Electronic Supplementary Material (ESI) for RSC Advances.

Bromophenazine Derivatives with Potent Inhibition, Dispersion and Eradication Activities against *Staphylococcus aureus* Biofilms

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Supporting Information

1.) General Information:

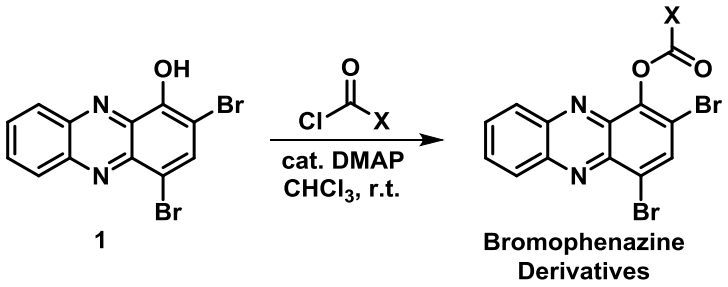
All reactions were carried out under an atmosphere of argon unless otherwise specified. All chemical reagents for synthesis were used without further purification. Analytical thin layer chromatography (TLC) was performed using 250 μm Silica Gel 60 F254 pre-coated plates (EMD Chemicals Inc.). Flash column chromatography was performed using 230-400 Mesh 60Å Silica Gel from Sorbent Technologies.

NMR experiments were recorded using broadband probes on a Varian Mercury-Plus-400 spectrometer via VNMR-J software (400 MHz for ^{1}H and 100 MHz for ^{13}C). All spectra are presented using MestReNova (Mnova) software and ^{1}H NMR are typically displayed from 10.0 to 1.7 ppm without the use of the signal suppression function (trace amounts of water, grease and TMS controls are omitted). Spectra were obtained in $CDCl_3$ with reference peaks: ^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77.23 ppm. All NMR experiments were performed at room temperature. Chemical shift values (δ) are reported in parts per million (ppm) for all ^{1}H NMR and ^{13}C NMR spectra. ^{1}H NMR multiplicities are reported as: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. High-resolution mass spectra were obtained for all new compounds from the Chemistry Department at the University of Florida.

All bromophenazine derivatives were stored as DMSO stocks at room temperature in the absence of light for several months at a time without observing any loss in biological activity. To ensure compound integrity of our DMSO stock solutions, we did not subject DMSO stocks of our bromophenazine derivatives to freeze-thaw cycles.

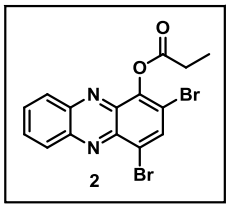
Microdilution minimum inhibitory concentration (MIC) experiments were carried out according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI). Luria-Bertani broth (LB) was used in MIC experiments against *S. aureus* (unless noted otherwise). Bacterial strains used: *Staphylococcus aureus* (ATCC 25923), MRSA-2 (methicillin-resistant *Staphylococcus aureus* clinical isolate from a patient treated at UF Shands Hospital), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (PAO1). Supporting images of representative MIC and biofilm experiments have been included. Bromophenazine **12** has been previously published (*Organic & Biomolecular Chemistry*, **2014**, *12*, 881-886.) and was used in ester hydrolysis experiments. Dose-response curves were generated to determine IC_{50} and EC_{50} values for *S. aureus* biofilm inhibitors and dispersal agents using GraphPad Prism 5.0 software.

2.) Chemical Synthesis Supporting Information:

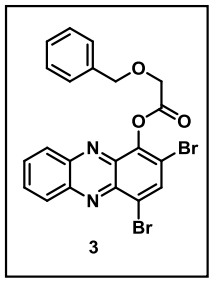


General procedure for derivative synthesis from 1-hydroxy-2,4-dibromophenazine 1: To a stirring solution of 1-hydroxy-2,4-dibromophenazine **1** (35 mg, 0.100 mmol), triethylamine (4.0 equivalents), and a catalytic amount of 4-dimethlyaminopyridine in chloroform (3 mL) was added the respective acid chloride or chloroformate reagent (1.2 equivalents) at room temperature. The reaction was allowed to stir for one hour before being quenched with an aqueous solution of saturated sodium bicarbonate. The resulting mixture was then transferred to a separatory funnel and ethyl acetate was added to extract the product. The organic layer was sequentially washed with sodium bicarbonate and brine before the organic layer was collected. The organic layer was then dried with anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. The respective bromophenazine derivative was then purified via flash column chromatography using hexanes:ethyl acetate:dichloromethane (91:1:8 to 82:10:8) to elute giving pure bromophenazine derivatives **2-11** as a yellow solid.

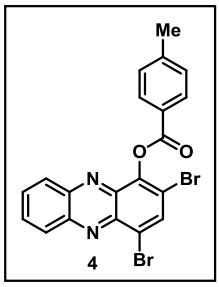
Characterization data for bromophenazine derivatives 2-11:



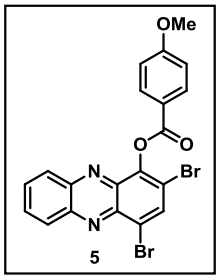
Yield: 76% yield; 49.3 mg of **2** was isolated as a yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.36 - 8.30 (m, 1H), 8.33 (s, 1H), 8.23 - 8.18 (m, 1H), 7.93 - 7.82 (m, 2H), 2.93 (q, *J* = 7.6 Hz, 2H), 1.46 (t, *J* = 7.6 Hz, 3H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 171.9, 145.2, 143.6, 143.5, 140.3, 137.7, 135.8, 132.2, 132.0, 130.1, 129.9, 122.1, 117.1, 27.7, 9.6.
HRMS (DART): calc. for $C_{15}H_{11}Br_2N_2O_2$ $[M+H]^+$: 410.9162, found: 410.9165.
MP: 126 - 127 °C.



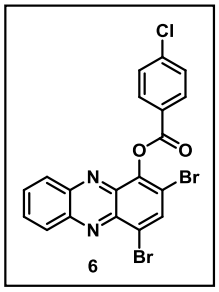
Yield: 88% yield; 68.9 mg of **3** was isolated as a yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.37 - 8.33 (m, 1H), 8.35 (s, 1H), 8.18 (m, 1H), 7.94 - 7.86 (m, 2H), 7.52 - 7.48 (m, 2H), 7.44 - 7.33 (m, 3H), 4.90 (s, 2H), 4.74 (s, 2H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 168.1, 144.6, 143.8, 143.5, 140.3, 137.4, 137.1, 135.7, 132.5, 132.1, 130.2, 129.8, 128.8, 128.6, 128.4, 122.6, 117.1, 73.7, 67.0.
HRMS (DART): calc. for $C_{21}H_{15}Br_2N_2O_3$ $[M+H]^+$: 502.9425, found: 502.9413.
MP: 99 - 101 °C.



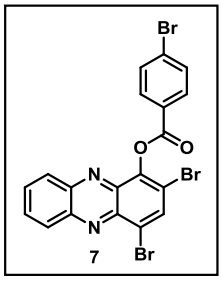
Yield: 96% yield; 19.3 mg of **4** was isolated as a yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.39 (s, 1H), 8.35 (ddd, *J* = 8.7, 1.5, 0.7 Hz, 1H), 8.31 - 8.26 (m, 2H), 8.15 (ddd, *J* = 8.6, 1.5, 0.7 Hz, 1H), 7.90 (ddd, *J* = 8.7, 6.6, 1.5 Hz, 1H), 7.83 (ddd, *J* = 8.6, 6.6, 1.5 Hz, 1H), 7.44 - 7.38 (m, 2H), 2.52 (s, 3H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 164.2, 145.5, 145.2, 143.6, 143.6, 140.3, 138.0, 135.8, 132.1, 131.9, 131.0, 130.1, 130.1, 129.6, 126.0, 122.2, 117.3, 22.1.
HRMS (DART): calc. for $C_{20}H_{13}Br_2N_2O_2$ $[M+H]^+$: 472.9319, found: 472.9314.
MP: 213 - 214 °C.



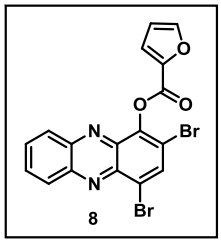
Yield: 83% yield; 37.7 mg of **5** was isolated as a yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.39 (s, 1H), 8.38 - 8.31 (m, 3H), 8.15 (ddd, *J* = 8.7, 1.6, 0.8 Hz, 1H), 7.90 (ddd, *J* = 8.8, 6.6, 1.6 Hz, 1H), 7.84 (ddd, *J* = 8.1, 6.7, 1.5 Hz, 1H), 7.11 - 7.05 (m, 2H), 3.95 (s, 3H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 164.6, 163.9, 145.6, 143.7, 143.7, 140.4, 138.2, 135.9, 133.2, 132.1, 132.0, 130.2, 130.1, 122.2, 121.1, 117.4, 114.3, 55.8.
HRMS (DART): calc. for $C_{20}H_{13}Br_2N_2O_3$ $[M+H]^+$: 488.9268, found: 488.9253.
MP: 215 - 216 °C.



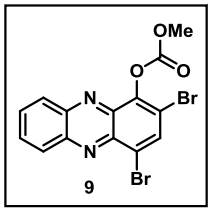
Yield: >99% yield; 16.4 mg of **6** was isolated as a yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.39 (s, 1H), 8.36 (m, 1H), 8.33 (d, *J* = 8.4 Hz, 2H), 8.13 (d, *J* = 8.7 Hz, 1H), 7.91 (ddd, *J* = 8.5, 6.6, 1.6 Hz, 1H), 7.85 (ddd, *J* = 8.3, 6.6, 1.5 Hz, 1H), 7.59 (d, *J* = 8.8 Hz, 2H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 163.4, 145.2, 143.7, 143.6, 140.9, 140.3, 137.8, 135.8, 132.3, 132.3, 132.1, 130.2, 130.1, 129.4, 127.3, 122.6, 117.3.
HRMS (DART): calc. for $C_{19}H_{10}Br_2ClN_2O_2$ $[M+H]^+$: 492.8771, found: 492.8767.
MP: 228 - 229 °C.



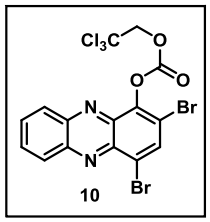
Yield: >99% yield; 20.5 mg of **7** was isolated as a yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.39 (s, 1H), 8.36 (d, *J* = 8.7 Hz, 1H), 8.25 (d, *J* = 7.7 Hz, 2H), 8.13 (d, *J* = 8.8 Hz, 1H), 7.91 (m, 1H), 7.85 (m, 1H), 7.76 (d, *J* = 8.0 Hz, 2H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 163.6, 145.2, 143.8, 143.6, 140.3, 137.8, 135.8, 132.4, 132.4, 132.3, 132.1, 130.2, 130.1, 129.7, 127.7, 122.6, 117.3.
HRMS (DART): calc. for $C_{19}H_{10}Br_3N_2O_2$ $[M+H]^+$: 538.8248, found: 538.8252.
MP: 232 - 235 °C.



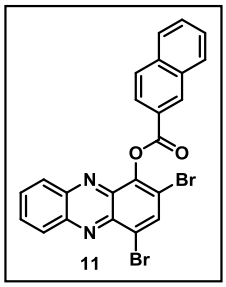
Yield: 75% yield; 58.4 mg of **8** was isolated as a yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.38 (s, 1H), 8.34 (ddd, *J* = 8.5, 1.6, 0.7 Hz, 1H), 8.18 (ddd, *J* = 8.5, 1.6, 0.7 Hz, 1H), 7.90 (ddd, *J* = 8.6, 6.6, 1.6 Hz, 1H), 7.85 (ddd, *J* = 8.2, 6.6, 1.6 Hz, 1H), 7.80 (dd, *J* = 1.8, 0.9 Hz, 1H), 7.64 (dd, *J* = 3.6, 0.9 Hz, 1H), 6.71 (dd, *J* = 3.5, 1.8 Hz, 1H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 155.6, 148.0, 144.5, 143.7, 143.6, 143.4, 140.3, 137.8, 135.8, 132.3, 132.1, 130.1, 130.1, 122.7, 121.0, 117.5, 112.7.
HRMS (DART): calc. for $C_{17}H_9Br_2N_2O_3$ $[M+H]^+$: 448.8955, found: 448.8957.
MP: 168 - 169 °C.



Yield: 65% yield; 41.4 mg of **9** was isolated as a yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.36 (m, 1H), 8.35 (s, 1H), 8.29 (m, 1H), 7.96 - 7.88 (m, 2H), 4.05 (s, 3H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 153.0, 144.6, 143.8, 143.6, 140.3, 137.7, 135.8, 132.5, 132.2, 130.2, 130.0, 122.7, 116.9, 56.6.
HRMS (DART): calc. for $C_{14}H_9Br_2N_2O_3$ $[M+H]^+$: 412.8955, found: 412.8953.
MP: 167 - 168 °C.



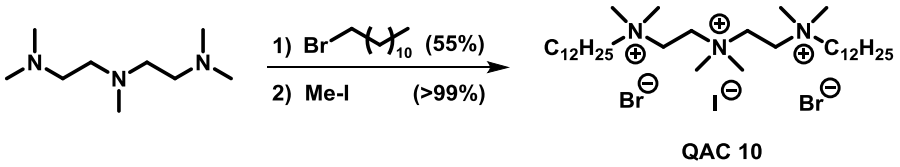
Yield: 82% yield; 32.8 mg of **10** was isolated as a yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.39 - 8.34 (m, 1H), 8.36 (s, 1H), 8.22 (m, 1H), 7.97 - 7.89 (m, 2H), 5.01 (s, 2H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 151.5, 144.1, 143.9, 143.6, 140.2, 137.3, 135.7, 132.7, 132.3, 130.2, 129.9, 123.3, 116.9, 94.3, 78.0.
HRMS (DART): calc. for $C_{15}H_8Br_2Cl_3N_2O_3$ $[M+H]^+$: 530.7915, found: 530.7900.
MP: 135 - 136 °C.



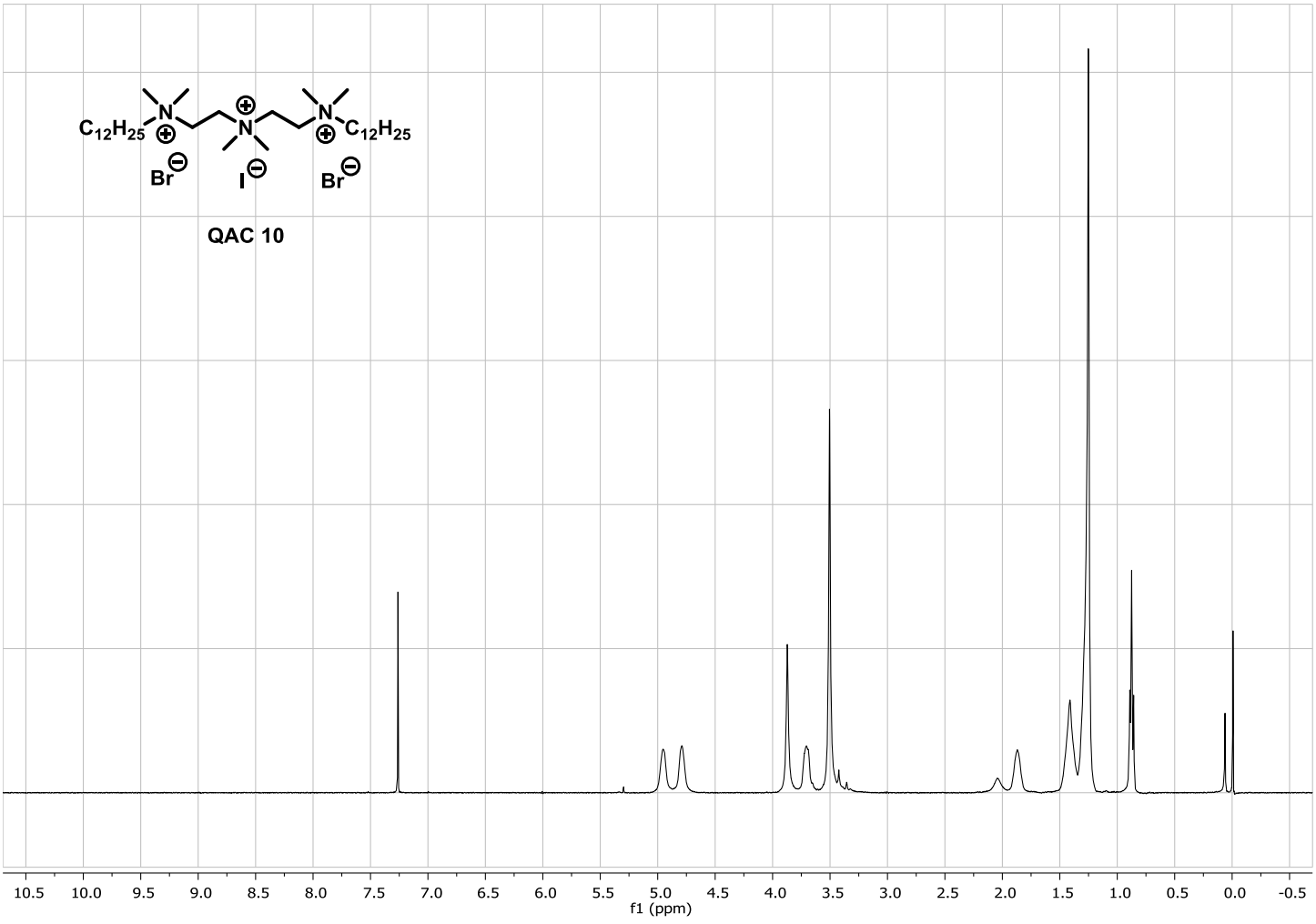
Yield: 97% yield; 80.6 mg of **11** was isolated as a yellow solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ 9.02 (d, *J* = 1.6 Hz, 1H), 8.42 (s, 1H), 8.36 (ddd, *J* = 8.7, 1.4, 0.7 Hz, 1H), 8.35 (dd, *J* = 8.7, 1.8 Hz, 1H), 8.13 (ddd, *J* = 8.7, 1.5, 0.7 Hz, 1H), 8.07 (ddd, *J* = 8.6, 1.2, 0.6 Hz, 1H), 8.04 (d, *J* = 8.6, 1H), 7.98 (dd, *J* = 8.0, 0.4 Hz, 1H), 7.90 (ddd, *J* = 8.7, 6.6, 1.5 Hz, 1H), 7.82 (ddd, *J* = 8.6, 6.6, 1.5 Hz, 1H), 7.69 (ddd, *J* = 8.2, 6.9, 1.4 Hz, 1H), 7.62 (ddd, *J* = 8.1, 6.9, 1.3 Hz, 1H).
^{13}C NMR (100 MHz, $CDCl_3$): δ 164.4, 145.5, 143.7, 143.6, 140.4, 138.0, 136.3, 135.9, 132.9, 132.7, 132.2, 132.0, 130.1, 130.1, 129.8, 129.1, 128.8, 128.1, 127.2, 126.0, 126.0, 122.4, 117.4.
HRMS (DART): calc. for $C_{23}H_{13}Br_2N_2O_2$ $[M+H]^+$: 508.9319, found: 508.9307.
MP: 220 - 221 °C.

