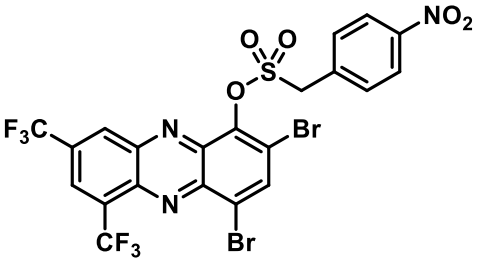
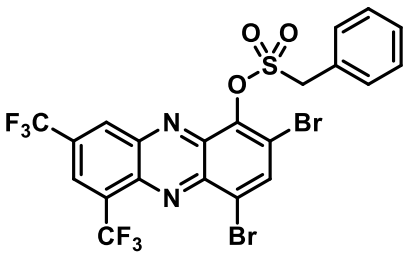
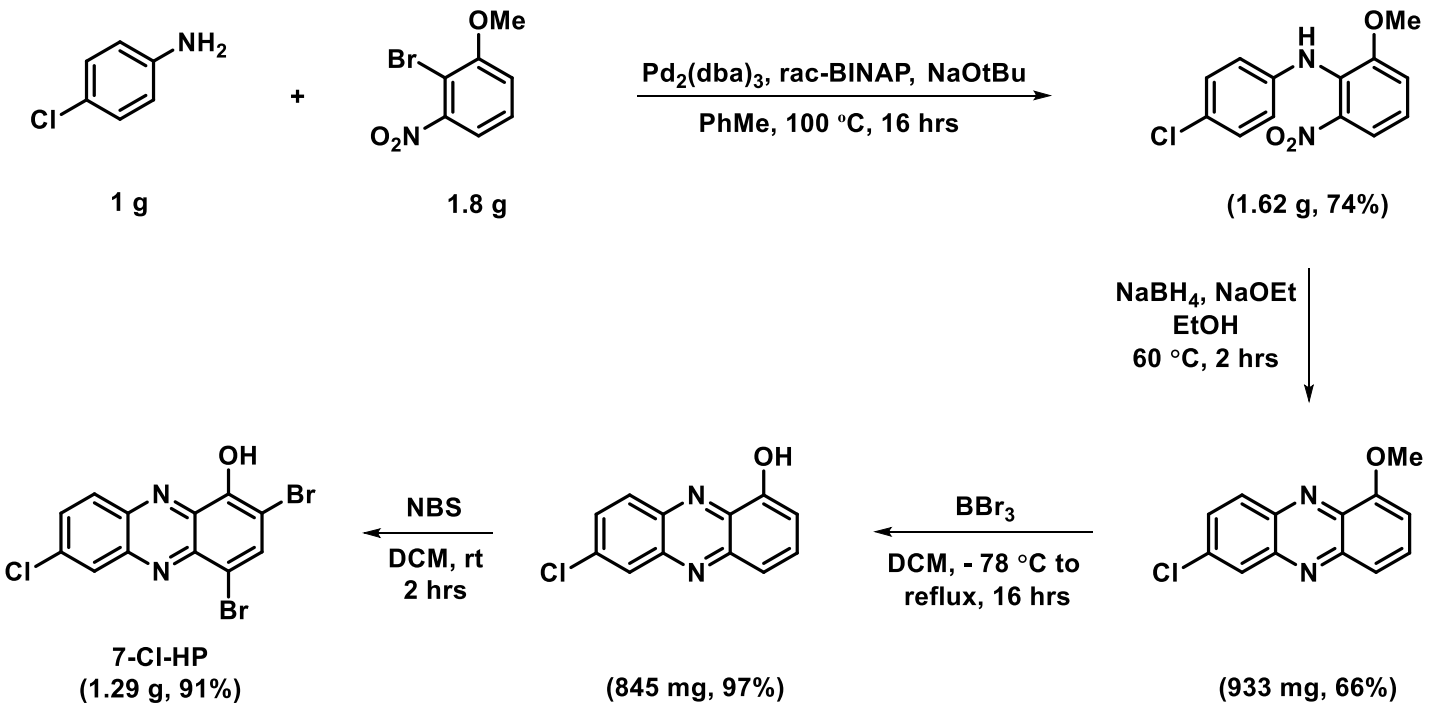
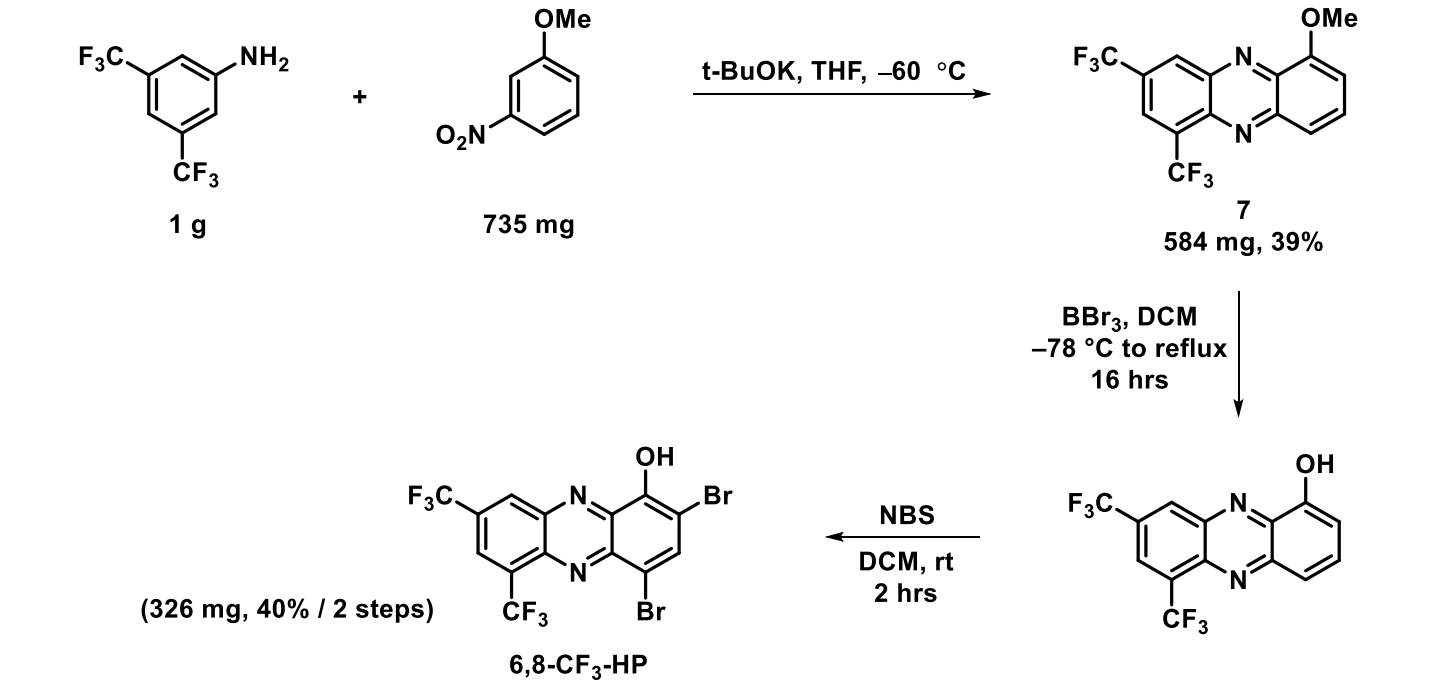
1.) Supporting Figure 1: Compounds Evaluated During These Studies.

Parent HP	NTR Prodrug	Control Compound
		
HP-1	HP-1-N	HP-1-SE
		
HP-29	HP-29-N	HP-29-SE
		
7-Cl-HP	7-Cl-HP-N	7-Cl-HP-SE
		
6,8-CF_3-HP	6,8-CF_3-HP-N	6,8-CF_3-HP-SE

2.) Supporting Figure 2: Scale Up Synthesis Towards Potent Halogenated Phenazines.



Scale up was performed during these studies. Protocols used are described in our previous work, reference: J. Med. Chem. 2018, 61, 3962-3983.



Note: For the synthesis of phenazine **7**, the desired nitrosoaniline intermediate (not shown) generated from reaction of **5** and **6** using potassium *tert*-butoxide (^{t}BuOK) spontaneously cyclized to **7** after the reaction solvent was removed (BSA not always required).

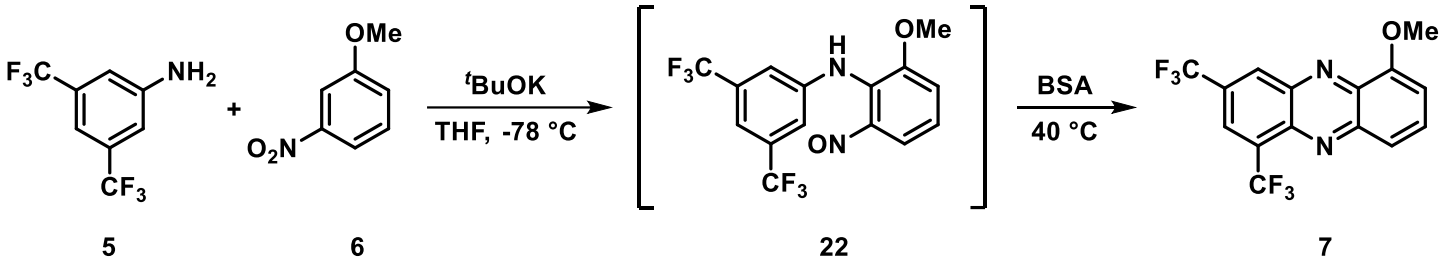
3.) General Information.

All synthetic reactions were carried out under an inert atmosphere of argon unless otherwise specified and all chemical reagents were purchased from commercial sources (at ≥ 95% purity) and used without further purification. Analytical thin layer chromatography (TLC) was performed using 250 μm Silica Gel 60 F254 pre-coated plates (EMD Chemicals Inc.). Flash column chromatography was performed using 230-400 Mesh 60Å Silica Gel from Sorbent Technologies. All melting points were obtained, uncorrected, using a Mel-Temp capillary melting point apparatus from Laboratory Services, Inc.

NMR experiments were recorded using broadband probes on a Bruker Avance II (600 MHz for ^{1}H NMR; 151 MHz for ^{13}C NMR). All spectra are presented using MestReNova 11.0 (Mnova) software and are displayed without the use of the signal suppression function. Spectra were obtained in the following solvents (reference peaks also included for ^{1}H and ^{13}C NMRs): $CDCl_3$ (^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77.23 ppm), and d_6-DMSO (^{1}H NMR: 2.50 ppm; ^{13}C NMR: 39.52 ppm). All NMR experiments were performed at room temperature. Chemical shift values (δ) are reported in parts per million (ppm) for ^{1}H NMR and ^{13}C NMR spectra. ^{1}H NMR multiplicities are reported as: s = singlet, d = doublet, q = quartet, m = multiplet. High-Resolution Mass Spectrometry (HRMS) data were obtained from the Chemistry Department at the University of Florida.

Nitroreductase (NTR) from *Escherichia coli* was ordered from Sigma-Aldrich (Product number: N9284) and stored at -20 °C. The NTR was received as solid and freshly dissolved in Tris buffer (pH 7.4) to reach a final concentration of 1 mg/mL before use. Bacterial strains used during these investigations include: methicillin-resistant *Staphylococcus aureus* (ATCC strains: BAA-1707, BAA-44; Clinical Isolates from Shands Hospital in Gainesville, FL: MRSA-1, MRSA-2, *S. aureus* 129, *S. aureus* 138, *S. aureus* 147, *S. aureus* 156), methicillin-sensitive *Staphylococcus epidermidis* (ATCC 12228), methicillin-resistant *Staphylococcus epidermidis* (MRSE, ATCC strain 35984; Clinical Isolate CL-1 from Shands Hospital), *Enterococcus faecalis* (OG1RF), vancomycin-resistant *Enterococcus faecium* (VRE, ATCC strain 700221), and *Streptococcus pneumoniae* (ATCC 6303). Compounds were dissolved in DMSO and aliquots of prodrug solutions were frozen at -80 °C until needed for biological study.

4.) Synthetic Procedures and Characterization Data.



5 6 22 7

Step 1. A solution of 6,8-bis(trifluoromethyl)aniline **5** (345 µL, 2.75 mmol) dissolved in 1 mL tetrahydrofuran was added to a stirring solution of potassium *tert*-butoxide (721 mg, 7.50 mmol) in tetrahydrofuran (12 mL) at a temperature of -78°C. Next, a solution of 3-nitroanisole **6** (421 mg, 2.75 mmol) dissolved in 2 mL tetrahydrofuran was added to the reaction mixture which was then stirred at -78 °C until **5** was consumed (monitored by TLC, reaction time was 4 hours). Upon completion, the reaction mixture was then transferred to a separatory funnel containing brine and the crude product was extracted with ethyl acetate (3 × 30 mL). The resulting organic layer was then dried with sodium sulfate, filtered and concentrated *in vacuo* resulting in crude nitroso intermediate **22**.

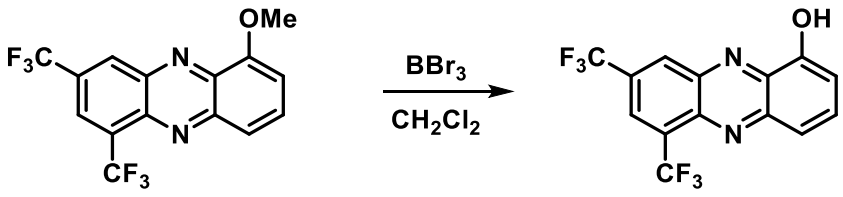
Step 2. The nitroso intermediate **22** was taken to the next step without purification. Nitroso **22** was dissolved in *N*,*N*-dimethylformamide (10 mL) before *N,O*-bis(trimethylsilyl)acetamide (3 mL, 12.5 mmol) was added to the mixture and the reaction was allowed to stir at 40 °C for 19 hours. Upon completion, the reaction contents were transferred to a separated funnel containing brine (50 mL) and the crude product was extracted with ethyl acetate. The organic layer was then washed with water (3 x 50 mL), dried over sodium sulfate, filtered and concentrated *in vacuo*. The resulting material was purified via column chromatography using 5:1 to 3:1 hexanes:ethyl acetate to afford 315 mg of phenazine **7** (36% yield) as a yellow solid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.92 (s, 1H), 8.32 (s, 1H), 7.93 (dd, *J* = 8.8, 1.0 Hz, 1H), 7.88 (dd, *J* = 8.8, 7.5 Hz, 1H), 7.16 (dd, *J* = 7.5, 1.0 Hz, 1H), 4.20 (s, 3H).

^{13}C NMR (151 MHz, $CDCl_3$): δ 155.3, 145.2, 140.7, 140.5, 137.9, 133.3, 133.0 (q, *J* = 4.5 Hz), 130.1 (q, *J* = 34.0 Hz), 129.9 (q, *J* = 30.8 Hz), 125.0 (m), 123.3 (q, *J* = 272.7 Hz), 123.0 (q, *J* = 274.0 Hz), 122.3, 108.2, 56.9.

HRMS (ESI): calc. for $C_{15}H_9F_6N_2O$ $[M+H]^+$: 347.0614, found: 347.0603.

MP: 148 - 150 °C.



7 23

Procedure. To a round bottom flask, **7** (120 mg, 0.347 mmol) was dissolved in anhydrous dichloromethane (25 mL) and cooled to -78 °C before the dropwise addition of 1M boron tribromide solution (2.48 mL, 2.48 mmol in

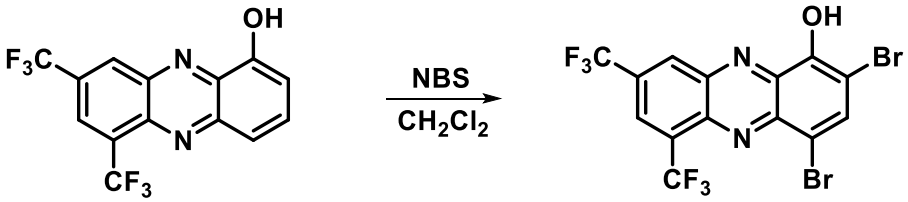
dichloromethane). The reaction was then allowed to stir at -78 °C for 10 minutes, then heated to reflux until complete (monitored by TLC; reaction time was 23 hours). Upon completion of the reaction, brine (50 mL) was added to quench and the contents were transferred to a separated funnel and extracted with dichloromethane. The resulting organic layers were then combined, dried with sodium sulfate, filtered and concentrated *in vacuo*. The resulting solid was purified via column chromatography using 1:1 hexane:dichloromethane to afford 109 mg of compound **23** as a yellow solid (95% yield).

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.76 (s, 1H), 8.35 (s, 1H), 8.02 (s, 1H), 7.95 - 7.91 (m, 2H), 7.36 (dd, *J* = 6.1, 2.5 Hz, 1H).

^{13}C NMR (151 MHz, $CDCl_3$): δ 151.8, 144.8, 141.2, 139.5, 135.6, 134.6, 131.9 (q, *J* = 4.5 Hz), 130.5 (q, *J* = 34.0 Hz), 130.4 (q, *J* = 31.1 Hz), 125.0 (m), 123.2 (q, *J* = 271.8 Hz), 123.0 (q, *J* = 274.3 Hz), 121.1, 111.1.

HRMS (ESI): calc. $C_{14}H_7F_6N_2O$ for $[M+H]^+$: 333.0457, found: 333.0447.

MP: 176 - 178 °C.



23 **6,8-CF_3-HP (8)**

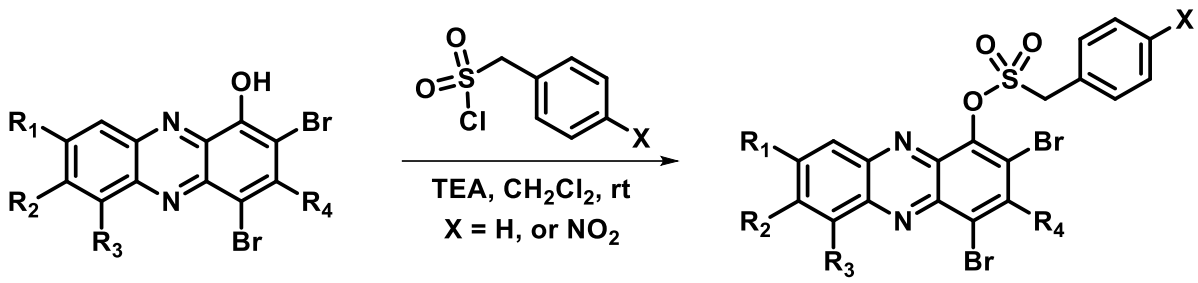
Procedure. Compound **23** (137 mg, 0.41 mmol) and *N*-bromosuccinimide (165 mg, 0.93 mmol) were dissolved in dichloromethane (8 mL) and allowed to stir at room temperature for 3 hours. Upon completion, the reaction was quenched upon adding a solution of 10% sodium thiosulfate (20 mL) and then extracted with dichloromethane. The extracts were then dried with sodium sulfate, filtered, and concentrated *in vacuo*. Finally, the crude material was purified via column chromatography using 5:1 to 4:1 hexane:ethyl acetate elute 197 mg of **6,8-CF_3-HP** (98% yield) as a yellow solid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.80 (s, 1H), 8.41 (s, 1H), 8.38 (s, 1H), 8.29 (s, 1H).

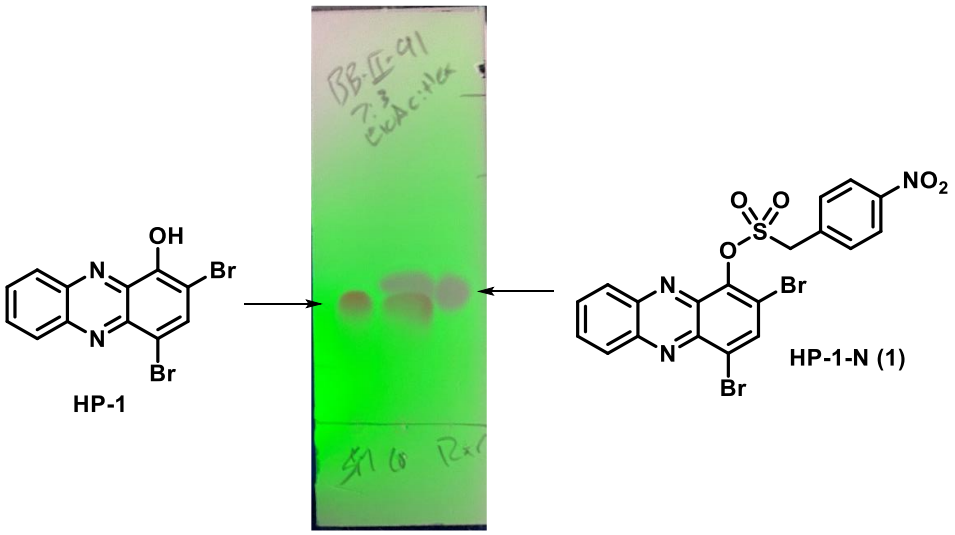
^{13}C NMR (151 MHz, $CDCl_3$): δ 149.0, 140.9, 140.7, 140.0, 139.9, 135.1, 132.2 (q, *J* = 34.3 Hz), 131.3 (q, *J* = 4.4 Hz), 130.9 (q, *J* = 31.8 Hz), 125.8 (m), 122.9 (q, *J* = 273.4 Hz), 122.6 (q, *J* = 274.5 Hz), 114.1, 106.0.

HRMS (ESI): calc. $C_{14}H_3Br_2F_6N_2O$ for $[M-H]^-$: 488.8502, found: 488.8524.

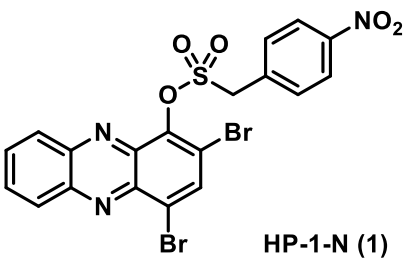
MP: 177 - 179 °C.



General Procedure for Halogenated Phenazine Sulfonate Esters. HP-1 (45 mg, 0.13 mmol) was added to a flame-dried round-bottom flask and dissolved in dry dichloromethane (5 mL). Next, triethylamine (18 µL, 0.13 mmol) was added drop-wise to the solution followed by the addition of (4-Nitrophenyl)methanesulfonyl chloride (31 mg, 0.13 mmol) in one portion. The reaction was then stirred at room temperature for 1 hour. Upon completion (by TLC analysis), the reaction was quenched with brine and the reaction contents were transferred to a separatory funnel and extracted with dichloromethane. The resulting organic layers were collected, dried with sodium sulfate, filtered and concentrated via rotavap. The crude product was purified using flash column chromatography using a gradient of 100% hexanes to 1:1 hexanes:ethyl acetate to afford **HP-1-N** (63.2 mg, 89%).



Note: TLC image for the reaction above was taken by Dr. Beau Brummel.



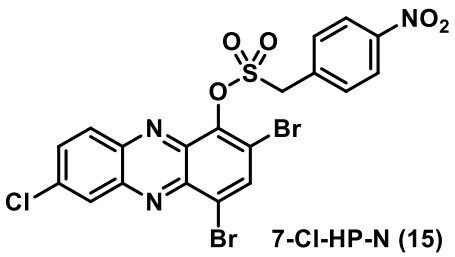
Yield: 89%; 63.2 mg of **HP-1-N** was isolated as a yellow solid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.45 (m, 1H), 8.42 (s, 1H), 8.32 (d, *J* = 8.8 Hz, 2H), 8.28 (m, 1H), 8.03 - 7.97 (m, 2H), 7.99 (d, *J* = 8.8 Hz, 2H), 5.51 (s, 2H).

^{13}C NMR (151 MHz, $CDCl_3$): δ 148.8, 144.0, 143.8, 143.4, 140.6, 138.2, 136.3, 135.2, 133.4, 132.5, 132.5, 130.7, 128.9, 124.4, 124.0, 120.6, 58.7.

