

Supplementary Information

Efforts in Redesigning the Antileukemic Drug 6-Thiopurine: Decreasing Toxic Side Effects while Maintaining Efficacy

Arnaldo X. Torres Hernandez,^{‡ab} Chamitha J. Weeramange,^{‡b} Prathibha Desman,^b Anthony Fatino,^b Olivia Haney,^b and Ryan J. Rafferty^{*b}

^aDepartment of Chemistry, Pontifical Catholic University of Puerto Rico, 2250 Boulevard Luis A. Ferré Aguayo, Suite 626 Ponce, PR 00717-0777

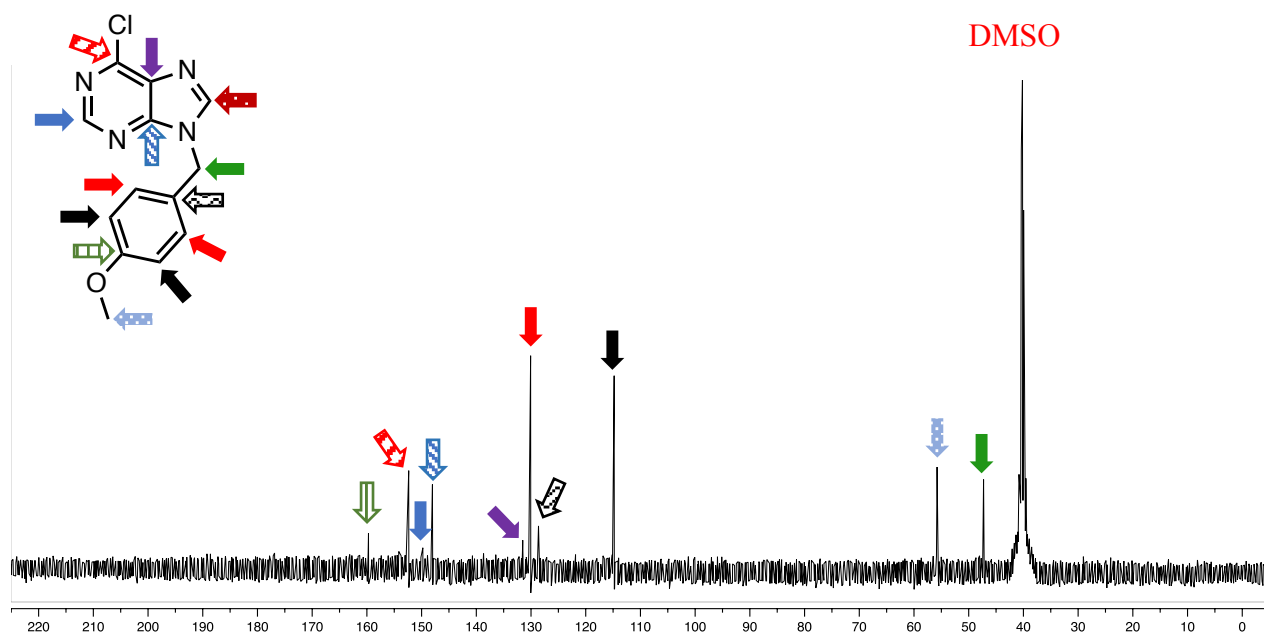
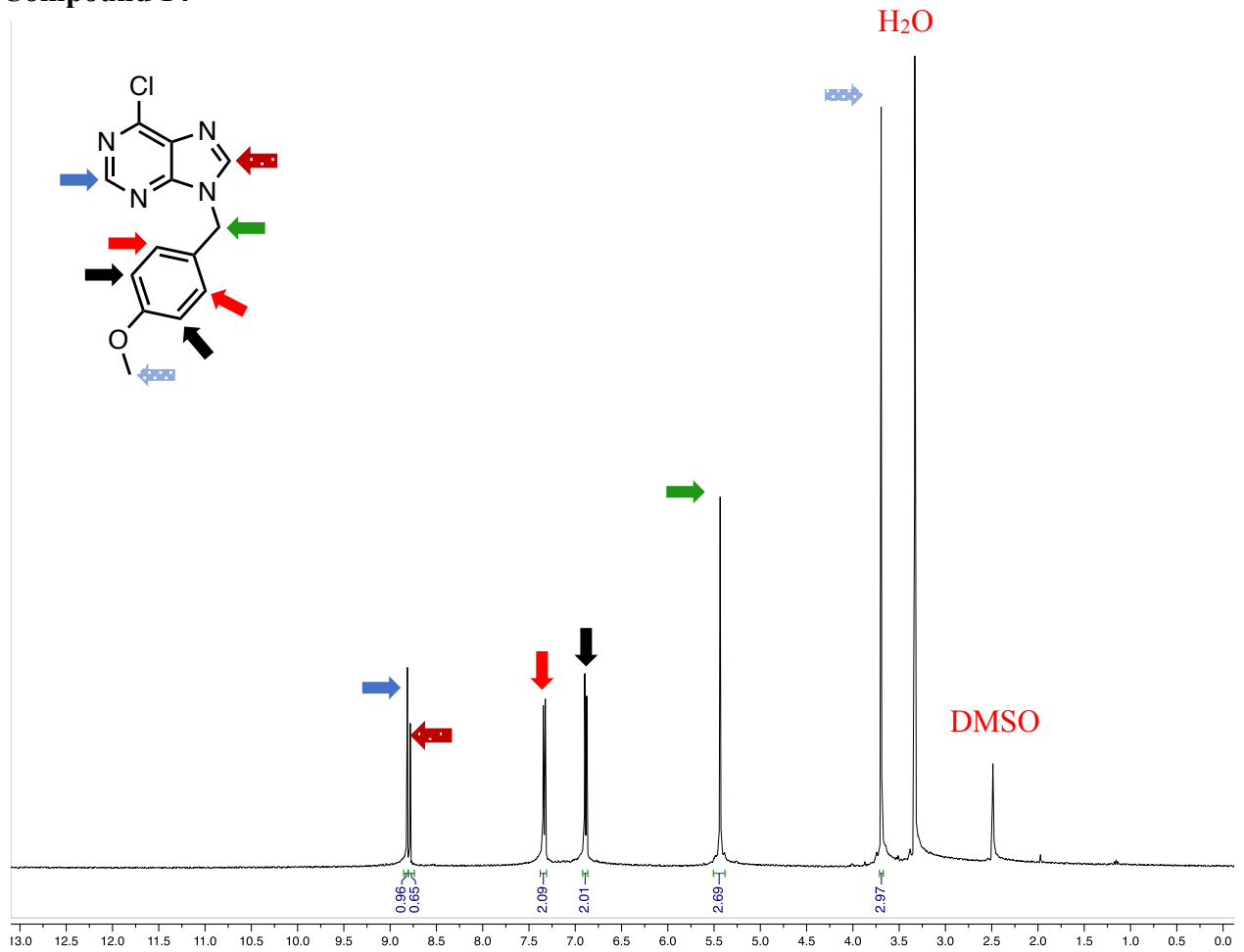
^bDepartment of Chemistry, Kansas State University, 1212 Mid-Campus Drive North, Manhattan, KS 66506

[‡]These authors contributed equally to the work.