Synthesis of QAC 10:

We synthesized known biofilm-eradicating quaternary ammonium cation **QAC 10** reported by Wuest and co-workers to serve as a positive control in our biofilm eradication assays against MRSA-2. We were able to follow the reported two-step synthesis from available materials to obtain 226 milligrams (55% yield over 2 steps) of **QAC 10**. Our ^{1}H NMR data is identical to previously reported values (*ChemBioChem* 2014, **15**, 2211.).



QAC 10



QAC 10

3.) *Staphylococcus aureus* Supporting Information:

***S. aureus* (ATCC 25923) Minimum Inhibitory Concentration (MIC) Susceptibility Assay Protocol:**

The minimum inhibitory concentration (MIC) for each phenazine 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 Luria Broth (one column served as a blank). Each well was inoculated with ~10^5 bacterial cells at the initial time of incubation, prepared from a fresh log phase culture (OD_{600} ~ 0.5). The MIC is defined as the lowest concentration of compound required to completely inhibit bacterial growth following 16 to 20 hours of incubation at 37 °C. The concentration range tested for each bromophenazine during this study was 0.10 to 100 µM. All phenazine compounds were evaluated from 10 mM DMSO stock solutions that were stored at room temperature in the absence of light. DMSO served as our vehicle and negative control in each microdilution MIC assay. DMSO was serially diluted at the same concentration as the bromophenazine compounds with a top concentration of 1% v/v.

Image of MIC experiment for new bromophenazine against *S. aureus* in LB medium

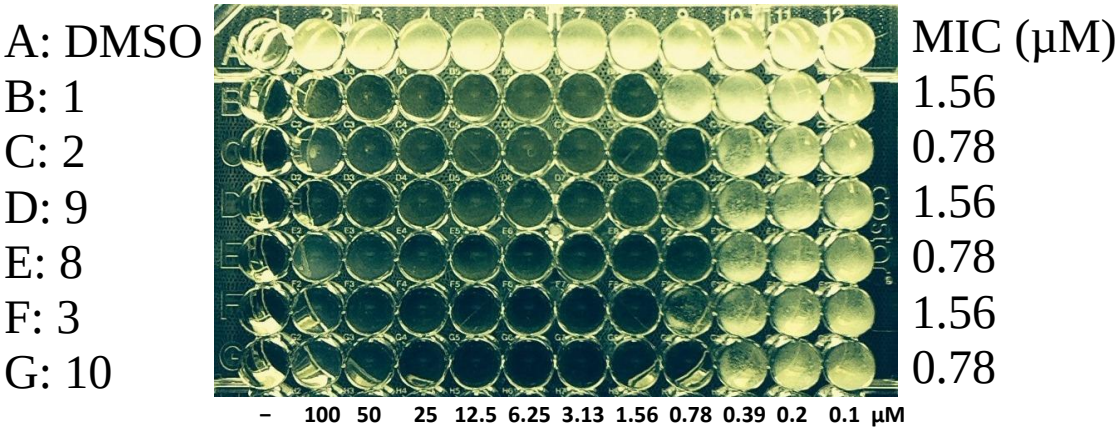
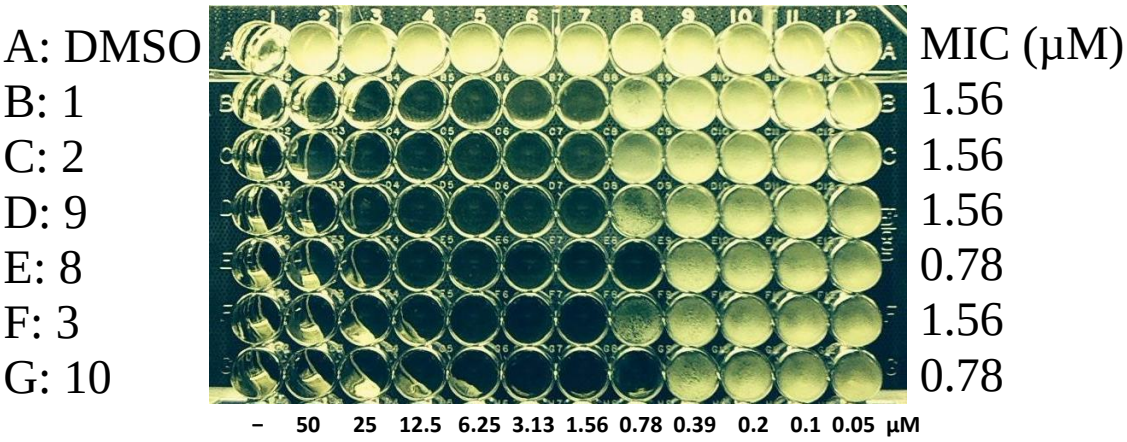


Image of MIC experiment for new bromophenazine against *S. aureus* in TSB medium

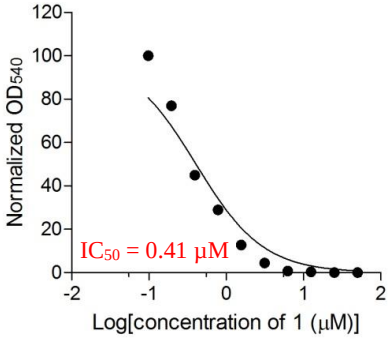


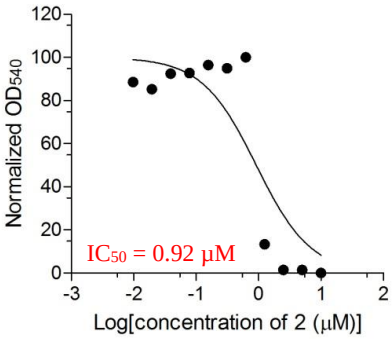
NOTE: The same MIC assay conditions were used to test bromophenazines **1-11** against *A. baumannii* (ATCC 19606) and *P. aeruginosa* (PAO1). A summary of all biological investigations reported here are on page S15.

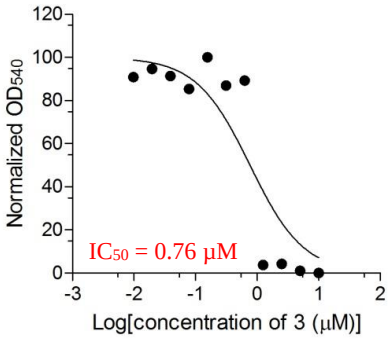
Biofilm Inhibition Protocol for *S. aureus* (ATCC 25923):

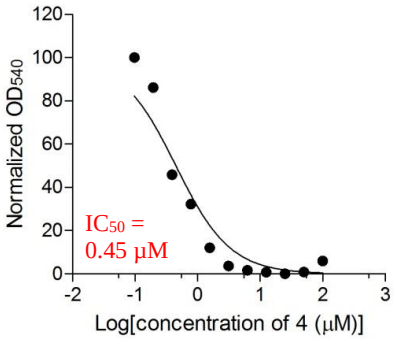
A serial two-fold dilution of 2X bromophenazine small molecule concentration was made in 100 µL tryptic soy broth (TSB) medium with 0.5% glucose was delivered into 0.1% gelatin (Millipore) coated 96-well tissue culture plates. The same volume of DMSO (vehicle control), was used as a negative control and did not go over 1% v/v in biofilm inhibition assays. To each microtiter well, 100 µL of TSB with 0.5% glucose containing 2×10^6 $CFUmL^{-1}$ *Staphylococcus aureus* cells, prepared from fresh culture (OD_{600} ~ 0.8), was added. The plates were incubated at 37 °C for 24 hours. The wells were gently rinsed by submerging the entire plates in a tub of cold, running tap water. The wells were then fixed with 200 µL methanol for 15 minutes. After the plates were air dried, the biofilms were stained with 100 µL of 1% crystal violet for 10 minutes. The plates were again rinsed with running water. After drying in air, quantitative assessment of biofilm formation was obtained by extracting the crystal violet associated with the remaining biofilm with 100 µL per well of the following bleaching solution (methanol:glacial acetic acid:water (v/v/v) = 4:1:5). This bleaching solution dissolved the bound crystal violet and produced a violet-colored solution in each well. The intensity of coloration was determined by measuring the absorbance at 540 nm.

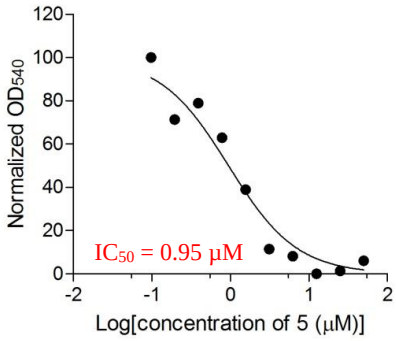
Dose-response curves for biofilm inhibition against *S. aureus* ATCC 25923 from preliminary screen (single replicate data):

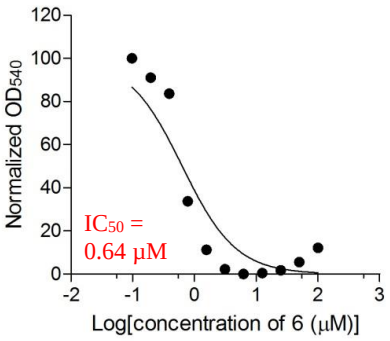


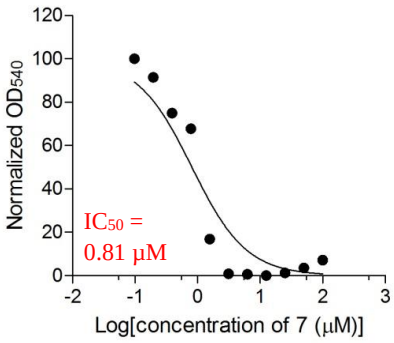


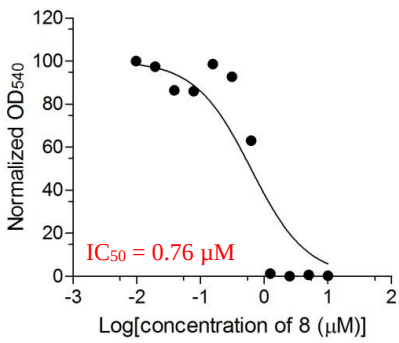


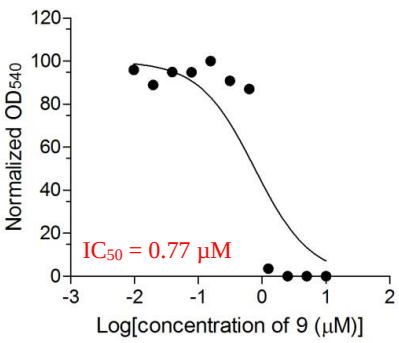


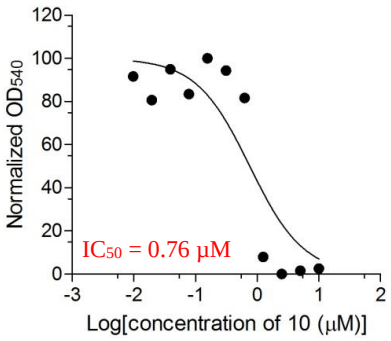






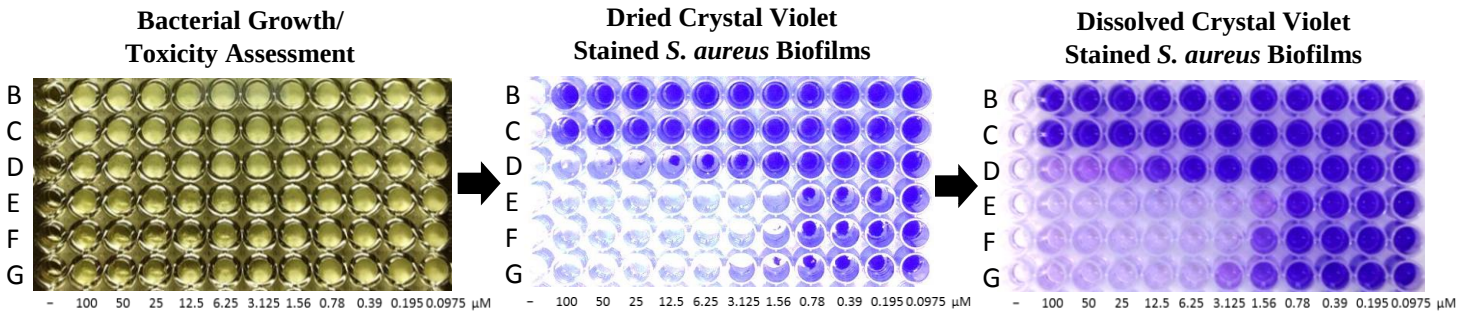






Biofilm Inhibition Assays (in triplicate) for Lead Biofilm Inhibitors against *S. aureus* (ATCC 25923):

Images of a single biofilm inhibition assay from planktonic growth assessment to crystal violet staining of *S. aureus* biofilms.