HRMS (ESI): Cal. for $C_{19}H_{12}Br_2N_3O_5S$ $[M+H]^+$: 553.8839, found: 553.8829.

MP: 210 - 212 °C.



7-Cl-HP-N (15)

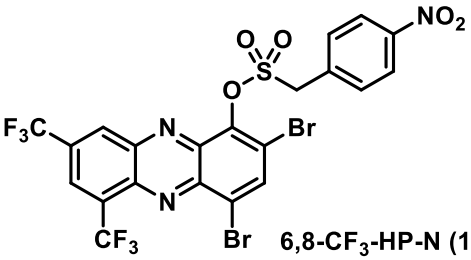
Yield: 90%; 166 mg of **7-Cl-HP-N** was isolated as a yellow solid.

^{1}H NMR (600 MHz, *d*$_6$-DMSO): δ 8.81 (s, 1H), 8.55 (d, *J* = 9.3 Hz, 1H), 8.51 (d, *J* = 2.3 Hz, 1H), 8.33 (d, *J* = 8.8 Hz, 2H), 8.14 (dd, *J* = 9.3, 2.3 Hz, 1H), 8.00 (d, *J* = 8.8 Hz, 2H), 5.78 (s, 2H).

^{13}C NMR (151 MHz, *d*$_6$-DMSO): δ 147.9, 143.2, 143.0, 141.5, 140.0, 137.8, 137.6, 136.6, 136.0, 134.3, 132.7, 131.3, 127.8, 123.8, 122.9, 120.4, 57.7.

HRMS (ESI): Cal. $C_{19}H_{11}Br_2ClN_3O_5S$ for $[M+H]^+$: 587.8448, found: 587.8438.

MP: 203 - 205 ºC.



6,8-CF_3-HP-N (16)

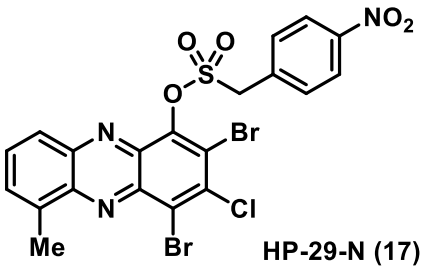
Yield: 98%; 109.2 mg of **6,8-CF_3-HP-N** was isolated as a red solid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.75 (s, 1H), 8.56 (s, 1H), 8.46 (s, 1H), 8.33 (d, *J* = 8.8 Hz, 2H), 7.89 (d, *J* = 8.8 Hz, 2H), 5.33 (s, 2H).

^{13}C NMR (151 MHz, $CDCl_3$): δ 148.9, 143.5, 141.8, 141.3, 140.5, 139.2, 138.6, 134.4, 133.2 (q, *J* = 34.4 Hz), 132.4, 131.4 (q, *J* = 4.3 Hz), 131.2 (q, *J* = 32.1 Hz), 126.4 (m), 124.9, 124.5, 122.8 (q, *J* = 272.9 Hz), 122.6, 122.5 (q, *J* = 274.6 Hz), 59.3.

HRMS (ESI): Cal. for $C_{21}H_{10}Br_2F_6N_3O_5S$ $[M+H]^+$: 689.8587, found: 689.8595.

MP: 199 - 201 ºC.



HP-29-N (17)

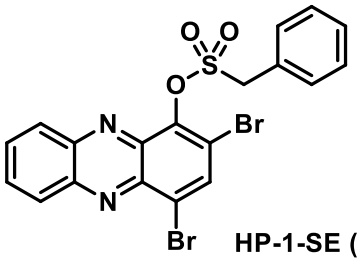
Yield: 97%; 101.7 mg of **HP-29-N** was isolated as a yellow solid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.32 (d, *J* = 8.8 Hz, 2H), 8.09 (d, *J* = 8.7 Hz, 1H), 7.98 (d, *J* = 8.8 Hz, 2H), 7.89 (dd, *J* = 8.7, 6.8 Hz, 1H), 7.84 (complex m, 1H), 5.54 (s, 2H), 3.01 (s, 3H).

^{13}C NMR (151 MHz, $CDCl_3$): δ 148.8, 144.4, 144.1, 143.5, 139.6, 139.6, 137.2, 135.9, 135.2, 133.6, 132.5, 131.9, 126.4, 125.5, 124.4, 123.3, 58.8, 17.6.

HRMS (ESI): Cal. $C_{20}H_{13}Br_2ClN_3O_5S$ for $[M+H]^+$: 601.8605, found: 601.8603.

MP: 185 - 187 ºC.



HP-1-SE (18)

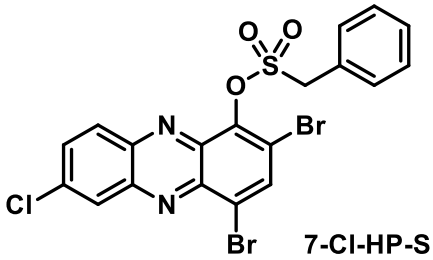
Yield: 99%; 51.5 mg of **HP-1-SE** was isolated as a yellow solid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.43 (m, 1H), 8.41 (s, 1H), 8.31 (m, 1H), 8.02 - 7.95 (m, 2H), 7.76 - 7.71 (m, 2H), 7.47 - 7.42 (m, 3H), 5.41 (s, 2H).

^{13}C NMR (151 MHz, $CDCl_3$): δ 144.2, 143.9, 143.5, 140.5, 138.5, 136.3, 133.1, 132.4, 131.4, 130.6, 129.6, 129.3, 129.2, 128.0, 123.6, 120.3, 59.7.

HRMS (DART): Cal. for $C_{19}H_{13}Br_2N_2O_3S$ $[M+H]^+$: 508.8988, found: 508.9013.

MP: 204 - 206 ºC.



7-Cl-HP-SE (19)

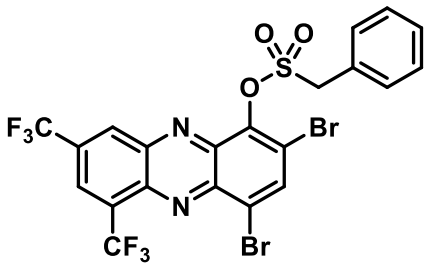
Yield: 97%; 20.3 mg of **7-Cl-HP-SE** was isolated as a yellow solid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.44 (d, *J* = 2.2 Hz, 1H), 8.42 (s, 1H), 8.25 (d, *J* = 9.3 Hz, 1H), 7.89 (dd, *J* = 9.3, 2.2 Hz, 1H), 7.72 - 7.68 (m, 2H), 7.46 - 7.42 (m, 3H), 5.34 (s, 2H).

^{13}C NMR (151 MHz, $CDCl_3$): δ 144.1, 143.7, 142.0, 140.9, 138.8, 138.4, 137.0, 134.6, 131.4, 130.4, 129.7, 129.3, 128.9, 127.8, 123.5, 120.5, 59.9.

HRMS (ESI): Cal. for $C_{19}H_{12}Br_2ClN_2O_3S$ $[M+H]^+$: 542.8597, found: 542.8598.

MP: 192 - 194 ºC.



6,8-CF_3-HP-SE (20)

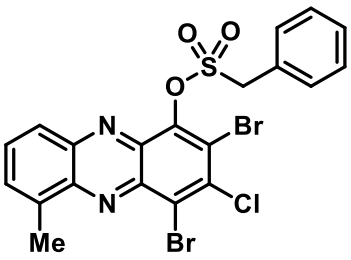
Yield: 100%; 39.7 mg of **6,8-CF_3-HP-SE** was isolated as a yellow solid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.77 (s, 1H), 8.54 (s, 1H), 8.44 (s, 1H), 7.68 - 7.64 (m, 2H), 7.48 - 7.45 (m, 3H), 5.23 (s, 2H).

^{13}C NMR (151 MHz, $CDCl_3$): δ 143.8, 141.9, 141.2, 140.4, 139.5, 138.6, 132.9 (q, *J* = 34.3 Hz), 131.7 (q, *J* = 4.3 Hz), 131.3, 131.0, 129.8, 129.4, 127.3, 126.3 (m), 124.4, 122.8 (q, *J* = 273.2 Hz), 122.6 (q, *J* = 274.8 Hz), 122.4, 60.2.

HRMS (ESI): Cal. for $C_{21}H_{11}Br_2F_6N_2O_3S$ $[M+H]^+$: 644.8736, found: 644.8758.

MP: 169 - 171 ºC.



HP-29-SE (21)

Yield: 98%; 40.5 mg of **HP-29-SE** was isolated as a yellow solid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.13 (complex m, 1H), 7.86 (dd, *J* = 8.6, 6.8 Hz, 1H), 7.81 (complex m, 1H), 7.75 - 7.71 (m, 2H), 7.46 - 7.42 (m, 3H), 5.44 (s, 2H), 3.00 (s, 3H).

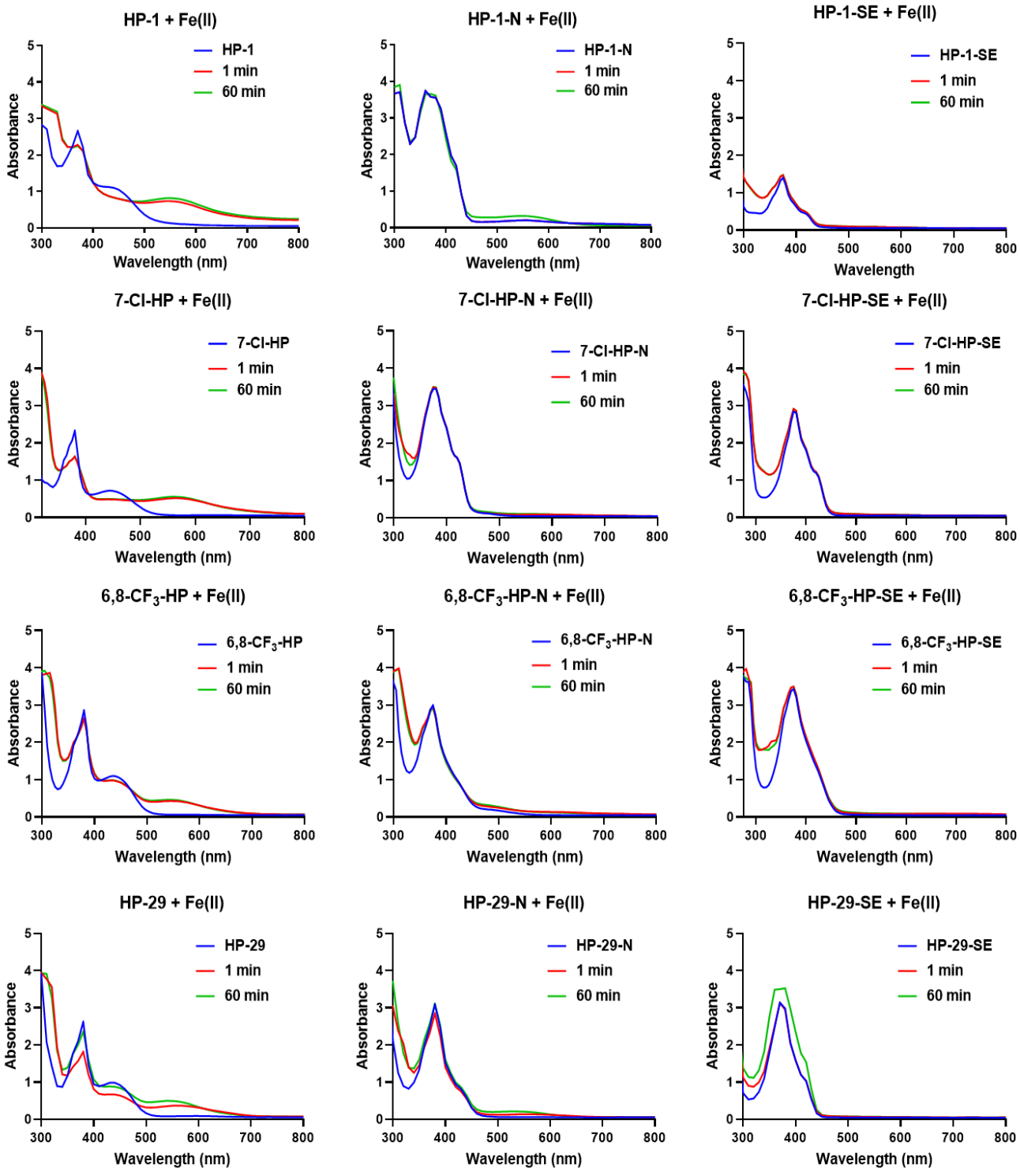
^{13}C NMR (151 MHz, $CDCl_3$): δ 144.8, 144.0, 143.5, 139.6, 139.4, 137.2, 136.2, 133.2, 131.8, 131.4, 129.6, 129.3, 127.9, 126.8, 125.0, 123.0, 59.9, 17.6.

HRMS (ESI): Cal. for $C_{20}H_{14}Br_2ClN_2O_3S$ $[M+H]^+$: 556.8754, found: 556.8736.

MP: 203 - 205 ºC.

5.) UV-Vis Spectroscopy to Determine Iron(II)-Binding.

The formation of phenazine-iron(II) complex were independently evaluated via UV-vis spectrometry. Initially, 950 µL of dimethyl sulfoxide and 50 µL of test compound (10 mM DMSO stock) were added into a 1.5 mL cuvette. In a separate cuvette, 925 µL of dimethyl sulfoxide, 50 µL of test compound (10 mM DMSO stock), and 25 µL of ammonium iron(II) sulfate hexahydrate (10 mM solution in water) were added. Following this, the contents were thoroughly mixed for 30 seconds and spectral scanning was performed from 300 to 800 nm in 2 nm increments after 1 and 60 minutes. Experimental results were plotted using GraphPad Prism.



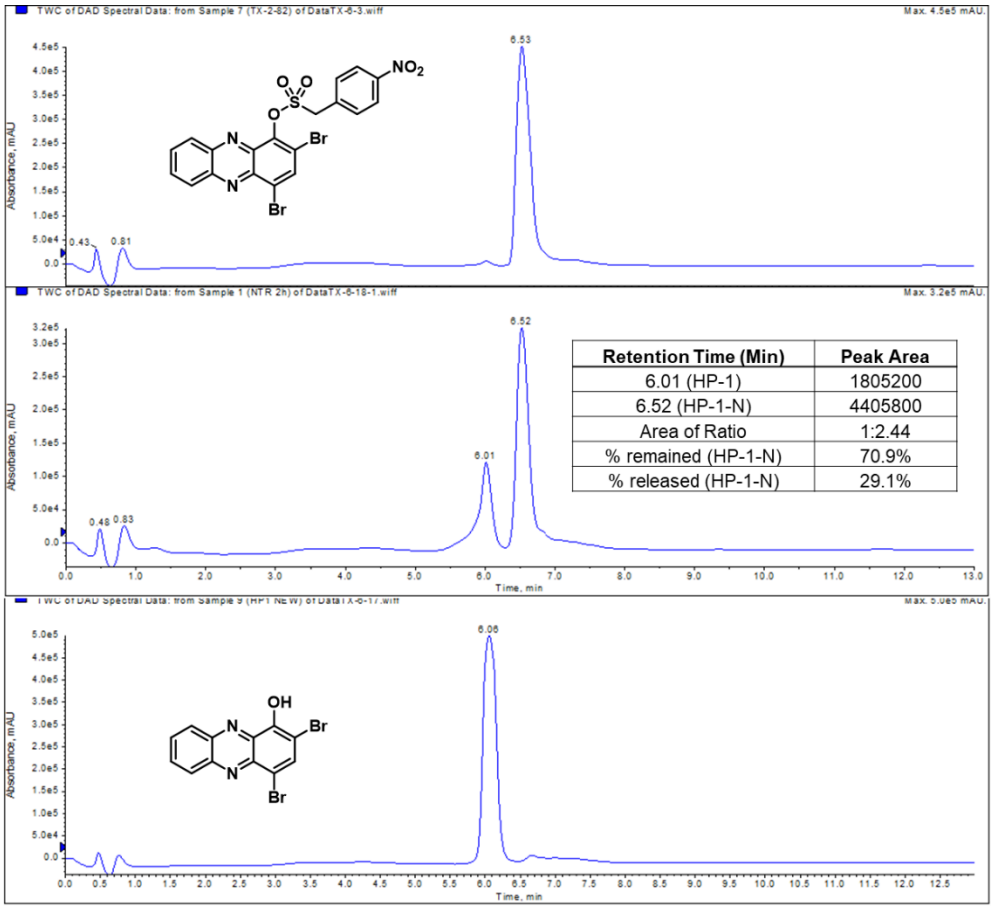
6.) Nitroreductase (NTR) Release Assay.

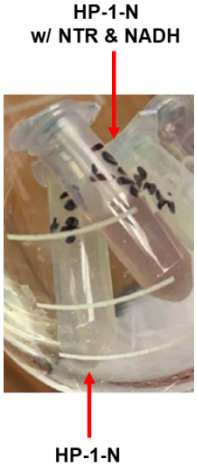
HP-1-N was dissolved in DMSO (stored as 10 mM stock solution). NADH and NTR were freshly dissolved in Tris buffer (pH 7.4) to reach final concentrations of 100 mM and 1 mg/mL, respectively. The reaction mixture was prepared in 2 mL Eppendorf tubes according to the table below. After incubation at 37 °C for 2 or 16 hours, each group was diluted with ethyl acetate (1 mL) and washed with brine (3 x 1 mL). The organic layer was collected and concentrated. The resulting residue was then dissolved in LC-MS grade acetonitrile (1 mL), filtered and ready for LC-MS analysis.

Group	10 mM **HP-1-N**	100 mM NADH	1 mg/mL NTR	Tris Buffer	Total Volume
w/ NTR	100 µL	100 µL	100 µL	700 µL	1000 µL
w/o NTR	100 µL	0	0	900 µL	1000 µL

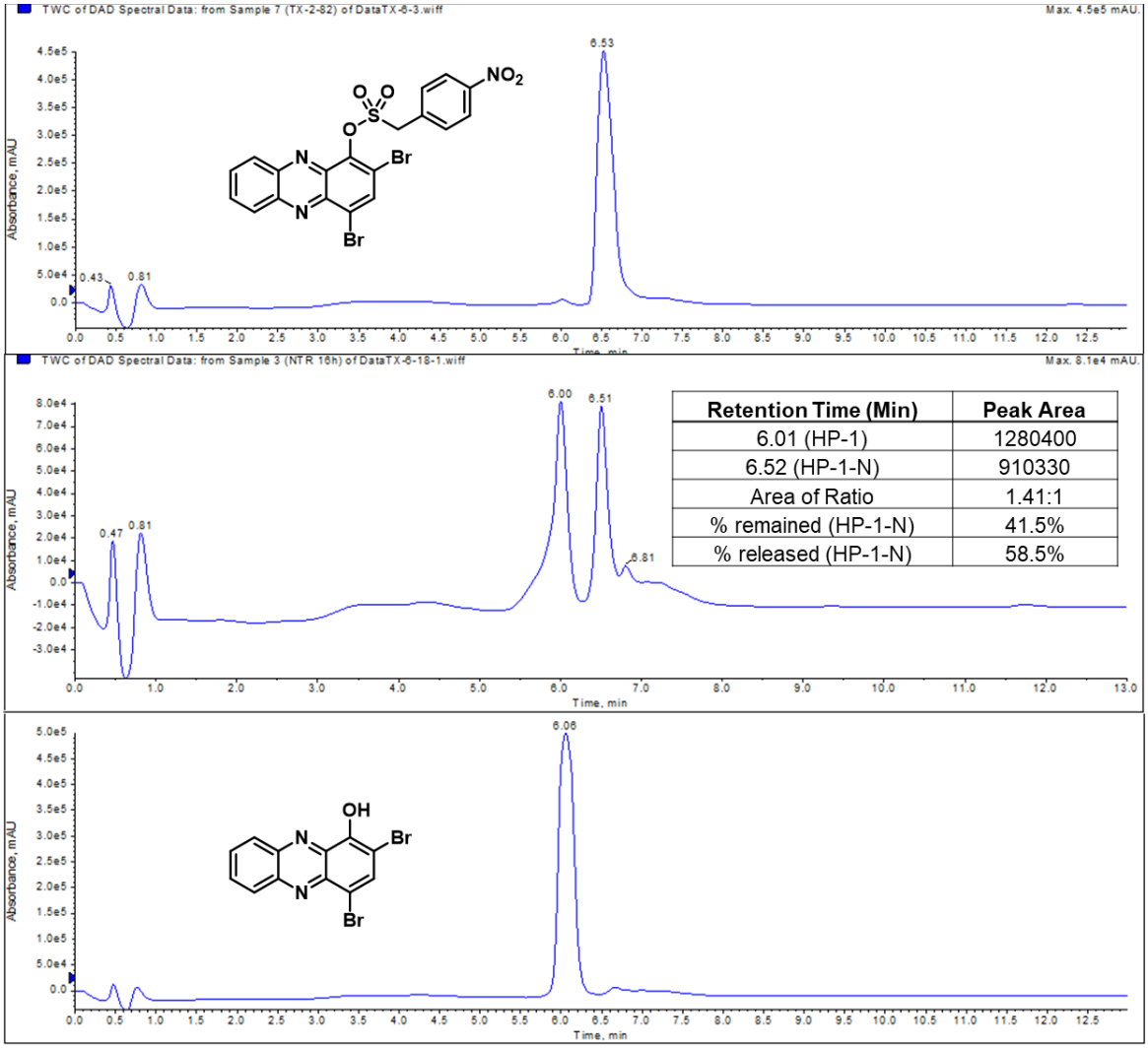
The LC-MS experiment was carried out over 13 minutes, using a Shimadzu Prominence HPLC system, AB Sciex 3200 QTRAP spectrometer and a Kinetex EVO C18 column (30 mm × 2.1 mm × 2.6 µm) with a gradient from 10% to 95% acetonitrile in 0.1% formic acid at a flow rate of 0.3 mL/min. The LC-MS spectrum of select experiments are shown below. Note: This protocol was used for all NTR-promoted release assays reported in this study.

(A) **HP-1-N** after 2 hour treatment with NTR and NADH.