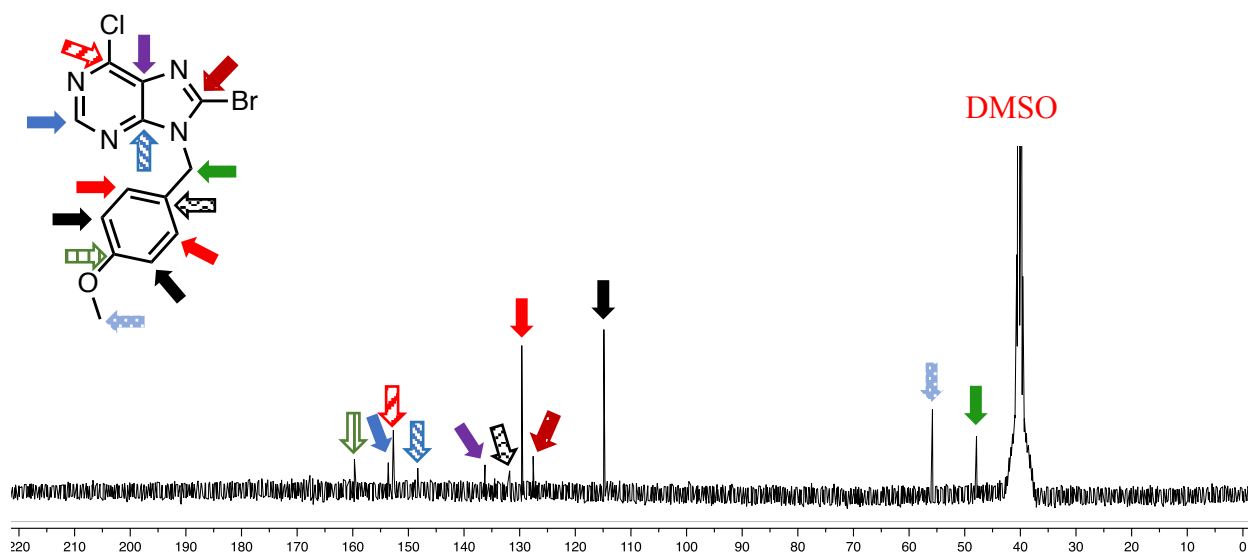
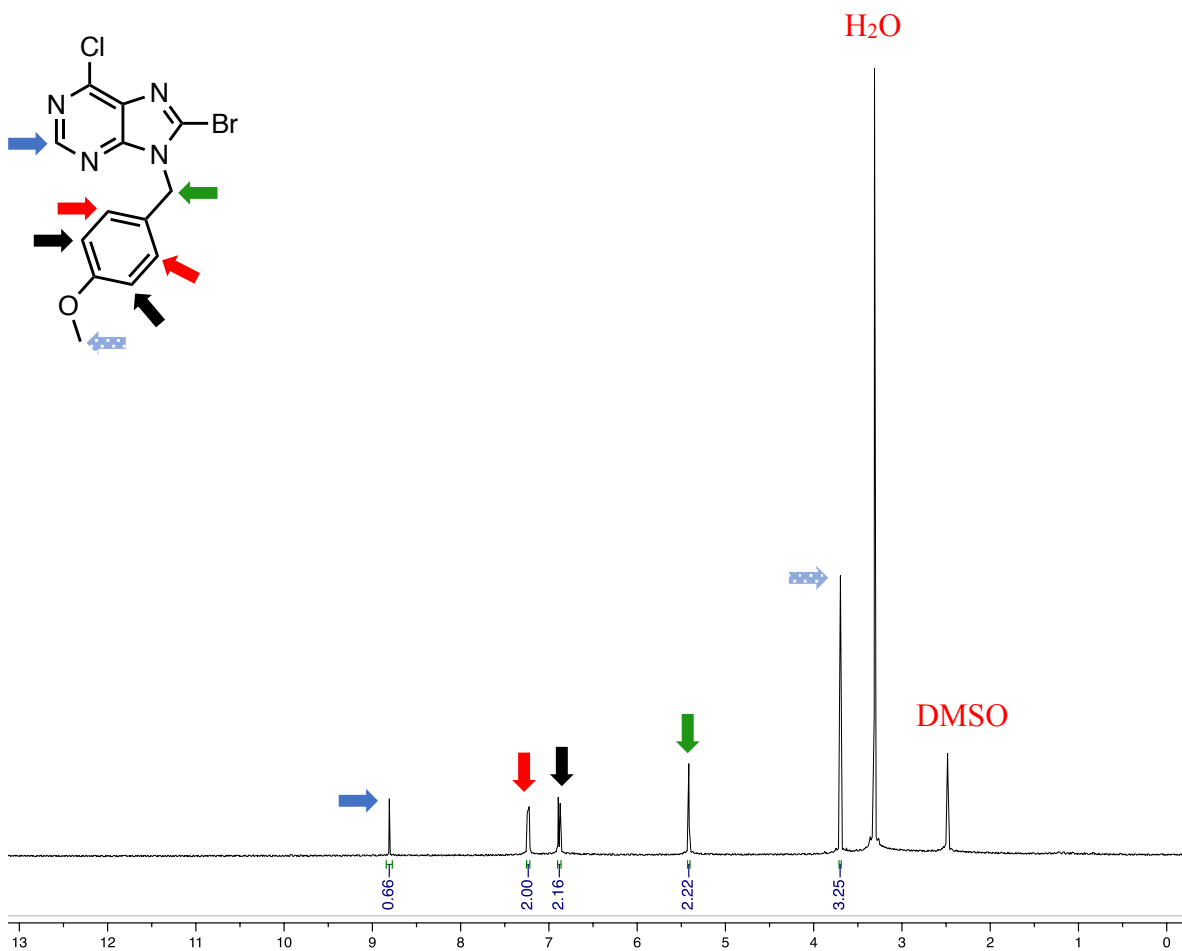