Key to images of *S. aureus* biofilm inhibition

B. no chemical
C. DMSO
D. Bromophenazine 5 (planktonic growth/biofilm inhibition; MIC >100 µM / IC_{50} = 10.3 µM)
E. Bromophenazine 4 (planktonic growth/biofilm inhibition; MIC >100 µM / IC_{50} = 0.55 µM)
F. Bromophenazine 6 (planktonic growth/biofilm inhibition; MIC >100 µM / IC_{50} = 0.77 µM)
G. Bromophenazine 7 (planktonic growth/biofilm inhibition; MIC >100 µM / IC_{50} = 1.13 µM)

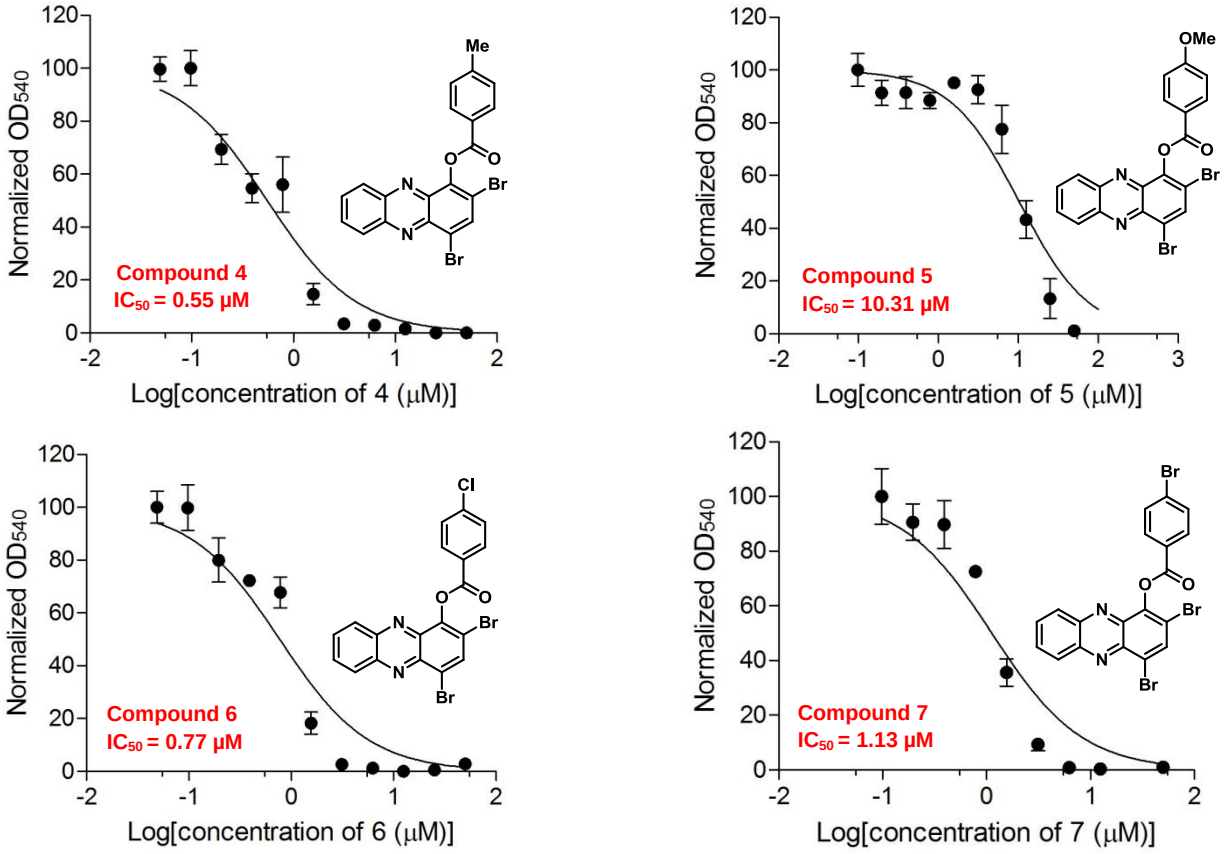
Spectrophotometer readings of crystal violet staining (OD540)

Plate01	1	2	3	4	5	6	7	8	9	10	11	12
Blank	0.056	0.056	0.057	0.06	0.055	0.061	0.066	0.058	0.062	0.065	0.057	0.054
No chemical	0.077	1.314	1.158	1.139	1.115	1.222	1.157	1.105	1.141	1.16	1.178	1.495
DMSO	0.083	1.182	1.204	1.195	1.112	1.205	1.243	1.22	1.113	1.145	1.103	1.442
5	0.089	0.26	0.312	0.363	0.677	1.063	1.137	1.254	1.153	1.077	1.102	1.398
4	0.081	0.129	0.126	0.131	0.151	0.153	0.162	0.239	0.854	0.956	1.019	1.588
6	0.082	0.201	0.158	0.12	0.115	0.137	0.138	0.469	1.148	1.085	1.377	1.645
7	0.089	0.214	0.145	0.135	0.157	0.148	0.314	0.605	1.014	1.458	1.415	1.609
blank	0.054	0.063	0.06	0.061	0.058	0.053	0.061	0.058	0.065	0.059	0.058	0.054

Plate02	1	2	3	4	5	6	7	8	9	10	11	12
Blank	0.059	0.055	0.057	0.059	0.057	0.057	0.08	0.06	0.062	0.061	0.061	0.068
No chemical	0.075	1.14	1.231	1.208	1.227	1.227	1.193	1.215	1.273	1.199	1.142	1.215
DMSO	0.083	1.188	1.105	1.192	1.486	1.225	1.237	1.178	1.199	1.149	1.226	1.293
5	0.081	0.276	0.241	0.28	0.585	0.88	1.158	1.232	1.214	1.205	1.195	1.252
4	0.085	0.16	0.125	0.157	0.187	0.212	0.209	0.313	0.623	0.847	0.904	1.371
6	0.105	0.309	0.146	0.142	0.145	0.156	0.198	0.317	0.98	1.008	1.027	1.32
7	0.106	0.222	0.157	0.163	0.143	0.166	0.239	0.464	1.016	1.15	1.179	1.196
Blank	0.054	0.052	0.055	0.051	0.055	0.054	0.054	0.055	0.055	0.057	0.059	0.075

Plate03	1	2	3	4	5	6	7	8	9	10	11	12
Blank	0.059	0.062	0.067	0.063	0.068	0.064	0.061	0.06	0.057	0.082	0.063	0.062
No chemical	0.081	1.226	1.307	1.46	1.302	1.308	1.409	1.182	1.189	1.469	1.138	1.224
DMSO	0.093	1.254	1.351	1.577	1.602	1.648	1.499	1.491	1.43	1.302	1.156	1.168
5	0.094	0.237	0.256	0.539	0.835	1.202	1.31	1.198	1.111	1.289	1.27	1.182
4	0.09	0.155	0.155	0.115	0.124	0.15	0.165	0.417	1.089	0.71	1.156	1.304
6	0.094	0.306	0.181	0.131	0.113	0.129	0.141	0.299	0.887	1.095	1.086	1.296
7	0.085	0.301	0.162	0.131	0.141	0.148	0.219	0.671	1.067	1.12	1.164	1.299
blank	0.057	0.057	0.058	0.054	0.059	0.057	0.064	0.056	0.056	0.061	0.055	0.056

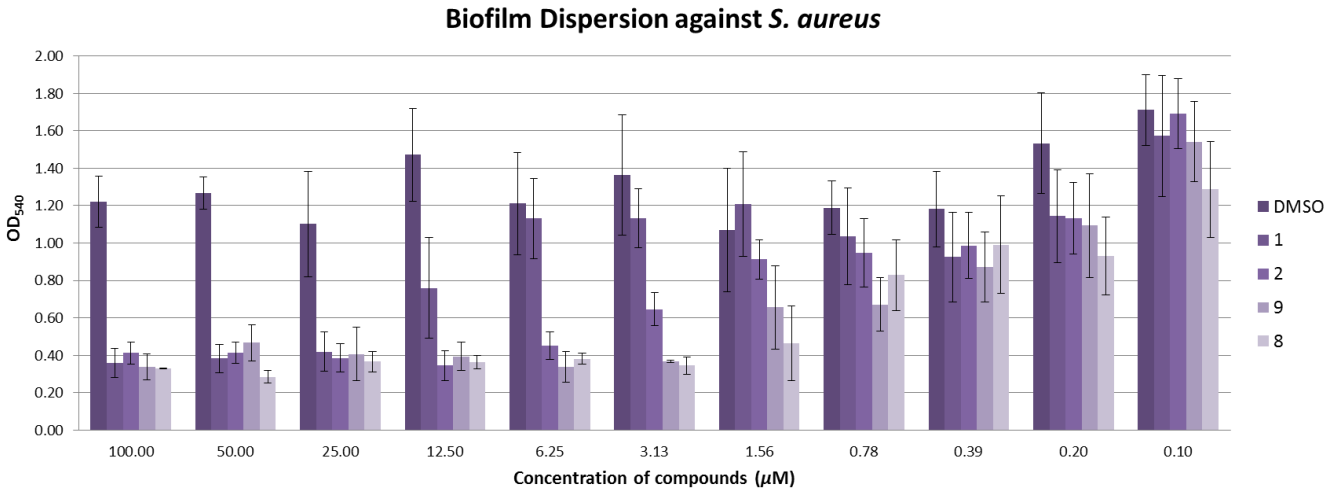
Dose-response curves for biofilm inhibition against *S. aureus* ATCC 25923 (triplicate data):



Biofilm Dispersion Protocols for *Staphylococcus aureus* ATCC 25923 and MRSA-2:

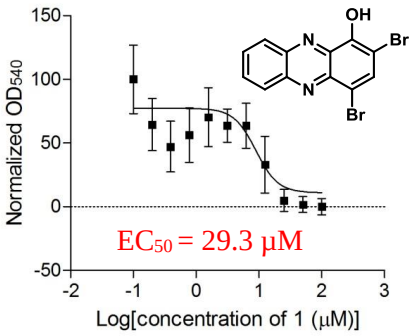
***S. aureus* ATCC 25923 Dispersion Assay:** A single colony grown on tryptic soy agar (TSA) solid medium was amplified in 2 milliliters of tryptic soy broth (TSB) medium with 0.5% glucose to an OD_{600} ~ 1.0. The bacterial suspension was then diluted to ~1×10^6 $CFUmL^{-1}$ in TSB with 0.5% glucose. Sterile 96-well flat-bottomed polystyrene plates (0.1% gelatin coated overnight) were then filled with 200 µL of this bacterial suspension. The plates were then covered and incubated for 24 hours at 37 °C. Following this, the contents of the wells were discarded and washed with 200 µL PBS (one time). Then serial, two-fold dilutions of bromophenazine small molecules in 200 µL PBS were delivered into each well. The plates were then covered and incubated for another 24 hours at room temperature. After this incubation time, the plates were gently washed with water three times (wells were rinsed by submerging the entire plates in a tub of cold, running tap water). Remaining biofilms were then fixed with 200 µL of methanol for 15 minutes. The plates were emptied and left to air dry. Upon drying, 100 µL of 1% crystal violet was added to each well for 10 minutes, then washed three times with water and again air dried. For quantitative assessment of biofilm formation 100 µL per well of bleaching solution (methanol:glacial acetic acid:water (v/v/v) = 4:1:5 with rotary shaking for 1 hour) was used. Remaining biofilm was measured using a spectrophotometer (absorbance readings at 540 nm). **NOTE:** The biofilm dispersion effectiveness of the dispersal active bromophenazines under these assay conditions was ~75% effective at 100 µM (highest concentration tested) and served as the "100%" dispersion in our dose response curves to determine our EC_{50} values (see Supporting Figure 1).

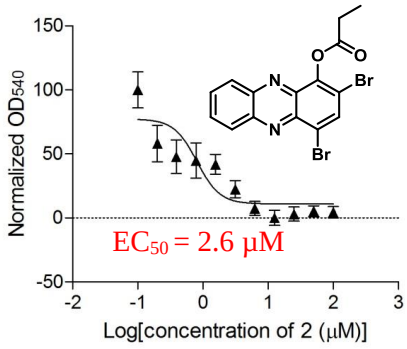
MRSA-2 Dispersion Assay: Biofilm dispersion assays were set up the same way as *S. aureus*; however, following biofilm establishment and wash, compounds were added to wells in 100 µL TSB media with 0.5% glucose and allowed to incubate at 37 °C for 24 hours. Following compound treatment, the contents of the 96-well plates were removed, washed, fixed with methanol, stained with crystal violet and quantified for biofilm dispersion as stated in the above biofilm dispersion procedure. **NOTE:** Complete biofilm dispersion of MRSA-2 was observed with bromophenazine **1** under these assay conditions.

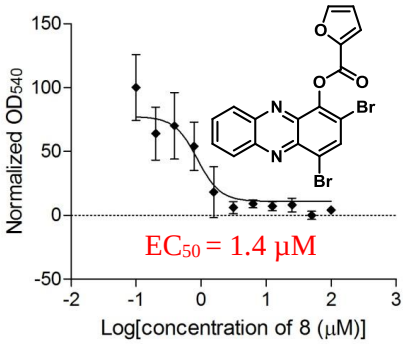


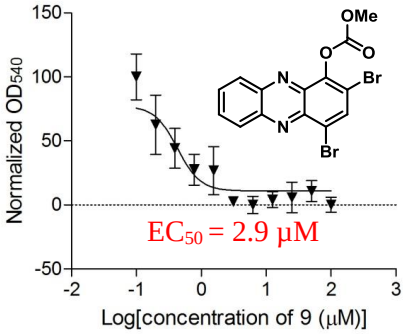
Supporting Figure 1. Biofilm dispersion activity of bromophenazines **1**, **2**, **9** and **8** against *S. aureus* (ATCC 25923) biofilms at each concentration tested (0.1-100 µM).

<u>Dose-response curves for Bromophenazines vs. *Staphylococcus aureus* ATCC 25923 from biofilm dispersion assays:</u>
Note: maximum biofilm dispersion was ~75% in these assays



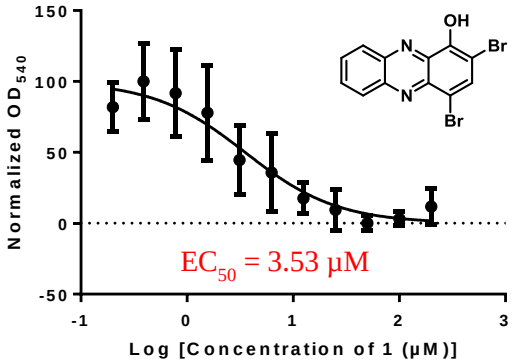






Dose-response curves for Bromophanezine 1 against methicillin-resistant *Staphylococcus aureus* MRSA-2 from biofilm dispersion assays:

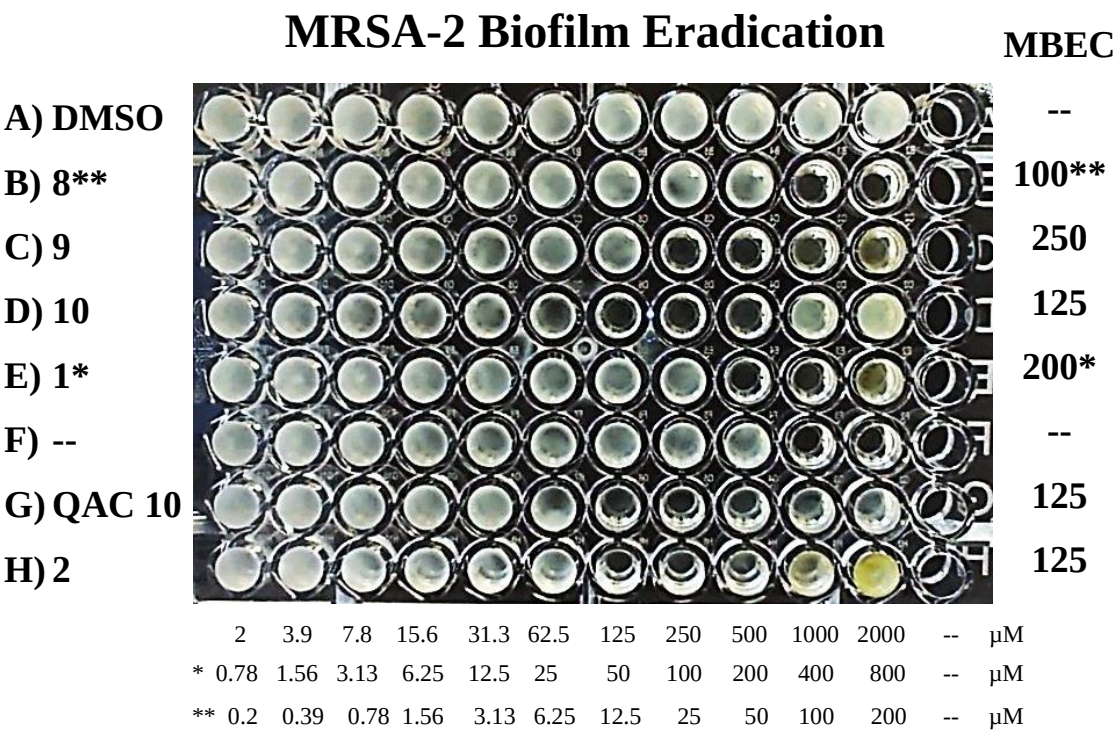
Note: observed complete biofilm dispersion against MRSA-2



Biofilm Eradication Protocol for methicillin-resistant *Staphylococcus aureus* (MRSA-2):

A single colony grown on LB agar solid medium was amplified in 2 milliliters of tryptic soy broth (TSB) medium with 0.5% glucose to an OD_{600} ~ 1.0. The bacterial suspension was then diluted to ~1×10^6 $CFUmL^{-1}$ in TSB with 0.5% glucose. Sterile 96-well flat-bottomed polystyrene plates (0.1% gelatin coated overnight) were then filled with 100 µL of this bacterial suspension. The plates were then covered and incubated for 24 hours at 37 °C. Following this, the contents of the wells were discarded and washed with water three times (wells were rinsed by submerging the entire plates in a tub of cold, running tap water). Then serial, two-fold dilutions of test compound in 100 µL TSB 0.5% Glucose were delivered into each well at concentrations ranging from 2 to 2,000 µM (DMSO did not exceed 2% v/v in these assays). The plates were then covered and incubated for 24 hours at 37 °C. After this time, the plates were gently washed with water three times (wells were rinsed by submerging the entire plates in a tub of cold, running tap water). The remaining biofilms were allowed to recover by adding 100 µL of TSB with 0.5 % glucose to each well for 24 hours at 37 °C. At the end of this final incubation, wells that were turbid result from live biofilms and completely eradicated biofilms resulted in wells that had no turbidity (see supporting image on next page). Minimum Biofilm Eradication Concentration (MBEC) values were determined as the lowest concentration of a test compound that resulted in no turbidity after final incubation.