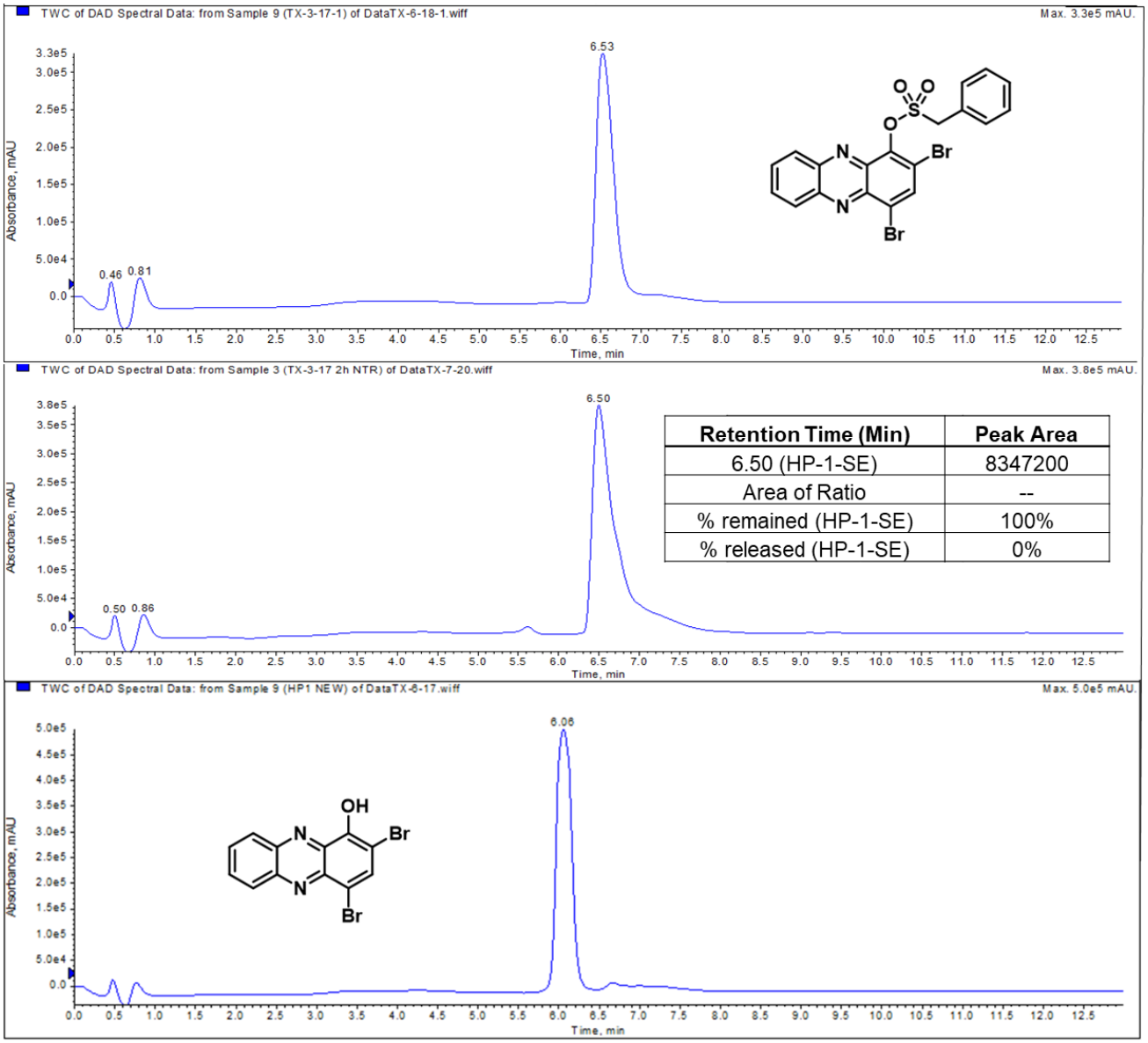


(B) **HP-1-N** after 16 hour treatment with NTR and NADH.



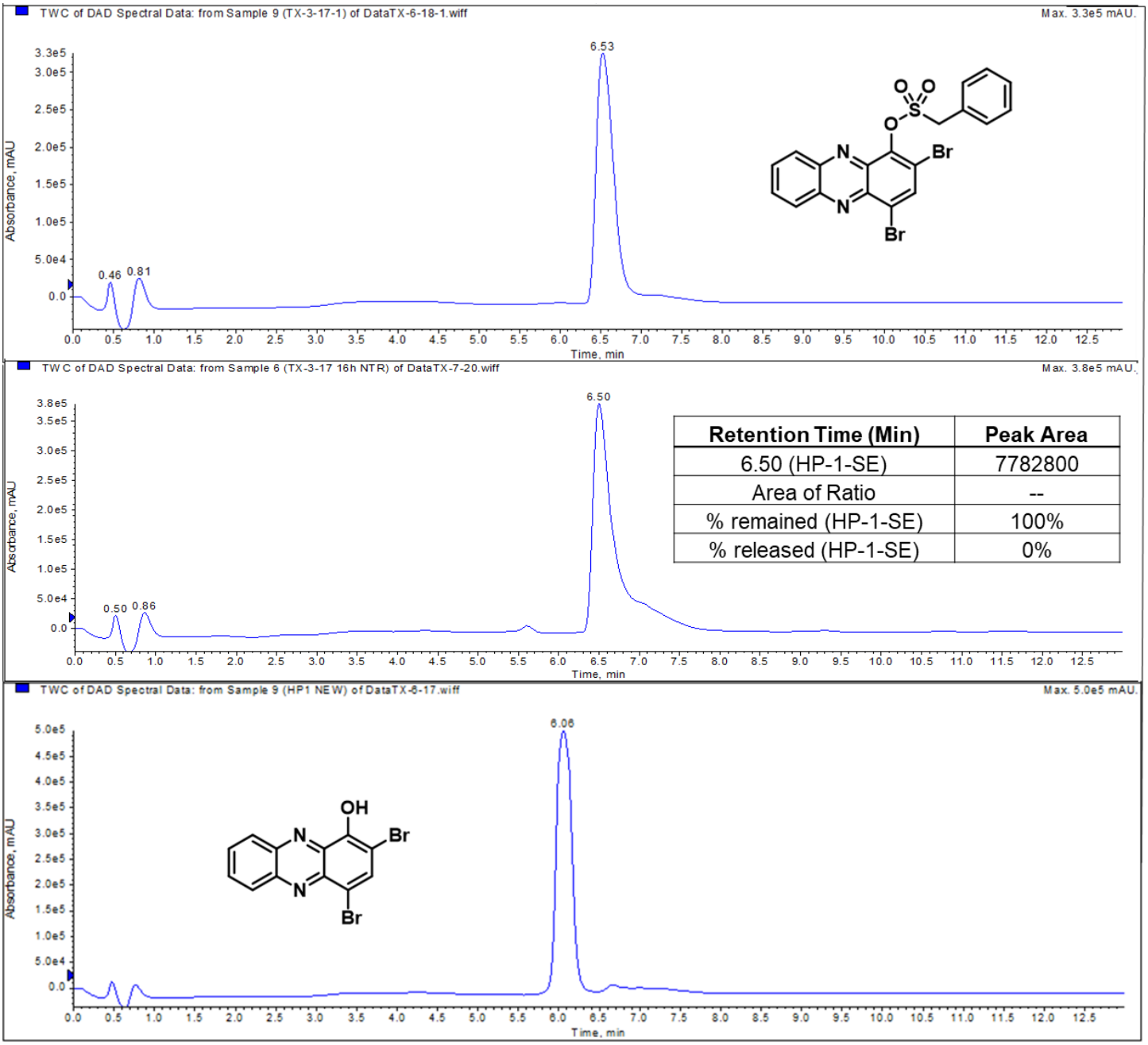
Retention Time (Min)	Peak Area
6.01 (HP-1)	1280400
6.52 (HP-1-N)	910330
Area of Ratio	1.41:1
% remained (HP-1-N)	41.5%
% released (HP-1-N)	58.5%

(C) **HP-1-SE** after 2 hour treatment with NTR and NADH.



Retention Time (Min)	Peak Area
6.50 (HP-1-SE)	8347200
Area of Ratio	--
% remained (HP-1-SE)	100%
% released (HP-1-SE)	0%

(D) **HP-1-SE** after 16 hour treatment with NTR and NADH.



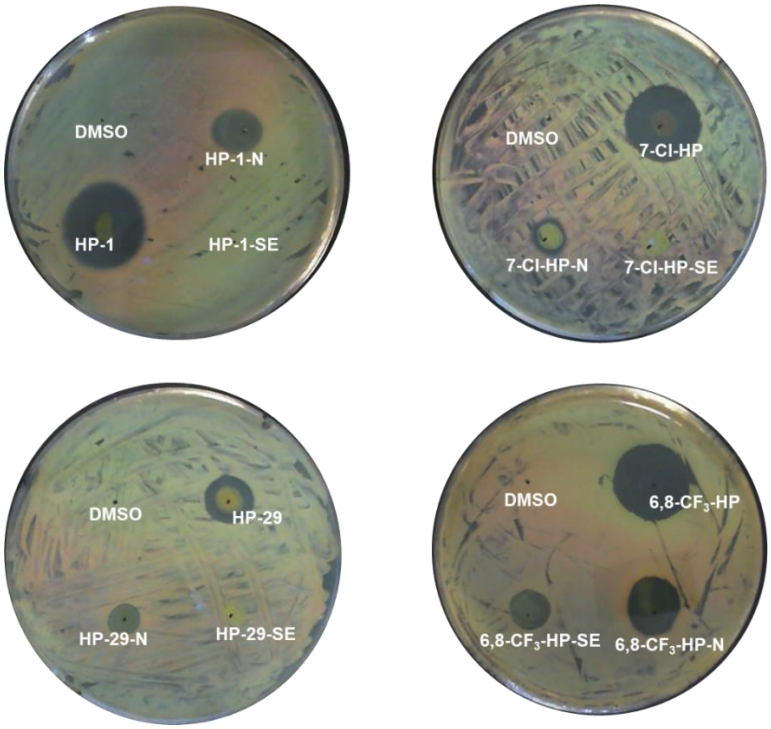
Retention Time (Min)	Peak Area
6.50 (HP-1-SE)	7782800
Area of Ratio	--
% remained (HP-1-SE)	100%
% released (HP-1-SE)	0%

7.) Antibacterial Assays.

Agar Diffusion Assay.

Agar diffusion assays for prodrug evaluation were performed according to the standard Kirby-Bauer disk diffusion susceptibility test protocol with some minor modifications. First, 100 µL of fresh MRSA 1707 inoculum (OD_{600} = 0.7, ~10^8 CFU) was spread on lysogeny broth (LB) agar plates. The plates were allowed to sit for 30 minutes before 10 µL of test compound solution (10 mM in DMSO) was gently pipetted onto the plate. The plates were incubated at 37°C for 16 hours to allow for bacterial growth. After this time, zones of inhibition were determined for test analogues. All zones of inhibition data are reported from three independent experiments.

Agar Diffusion Assay of Test Analogues against MRSA 1707:



Compound	Zone of Inhibition (cm^2)
HP-1	5.08 ± 0.52
HP-1-N	1.13 ± 0.24
HP-1-SE	0
7-Cl-HP	1.70 ± 0.07
7-Cl-HP-N	0.66 ± 0.07
7-Cl-HP-SE	0
6,8-CF_3-HP	4.58 ± 0.17
6,8-CF_3-HP-N	2.57 ± 0.19
6,8-CF_3-HP-SE	1.07 ± 0.08
HP-29	3.85 ± 0.18
HP-29-N	0.87 ± 0.09
HP-29-SE	0

Minimum Inhibitory Concentration (MIC) Susceptibility Assay.

The minimum inhibitory concentration (MIC) for each test compound was determined by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI). In a 96-well plate, eleven two-fold serial dilutions of each compound were made in a final volume of 100 µL LB (brain-heart infusion was used for *Enterococcus*). Each well was inoculated with ~10^5 bacterial cells at the initial time of incubation, prepared from a fresh log phase culture (OD_{600} of 0.6 to 0.8). The MIC was defined as the lowest concentration of compound that prevented bacterial growth after incubating for 16 hours at 37 °C (MIC values were determined by spectrophotometric readings of turbidity at OD_{600}). The concentration range for each test compound was 0.10 to 100 µM. DMSO served as our vehicle and negative control in each MIC assay. DMSO was serially diluted with a top concentration of 0.5% v/v. All compounds were tested in a minimum of three independent experiments.

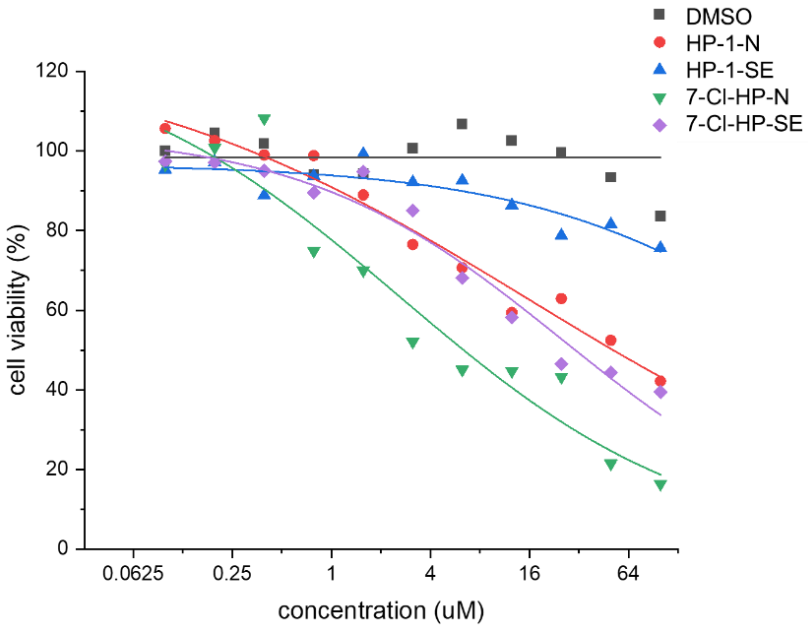
Supporting Table. The fold changes in MIC values when comparing the activity profiles of prodrugs with those of corresponding parents. All MIC results in this table are reported in micromolar (µM) concentrations.

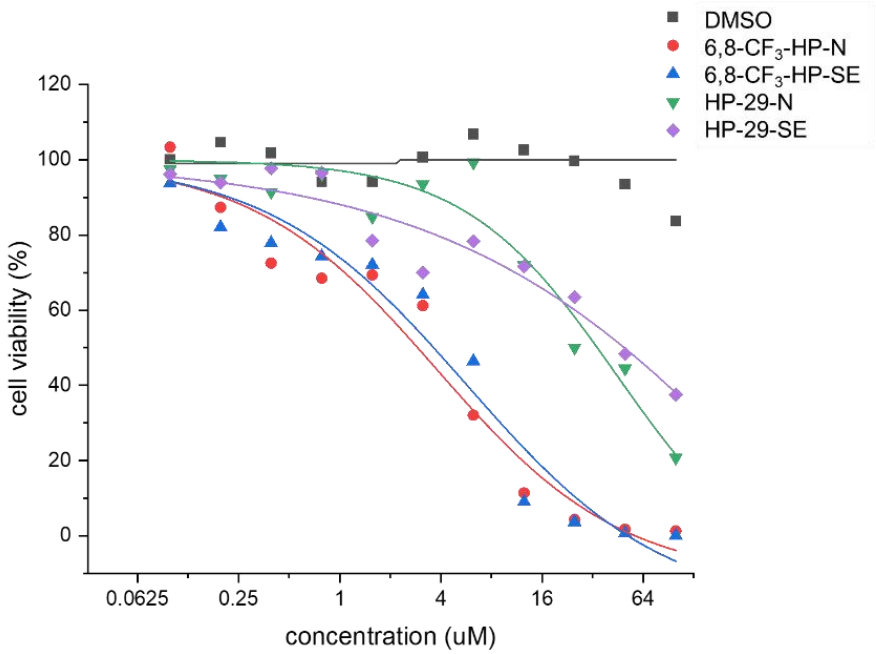
Compound	MRSA BAA-1707		MRSA BAA-44		MRSA 2		MRSE 35984		*S. epidermidis* 12228		VRE 700221	
	MIC	Fold*	MIC	Fold*	MIC	Fold*	MIC	Fold*	MIC	Fold*	MIC	Fold*
HP-1	1.17[a]	--	1.56	--	1.56	--	1.56	--	2.35[a]	--	4.69[a]	--
HP-1-N	0.78	-1.5	1.56	1.0	2.35[a]	1.5	4.69[a]	3.0	4.69[a]	2.0	9.38[a]	2.0
HP-1-SE	>100	>86	>100	>64	>100	>64	>100	>64	>100	>42	>100	>21
7-Cl-HP	0.075[a]	--	0.15[a]	--	0.15[a]	--	0.39	--	0.30[a]	--	0.39	--
7-Cl-HP-N	0.10[b]	1.3	0.15[a]	1.0	0.15[a]	-1.3	0.39	1.0	0.15[a]	-2.0	0.39	1.0
7-Cl-HP-SE	2.35[a]	31	1.17[a]	7.8	1.17[a]	7.8	4.69[a]	12	3.13	10.4	4.69[a]	12
6,8-CF_3-HP	0.15[a]	--	0.15[a]	--	0.78	--	0.05[c]	--	0.10[c]	--	0.20	--
6,8-CF_3-HP-N	1.56	10.4	4.69[a]	31.3	6.25	8	0.78	15.6	0.15[a]	1.5	1.56	7.8
6,8-CF_3-HP-SE	>100	>667	18.8[a]	125	>100	>128	1.56	31.2	0.15[a]	1.5	0.78	3.9
HP-29	0.10[b]	--	0.15[a]	--	0.15[a]	--	0.10[c]	--	0.10[c]	--	0.10[c]	--
HP-29-N	0.10[c]	1.0	0.30[a]	2.0	0.30[a]	2.0	0.15[a]	1.5	0.59[a]	5.9	0.15[a]	1.5
HP-29-SE	>100	>1000	>100	>667	>100	>667	>100	>1000	>100	>1000	>100	>1000

Note: [a]Midpoint value for 2-fold range in MIC values observed. [b]Lowest concentration tested. Each data point is the result of three independent experiments. *Fold change of MIC values compared to that of corresponding parent compound.

8.) Cytotoxicity Assessment against HEK-293 Cells.

MTT assay was performed to determine compound cytotoxicity against human embryonic kidney 293 (HEK-293) cells. HEK-293 cells (10^4 cells in 100 µL) were seeded onto a 96-well plate containing DMEM supplemented with 10% FBS, 100 U /mL penicillin and 100 µg/mL streptomycin. After overnight incubation at 37 °C in a humidified incubator under 5% CO_2, the medium was removed and cells were then treated with serial concentrations of compounds at 0, 0.10, 0.20, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µM. DMSO was used as negative control. After further incubation at 37 °C for 48 hours, 10 µL of MTT (5 mg/mL in PBS) was added to each well and incubated for 3 hours. Then the medium was aspirated and dimethyl sulfoxide (DMSO, 100 µL) was added to each well to dissolve the MTT and the plate was agitated for 30 minutes. After this time, the optical density (OD) was measured at 570 nm using a UV/vis microplate spectrophotometer (BioTek). Eight replications were performed per treatment and the data were analyzed by ORIGIN.

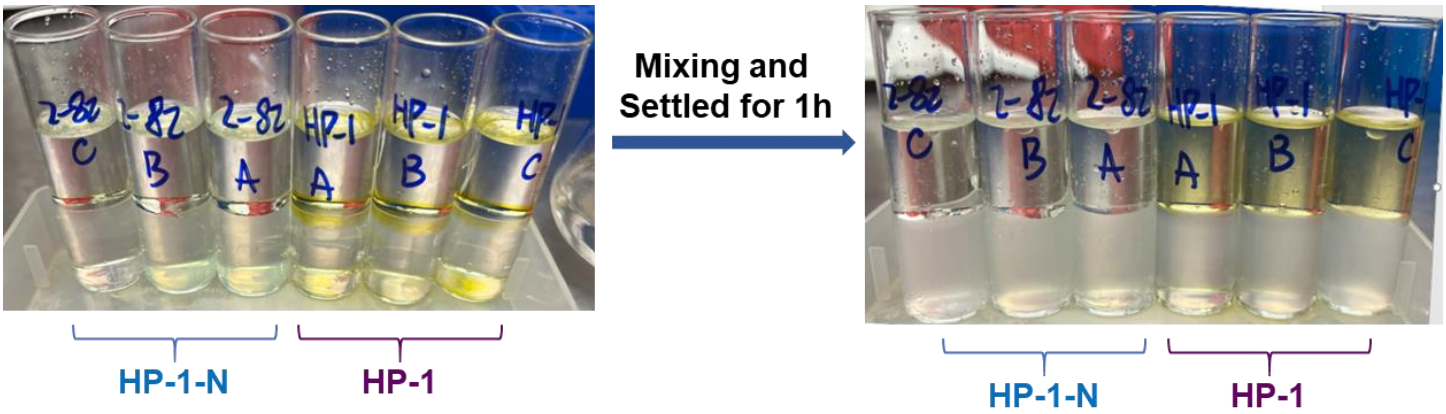




9.) Determination of LogP Values.

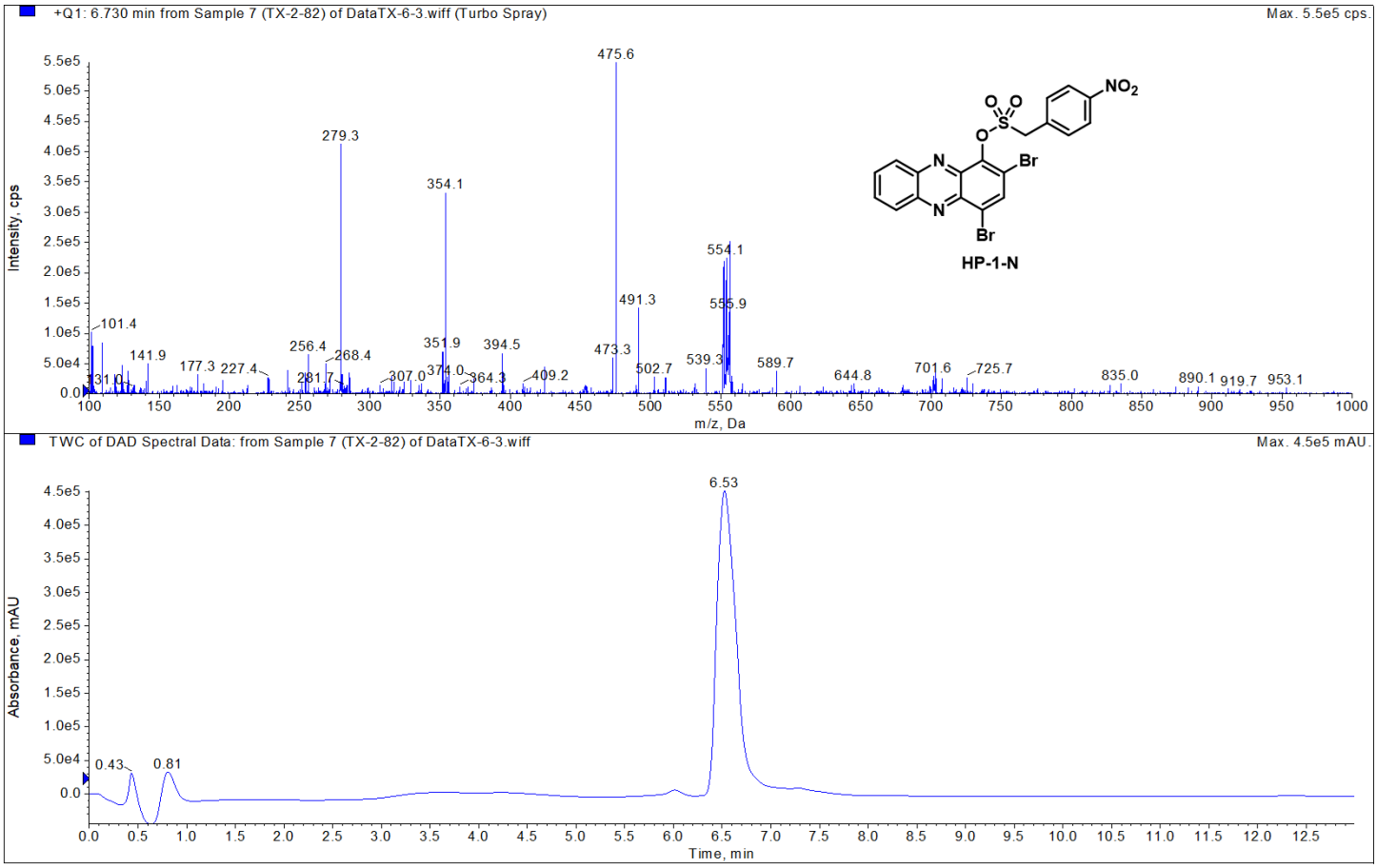
Determination of LogP Values. LogP values of select compounds were determined through the use of reported protocols with modifications. Compound solutions were prepared in octanol (1% v/v DMSO via 20 µL of 10mM DMSO stock solution transferred into 1.98 mL of octanol) with an initial concentration of 100 µM. The solution was then serially diluted 2-fold and the absorbance of each solution was measured by UV-Vis (HP-1 and HP-1-N: at 370 nm; HP-29 and HP-29-N: at 380 nm) to generate an absorbance-concentration standard curve. After this, 5 mL of water was added into 5 mL of octanol containing compound (1% v/v DMSO via 50 µL of 10mM DMSO stock solution transferred into 4.95 mL of octanol) and the resulting octanol-water mixture was vigorously stirred in a vial and allowed to stand for 1 h at room temperature to reach equilibration phase separation. The octanol phase was then measured by UV-Vis (HP-1 and HP-1-N: at 370 nm; HP-29 and HP-29-N: at 380 nm) to determine the concentration of compound in octanol (C_{oct}). The concentration of compound in water (C_w) was then calculated on the basis of mass balance. LogP was determined using the equation: $LogP = Log10(C_{oct}/C_w)$. Each LogP value reported below is the result of three independent experiments (standard error included). Note: The LogP of acetophenone was determined using this protocol and served as our positive control for these experiments (aligning with reported LogP = 1.66 and CLogP = 1.58 values). Acetophenone was evaluated using an initial concentration of 1000 mg/L in octanol (1% v/v DMSO) and absorbance was read at 306 nm. LogP values for select HP analogues and acetophenone are reported below.

