Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

An Ether-Linked Halogenated Phenazine-Quinone Prodrug Model for Antibacterial Applications

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Supporting Information

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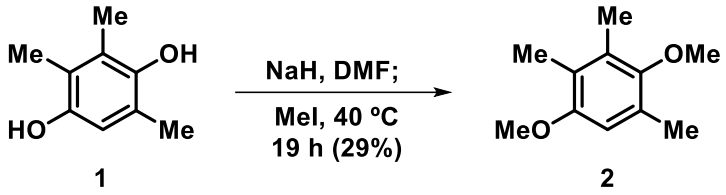
1.) General Information.

All synthetic reactions were carried out under an inert atmosphere of argon unless otherwise specified. All reagents for chemical synthesis were purchased from commercial sources and used without further purification. Commercially available comparators (antibiotics) were used in our biological investigations 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; 151 MHz for ^{13}C). 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 deuterated chloroform (reference peaks, ^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77.23 ppm) and 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 and d = doublet. High-Resolution Mass Spectrometry (HRMS) data was obtained for **HP-29-Q** from the Chemistry Department at the University of Florida.

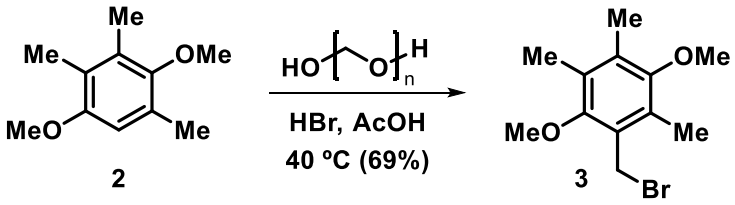
Bacterial strains used during these investigations include: methicillin-resistant *Staphylococcus aureus* (ATCC strains: BAA-1707, BAA-44; Clinical Isolates from Shands Hospital in Gainesville, FL: MRSA-1, MRSA-2, *S. aureus* 129, 147, 138 and 156), methicillin-resistant *Staphylococcus epidermidis* (MRSE ATCC 35984; *Staphylococcus epidermidis* 1, clinical Isolate from a patient treated at Shands Hospital in Gainesville, FL), vancomycin-resistant *Enterococcus* (VRE ATCC 700221), *Enterococcus faecalis* (OG1RF), and *Streptococcus pneumonia* (ATCC 6303). All compounds were stored as dimethyl sulfoxide (DMSO) stocks at room temperature in the absence of light. Some HPs demonstrated low DMSO solubilities and were subjected to gentle heating before assays. To ensure compound integrity of our DMSO stock solutions, we did not subject DMSO stocks of our test compounds to freeze-thaw cycles.

2.) Synthetic Procedures and Characterization Data.



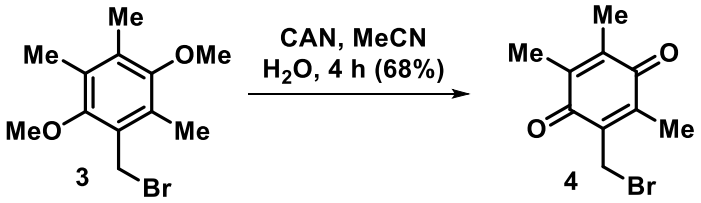
Procedure for the synthesis of 1,4-dimethoxy-2,3,5-trimethylbenzene (2). Sodium hydride (696 mg, 28.8 mmol, 60% dispersion in mineral oil) was dissolved in anhydrous *N,N*-dimethylformamide in a dry round bottom flask. A solution of 2,3,5-trimethylbenzene-1,4-diol **1** (2.0 g, 13.0 mmol) in *N,N*-dimethylformamide was added in over 20 minutes. After the evolution of hydrogen gas from this mixture, iodomethane (3.28 mL, 52.8 mmol) was then added dropwise to the reaction over 20 minutes. The resulting mixture was then heated to 40 °C for 40 hours until complete. After this time, the mixture was allowed to cool to room temperature before the reaction contents were transferred to a separatory funnel containing brine (50 mL). The organic material was then extracted with ethyl acetate (30 mL × 3) and the organic layers were combined, washed with brine (30 mL × 3), dried with sodium sulfate, filtered and concentrated *in vacuo*. The resulting crude oil was purified with a chromatography using hexanes:ethyl acetate (gradient from 100:1 to 10:1) as the eluent to yield **2** (768 mg, 29%) as a light yellow oil.[1]

Note: Our ^{1}H NMR tabulation matches those previously reported for 1,4-dimethoxy-2,3,5-trimethylbenzene **2** (CAS: 4537-09-1).[2]



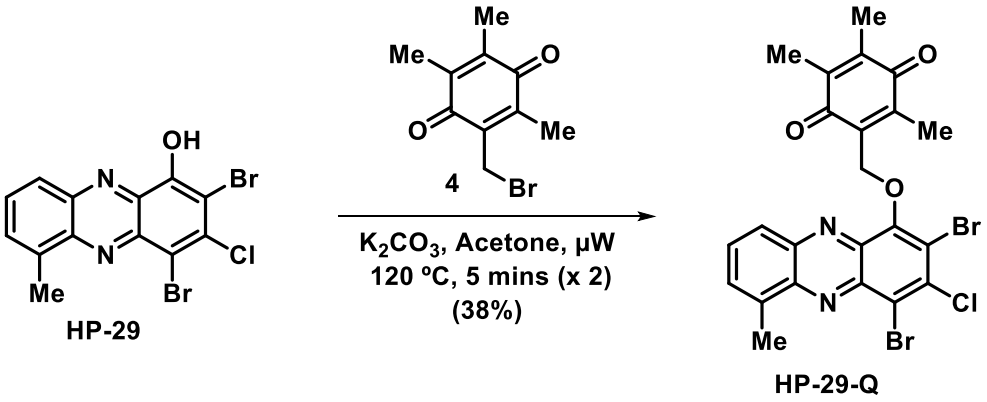
Procedure for the synthesis of 1-(bromomethyl)-2,5-dimethoxy-3,4,6-trimethylbenzene (3). The following materials were added to a round bottom flask: **2** (345 mg, 1.90 mmol), paraformaldehyde (86 mg, 2.87 mmol) and glacial acid (1.9 mL). Following this, a 33 wt. % hydrobromide solution in acetic acid (519 µL, 2.87 mmol) was added to the reaction mixture and allowed to stir at 50 °C for 14 hours. Upon completion, the reaction contents were poured into water and the precipitate was filtered and washed with water to yield **3** (358 mg, 69%) as a white solid.[3]

Note: Our ^{1}H NMR tabulation matches those previously reported for 1-(bromomethyl)-2,5-dimethoxy-3,4,6-trimethylbenzene (CAS:117574-75-1).[4]