MRSA-2 Biofilm Eradication Assay (Supporting Image):



NOTE: DMSO stock solutions were made at either 10, 40 or 100 mM for each compound tested in biofilm eradication assays. Some compounds were insoluble at the highest concentrations tested in this assay. The compound in lane F of this supporting image was not part of this study.

Complete Summary of Antibacterial and Antibiofilm Studies:

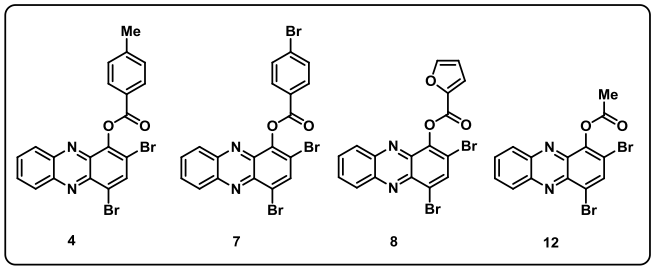
Compound	*S. aureus* MIC	MRSA-2 MIC	*A. baumannii* MIC	PAO1 MIC	*S. aureus* Biofilm Inhibition	*S. aureus* Biofilm Dispersion	MRSA-2 Biofilm Dispersion	MRSA-2 Biofilm Eradication
1	**1.56**	**1.56**	**50**	**>100**	**0.41**	**29.3**	**3.53**	**100-200**
2	**1.56**	**--**	**>100**	**>100***	**0.92**	**2.6**	**--**	**125**
3	**1.56**	**--**	**50**	**>100**	**0.76**	**>100**	**--**	**--**
4	**>100**	**--**	**>100**	**>100**	**0.55**	**>100**	**--**	**--**
5	**>100**	**--**	**>100**	**>100**	**10.3**	**>100**	**--**	**--**
6	**>100**	**--**	**>100**	**>100**	**0.77**	**>100**	**--**	**--**
7	**>100**	**--**	**>100**	**>100**	**1.13**	**>100**	**--**	**--**
8	**0.78**	**--**	**>100**	**>100***	**0.76**	**1.4**	**--**	**62.5-100**
9	**1.56**	**--**	**>100**	**>100***	**0.77**	**2.9**	**--**	**250**
10	**0.78**	**--**	**>100**	**>100***	**0.76**	**>100**	**--**	**125**
11	**>100**	**--**	**>100**	**>100**	**>100**	**>100**	**--**	**--**
QAC 10	**--**	**--**	**--**	**--**	**--**	**--**	**--**	**62.5-125**
vancomycin	**--**	**0.78**	**--**	**--**	**--**	**--**	**--**	**>2,000**

Note: All values are reported in µM. MIC values determined by turbidity. Biofilm inhibition is determined by crystal violet staining and reported via IC_{50} values. Biofilm dispersion is determined by crystal violet staining and reported via EC_{50} values. Biofilm eradication is determined by turbidity and reported via MBEC values. Bacterial strains: *S. aureus* (ATCC 25923), MRSA-2 (methicillin-resistant *Staphylococcus aureus* strain from a patient treated at UF Shands Hospital), *A. baumannii* (ATCC 19606), *P. aeruginosa* (PAO1). *MIC >1,000 µM when tested at higher concentrations. "--" is used for compounds that were not tested in the corresponding assay.

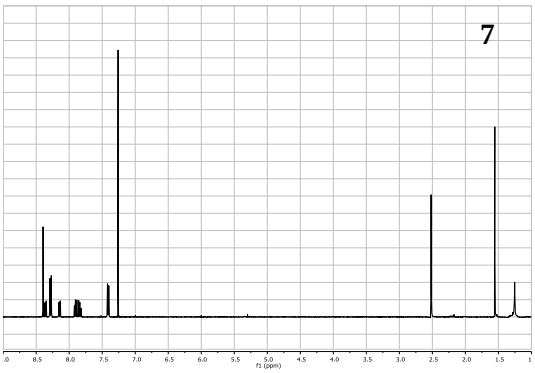
All data in this summary was generated from 2 to 4 independent experiments during these investigations.

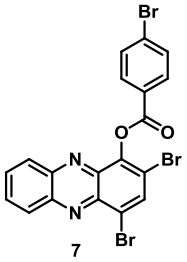
4.) Bromophenazine Ester Stability Test:

Attempts to hydrolyze bromophenazine esters **4**, **7**, **8** and **12** were made by treating 3.2 to 4.5 mgs of each bromophenazine derivative with 1 mL of sterile water. Each reaction was allowed to incubate for 24 hours at 37 °C under static conditions analogous to the *S. aureus* biofilm inhibition assays described in this work. After 24 hours, all reactions were extracted with dichloromethane and evaluated for hydrolysis of bromophenazine esters to bromophenazine **1** (via TLC and NMR). No ester hydrolysis or degradation was observed for any bromophenazine ester in this study (^{1}H NMR spectra below).

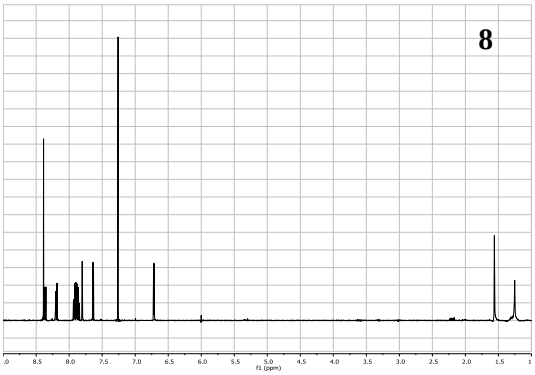


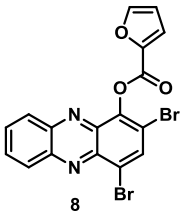
Crude ^{1}H NMRs after extraction.



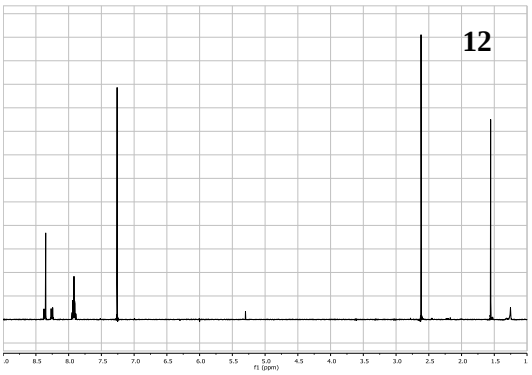


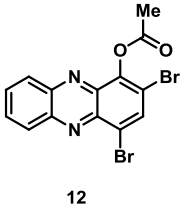
^{1}H NMR following hydrolysis attempt shows not hydrolysis or degradation of bromophenazine **7**.





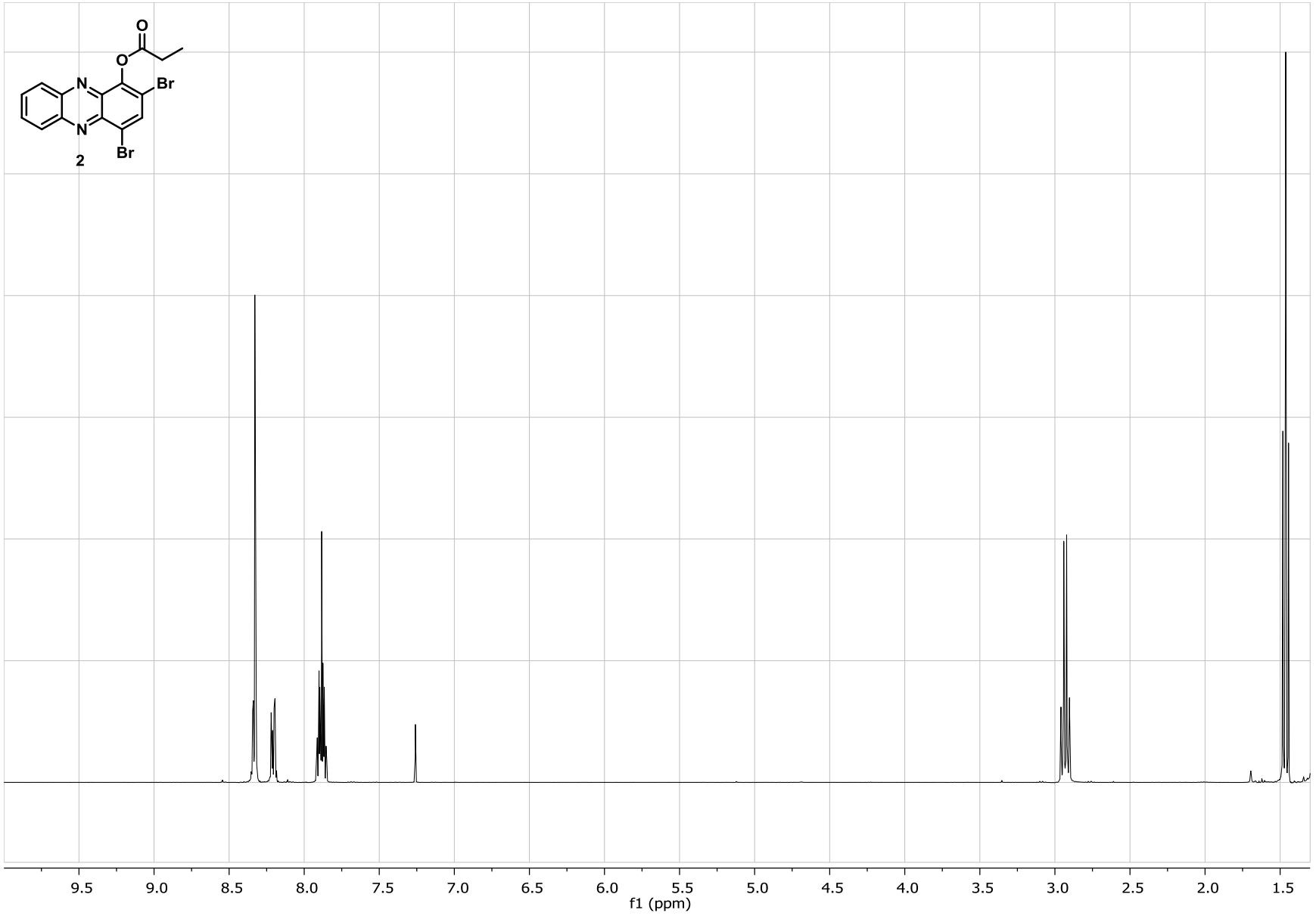
^{1}H NMR following hydrolysis attempt shows not hydrolysis or degradation of bromophenazine **8**.