Compound	LogP
HP-1	0.25 ± 0.09
HP-1-N	0.42 ± 0.18
HP-29	0.81 ± 0.16
HP-29-N	3.11 ± 0.27
Acetophenone	1.51 ± 0.16



10.) LC-MS Purity Analysis of Compounds.

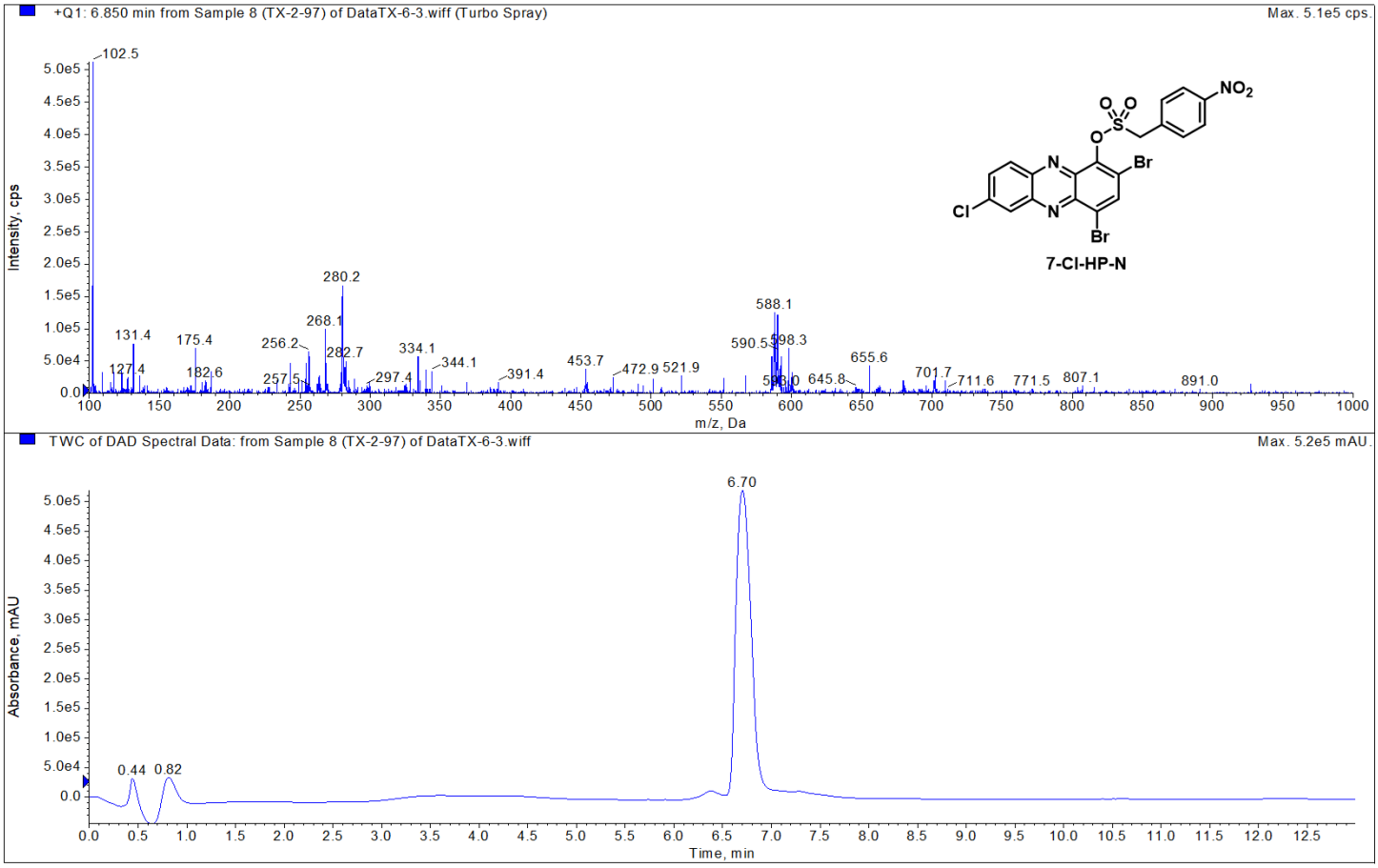
Each LC-MS experiment to determine test compound purity was carried out over 13 minutes, using a Shimadzu Prominence HPLC system, AB Sciex 3200 QTRAP spectrometer and a Kinetex EVO C18 column (30 mm × 2.1 mm × 2.6 µm) with a gradient from 10 to 95% acetonitrile in 0.1% formic acid at a flow rate of 0.3 mL/min. A blank acetonitrile run was used to remove background noise from the purity assessment for each compound. The purity range for these test compounds was determined to be 96.9 - 100% pure, based on LC-MS results.



LRMS (ESI): found for $C_{29}H_{28}Br_2N_5O_6$ $[M + H]^+$: 554.1.

Note: The (1:1) natural abundance of ^{79}Br:^{81}Br is observed in the mass spectrum.

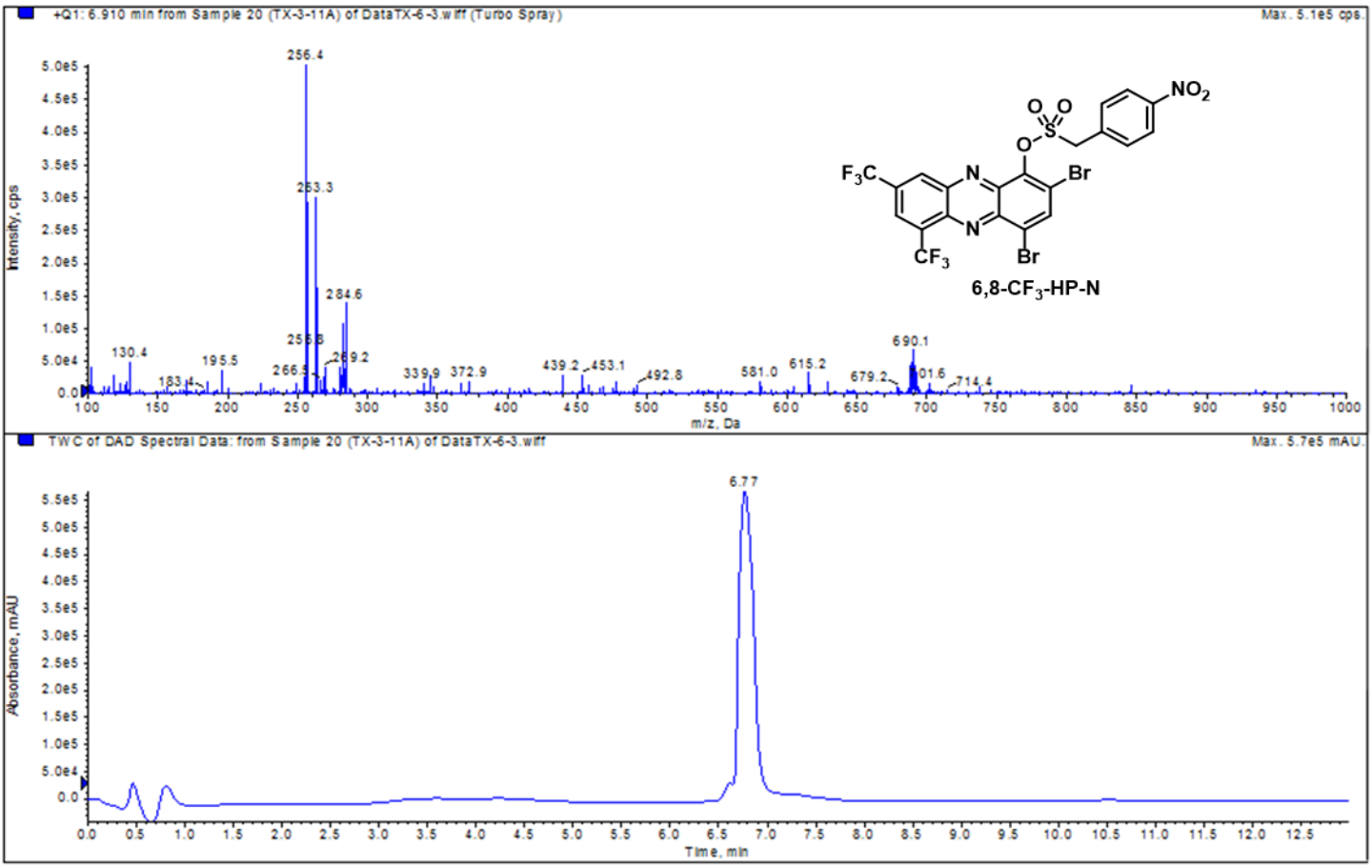
Retention Time (Min)	Peak Area
6.00	125450
6.53 **(compound)**	6460300
Sum of Area	6585750
% Purity	**98.1%**



LRMS (ESI): found for $C_{29}H_{28}Br_2N_5O_6$ $[M + H]^+$: 588.1.

Note: The (1:1) natural abundance of ^{79}Br:^{81}Br is observed in the mass spectrum.

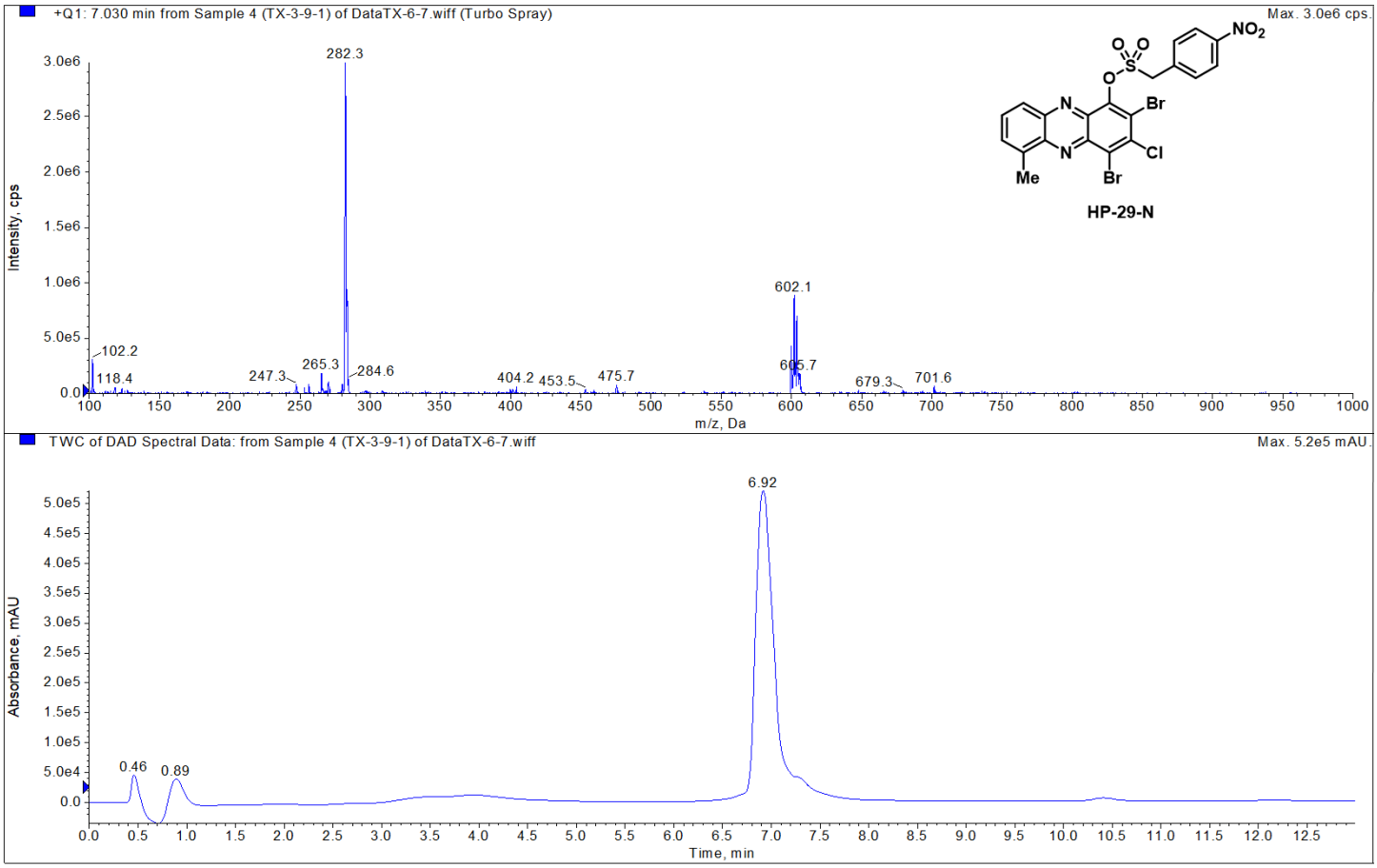
Retention Time (Min)	**Peak Area**
6.40	103420
6.70 **(compound)**	5696800
Sum of Area	5800220
% Purity	**98.2%**



LRMS (ESI): found for $C_{29}H_{28}Br_2N_5O_6$ $[M + H]^+$: 690.1.

Note: The (1:1) natural abundance of ^{79}Br:^{81}Br is observed in the mass spectrum.

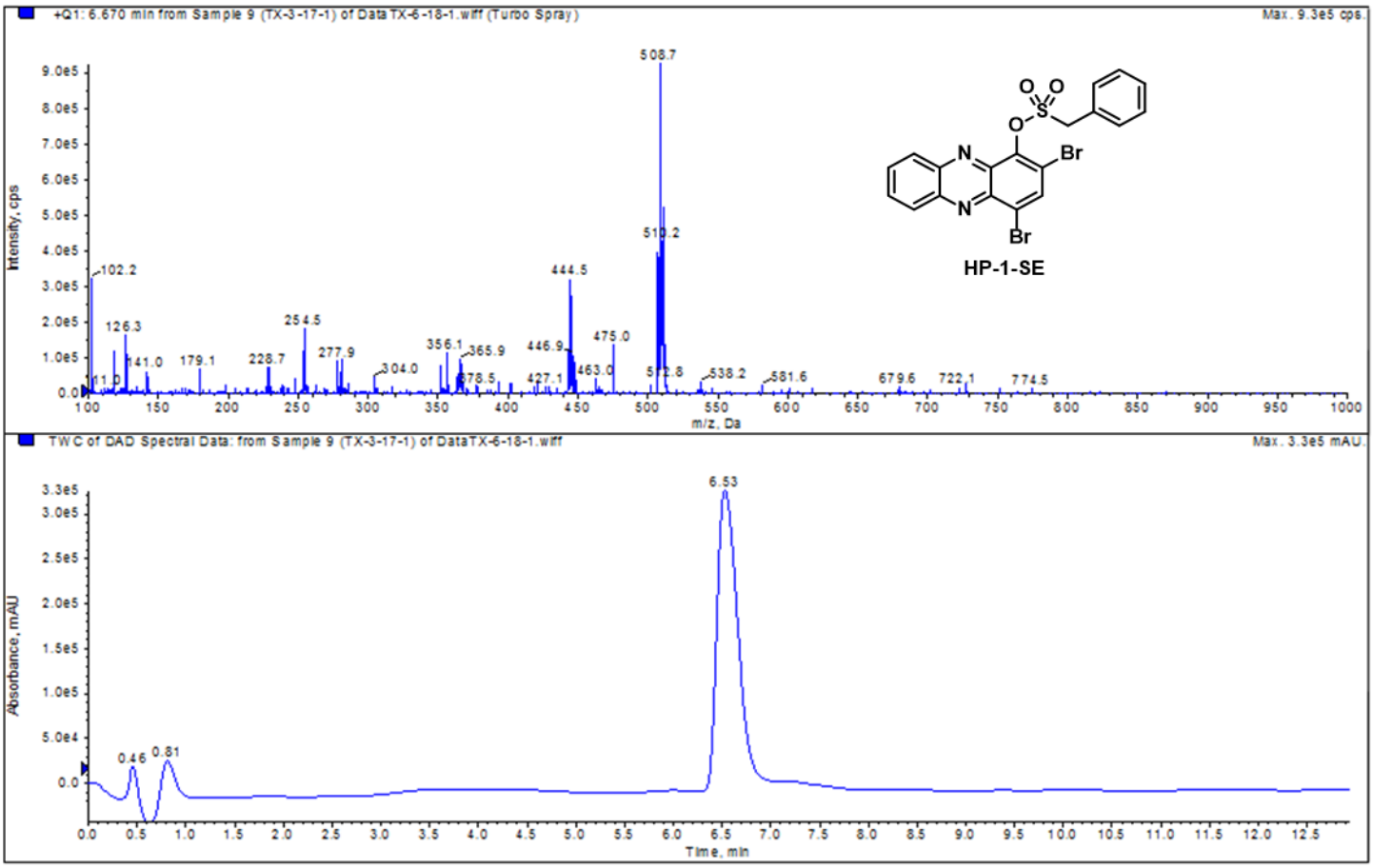
Retention Time (Min)	**Peak Area**
6.62	197560
6.77 **(compound)**	6137600
Sum of Area	6335160
% Purity	**96.9%**



LRMS (ESI): found for $C_{29}H_{28}Br_2N_5O_6$ $[M + H]^+$: 602.1.

Note: The (1:1) natural abundance of ^{79}Br:^{81}Br is observed in the mass spectrum.

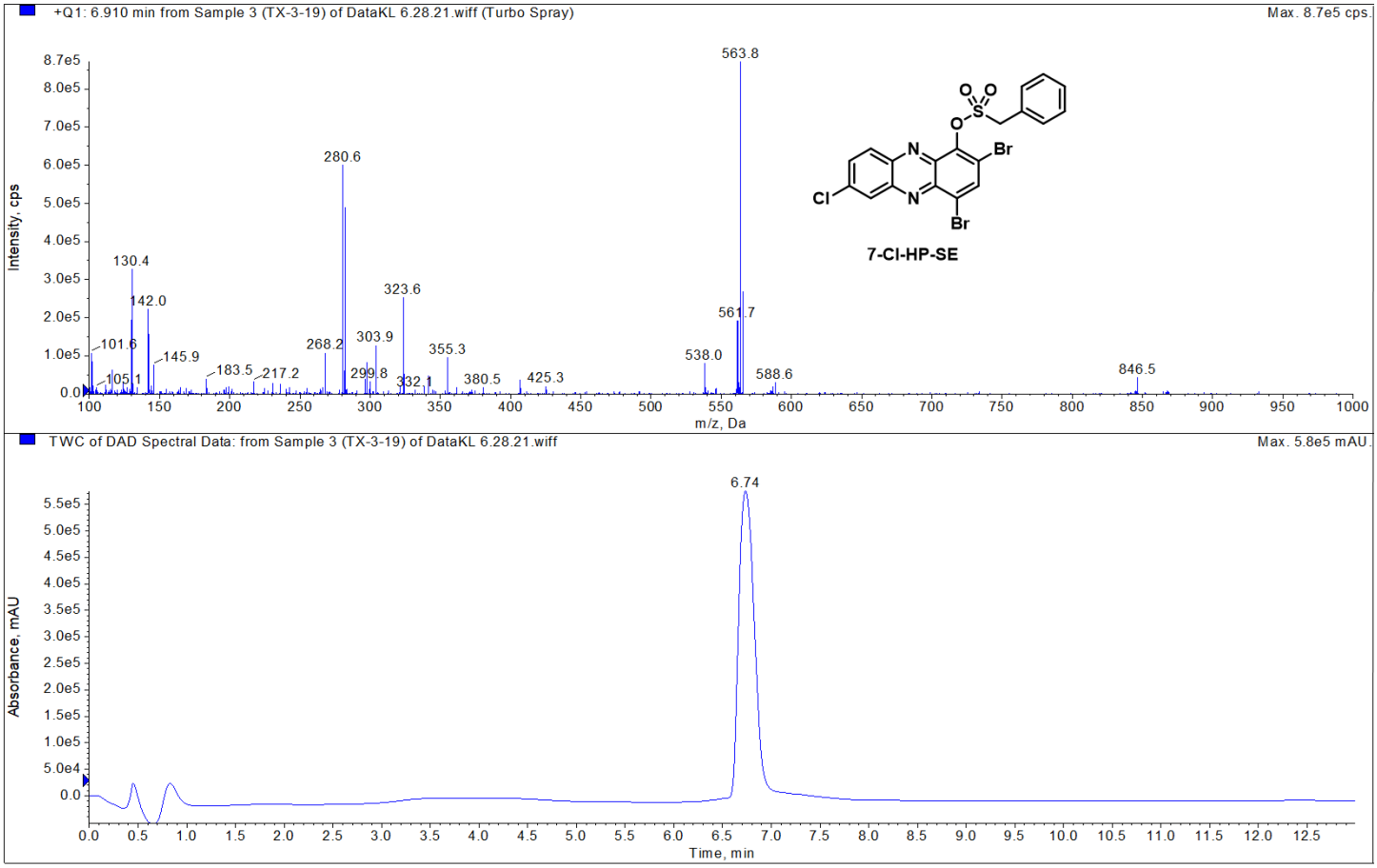
Retention Time (Min)	**Peak Area**
6.92	233610
7.30 (compound)	7316600
Sum of Area	7550210
% Purity	**96.9%**



LRMS (ESI): found for $C_{29}H_{28}Br_2N_5O_6$ $[M + H]^+$: 508.7.

Note: The (1:1) natural abundance of ^{79}Br:^{81}Br is observed in the mass spectrum.