Procedure for the synthesis of 2-(bromomethyl)-3,5,6-trimethylcyclohexa-2,5-diene-1,4-dione (4). Compound **3** (300 mg, 1.23 mmol) was dissolved in acetonitrile and added a stirring solution of ceric ammonium nitrate (CAN, 1.57 g, 2.9 mmol) dissolved in water (4 mL). The resulting mixture was allowed to stir at room temperature for 4 hours before being quenched (with 20 mL of water). Then, the mixture was transferred to a separatory funnel and extracted with dichloromethane (30 mL × 3). The organic layers were collected, dried over sodium sulfate and concentrated via rotavap. The crude material was then purified by silica chromatography using hexanes:ethyl acetate (100:1 to 50:1) to elute **4** (203 mg, 68%) as a yellow oil.[4]

Note: Our ^{1}H NMR tabulation matches those previously reported for 2-(bromomethyl)-3,5,6-trimethylcyclohexa-2,5-diene-1,4-dione (CAS: 178262-79-8).[4]



Procedure for the synthesis of HP-29-Q. Potassium carbonate (81.4 mg, 0.59 mmol) was added to a stirring solution of **HP-29** (47.5 mg, 0.12 mmol) in acetone (2 mL) in a microwave reaction vessel. The resulting mixture was allowed to stir at room temperature for 15 minutes before **4** (28.7 mg, 0.12 mmol) was added. Following this, the vessel was then sealed and heated to 120 °C for 5 minutes. After this time, a TLC showed that the starting material was not fully consumed, and the mixture was sealed and heated to 120 °C for another 5 minutes. After this time, TLC indicated full consumption of starting material. Then, the reaction mixture was transferred to a separatory funnel containing brine (30 mL) and extracted with dichloromethane. The organic layers were collected, dried with sodium sulfate, filtered and concentrated *in vacuo*. The resulting solid was purified by column using hexane:ethyl acetate (4:1 to 1:1) to elute **HP-29-Q** (25 mg, 38%) as a yellow solid.

^{1}H NMR (600 MHz, $CDCl_3$): δ 8.07 (d, *J* = 8.7 Hz, 1H), 7.80 (dd, *J* = 8.7, 6.7 Hz, 1H), 7.75 (d, *J* = 6.7 Hz, 1H), 5.56 (s, 2H), 2.97 (s, 3H), 2.41 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H).

^{13}C NMR (151 MHz, $CDCl_3$): δ 188.1, 186.3, 152.7, 145.6, 143.5, 142.9, 141.2, 141.0, 139.9, 138.8, 137.4, 137.2, 136.7, 132.2, 131.3, 127.4, 121.4, 119.0, 67.7, 17.5, 13.2, 12.7, 12.7.

HRMS (ESI): calc. for $C_{23}H_{18}Br_2ClN_2O_3$ $[M+H]^+$: 564.9347, found: 564.9350.

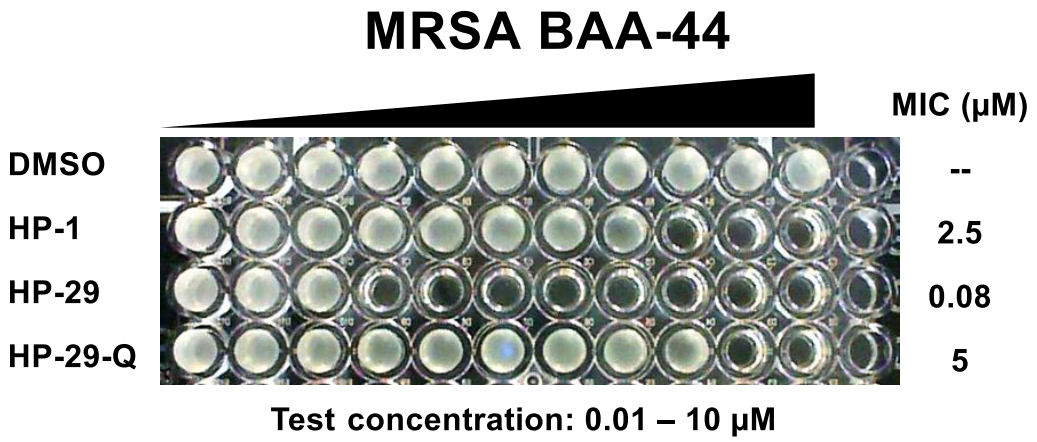
MP: 224 - 226 °C, decomposition.

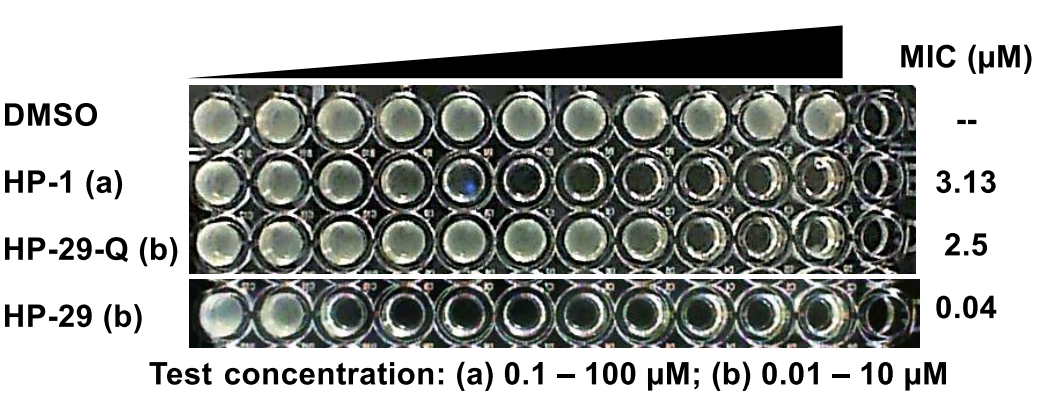
3.) Biological Methods.

A.) Minimum Inhibitory Concentration (MIC) Susceptibility Assay (in 96-well plate).