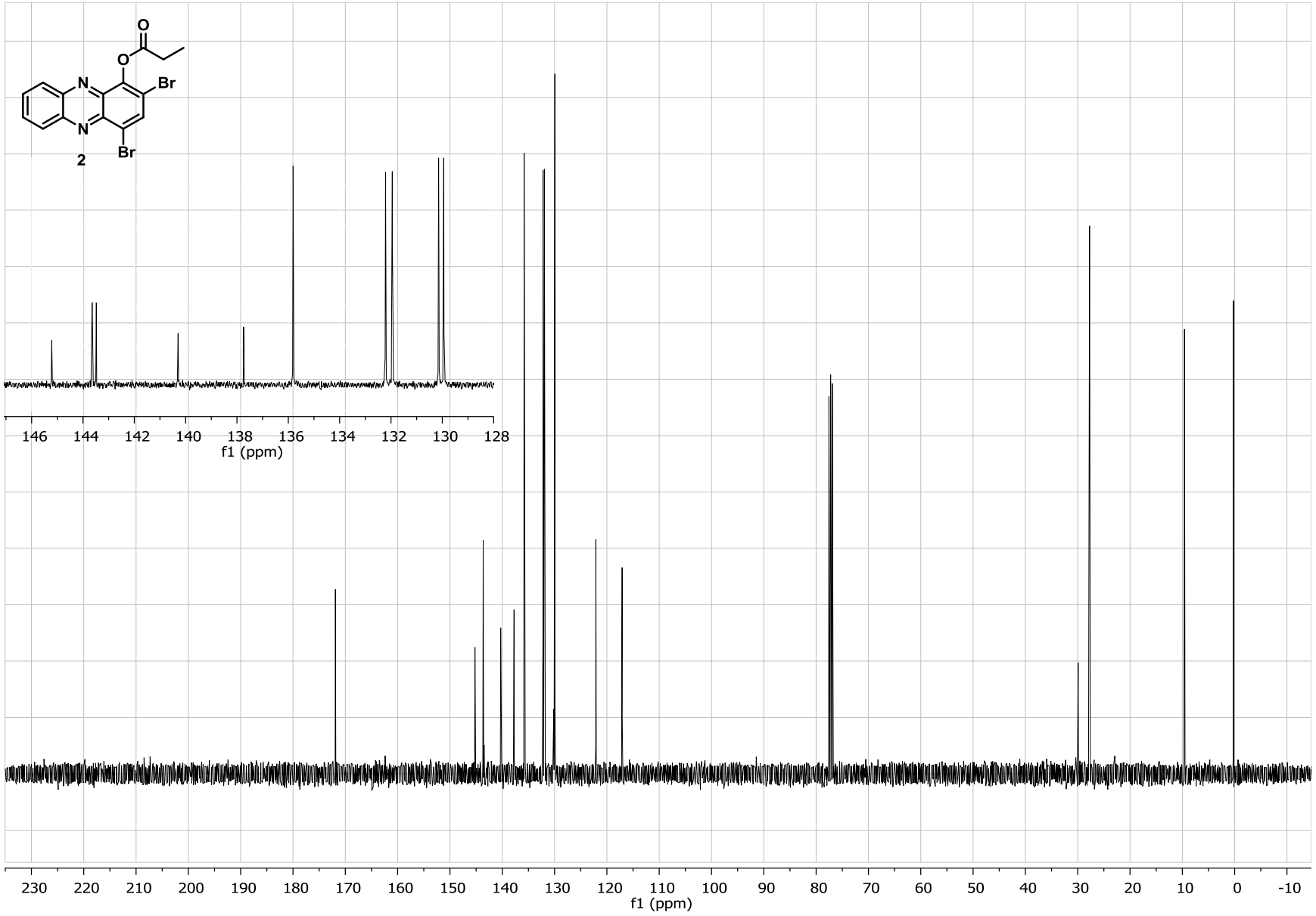


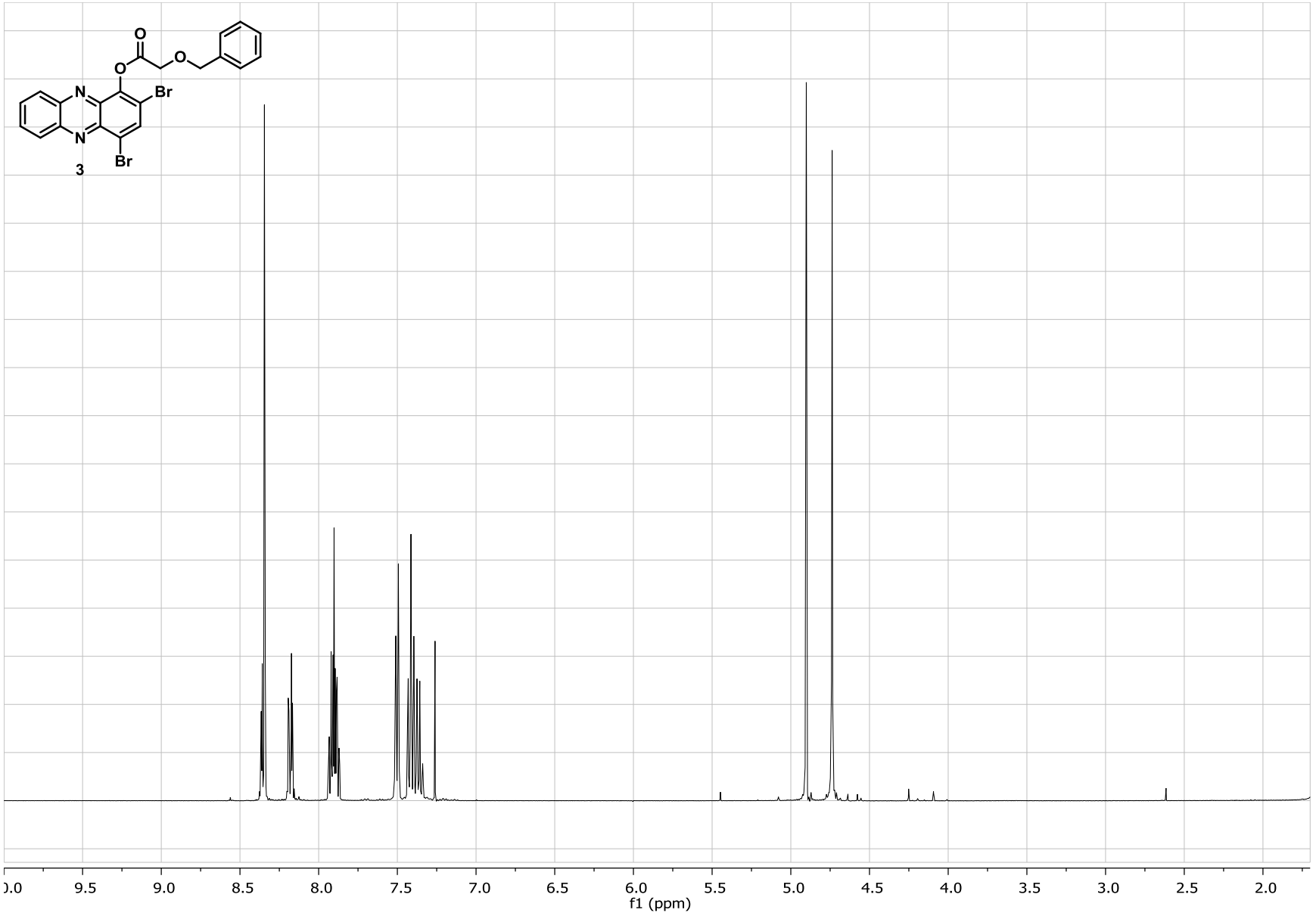


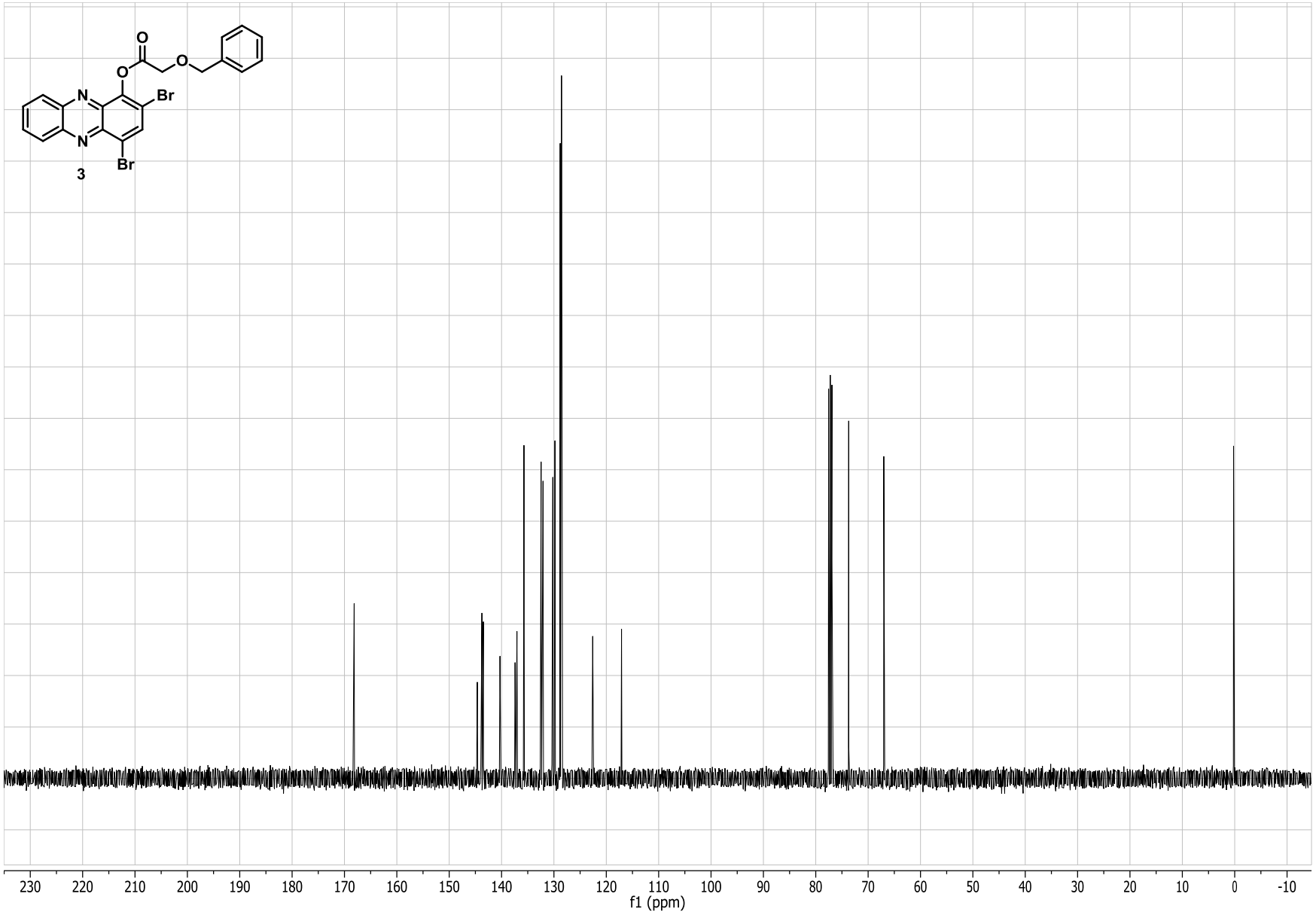
^{1}H NMR following hydrolysis attempt shows not hydrolysis or degradation of bromophenazine **12**.

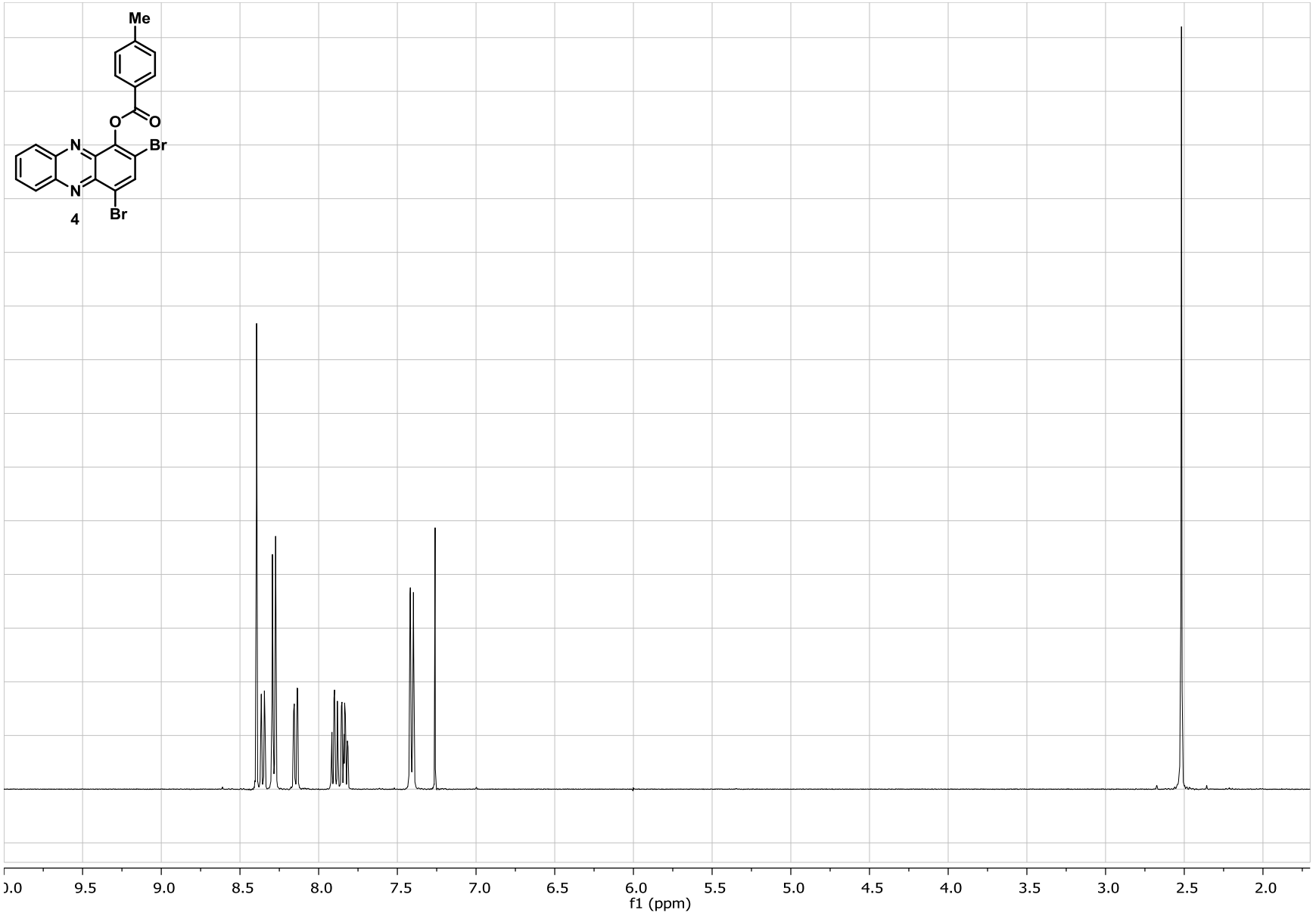
5.) ^{1}H NMR & ^{13}C NMR spectra for bromophenazine derivatives:

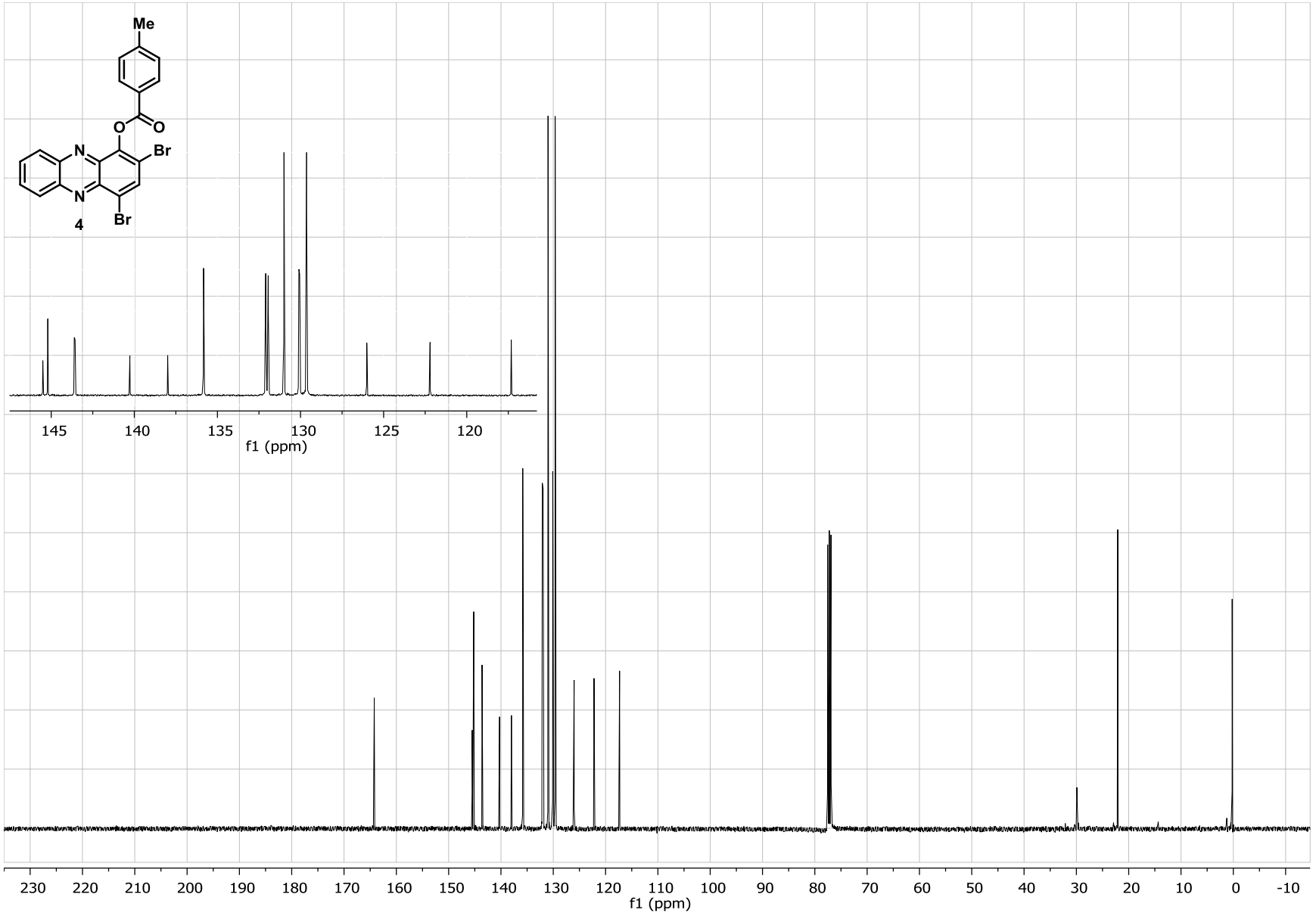

O
O
N
Br
N
Br
2
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
f1 (ppm)

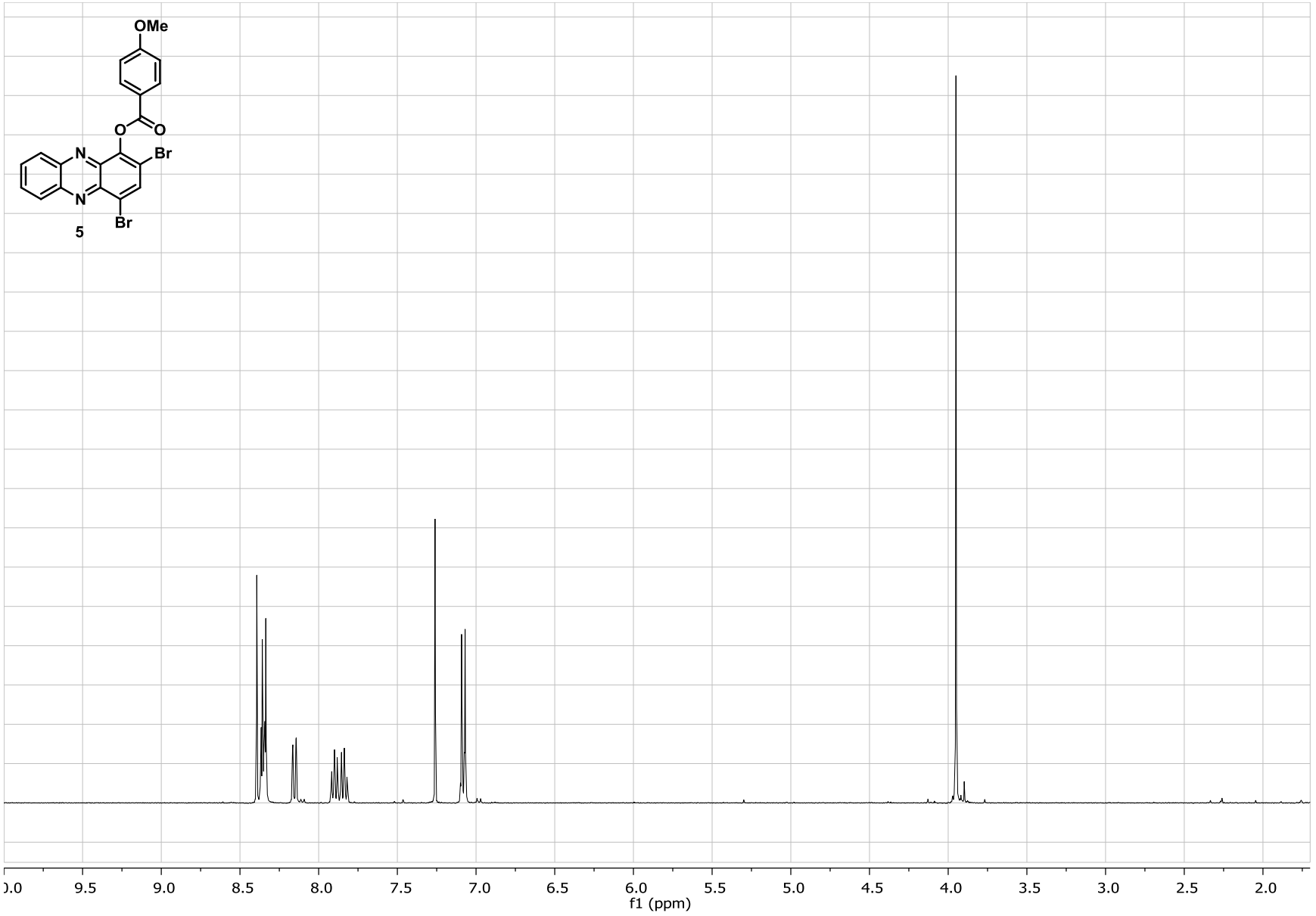

O
O
N
N
Br
Br
2
146
144
142
140
138
136
134
132
130
128
f1 (ppm)
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