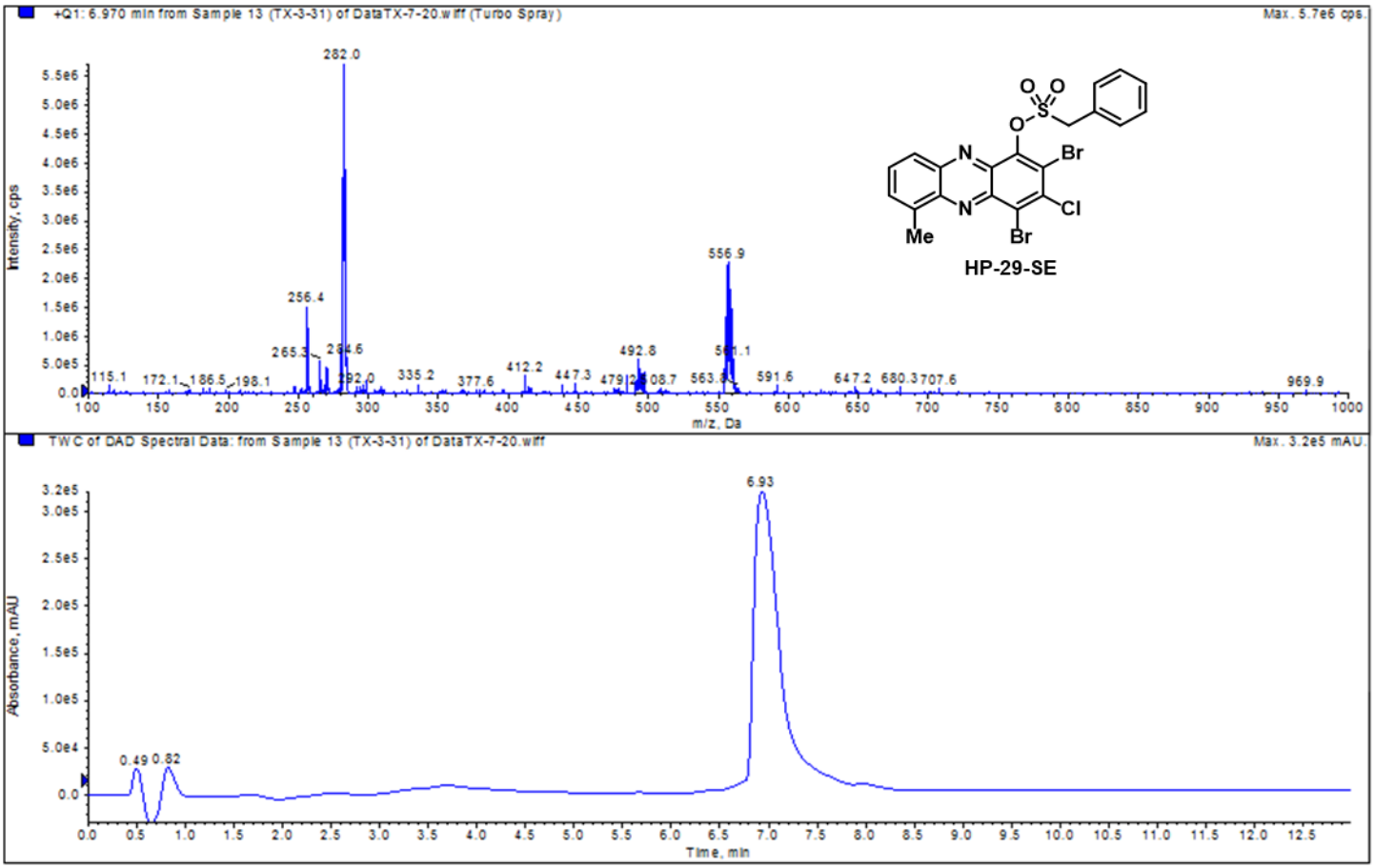
Retention Time (Min)	Peak Area
6.53 (compound)	5154400
7.23	24300
Sum of Area	5178700
% Purity	**99.5%**



LRMS (ESI): found for $C_{29}H_{28}Br_2N_5O_6$ $[M + Na]^+$: 563.8.

Note: The (1:1) natural abundance of ^{79}Br:^{81}Br is observed in the mass spectrum.

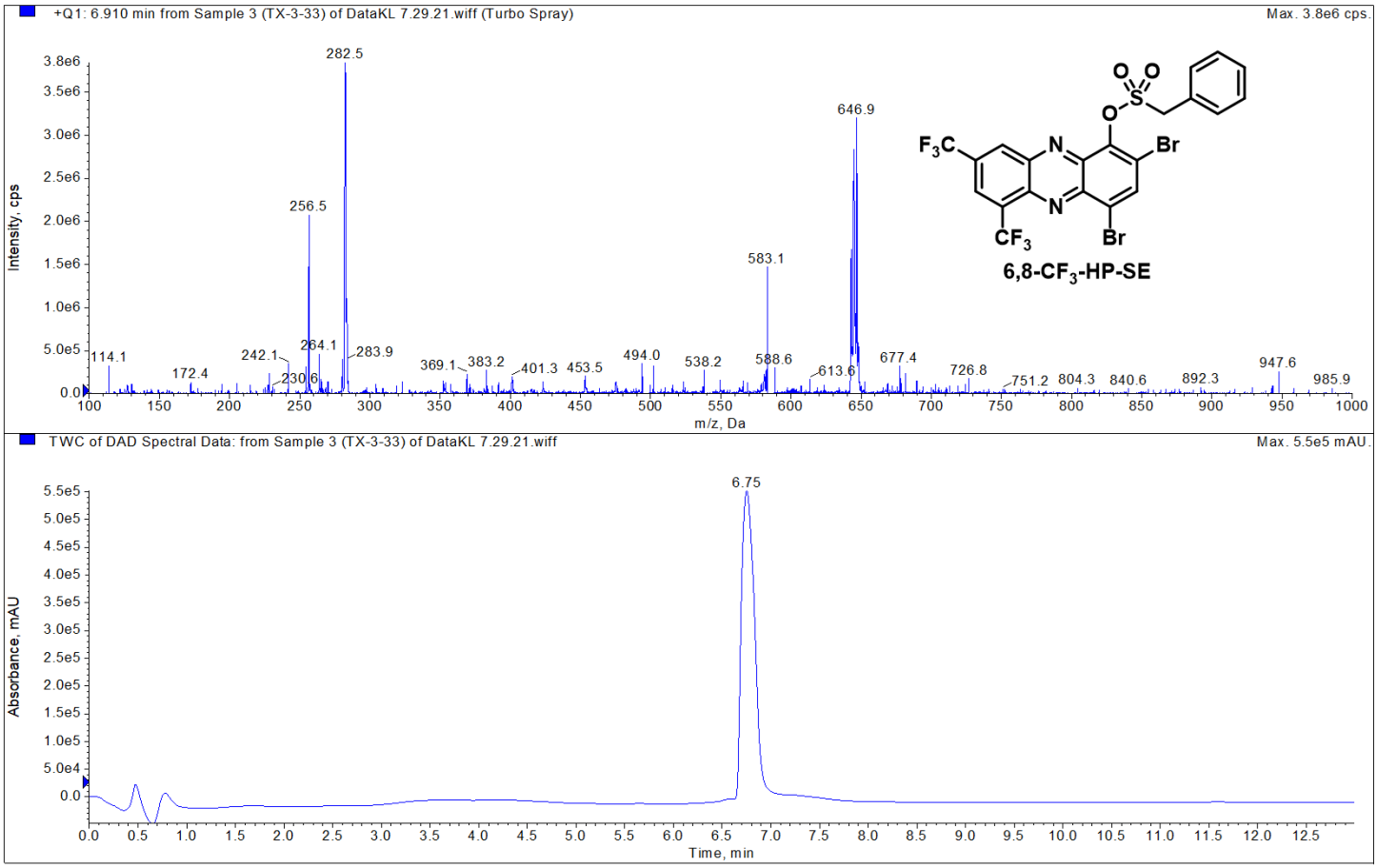
Retention Time (Min)	Peak Area
6.74 (compound)	7181700
Sum of Area	7181700
% Purity	**100%**



LRMS (ESI): found for $C_{29}H_{28}Br_2N_5O_6$ $[M + Na]^+$: 556.9.

Note: The (1:1) natural abundance of ^{79}Br:^{81}Br is observed in the mass spectrum.

Retention Time (Min)	**Peak Area**
6.93 (compound)	5881300
7.96	25173
Sum of Area	5906473
% Purity	**99.6%**

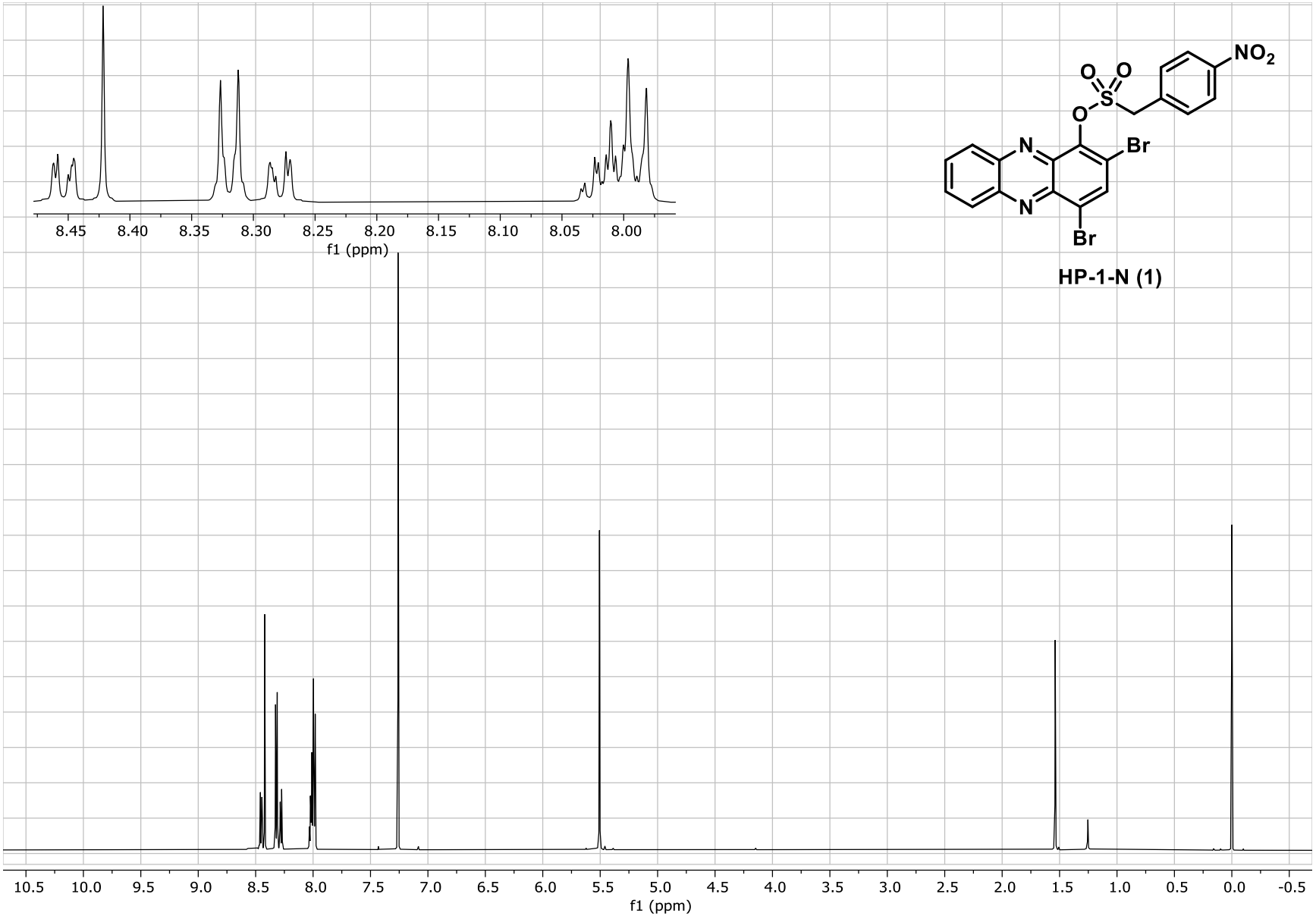


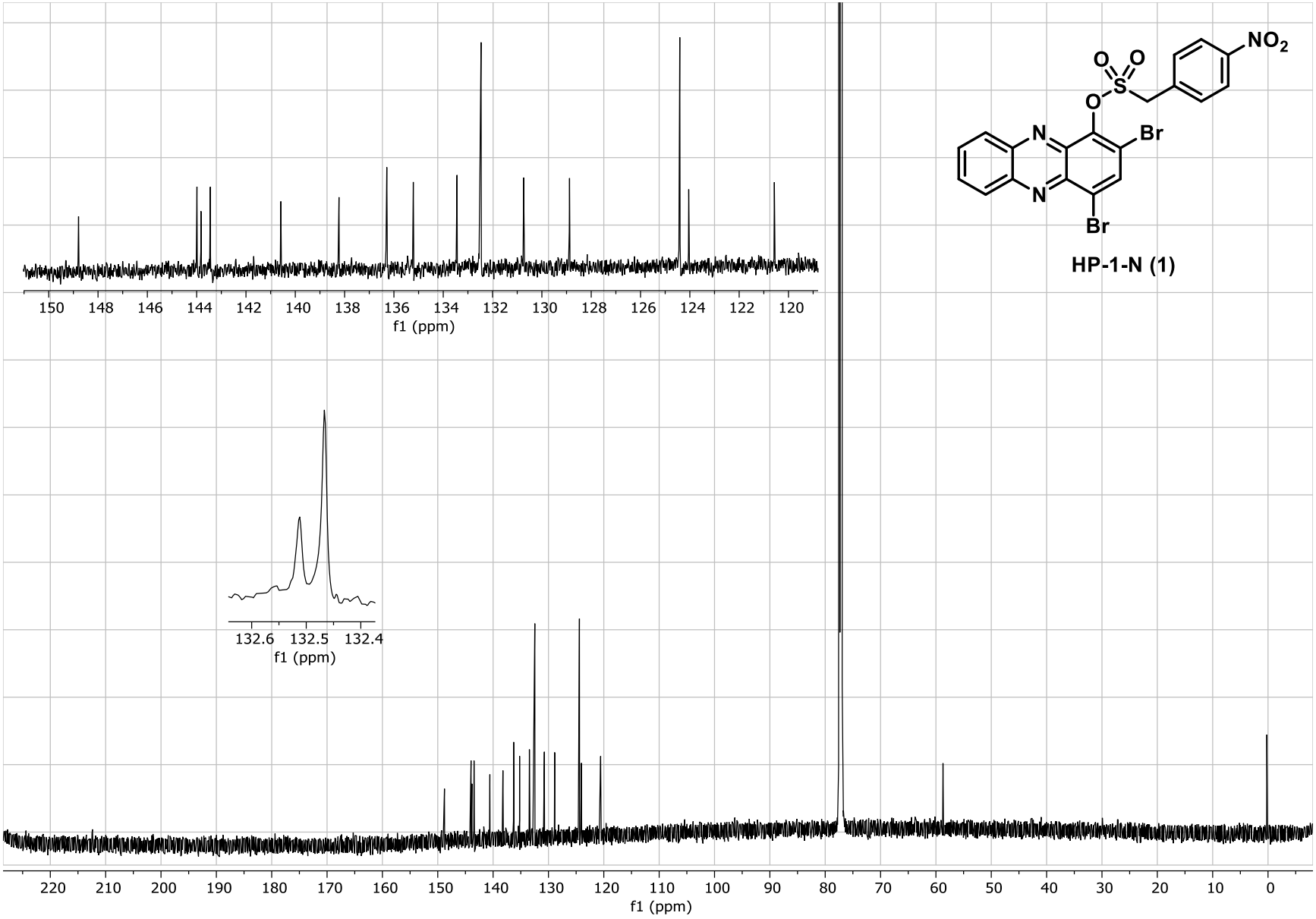
LRMS (ESI): found for $C_{29}H_{28}Br_2N_4O_4$ $[M + H]^+$: 646.9.

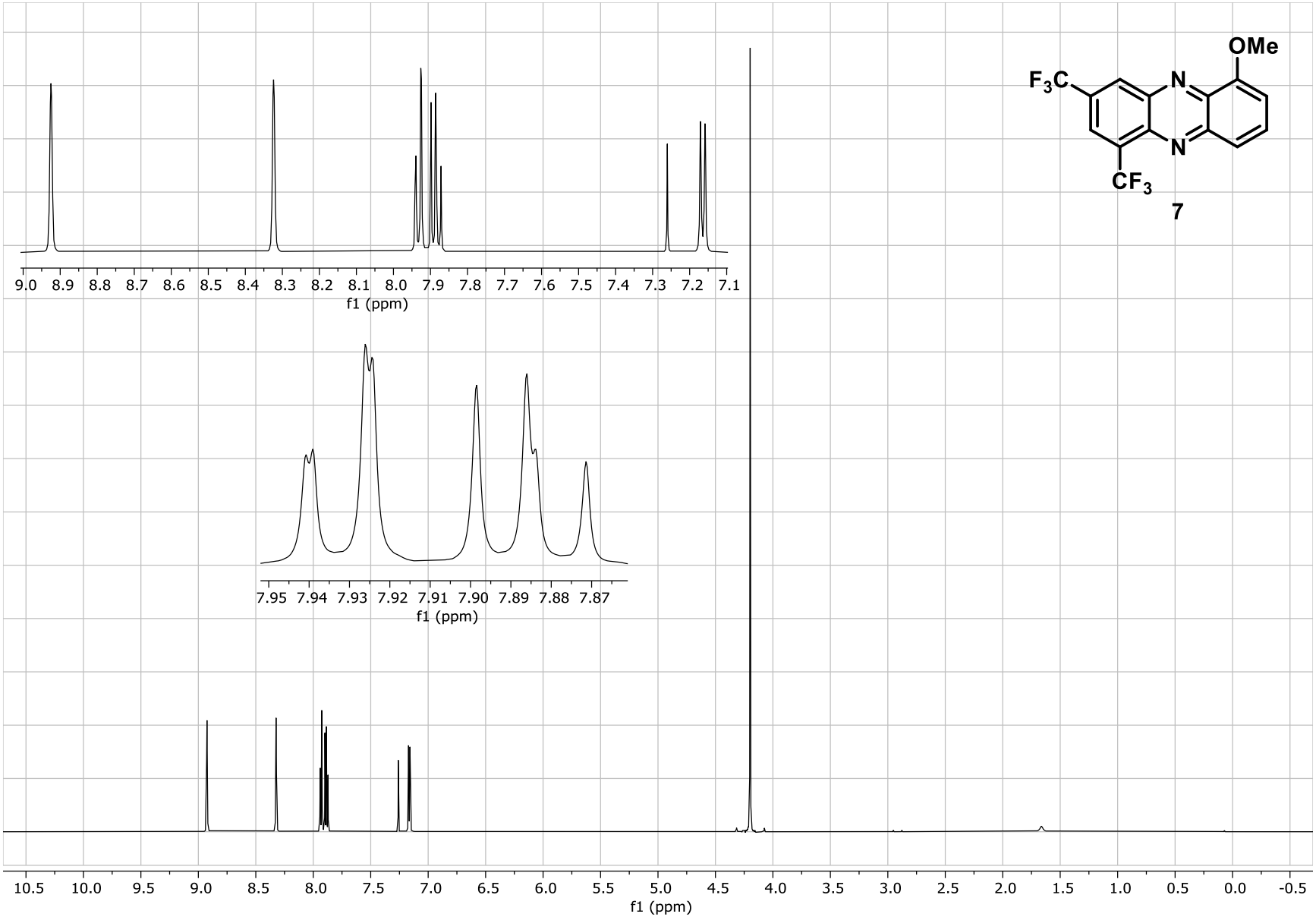
Note: The (1:1) natural abundance of ^{79}Br:^{81}Br is observed in the mass spectrum.

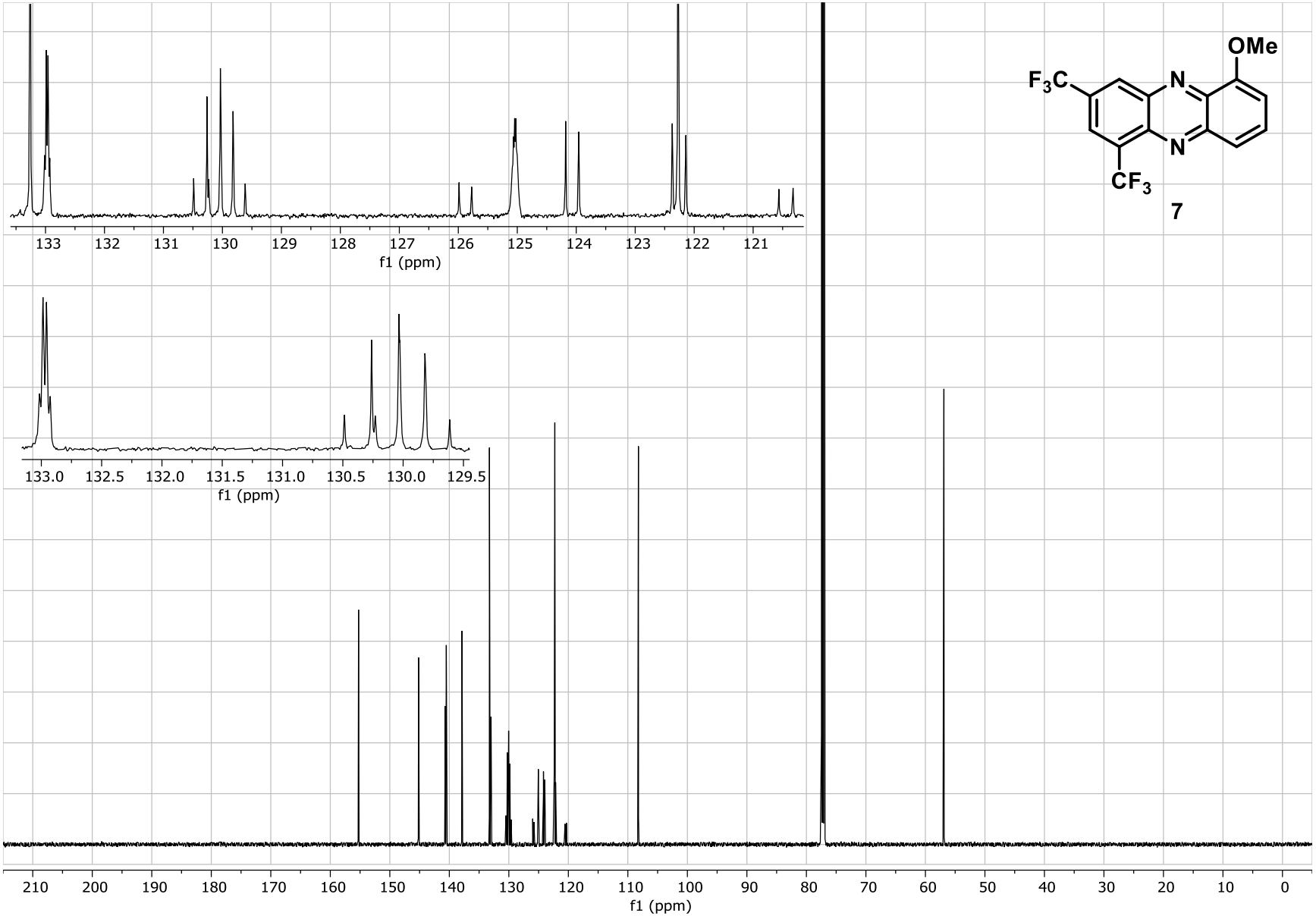
Retention Time (Min)	**Peak Area**
6.75 **(compound)**	5577500
7.35	25278
Sum of Area	5602778
% Purity	**99.5%**

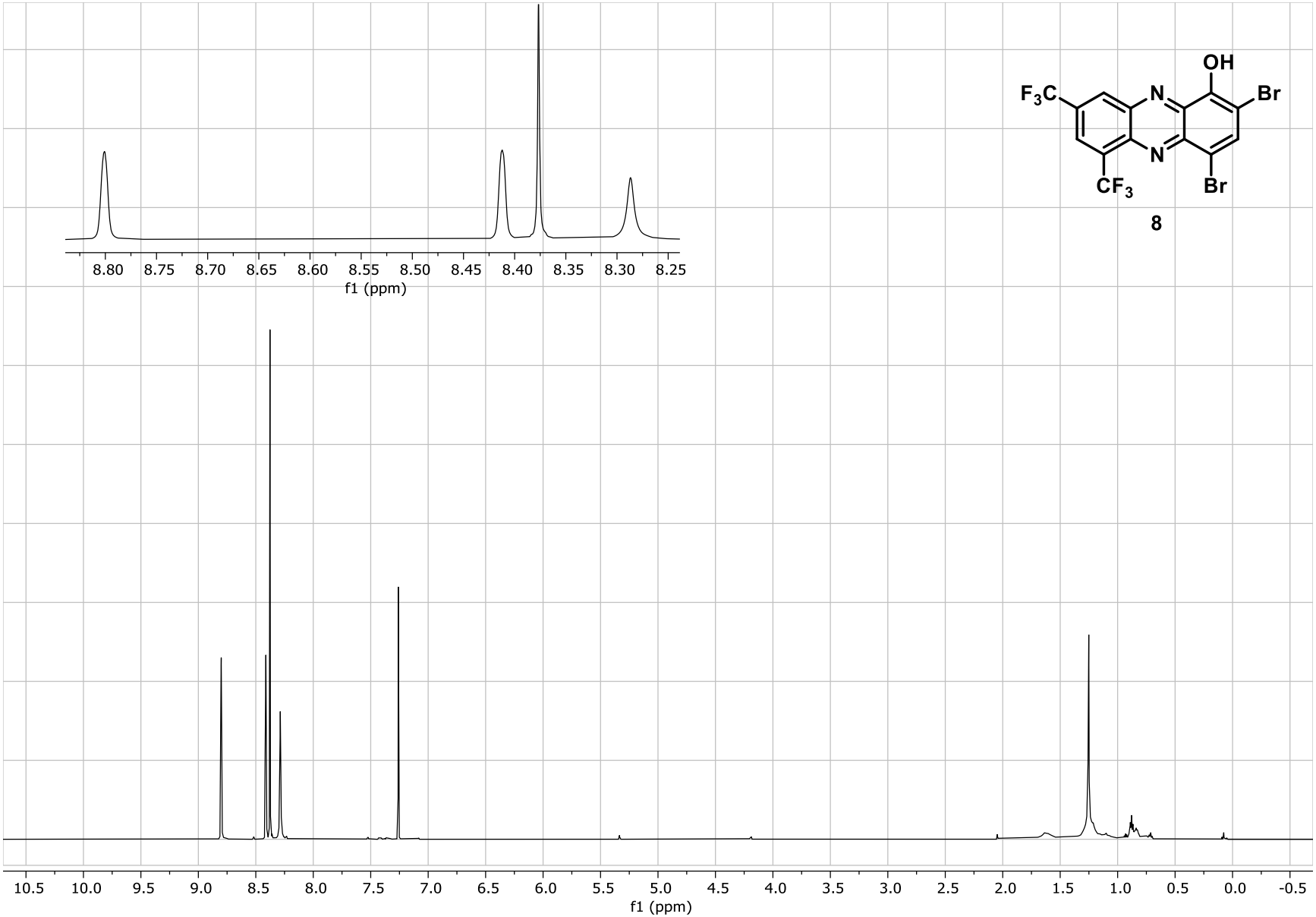
11.) NMR Spectra.