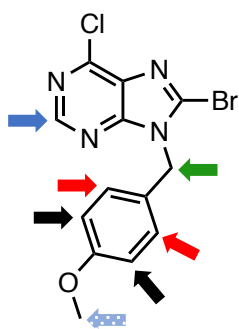
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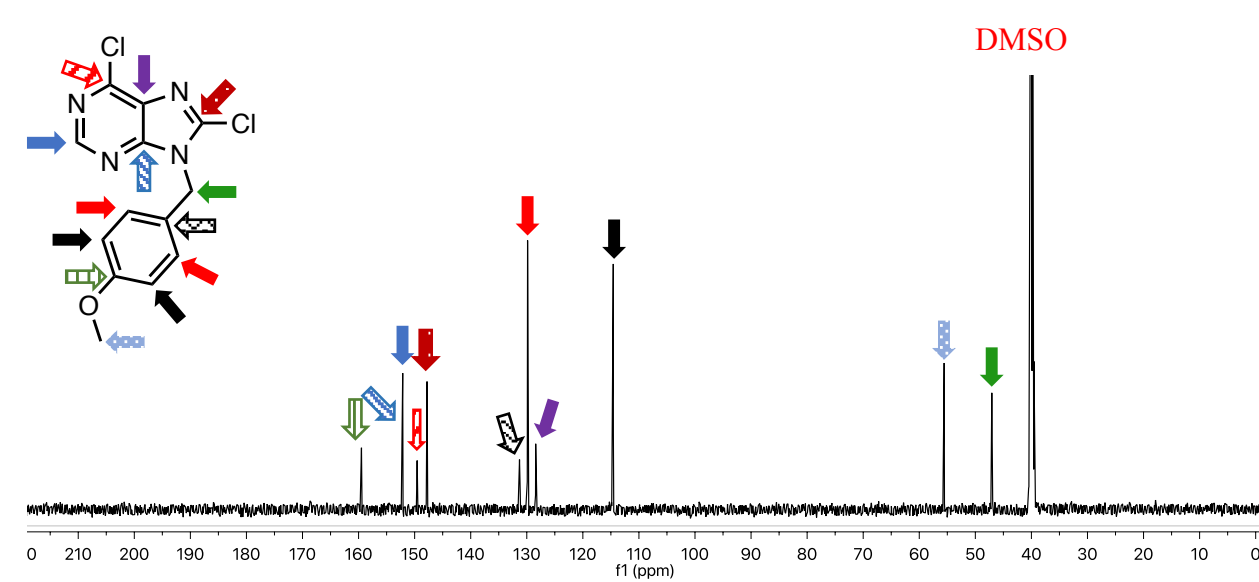
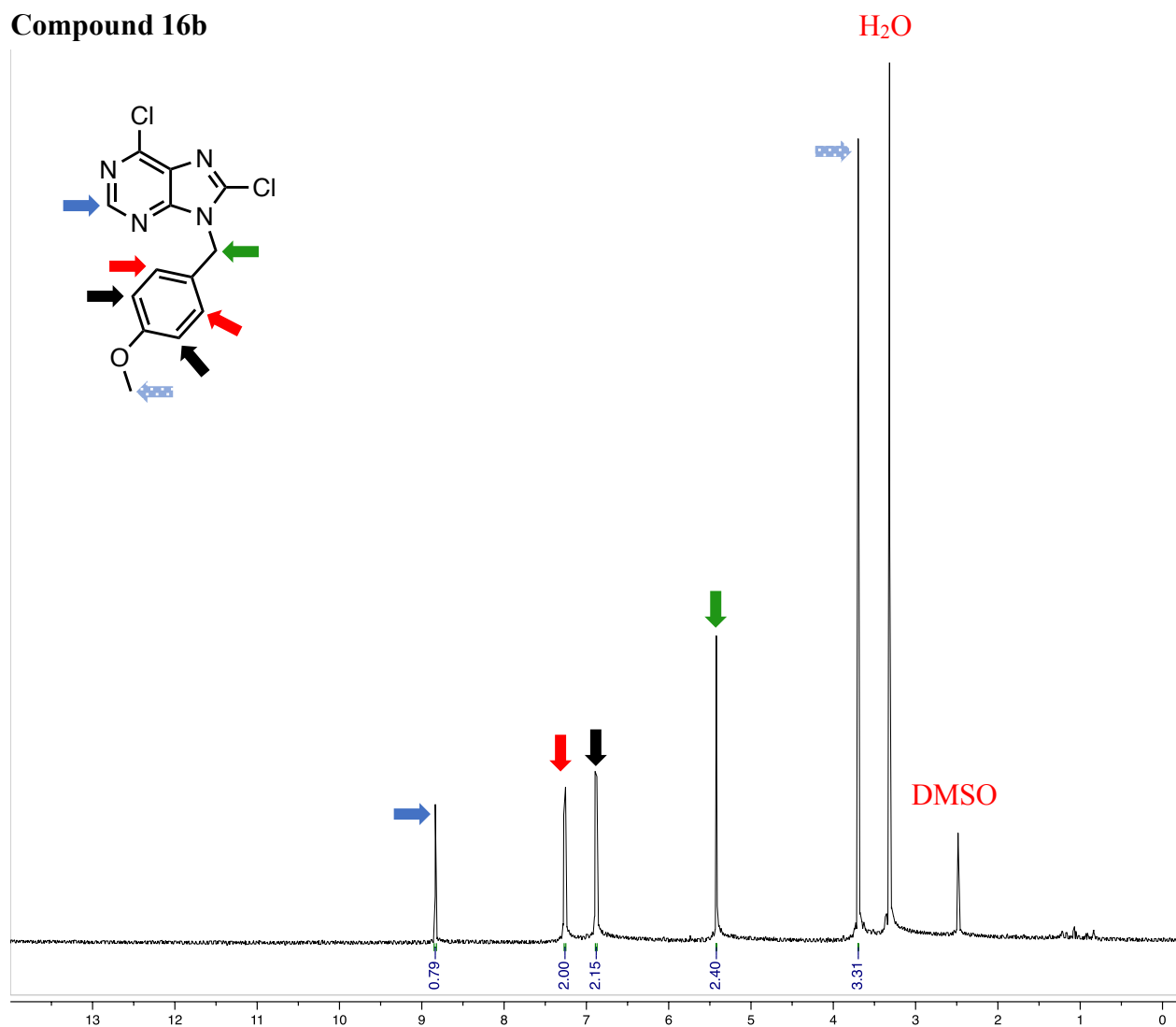
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