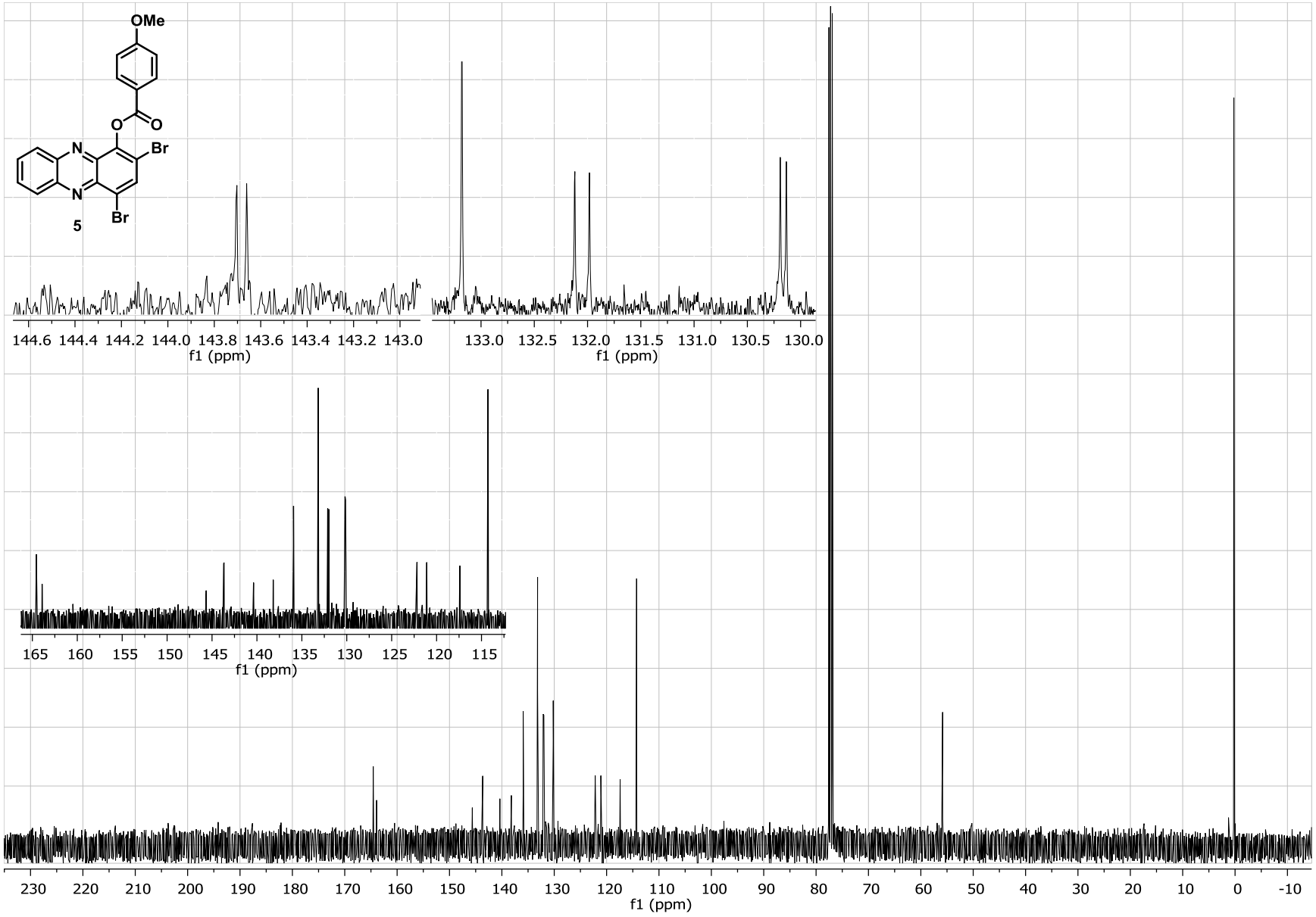

O
O
O
N
N
Br
Br
3
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
f1 (ppm)

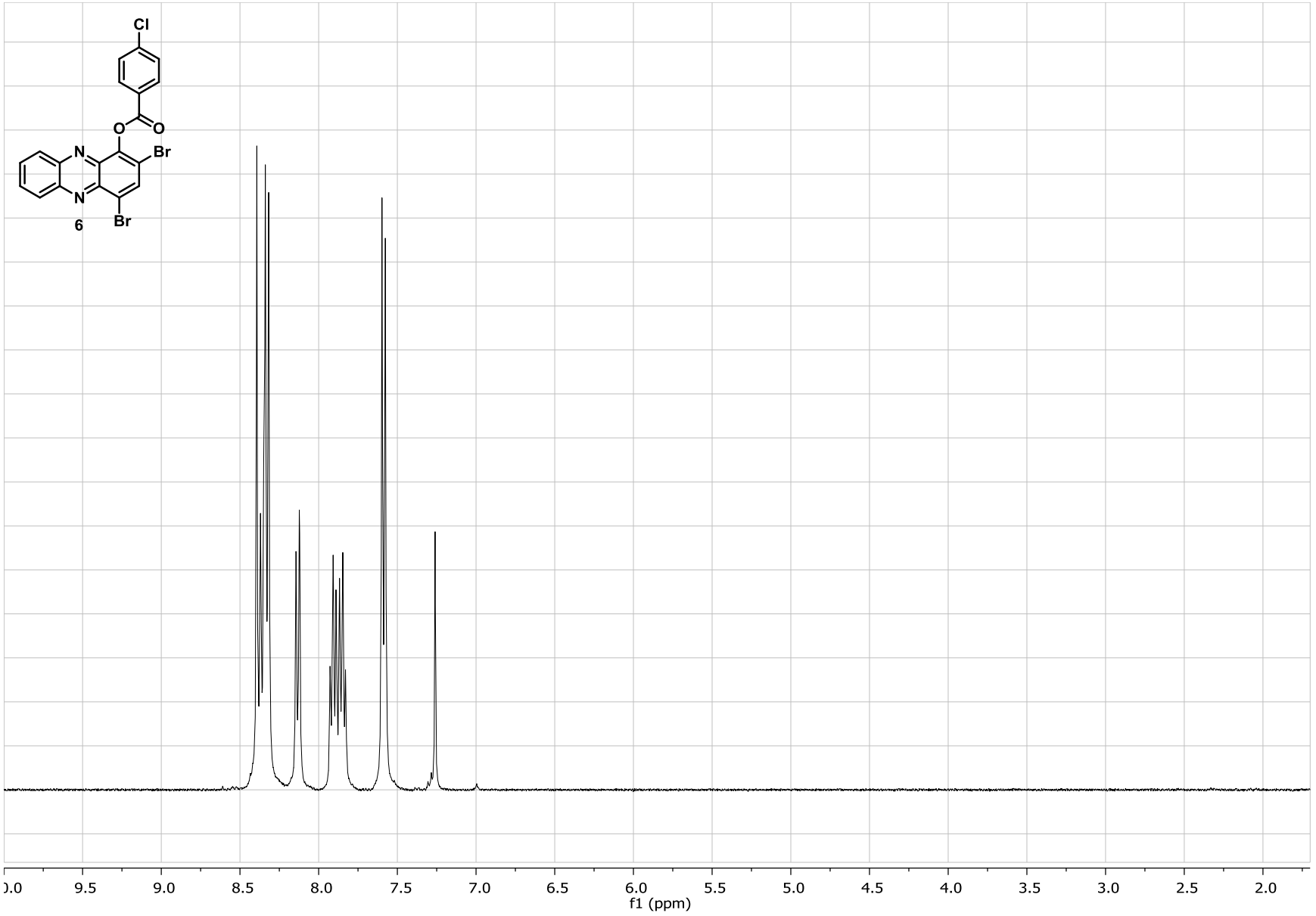

O
O
O
N
N
Br
Br
3
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

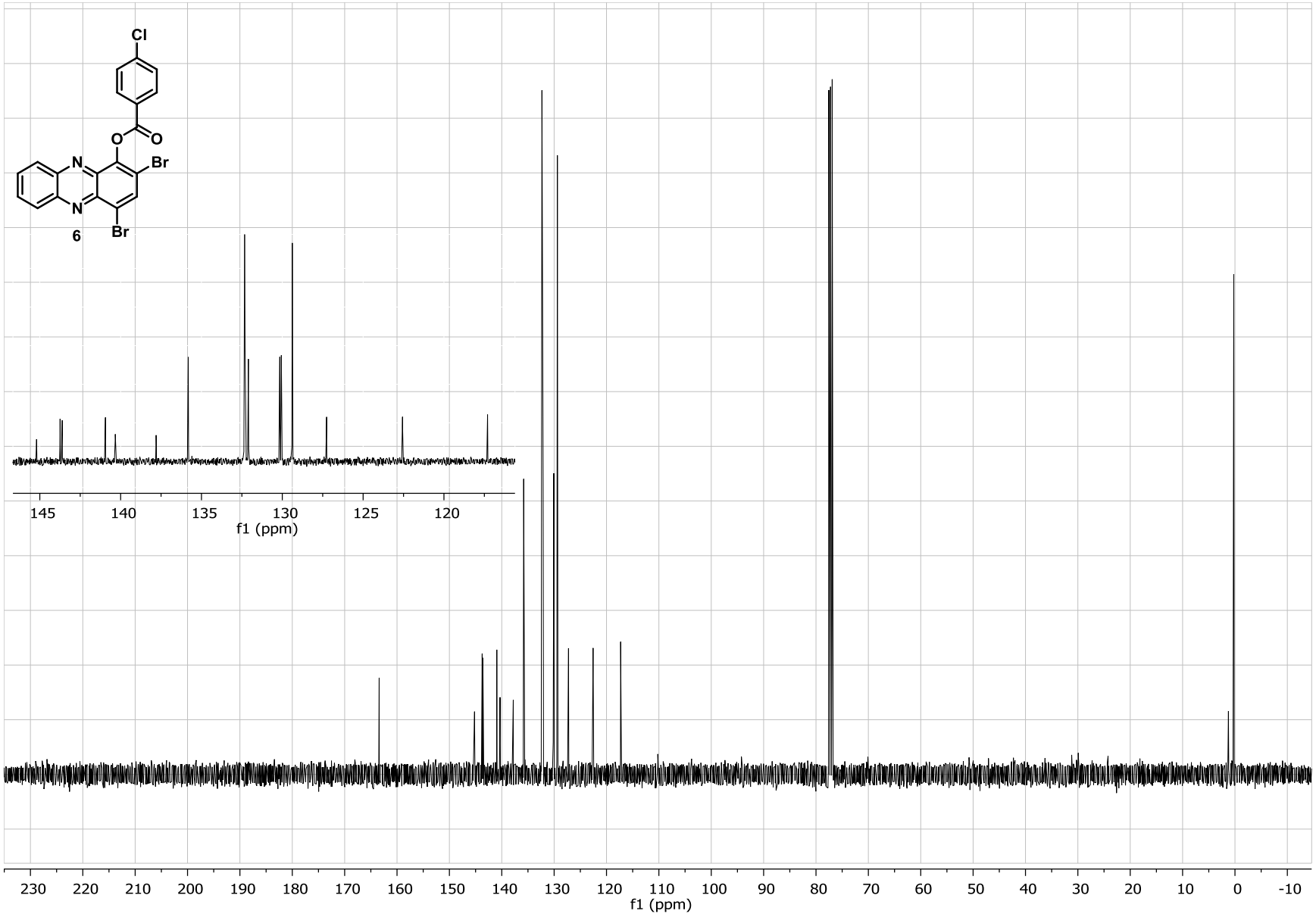

Me
O
O
N
Br
N
Br
4
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
f1 (ppm)

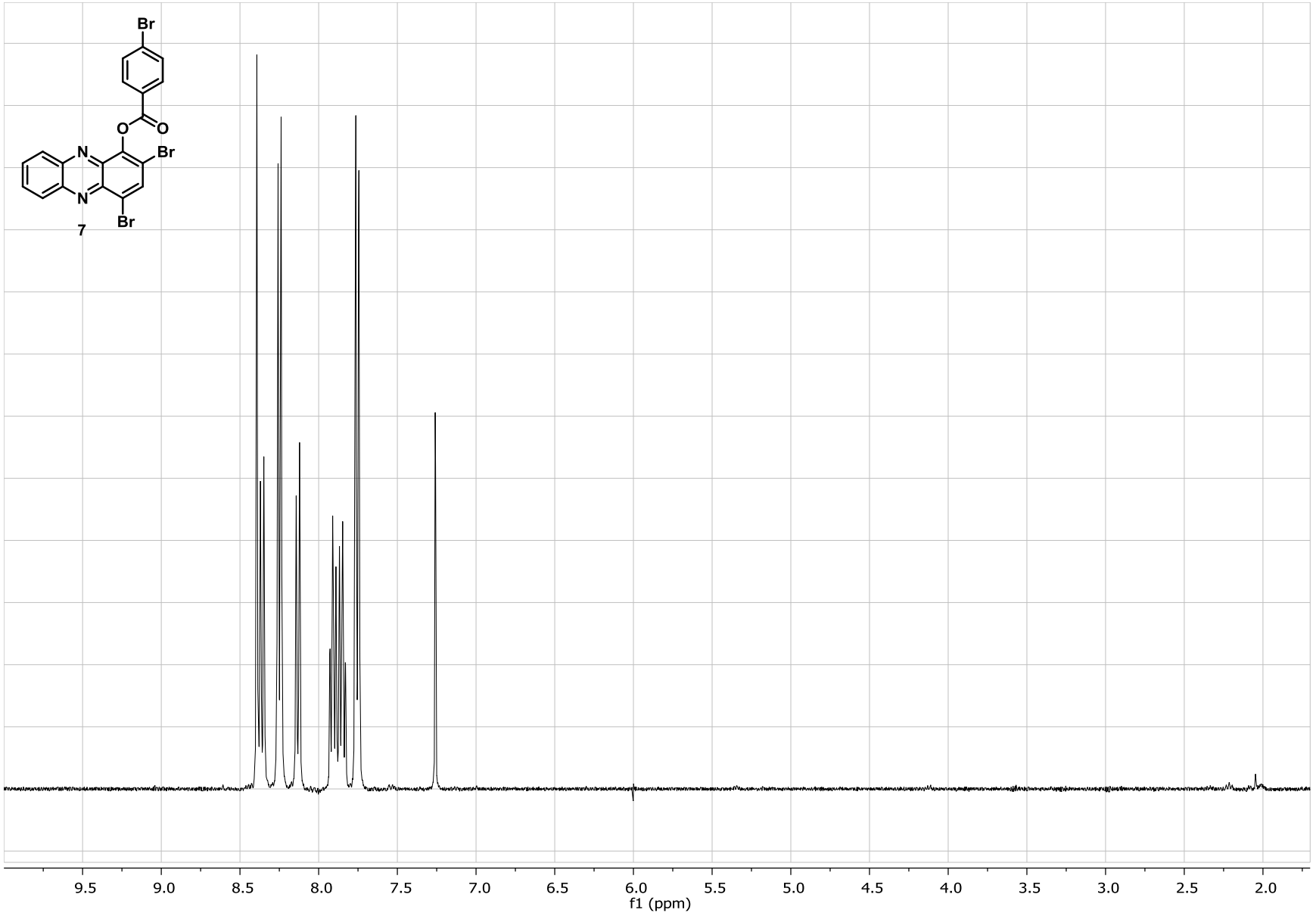

Me
O
O
N
Br
N
Br
4
145
140
135
130
125
120
f1 (ppm)
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