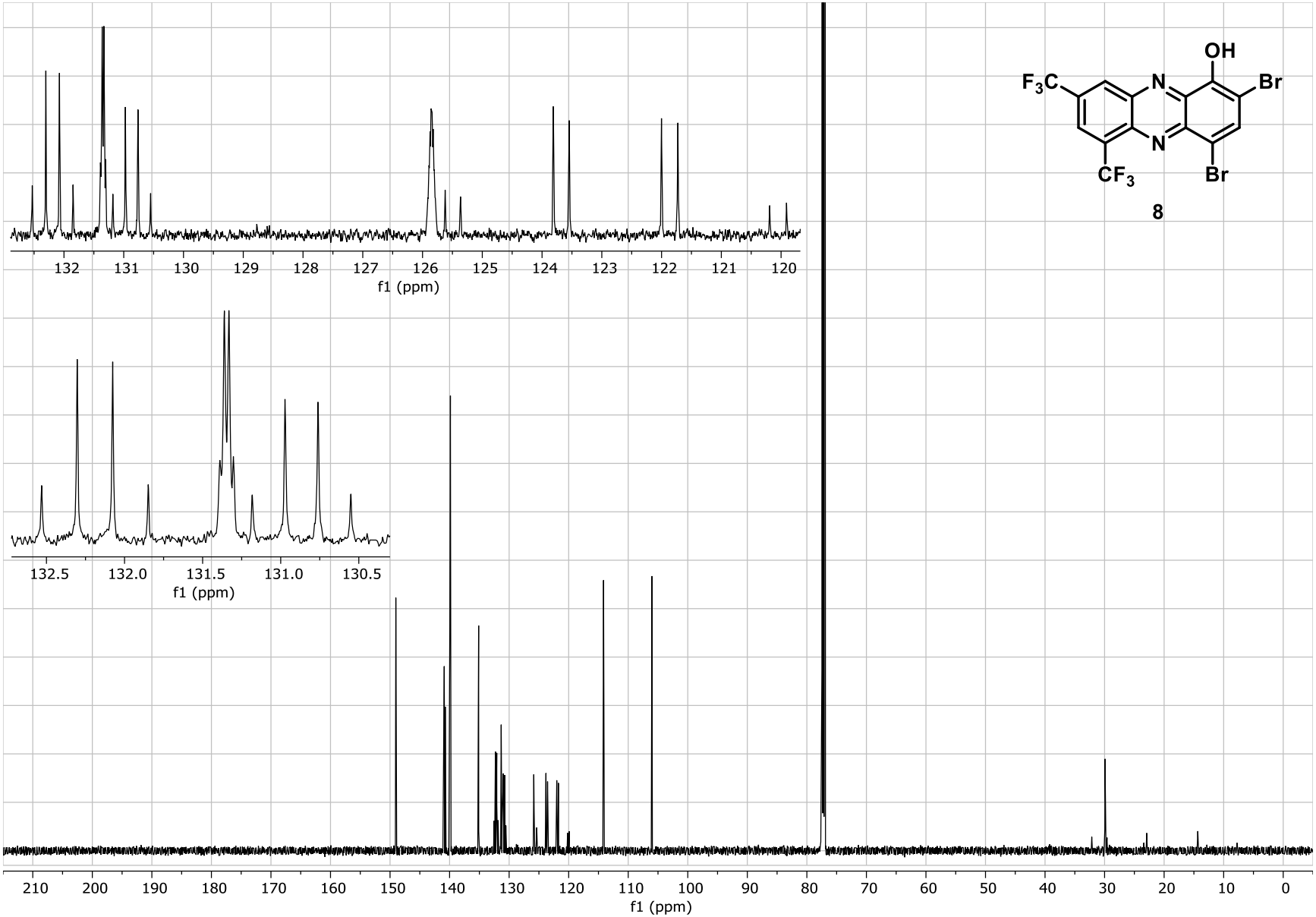

8.45
8.40
8.35
8.30
8.25
8.20
8.15
8.10
8.05
8.00
f1 (ppm)
NO2
O
O
S
O
N
Br
N
Br
HP-1-N (1)
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)

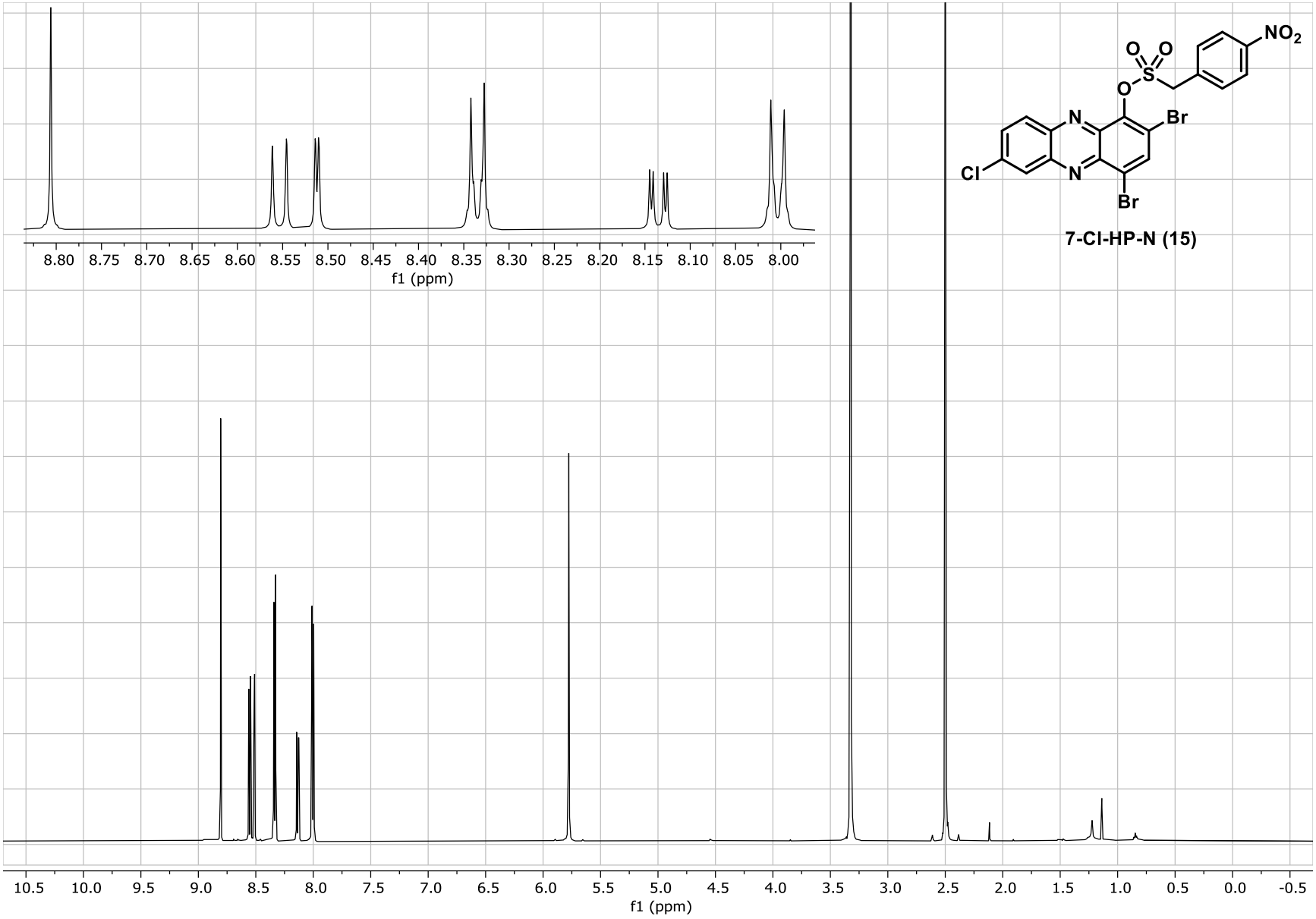

NO2
O
O
S
O
N
Br
N
Br
HP-1-N (1)
150 148 146 144 142 140 138 136 134 132 130 128 126 124 122 120
f1 (ppm)
132.6 132.5 132.4
f1 (ppm)
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

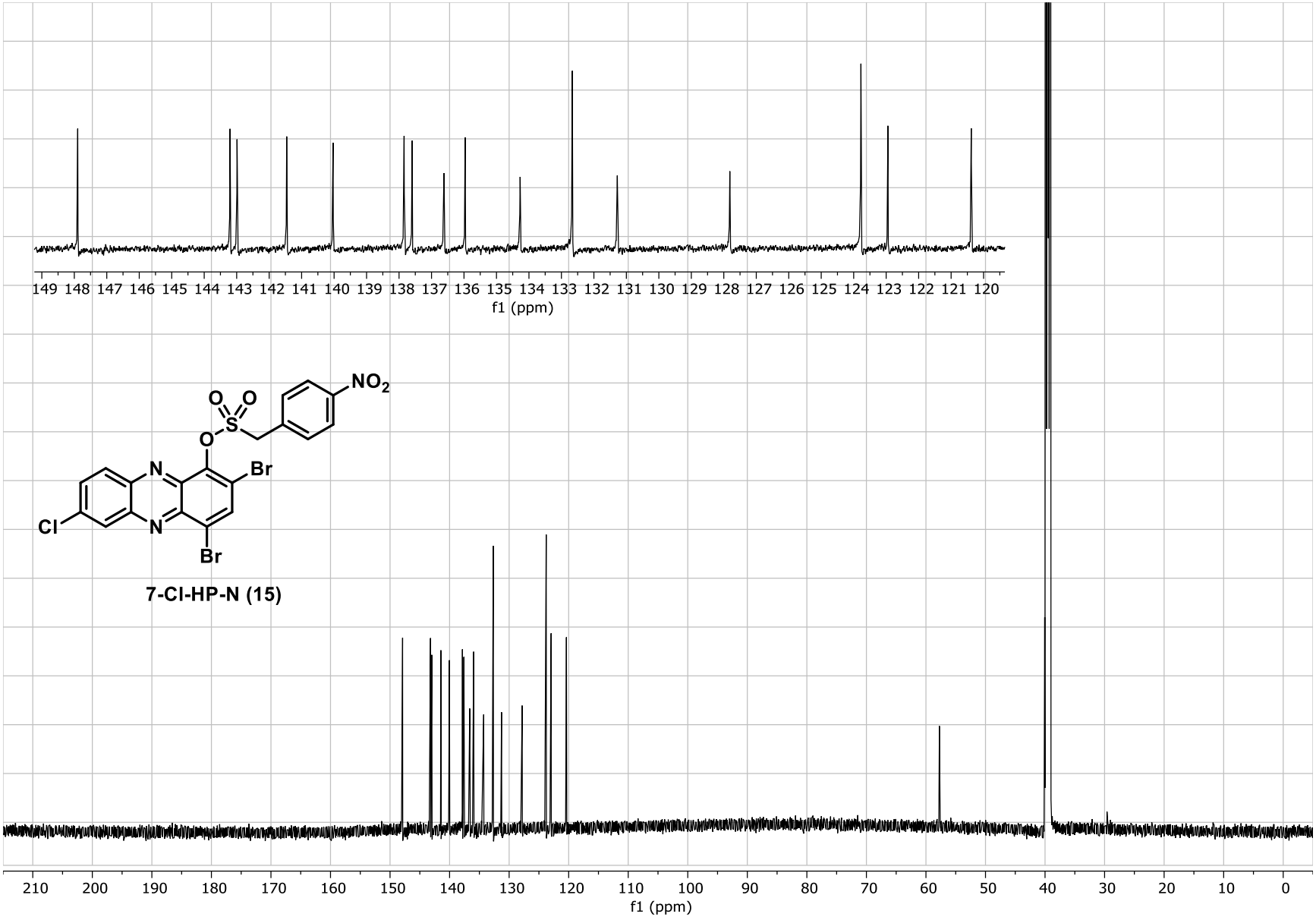

OMe
F3C
N
N
CF3
7
9.0 8.9 8.8 8.7 8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5 7.4 7.3 7.2 7.1
f1 (ppm)
7.95 7.94 7.93 7.92 7.91 7.90 7.89 7.88 7.87
f1 (ppm)
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

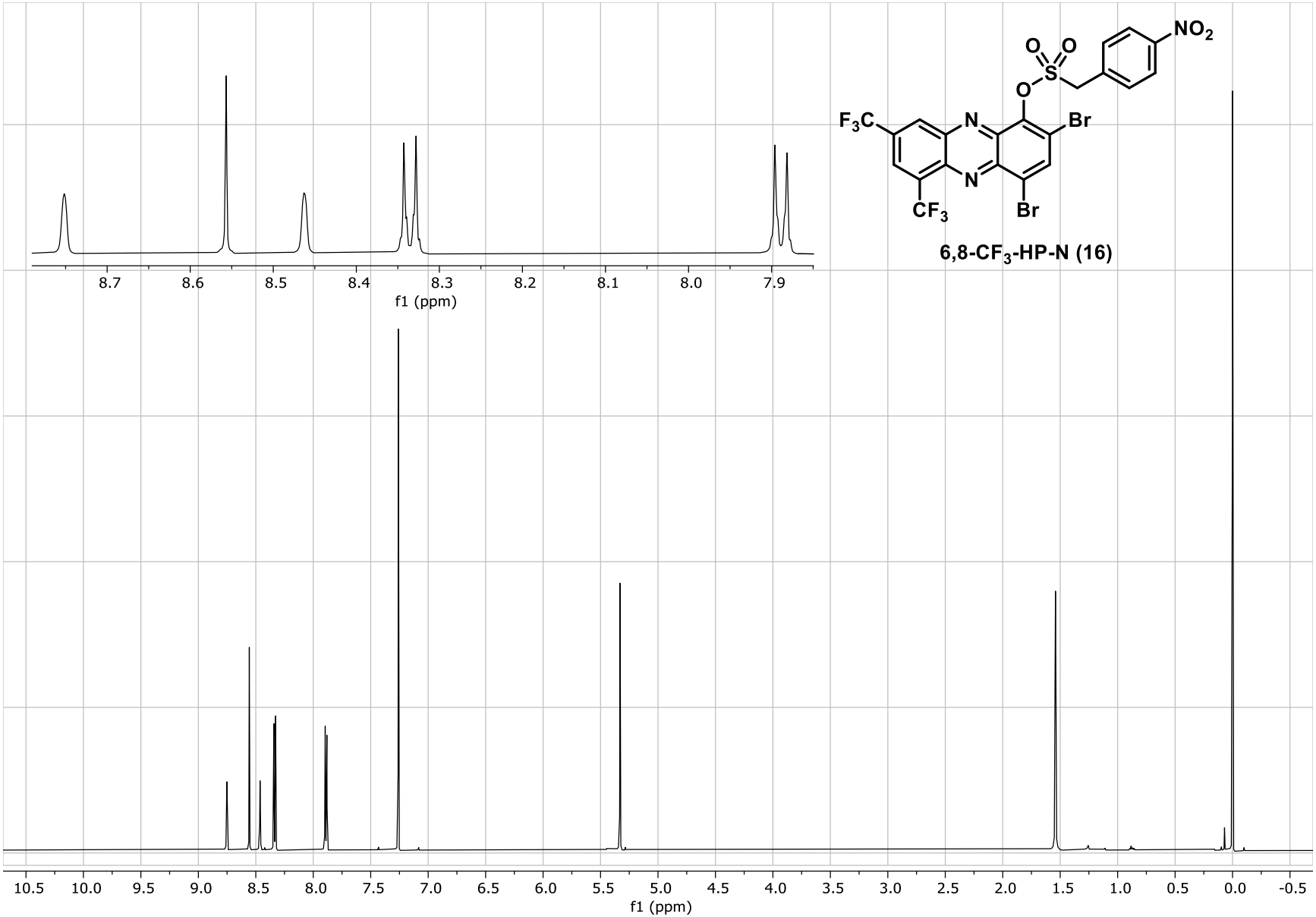

OMe
F3C
N
N
CF3
7
133 132 131 130 129 128 127 126 125 124 123 122 121
f1 (ppm)
133.0 132.5 132.0 131.5 131.0 130.5 130.0 129.5
f1 (ppm)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

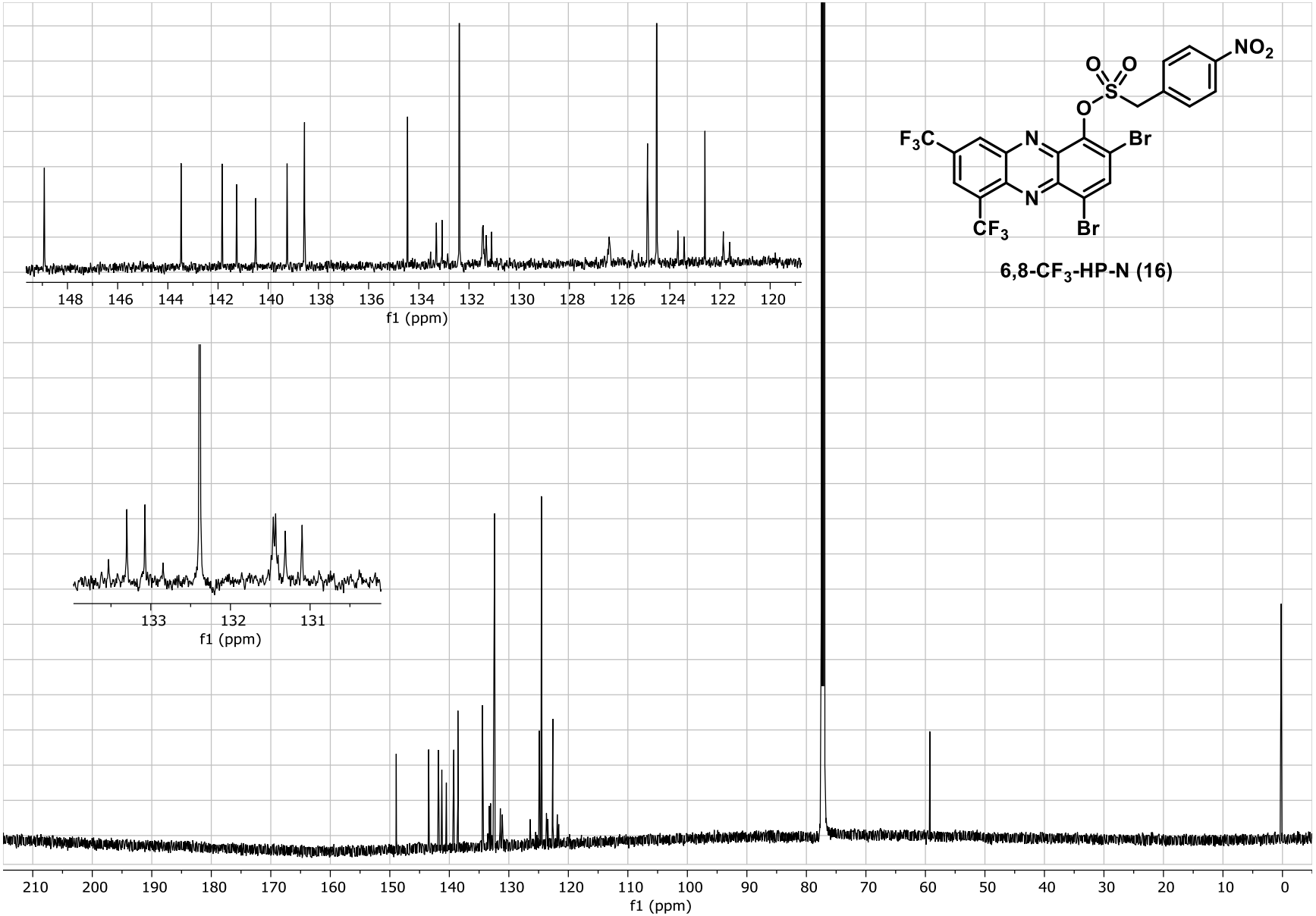

8.80 8.75 8.70 8.65 8.60 8.55 8.50 8.45 8.40 8.35 8.30 8.25
f1 (ppm)
OH
F3C
N
Br
N
CF3
Br
8
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