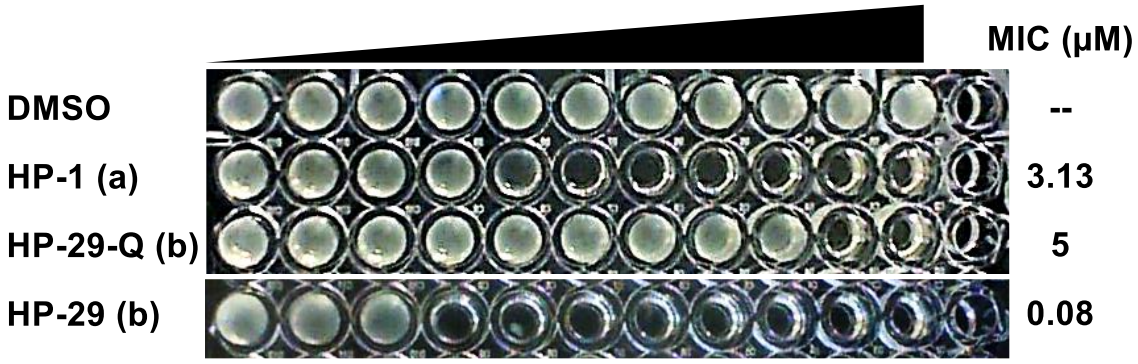
The minimum inhibitory concentration (MIC) for each test compound was determined by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI).[5] In a 96-well plate, two-fold serial dilutions of each test compound were made in a final volume of 100 µL broth (Lysogeny Broth, LB: *S. aureus*, *S. epidermidis* and *S. pneumonia*; Brain Heart Infusion, BHI: *E. faecium* and *E. faecalis*). Each microtiter well was inoculated with ~10^5 bacterial cells at the initial time of incubation, prepared from a fresh log phase culture (OD_{600} of 0.5 to 1.0). The MIC was defined as the lowest concentration of compound that prevented bacterial growth after incubating 16 hours at 37 °C (MIC values were determined by spectrophotometric readings at OD_{600} and visible inspection of turbidity). The concentration range tested during this study was either 0.01 to 10 µM or 0.1 to 100 µM (depending on solubility). DMSO served as our vehicle and negative control in each microdilution MIC assay. DMSO was serially diluted with a top concentration of 1% v/v. All compounds were tested in a minimum of three independent experiments.

Images of representative MIC assays performed during these studies.



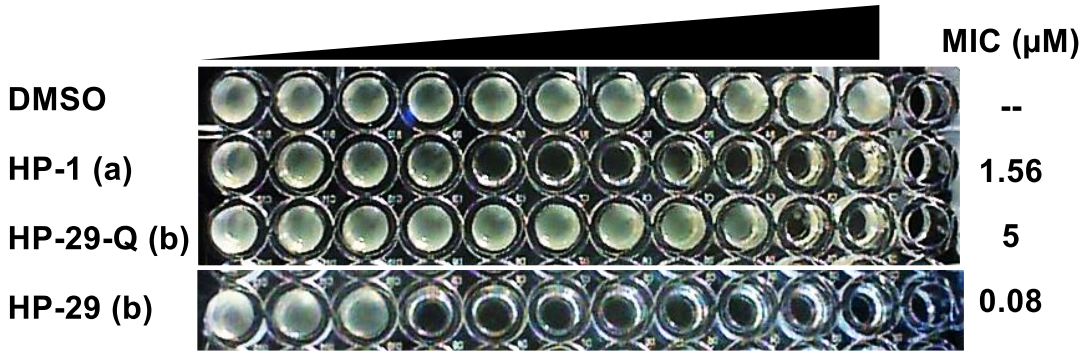


S. aureus 138



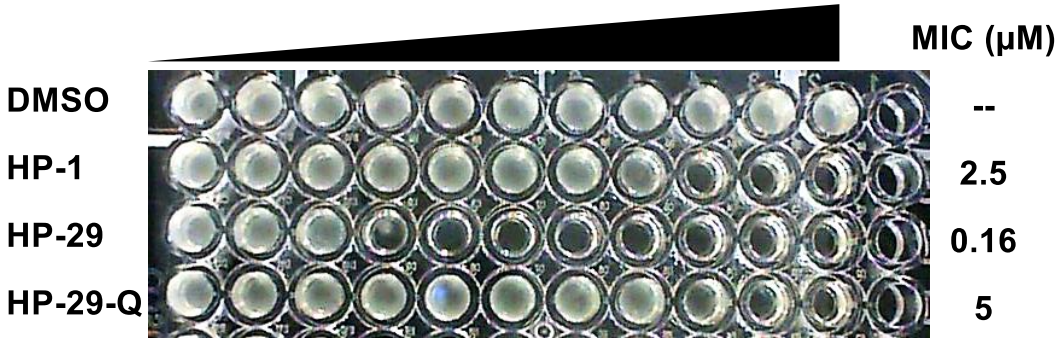
Test concentration: (a) 0.1 – 100 μM; (b) 0.01 – 10 μM

S. aureus 156

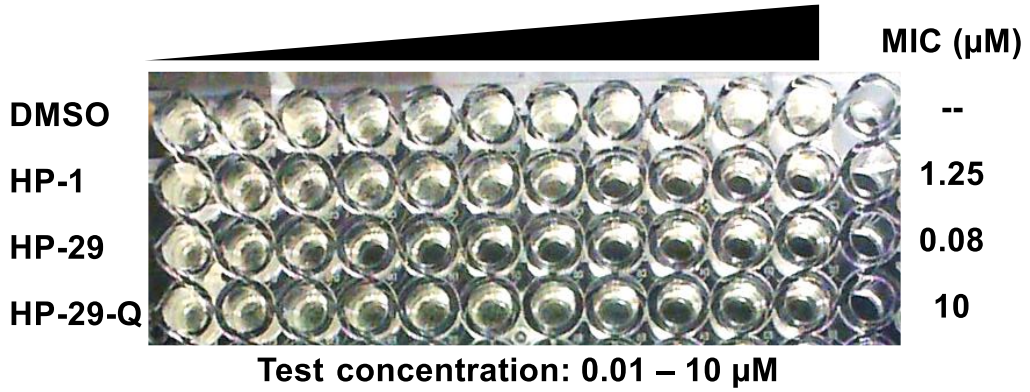


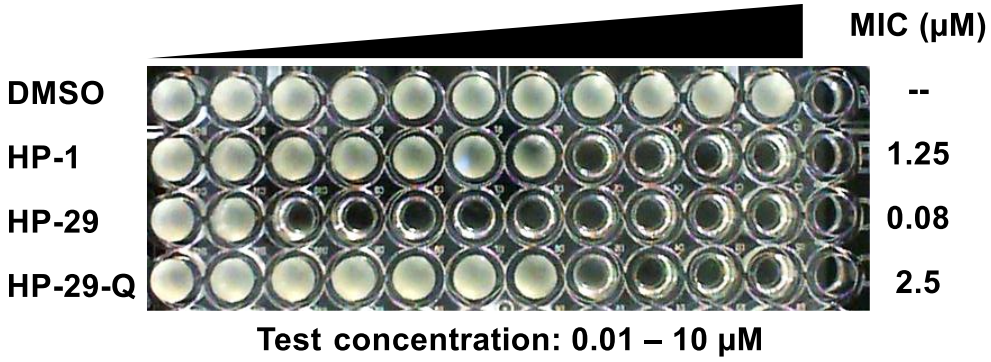
Test concentration: (a) 0.1 – 100 μM; (b) 0.01 – 10 μM