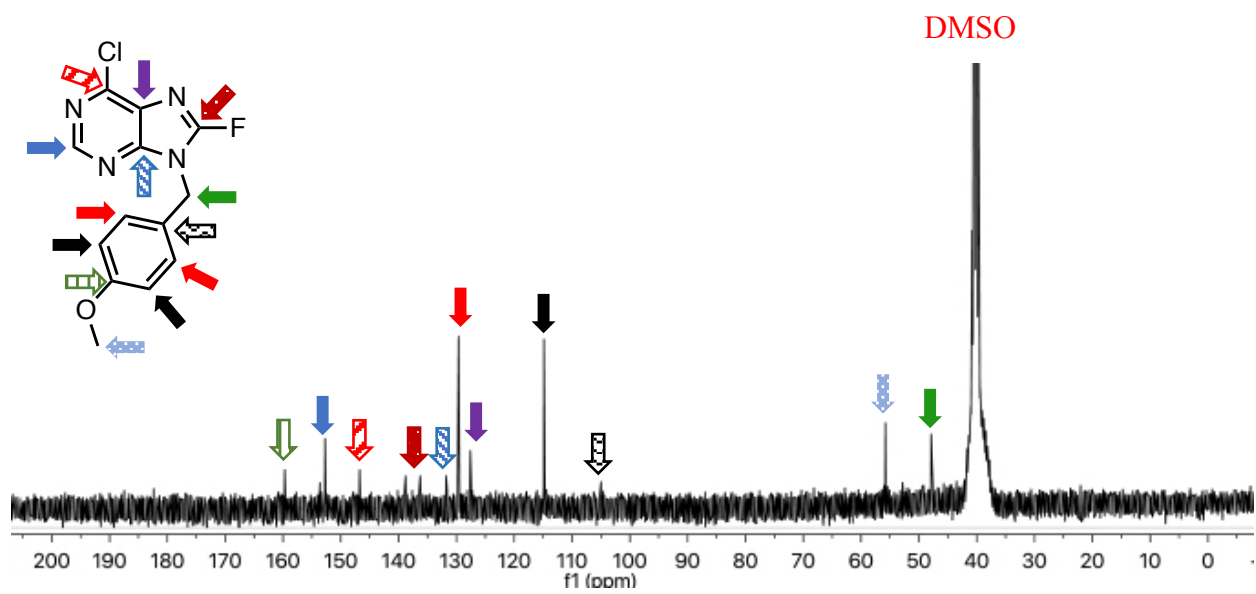
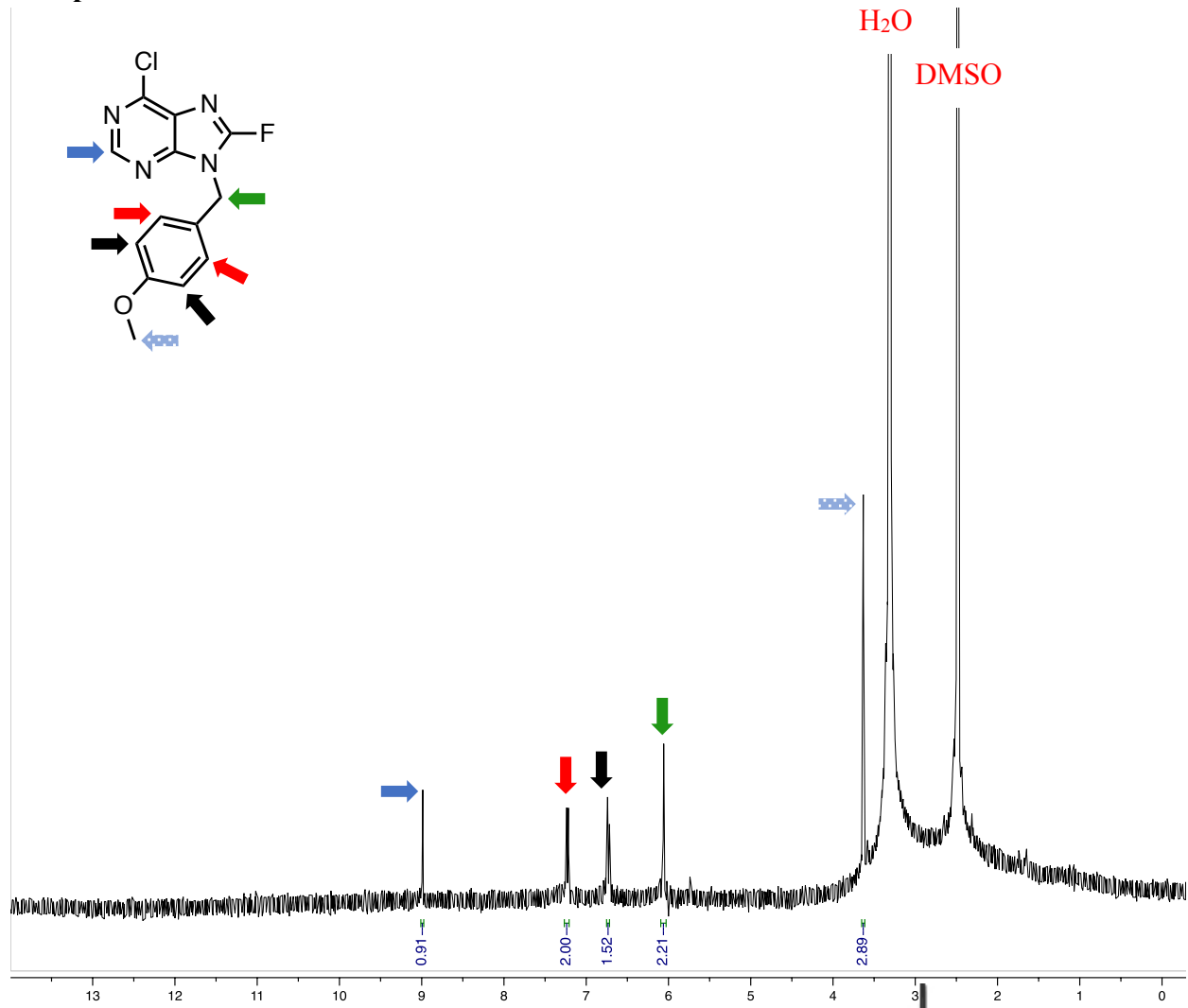