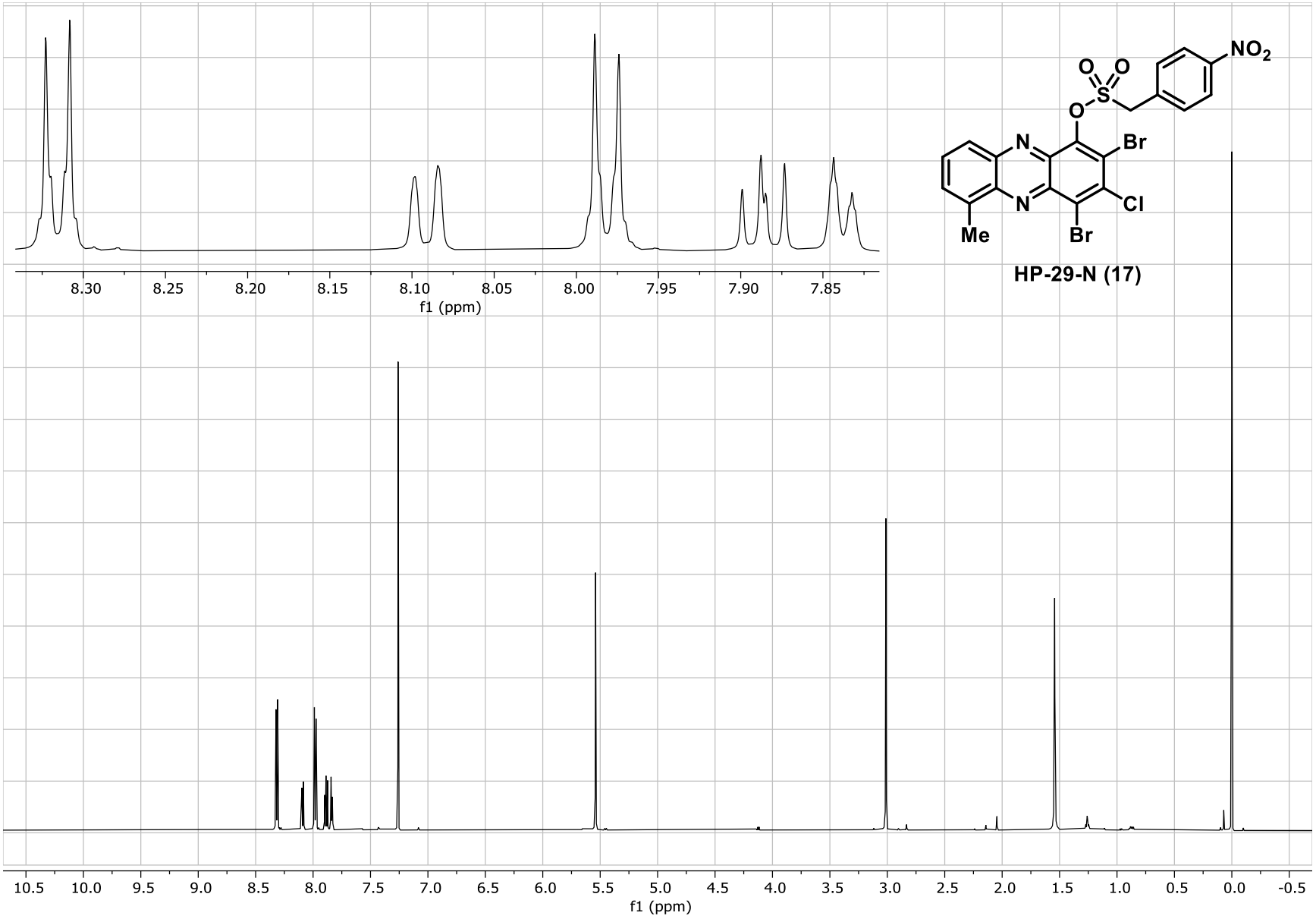

OH
F3C
N
Br
N
CF3
Br
8
132 131 130 129 128 127 126 125 124 123 122 121 120
f1 (ppm)
132.5 132.0 131.5 131.0 130.5
f1 (ppm)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

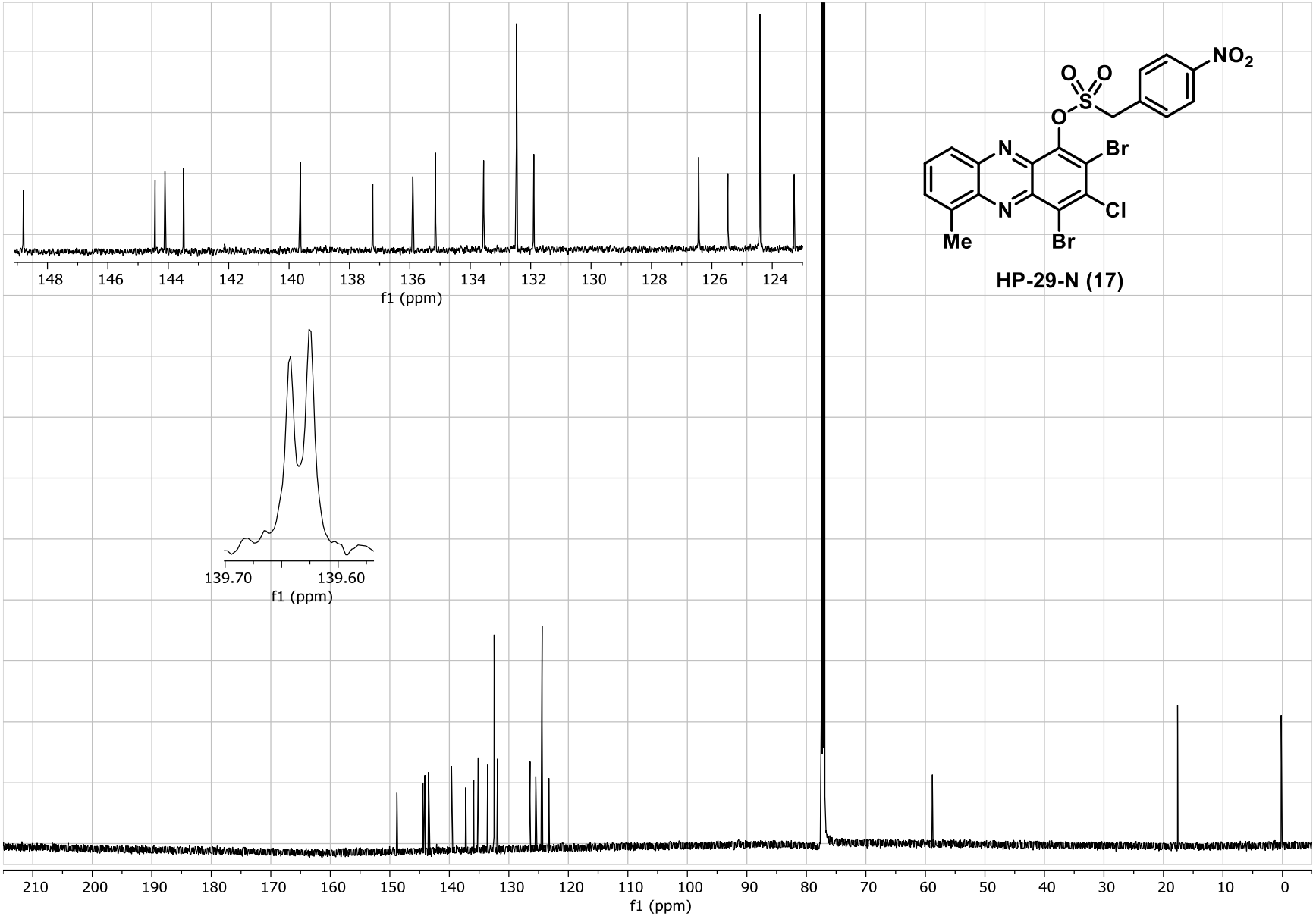

8.80 8.75 8.70 8.65 8.60 8.55 8.50 8.45 8.40 8.35 8.30 8.25 8.20 8.15 8.10 8.05 8.00
f1 (ppm)
NO2
O
O
S
O
N
Br
Cl
N
Br
7-Cl-HP-N (15)
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)

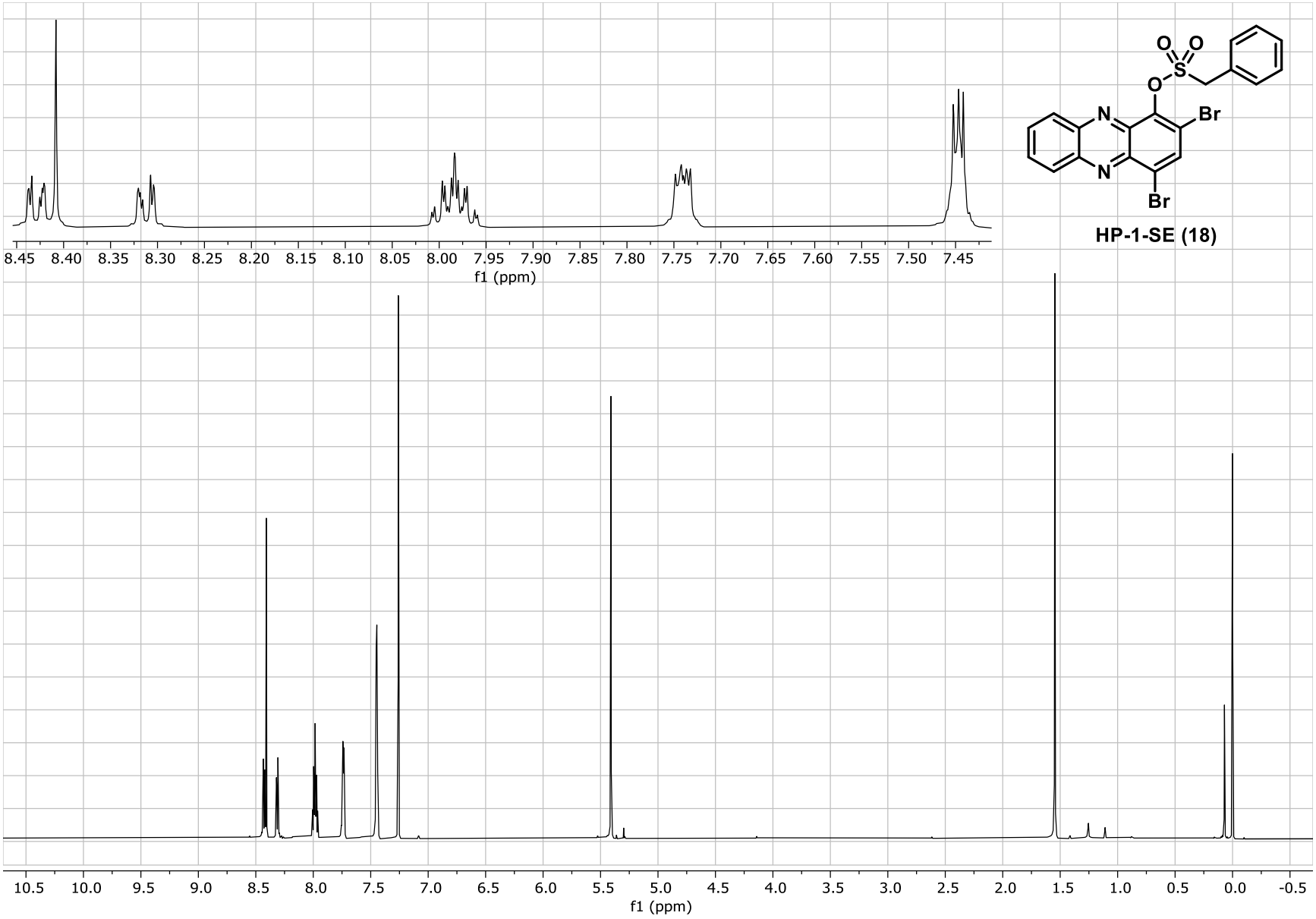

149 148 147 146 145 144 143 142 141 140 139 138 137 136 135 134 133 132 131 130 129 128 127 126 125 124 123 122 121 120
f1 (ppm)
O
O
S
O
NO2
N
Br
Cl
N
Br
7-Cl-HP-N (15)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

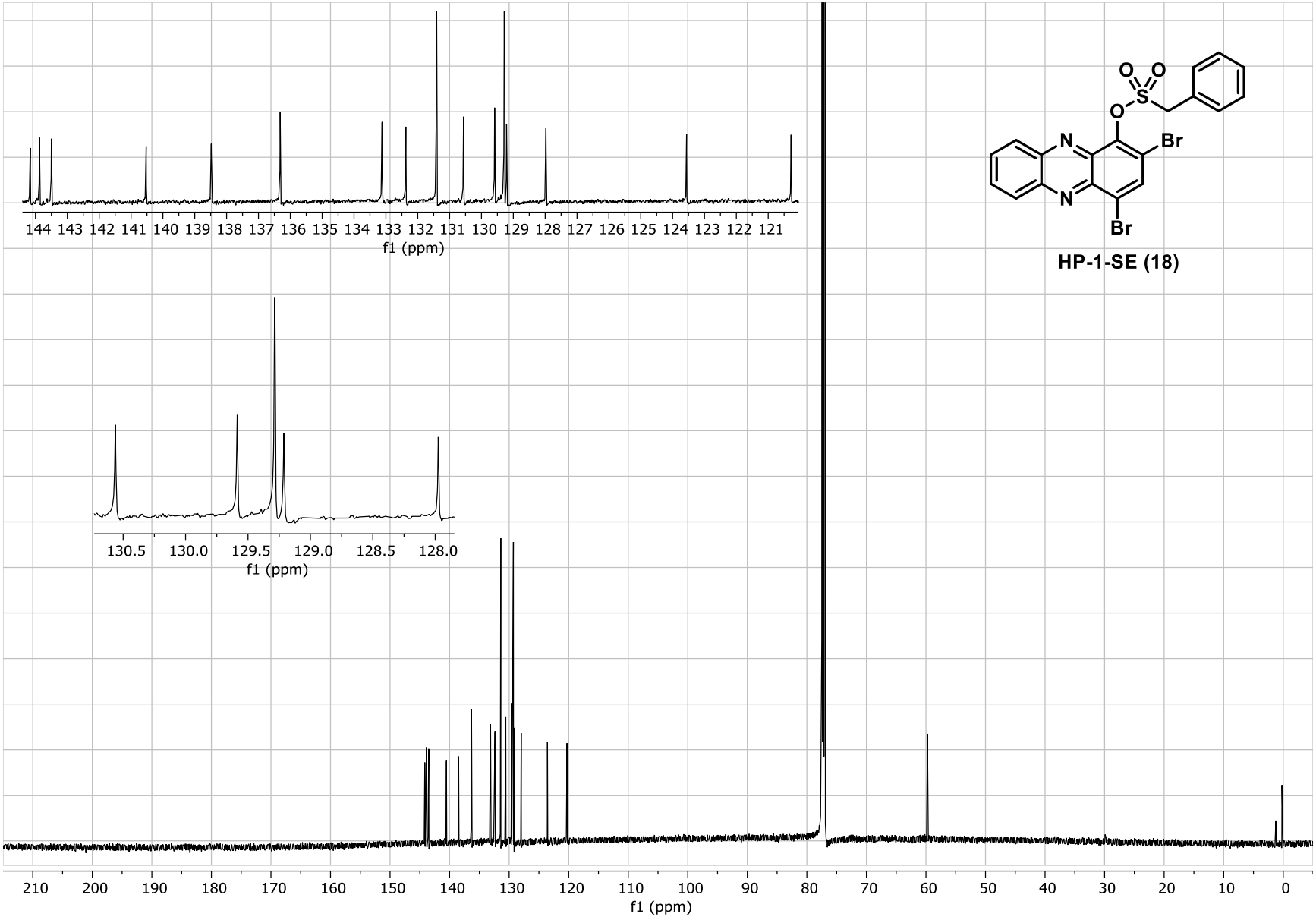

8.7
8.6
8.5
8.4
8.3
8.2
8.1
8.0
7.9
f1 (ppm)
NO2
O
O
S
O
F3C
N
Br
N
CF3
Br
6,8-CF3-HP-N (16)
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)

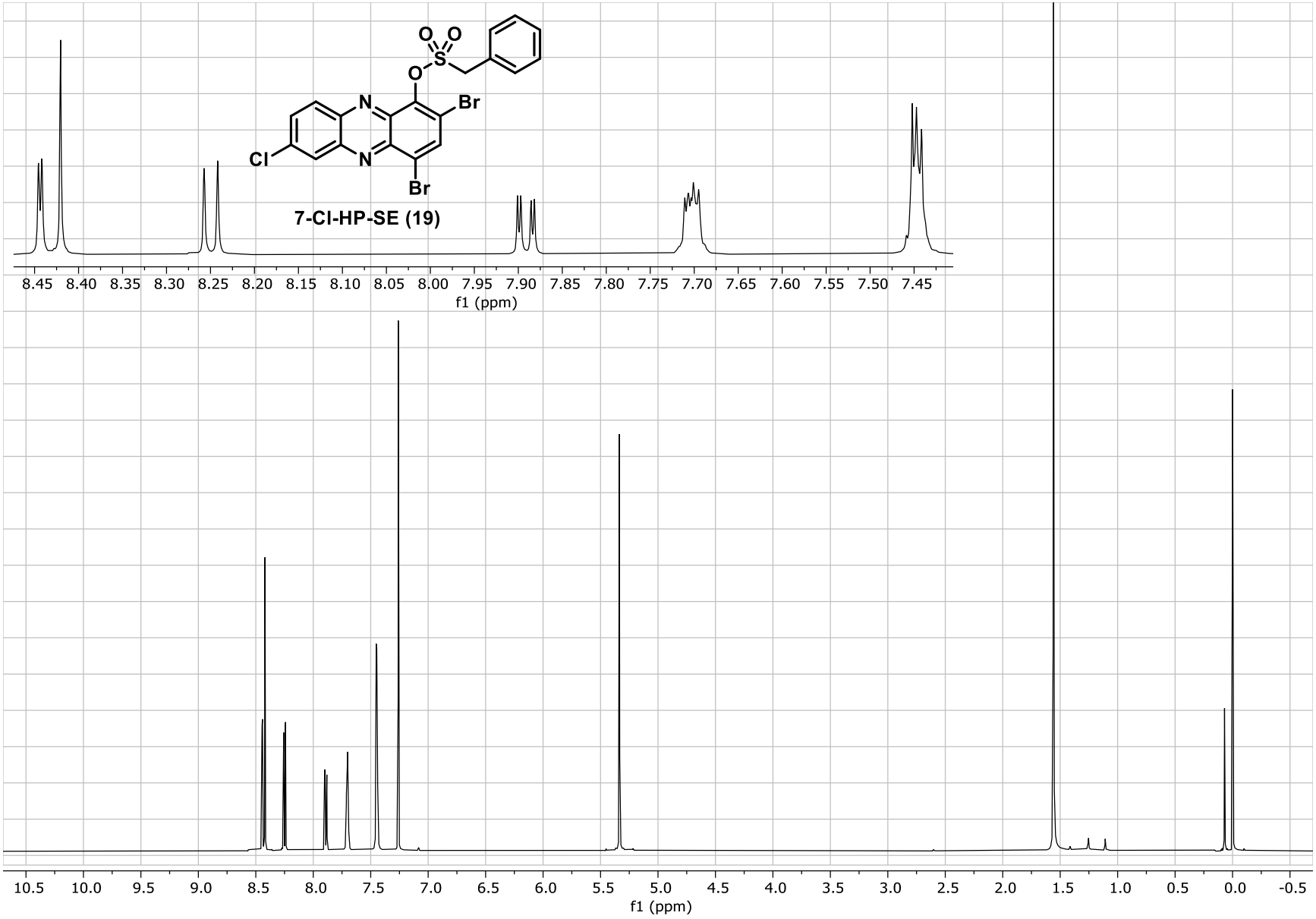

148 146 144 142 140 138 136 134 132 130 128 126 124 122 120
f1 (ppm)
133 132 131
f1 (ppm)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)
NO2
O
O
S
O
F3C
N
Br
N
CF3
Br
6,8-CF3-HP-N (16)

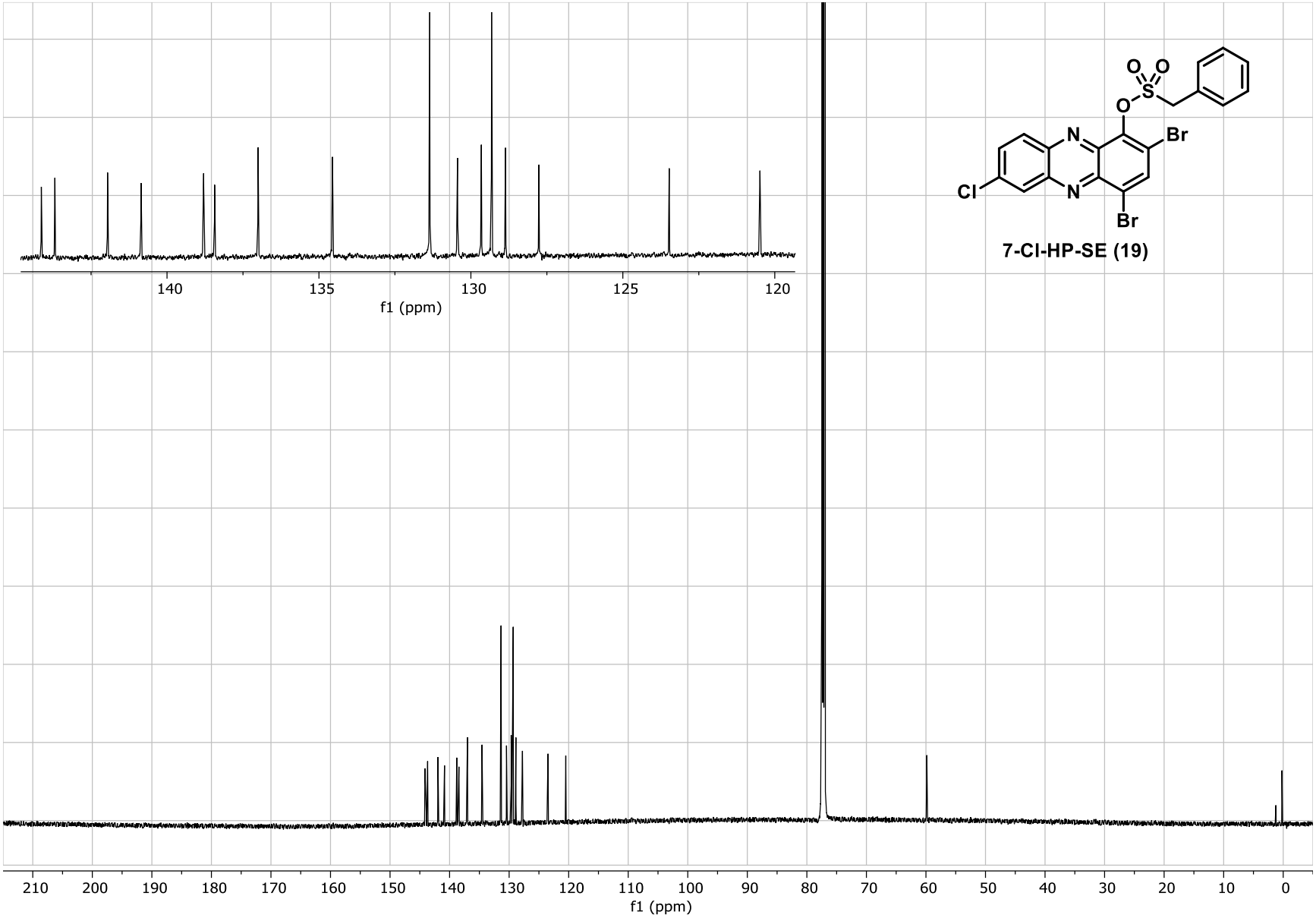

8.30
8.25
8.20
8.15
8.10
8.05
8.00
7.95
7.90
7.85
f1 (ppm)
NO2
O
O
S
O
N
N
Br
Cl
Br
Me
HP-29-N (17)
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)