MRSE 35984



Test concentration: 0.01 – 10 μM





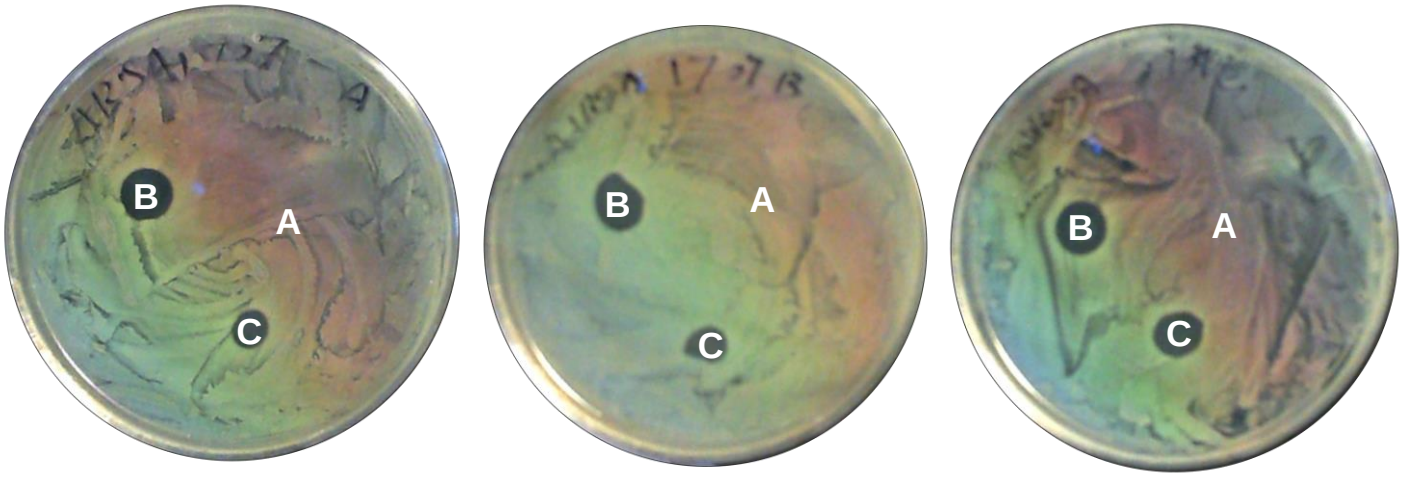
B.) MIC Assay with Glutathione Co-Treatment.

MIC assays with Glutathione (GSH) co-treatment was performed analogous to our standard MIC assays; however, 200 μM GSH was added to the media (in the bottom-half of the microtitier well plate). All data were obtained from three independent experiments.

C.) Agar Diffusion Assays to Determine Zone of Inhibition (Clearance).

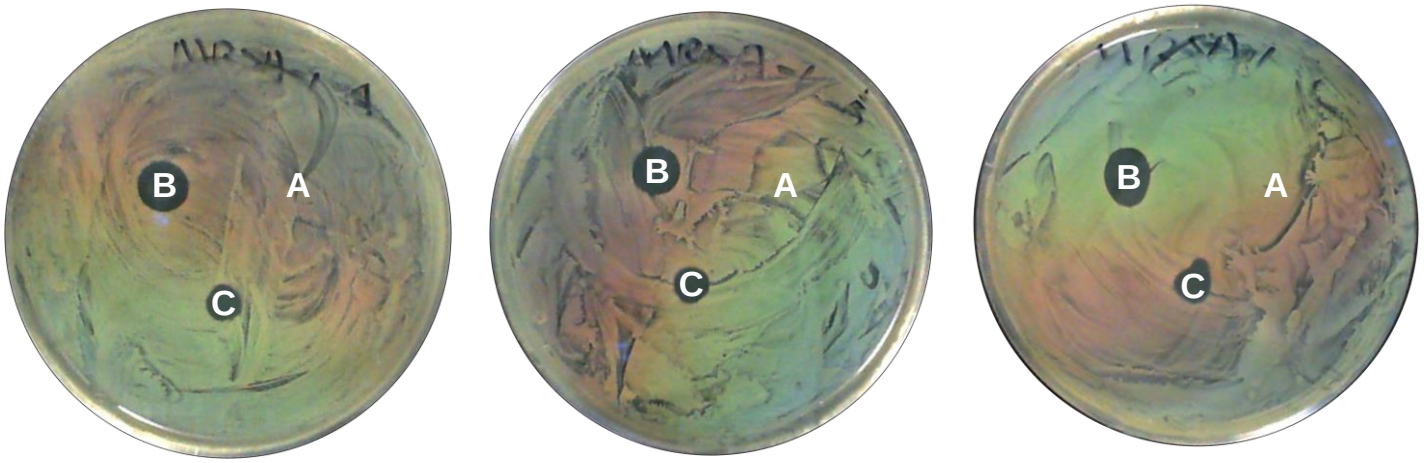
A bacterial culture (MRSA BAA-1707, MRSA-1, *S. aureus* 156, *S. aureus* 138, MRSE 35984 and *S. epidermidis* 1, OD_{600} = 0.7, ~10^8 CFU) was spread on LB agar plates in petri dishes and allowed to dry for 30 minutes. After this time, 3 μL of test compound from a 1 mM DMSO stock, or DMSO (vehicle control), was gently pipetted onto the petri dishes containing a lawn of bacteria at specified locations. The resulting petri dishes were then incubated at 37°C for 16 hours to allow visible growth of the bacterial lawn and clear zones of inhibition to be seen. After this time, images were taken of each experiment and zones of bacterial clearance (area) were measured and recorded in mm^2 using ImageJ software (NIH). The final zones of inhibition resulted from three independent experiments with DMSO (vehicle control), **HP-29** and **HP-29-Q** tested on the same petri dishes for direct comparison between these treatments (1 replicate each per petri dish; see following images).

Images of petri dish experiments against MRSA BAA-1707.