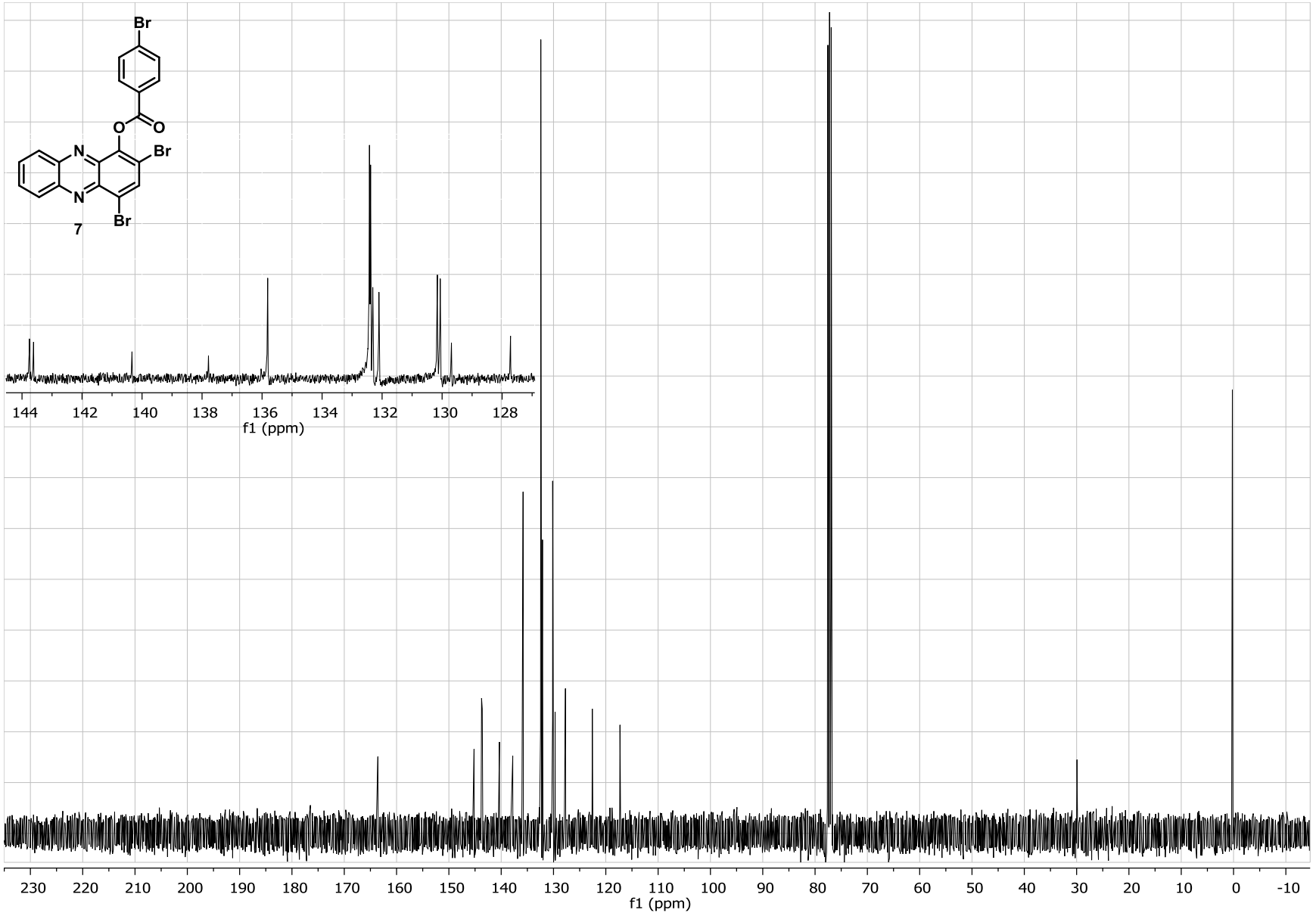

OMe
O
O
N
Br
N
Br
5
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
f1 (ppm)

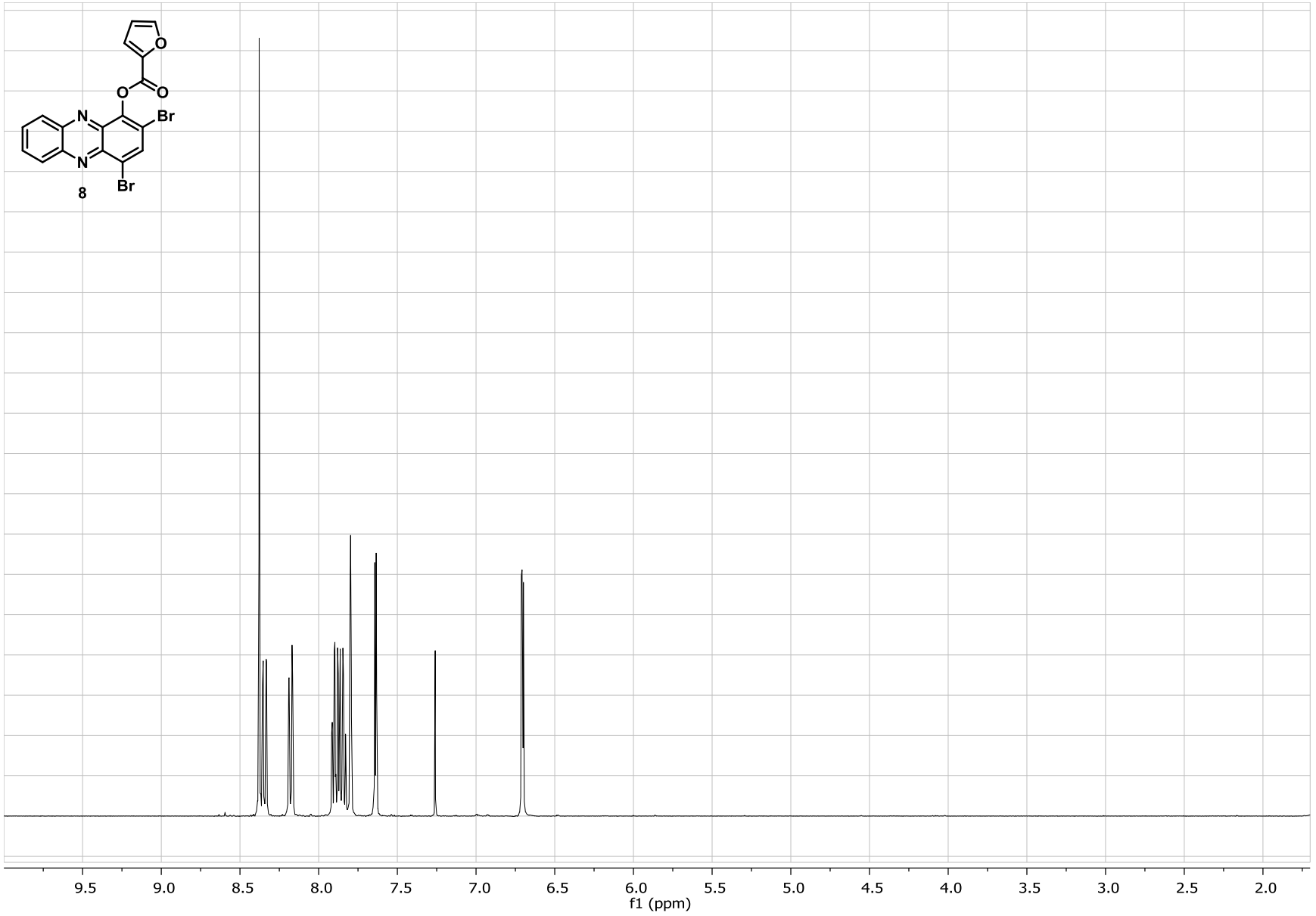

OMe
O
O
N
Br
N
Br
5
144.6 144.4 144.2 144.0 143.8 143.6 143.4 143.2 143.0
f1 (ppm)
133.0 132.5 132.0 131.5 131.0 130.5 130.0
f1 (ppm)
165 160 155 150 145 140 135 130 125 120 115
f1 (ppm)
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

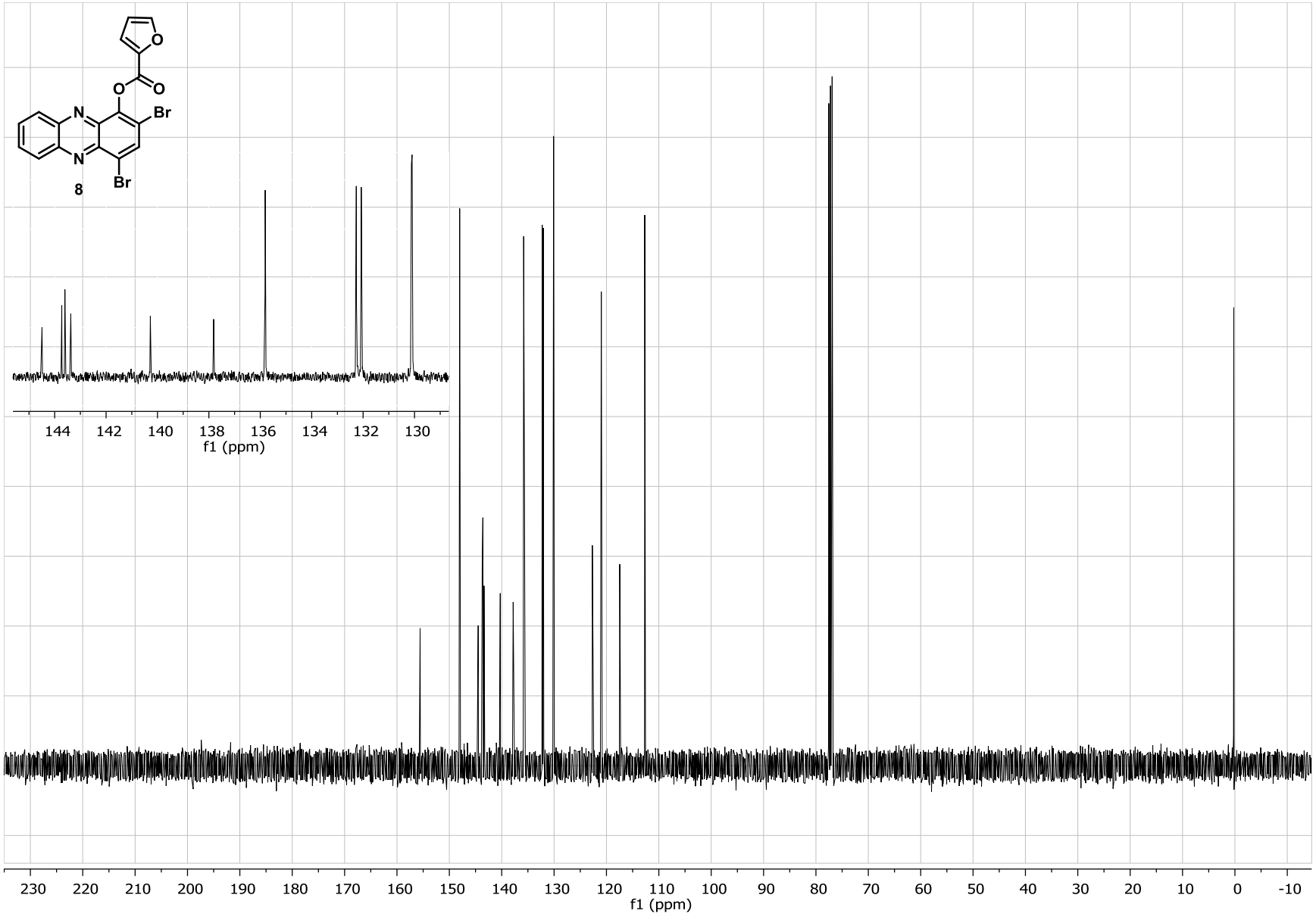

Cl
O
O
N
Br
N
Br
6
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
f1 (ppm)

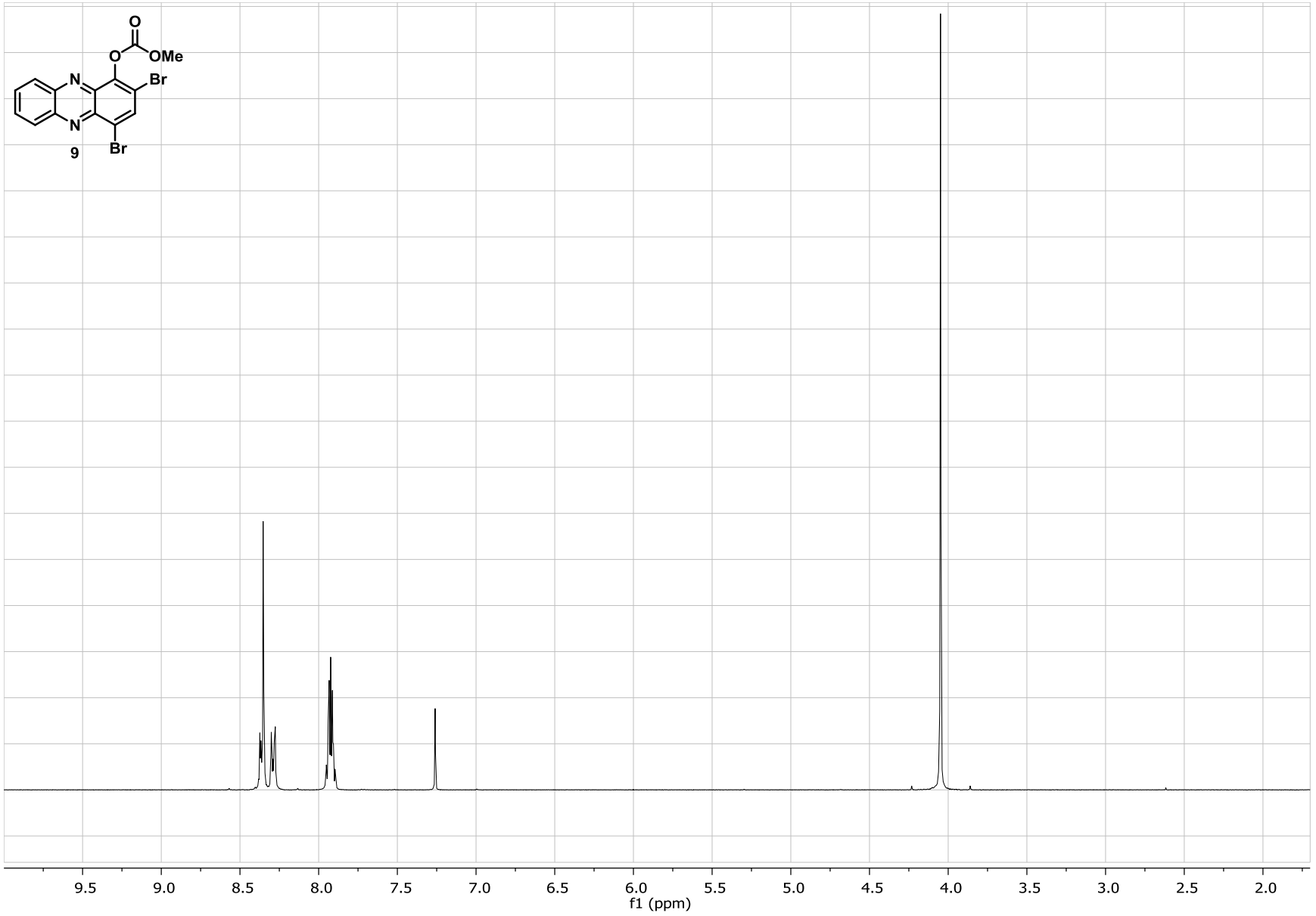

Cl
O
O
N
Br
N
Br
6
145
140
135
130
125
120
f1 (ppm)
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

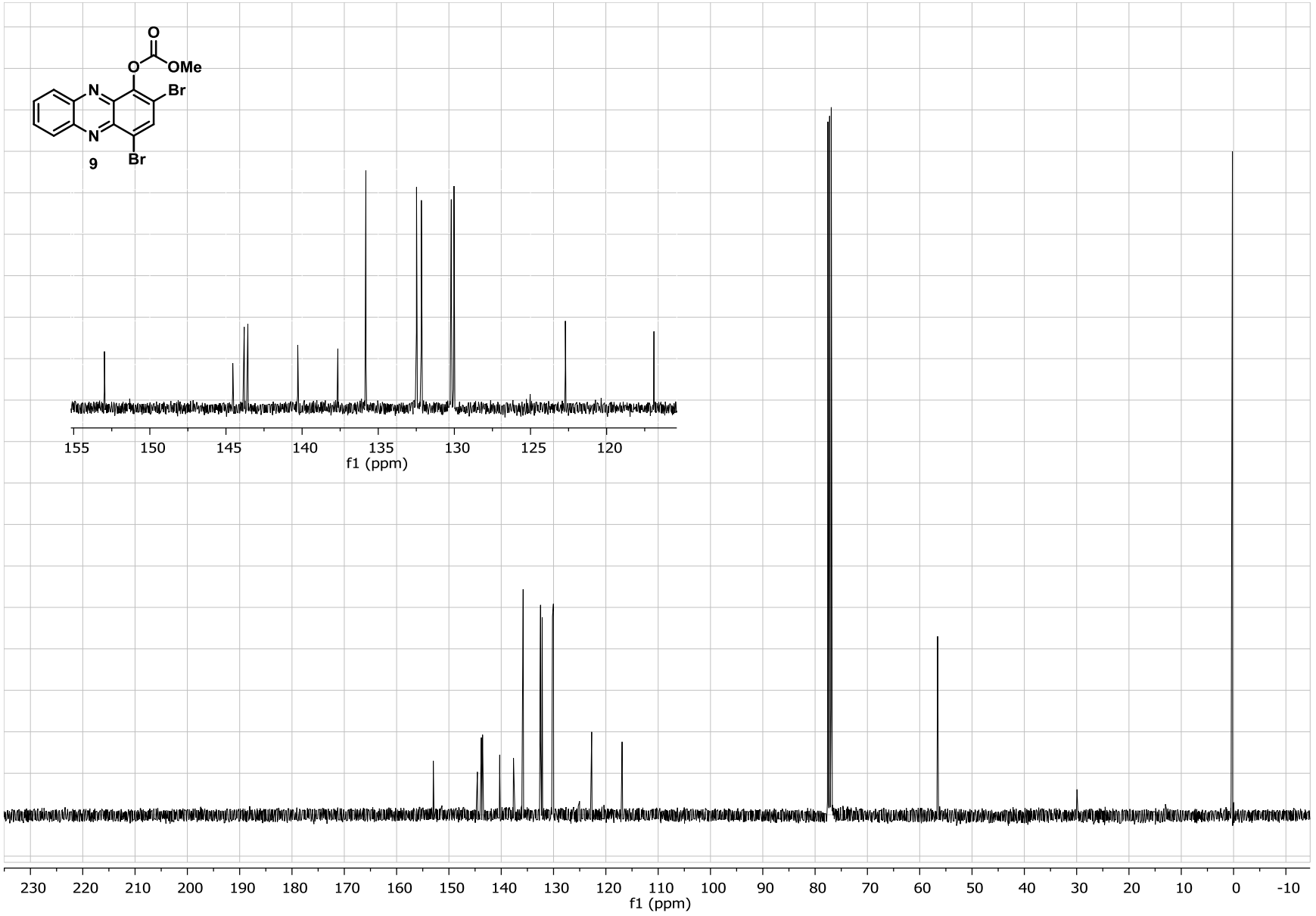

Br
O
O
N
Br
N
Br
7
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
f1 (ppm)