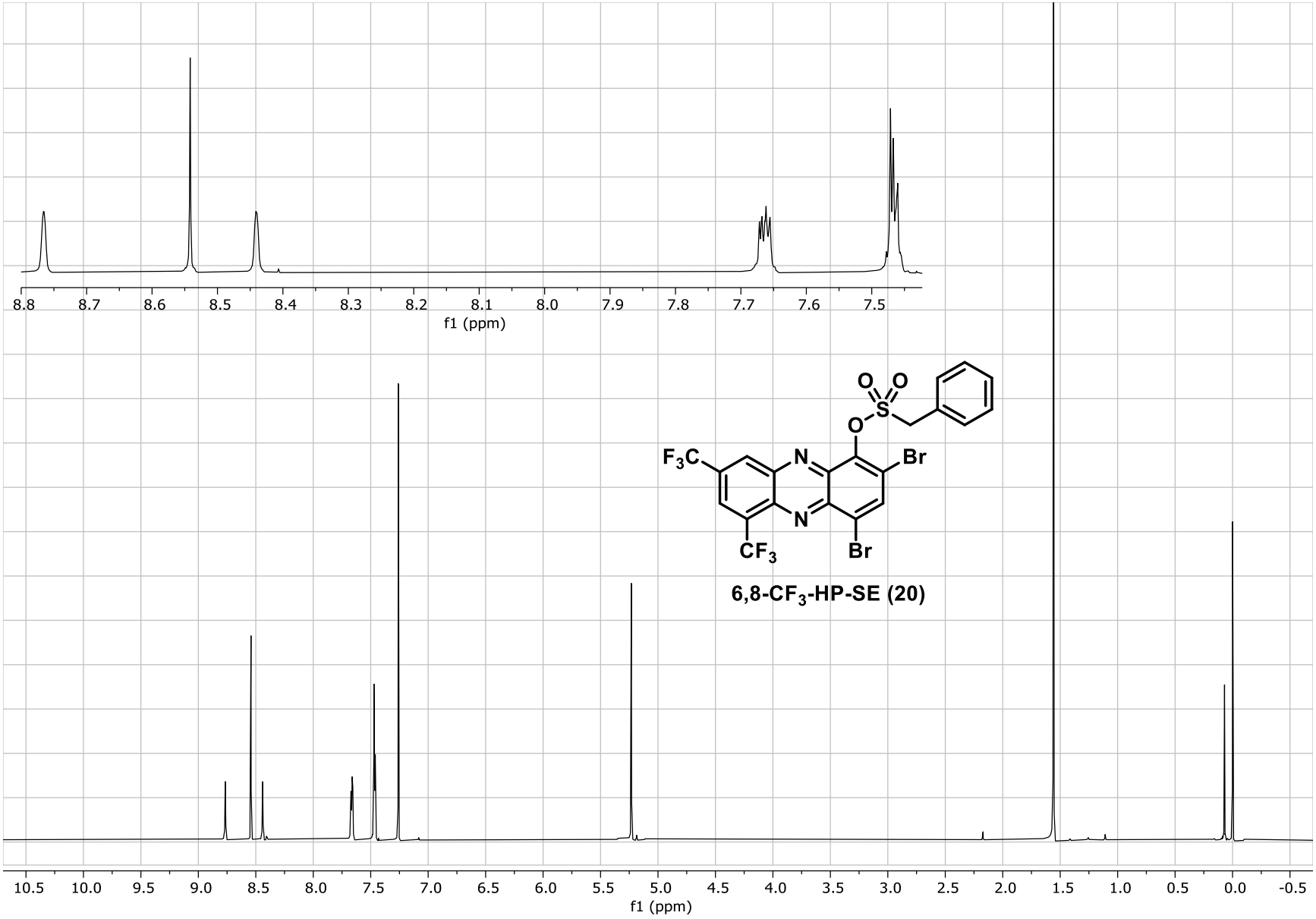

148 146 144 142 140 138 136 134 132 130 128 126 124
f1 (ppm)
139.70 139.60
f1 (ppm)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)
NO2
O
O
S
O
N
Br
N
Cl
Me
Br
HP-29-N (17)

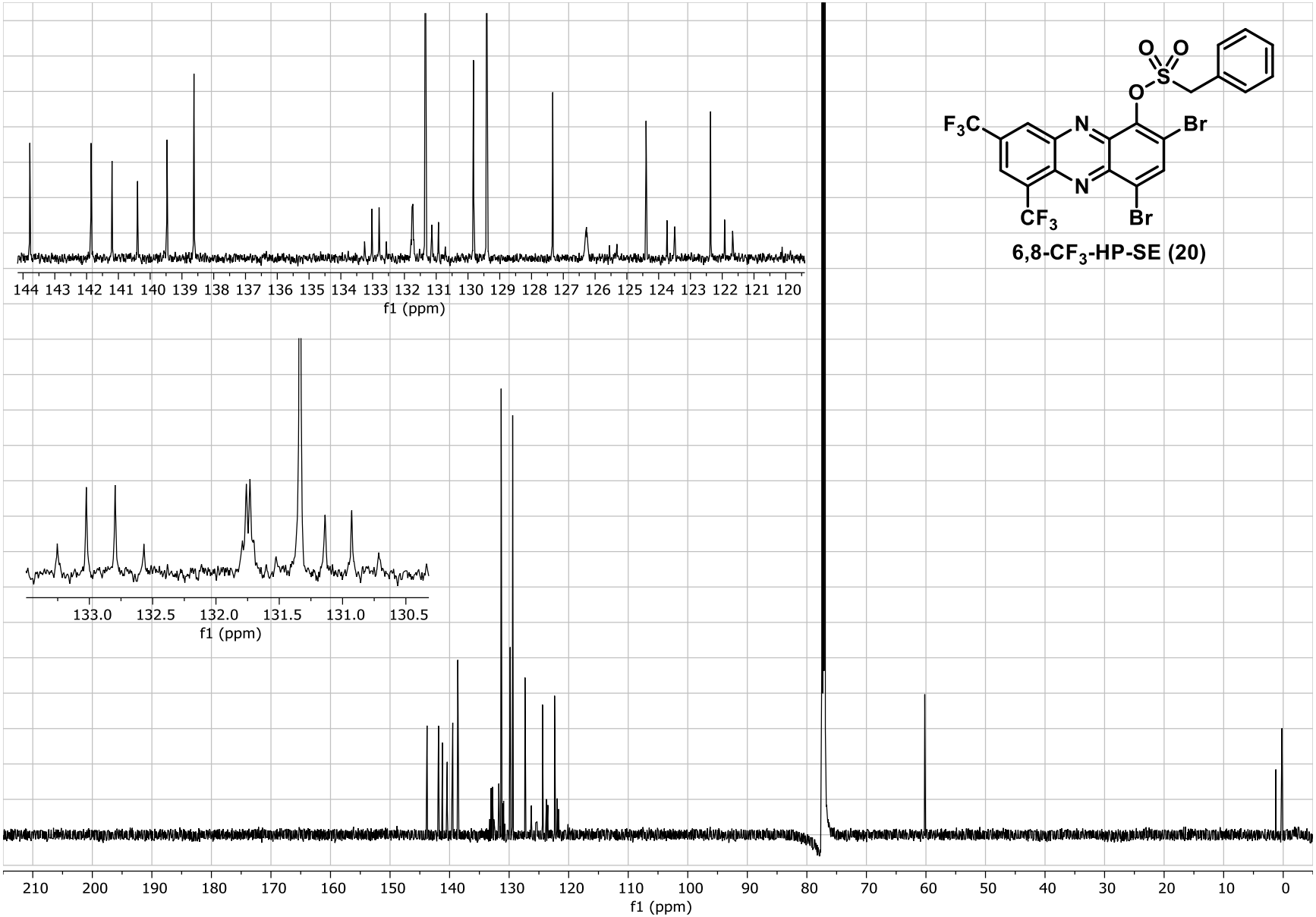

HP-1-SE (18)
f1 (ppm)
f1 (ppm)

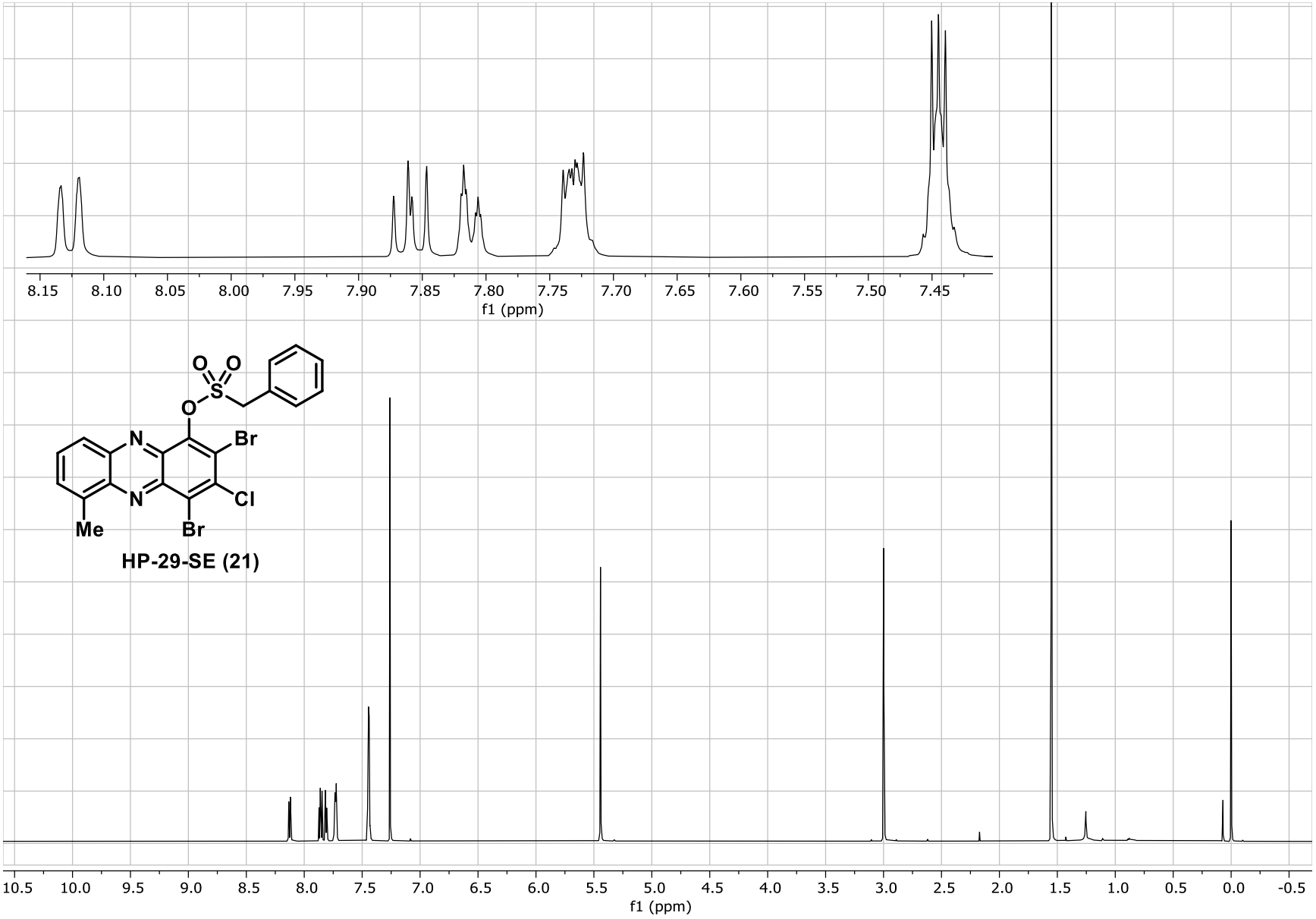

144 143 142 141 140 139 138 137 136 135 134 133 132 131 130 129 128 127 126 125 124 123 122 121
f1 (ppm)
130.5 130.0 129.5 129.0 128.5 128.0
f1 (ppm)
O
O
S
O
N
N
Br
Br
HP-1-SE (18)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

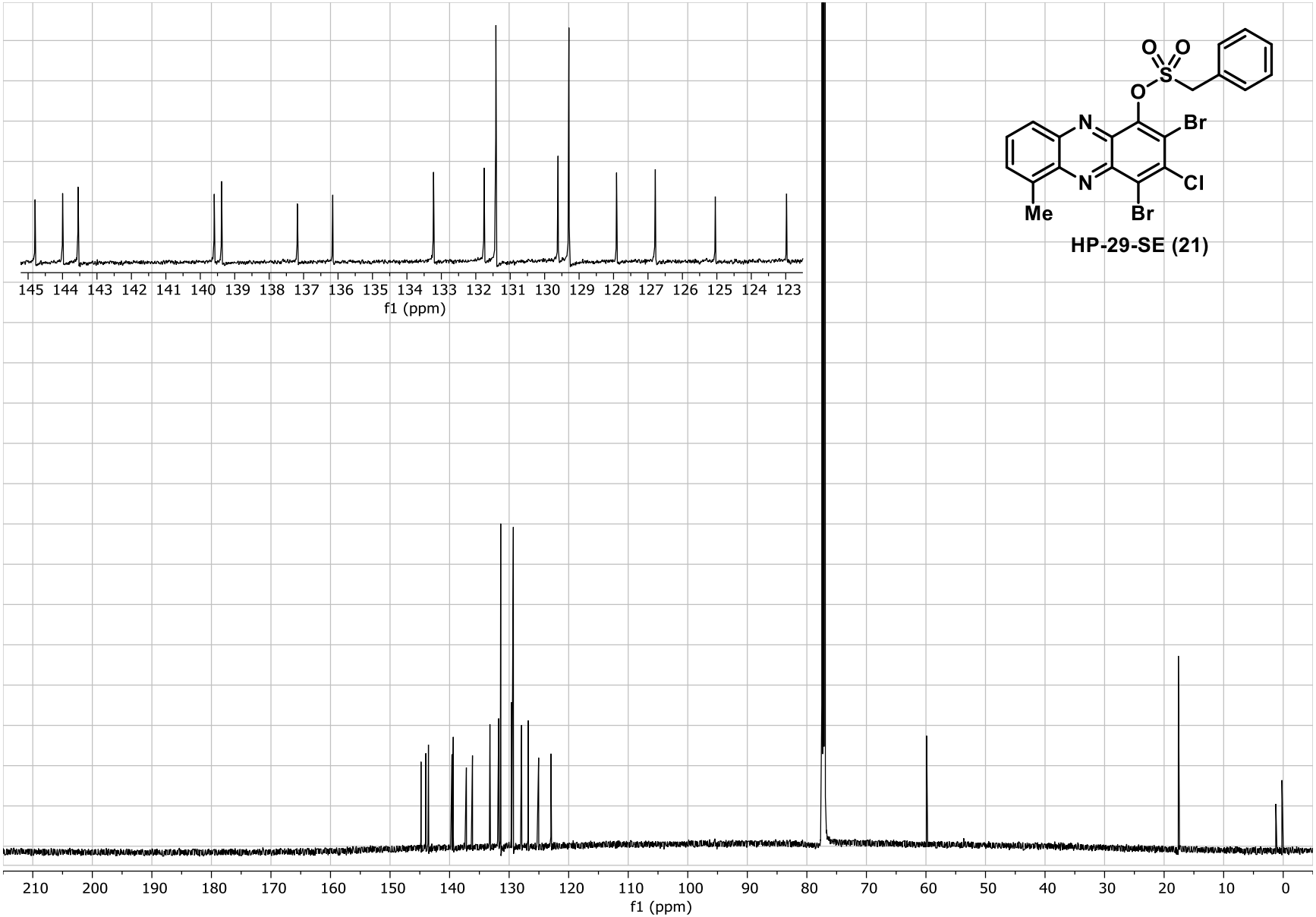

7-Cl-HP-SE (19)
f1 (ppm)
f1 (ppm)

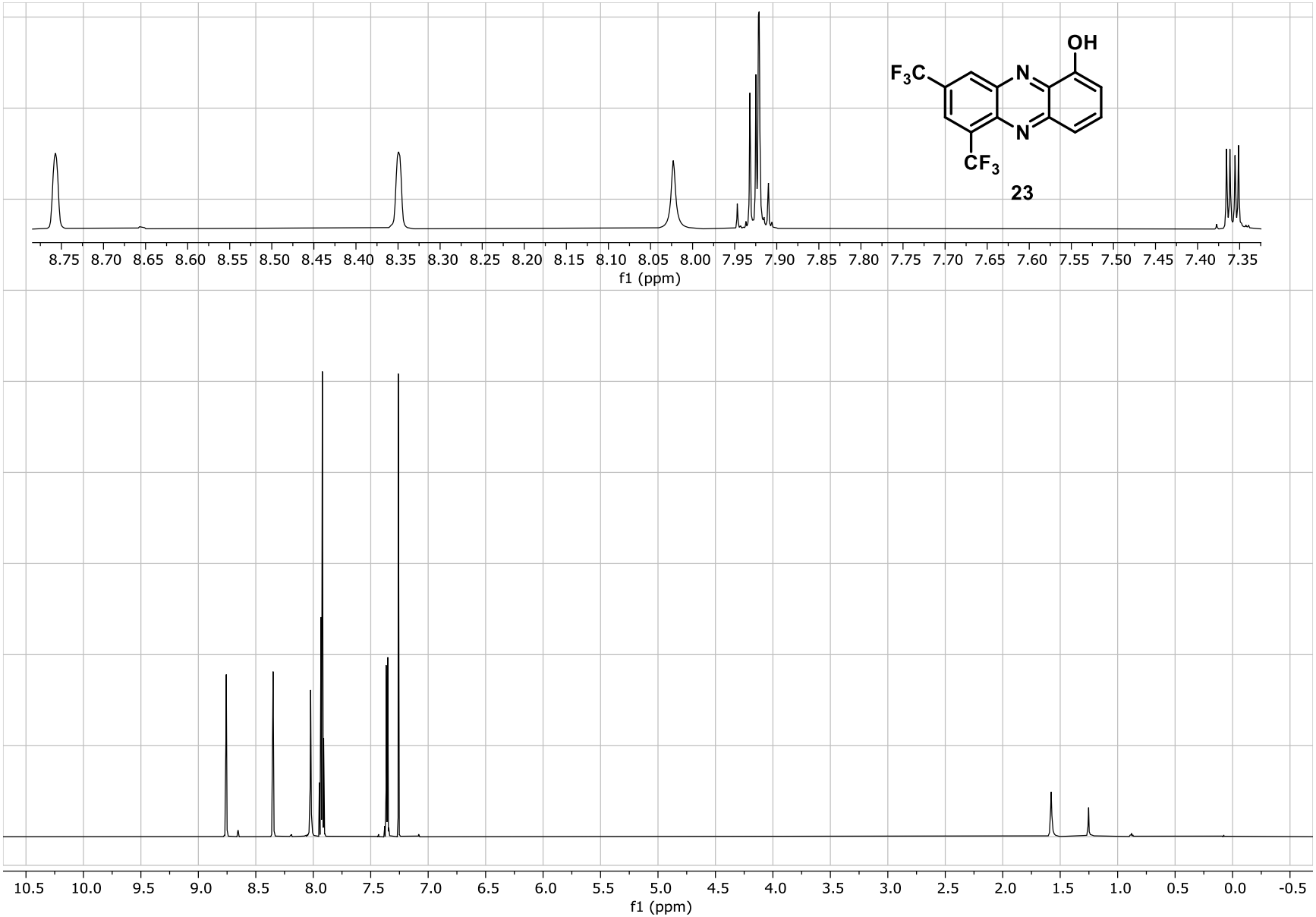

140
135
130
125
120
f1 (ppm)
O
O
S
O
N
Br
Cl
N
Br
7-Cl-HP-SE (19)
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

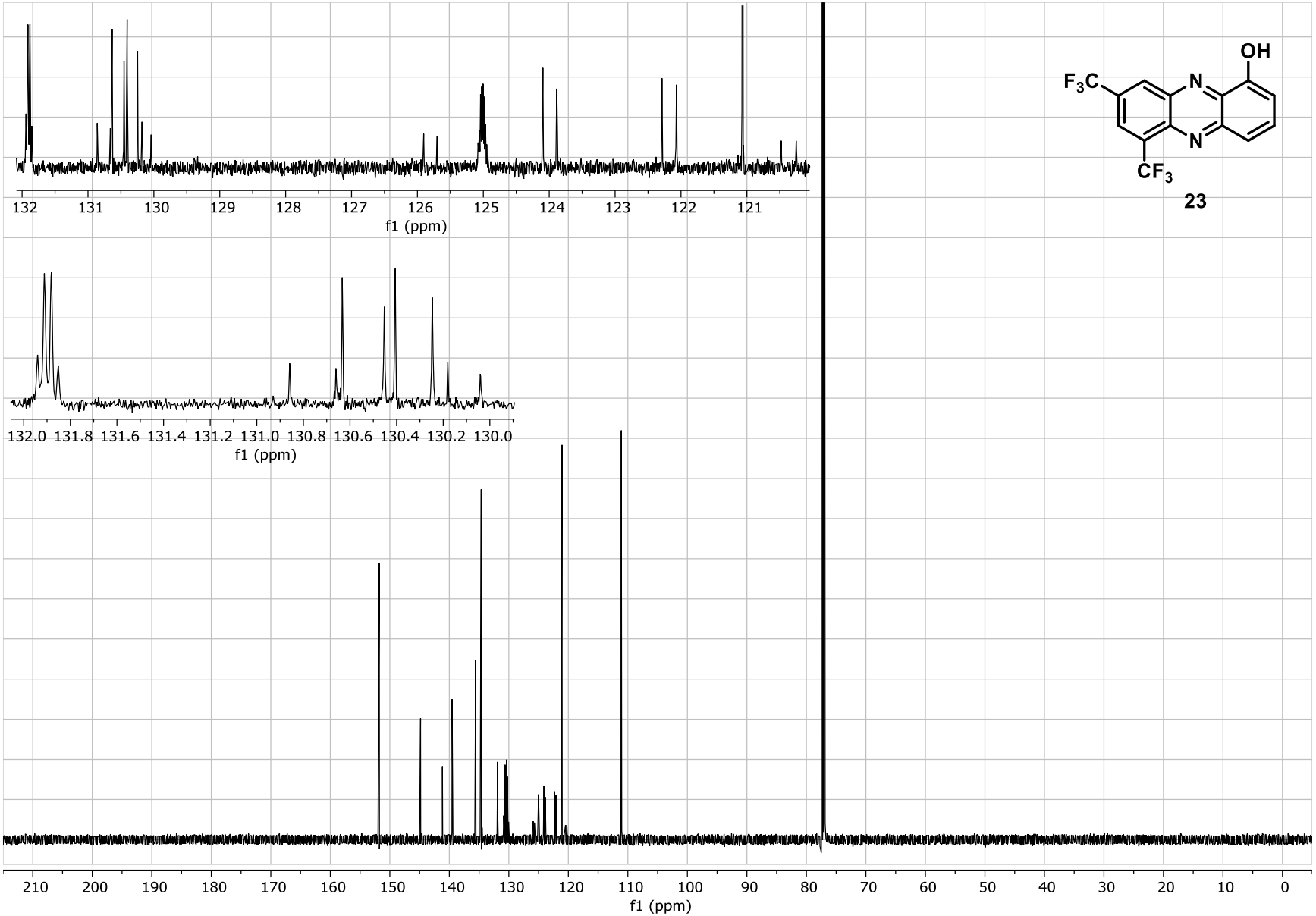

8.8 8.7 8.6 8.5 8.4 8.3 8.2 8.1 8.0 7.9 7.8 7.7 7.6 7.5
f1 (ppm)
O
O
S
O
F3C
N
Br
N
CF3
Br
6,8-CF3-HP-SE (20)
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)


144 143 142 141 140 139 138 137 136 135 134 133 132 131 130 129 128 127 126 125 124 123 122 121 120
f1 (ppm)
133.0 132.5 132.0 131.5 131.0 130.5
f1 (ppm)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)
O
O
S
O
F3C
N
Br
N
CF3
Br
6,8-CF3-HP-SE (20)


8.15
8.10
8.05
8.00
7.95
7.90
7.85
7.80
7.75
7.70
7.65
7.60
7.55
7.50
7.45
f1 (ppm)
O
O
S
O
N
Br
N
Cl
Me
Br
HP-29-SE (21)
10.5
10.0
9.5
9.0
8.5
8.0
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
0.0
-0.5
f1 (ppm)


O
O
S
O
N
Br
N
Cl
Me
Br
HP-29-SE (21)
145 144 143 142 141 140 139 138 137 136 135 134 133 132 131 130 129 128 127 126 125 124 123
f1 (ppm)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


OH
F3C
N
N
CF3
23
8.75 8.70 8.65 8.60 8.55 8.50 8.45 8.40 8.35 8.30 8.25 8.20 8.15 8.10 8.05 8.00 7.95 7.90 7.85 7.80 7.75 7.70 7.65 7.60 7.55 7.50 7.45 7.40 7.35
f1 (ppm)
10.5 10.0 9.5 9.0 8.5 8.0 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 0.0 -0.5
f1 (ppm)


OH
F3C
N
N
CF3
23
132 131 130 129 128 127 126 125 124 123 122 121
f1 (ppm)
132.0 131.8 131.6 131.4 131.2 131.0 130.8 130.6 130.4 130.2 130.0
f1 (ppm)
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)