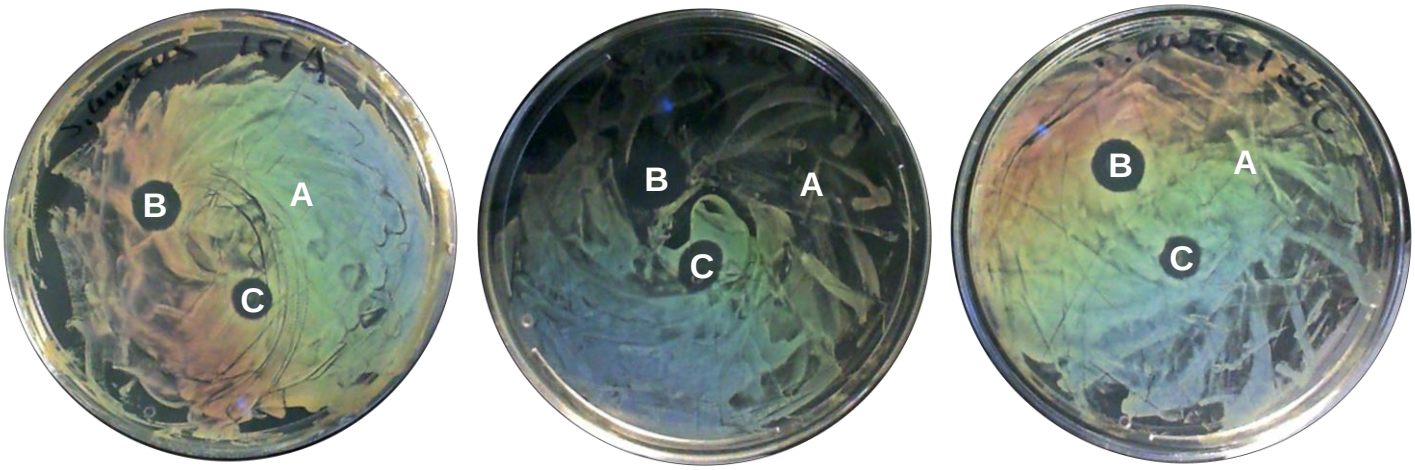
Agar diffusion assay results against MRSA BAA-1707: (A) DMSO (vehicle control, zone of inhibition = 0 mm^2), (B) **HP-29** (zone of inhibition = 104.4 ± 2.2 mm^2), (C) **HP-29-Q** (zone of inhibition = 64.9 ± 18.5 mm^2).

Images of petri dish experiments against MRSA-1.



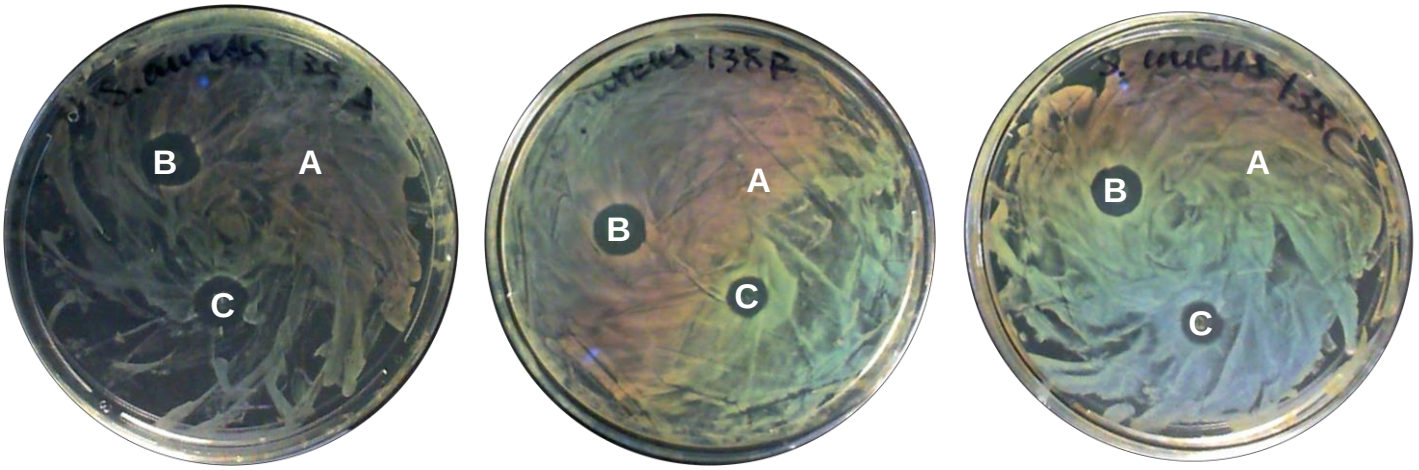
Agar diffusion assay results against MRSA-1: (A) DMSO (vehicle control, zone of inhibition = 0 mm^2), (B) **HP-29** (zone of inhibition = 105.9 ± 10.7 mm^2), (C) **HP-29-Q** (zone of inhibition = 58.2 ± 10.4 mm^2).

Images of petri dish experiments against S. aureus 156.



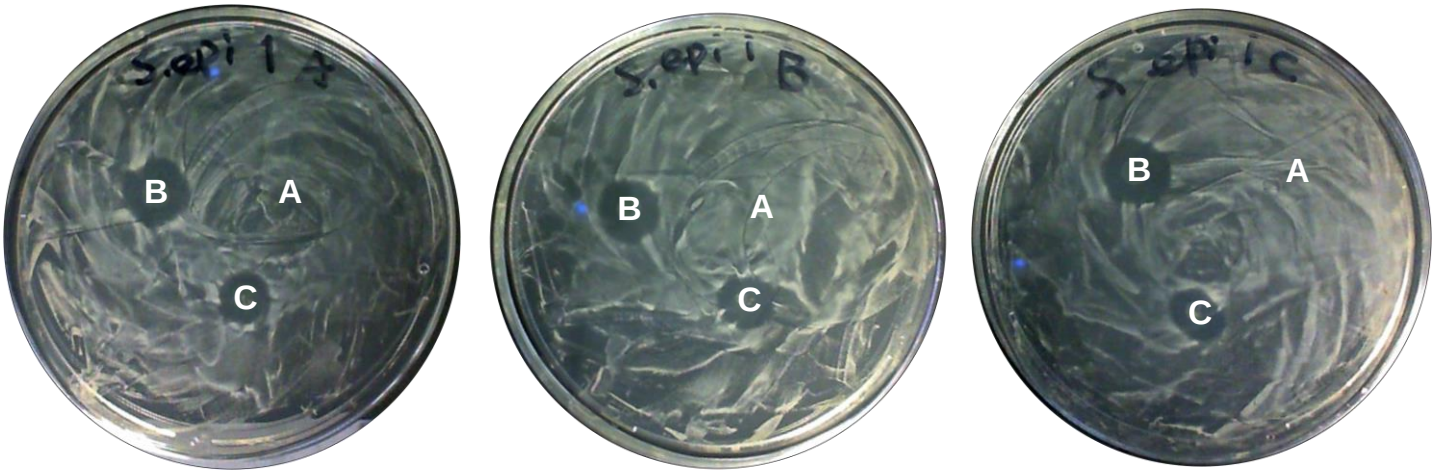
Agar diffusion assay results against S. aureus 156: (A) DMSO (vehicle control, zone of inhibition = 0 mm^2), (B) **HP-29** (zone of inhibition = 113.8 ± 14.9 mm^2), (C) **HP-29-Q** (zone of inhibition = 71.1 ± 12.6 mm^2).

Images of petri dish experiments against S. aureus 138.