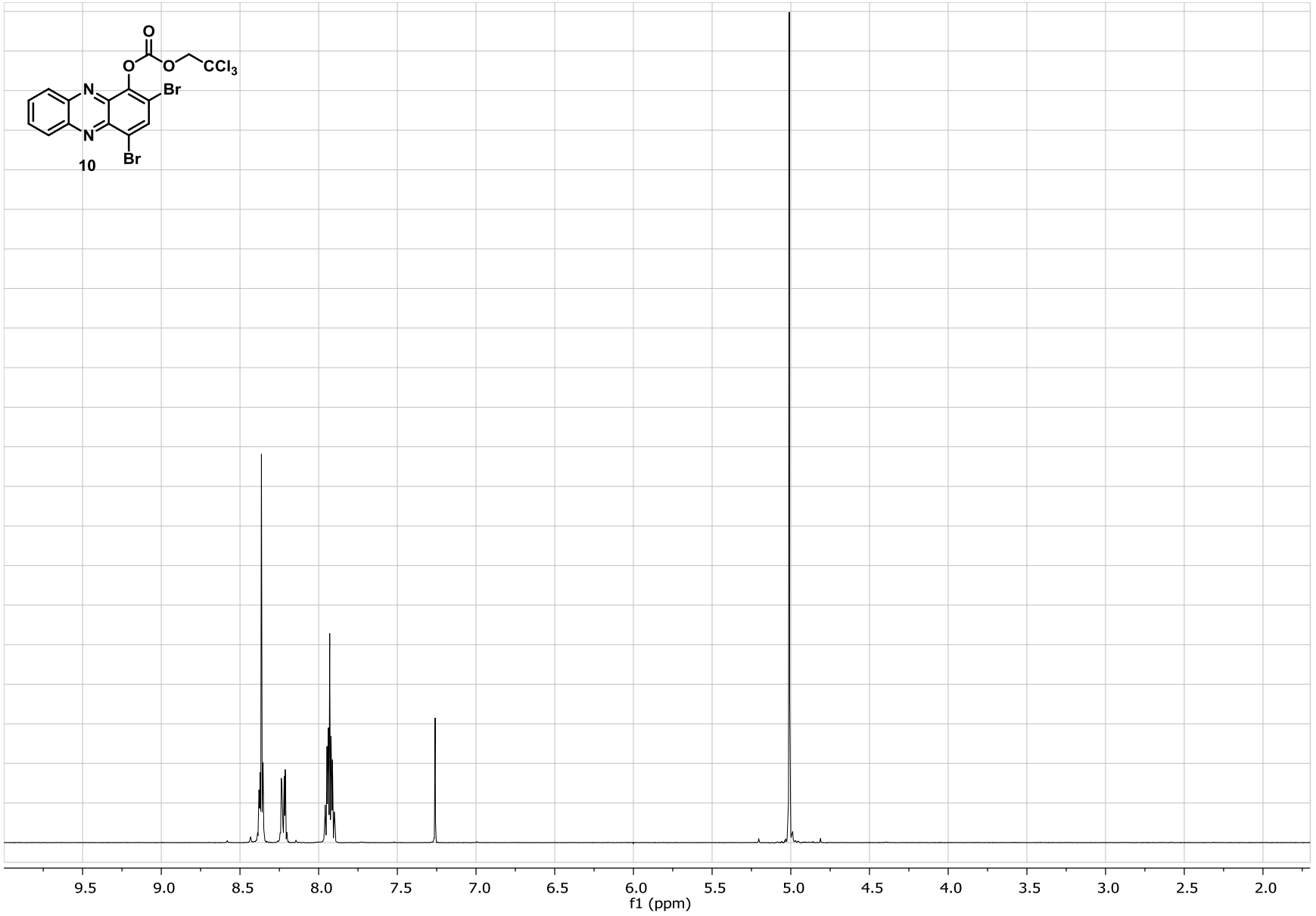

Br
O
O
N
Br
N
Br
7
144
142
140
138
136
134
132
130
128
f1 (ppm)
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

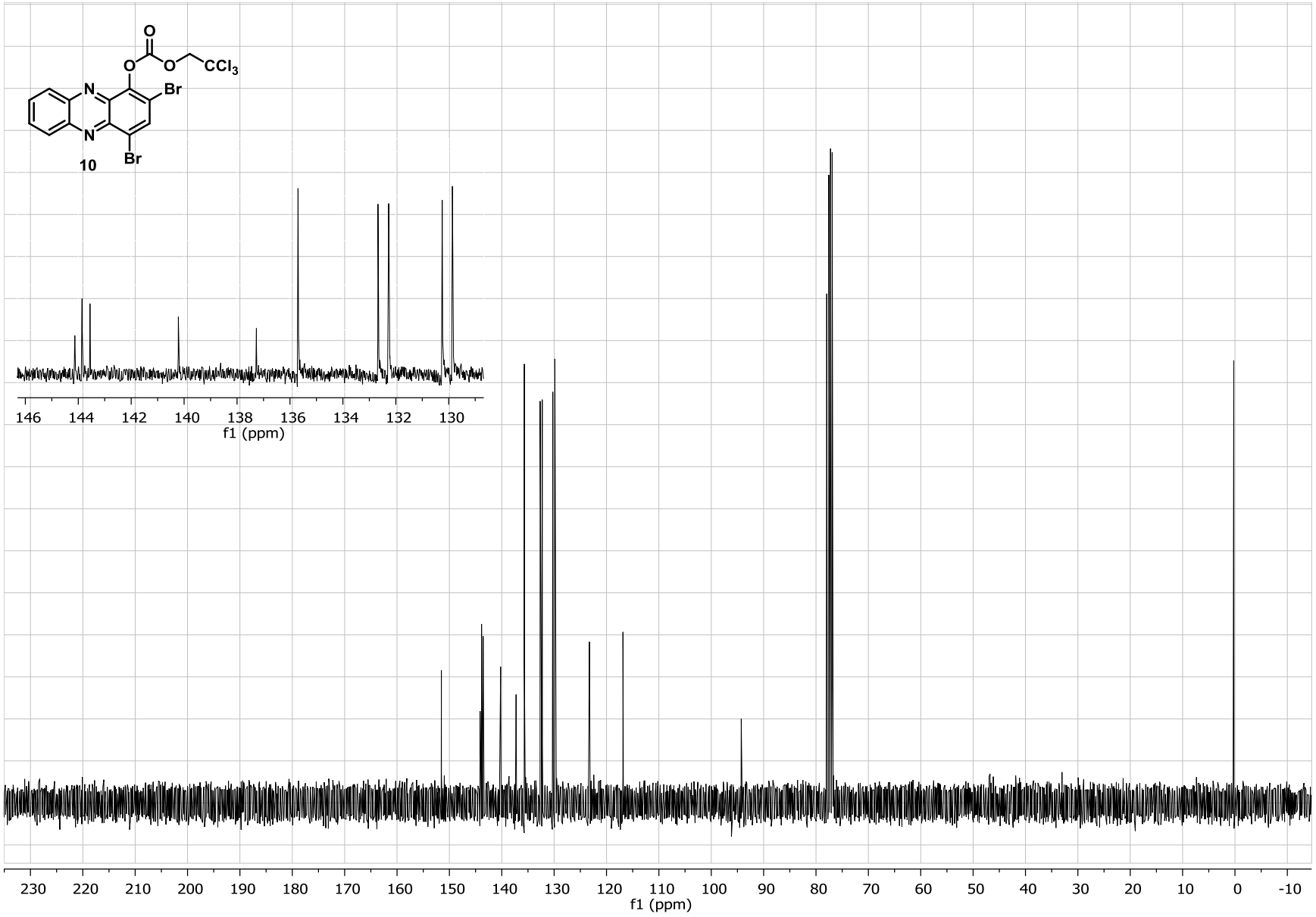

O
O
O
N
Br
N
Br
8
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
f1 (ppm)

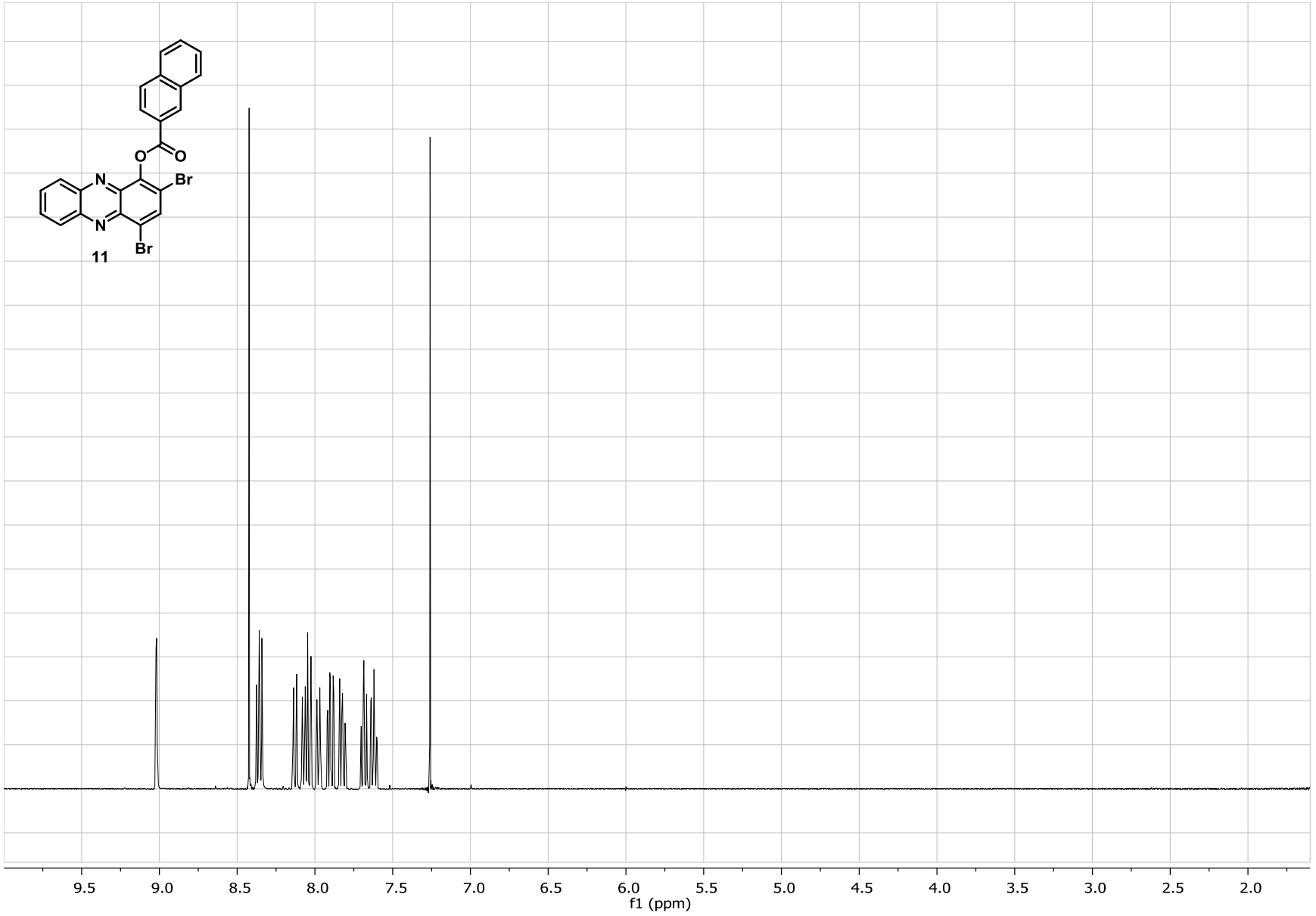

O
O
O
N
N
Br
Br
8
144
142
140
138
136
134
132
130
f1 (ppm)
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

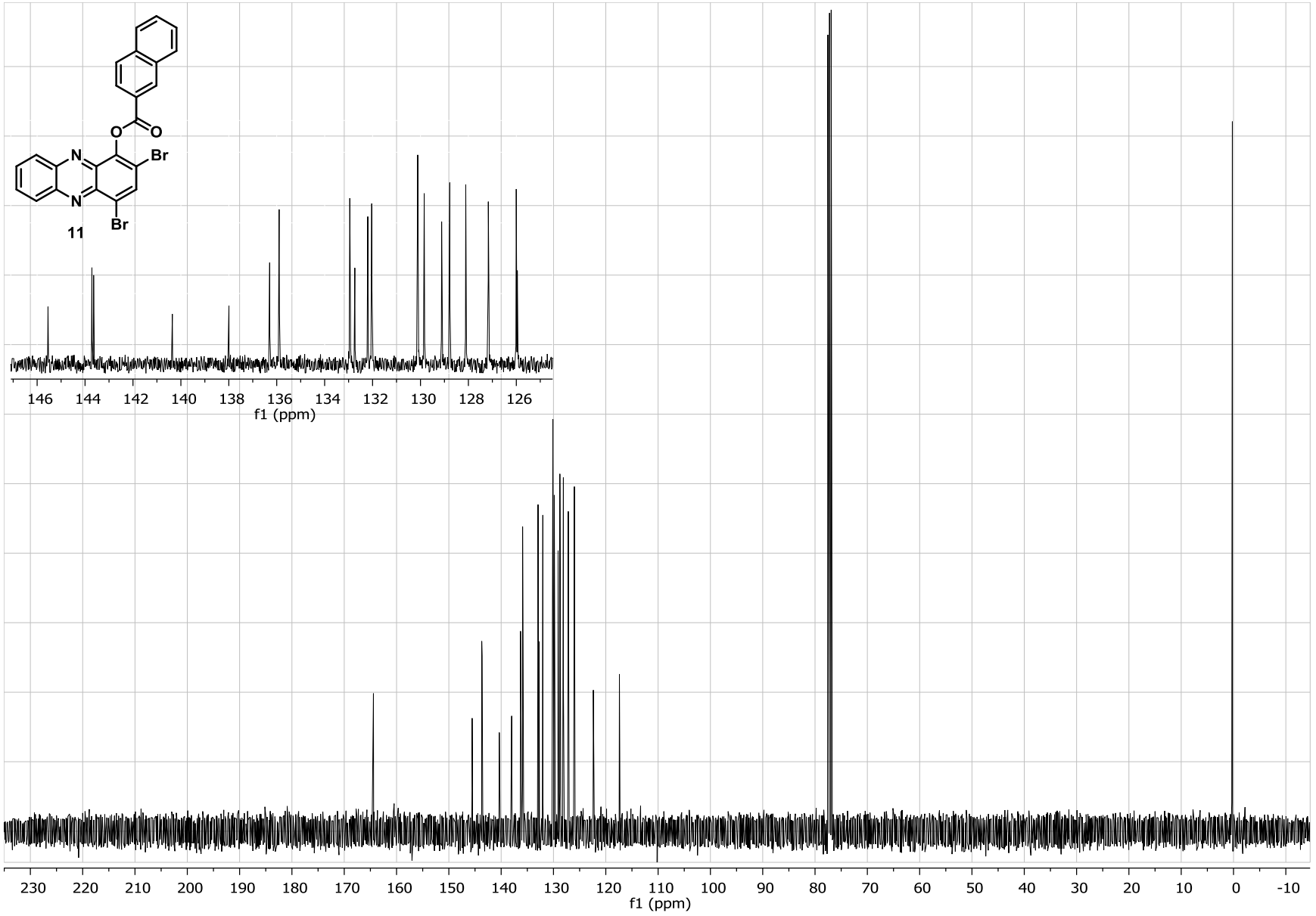

O
O
OMe
Br
N
N
Br
9
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
f1 (ppm)


O
O
OMe
N
Br
N
Br
9
155
150
145
140
135
130
125
120
f1 (ppm)
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)


O
O
O
CCl3
N
Br
N
Br
10
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
f1 (ppm)


O
O
O
CCl3
N
Br
N
Br
10
146
144
142
140
138
136
134
132
130
f1 (ppm)
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)


O
O
N
N
Br
Br
11
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
f1 (ppm)


O
O
N
N
Br
Br
11
146
144
142
140
138
136
134
132
130
128
126
f1 (ppm)
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)