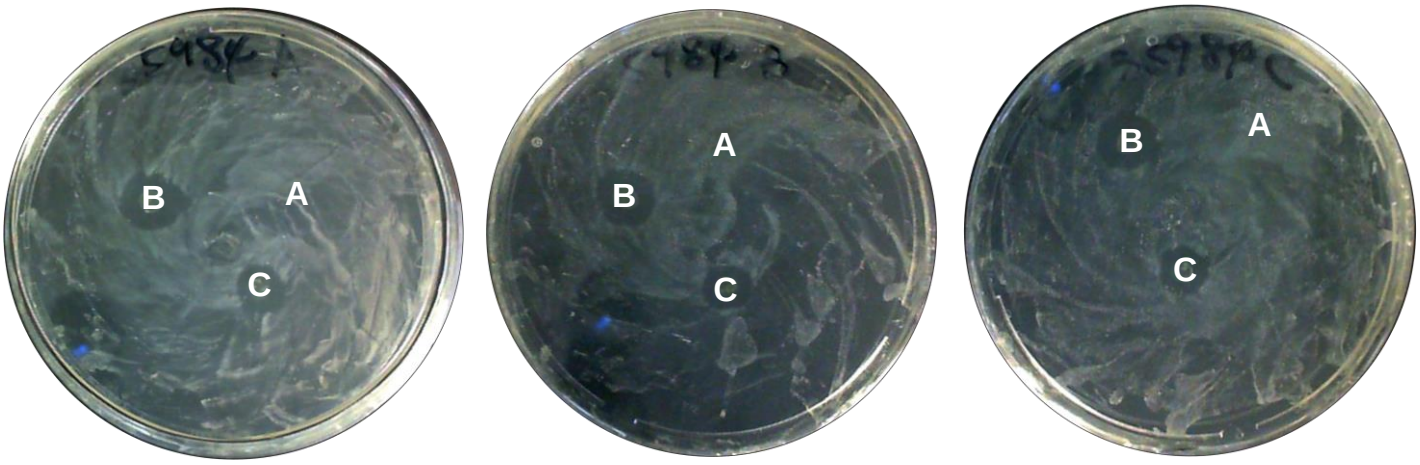
Agar diffusion assay results against S. aureus 138: (A) DMSO (vehicle control, zone of inhibition = 0 mm^2), (B) **HP-29** (zone of inhibition = 113.0 ± 7.0 mm^2), (C) **HP-29-Q** (zone of inhibition = 76.3 ± 16.9 mm^2).

Images of petri dish experiments against S. epidermidis 1.



Agar diffusion assay results against S. epidermidis 1: (A) DMSO (vehicle control, zone of inhibition = 0 mm^2), (B) **HP-29** (zone of inhibition = 169.9 ± 5.2 mm^2), (C) **HP-29-Q** (zone of inhibition = 99.6 ± 6.6 mm^2).

Images of petri dish experiments against MRSE 35984.

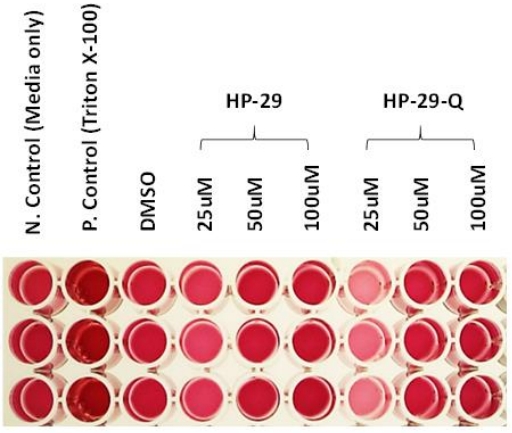


Agar diffusion assay results against MRSE 35984: (A) DMSO (vehicle control, zone of inhibition = 0 mm^2), (B) **HP-29** (zone of inhibition = 149.9 ± 3.6 mm^2), (C) **HP-29-Q** (zone of inhibition = 95.3 ± 6.1 mm^2).

D.) LDH Release Assay for HeLa Cytotoxicity Assessment.

HeLa cytotoxicity was assessed using the LDH release assay described by CytoTox96 (Promega G1780). HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum (FBS) at 37°C with 5% CO_2. When the HeLa cultures exhibited 70-80% confluence, DMSO and test compounds in DMSO stock (**HP-29** and **HP-29-Q**) were added to HeLa cells to make the final concentration to 25, 50 and 100 µM (2.5 µL, 5 µL and 10 µL). Triton X-100 (at 2% v/v) was used as the positive control for maximum lactate dehydrogenate (LDH) activity in this assay (i.e., complete cell death) while "medium only" lanes served as negative control lanes (i.e., no cell death). DMSO (at 5% v/v) was used as our vehicle control. HeLa cells were treated with compounds for 24 hours and then 50 µL of the supernatant was transferred into a fresh 96-well plate where 50 µL of the reaction mixture was added to the 96-well plate and incubated at room temperature for 30 minutes. Finally, Stop Solution (50 µL) was added to the incubating plates and the absorbance was measured at 490 nm. Results are from three independent experiments.

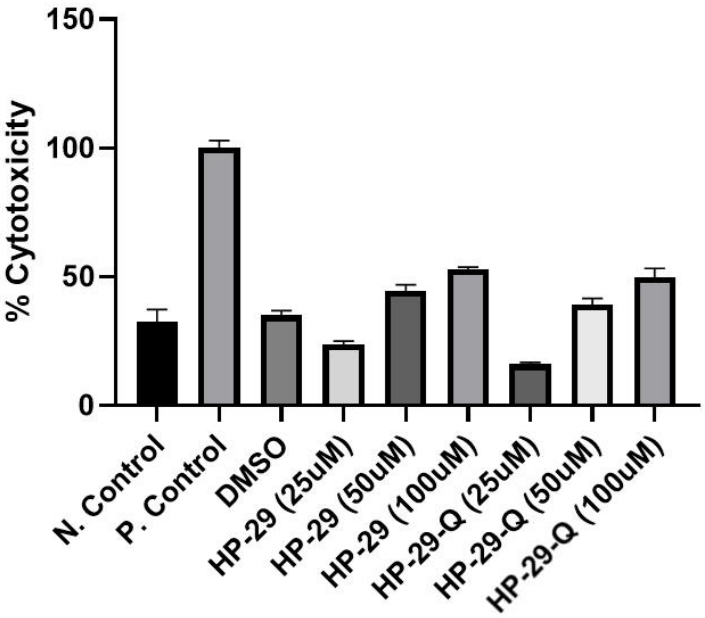
Halogenated Phenazine cytotoxicity results (Triton-X = 100% cell death; Medium Only: 0% cell death):



OD_{490nm}									
	1.54	5.13	1.70	1.28	2.26	2.67	0.81	2.00	2.33
	1.95	4.98	1.90	1.14	2.18	2.73	0.87	2.14	2.63
	1.51	5.27	1.78	1.22	2.42	2.75	0.80	1.90	2.68

% Cytotoxicity									
	30.1	100.0	33.2	25.0	44.0	52.0	15.7	39.0	45.5
	38.0	97.2	37.1	22.3	42.5	53.3	16.9	41.8	51.3
	29.4	102.9	34.7	23.7	47.2	53.6	15.6	37.0	52.2

$$\text{Percent cytotoxicity} = 100 \times \frac{\text{Experimental LDH Release } (OD_{490})}{\text{Maximum LDH Release } (OD_{490})}$$



4.) UV-Vis Spectroscopy.

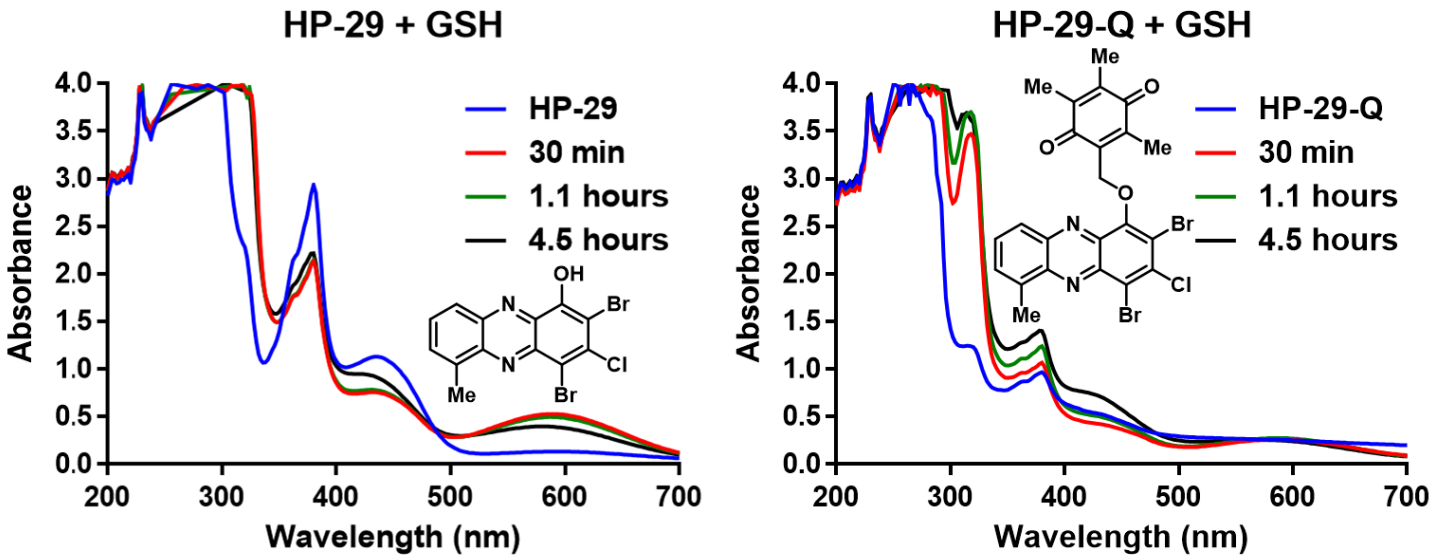
A.) Iron(II) Binding.

HP-29-Q and **HP-29** (500 µL of a 1 mM DMSO stock solution) was added to 1 mL DMSO in two separate vails. The solution was allowed to stir at room temperature to get homogenous solution. Then, 1 mL of each solution was aliquoted to obtain their UV-vis spectrum in the absence of iron(II). The solutions were then placed back into each vail and an ammonium iron(II) sulfate hexahydrate solution (25 µL; 10 mM in water) was added and the resulting mixtures were allowed to stir at room temperate for 1 minute to thoroughly mix and determine if **HP-29** and **HP-29-Q** could bind iron(II) directly. After one minute, spectral scanning was performed from 200 to 800 nm in 2 nm increments. Then the mixtures were then placed back to the corresponded vials and allowed to stir at room temperature for another 64 minutes. Spectral scanning was performed again to determine if there any change of the binding ability at 65 minutes. Results were plotted using GraphPad Prism.

B.) HP-29-Q Release with Glutathione.

HP-29-Q (or **HP-29**; 400 µL of a 1 mM DMSO stock solution) was added to a vail containing 8 mL DMSO and vortexed to mix. Then, 1 mL of each solution was aliquoted to obtain their UV-vis spectrum in the absence of glutathione (GSH). The solutions were then placed back in each vail, and 40 µL of GSH (10 mM solution in water; 1 equivalent GSH) were added to each vail. The mixtures were allowed to stir at room temperature and a 1 mL aliquot of each solution used to obtain a UV-vis spectrum at different time points. Spectral scanning was performed from 200 to 800 nm in 2 nm increments. Results were plotted using GraphPad Prism.

Note: UV-vis results for 1 equivalent of GSH are presented in the manuscript (experimental procedure above); however, the UV-vis results for **HP-29-Q** treated with 25 equivalents of GSH are included below (enabling a more rapid release of **HP-29**, as expected due to the appearance of **HP-29** peak at ~390 nm).



5.) Literature References.

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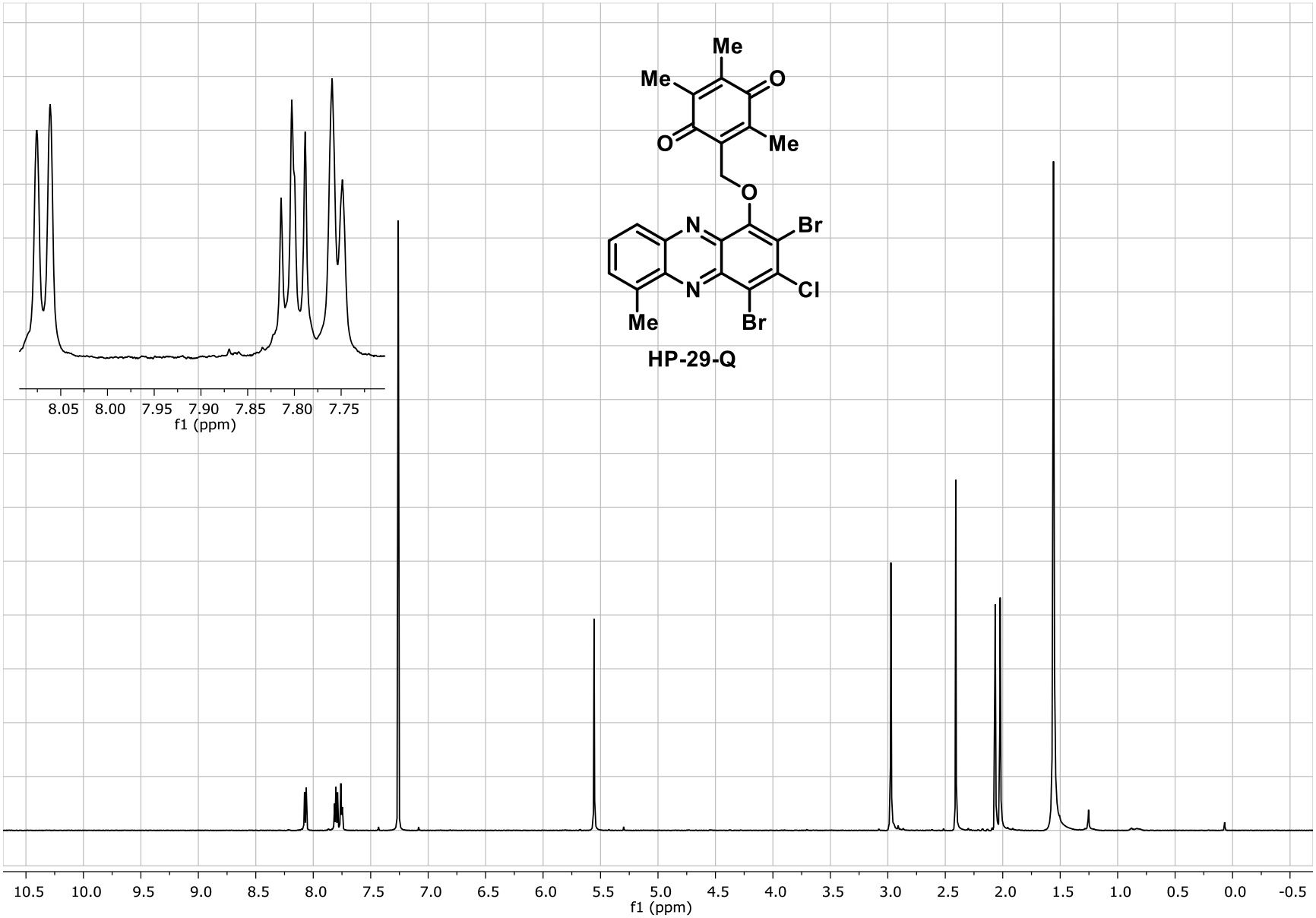
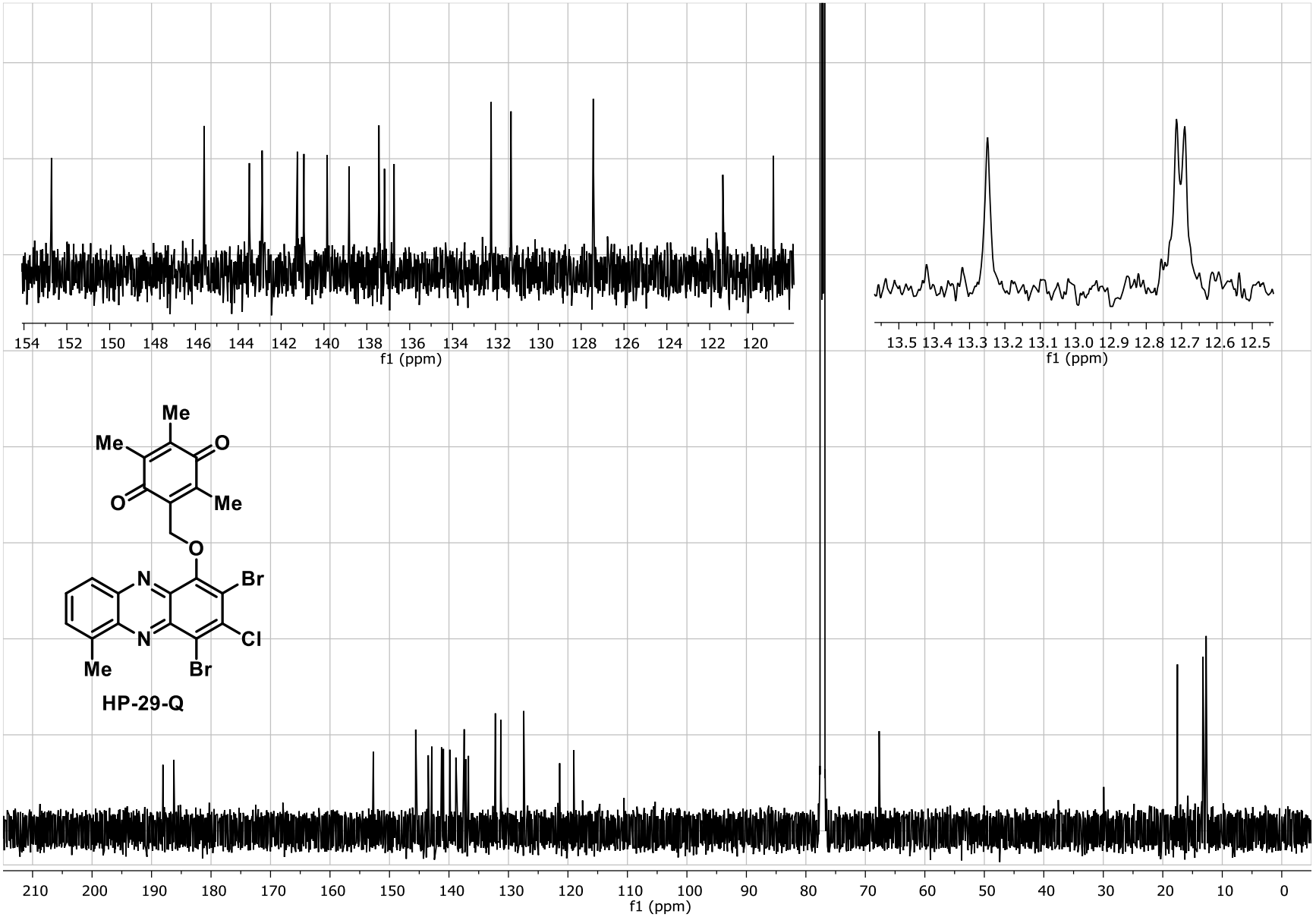
6.) NMR Spectra.


Me
Me
O
O
Me
O
N
Br
N
Cl
Me
Br
HP-29-Q
8.05 8.00 7.95 7.90 7.85 7.80 7.75
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)


154 152 150 148 146 144 142 140 138 136 134 132 130 128 126 124 122 120
f1 (ppm)
13.5 13.4 13.3 13.2 13.1 13.0 12.9 12.8 12.7 12.6 12.5
f1 (ppm)
Me
Me
O
O
Me
O
N
Br
N
Cl
Me
Br
HP-29-Q
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)