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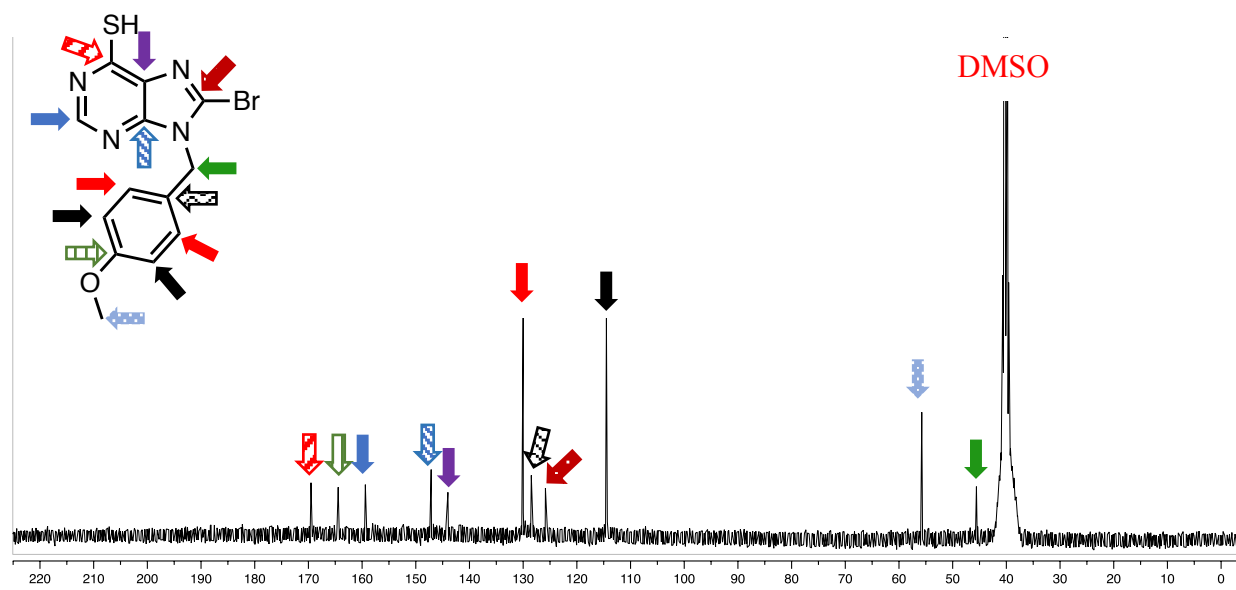
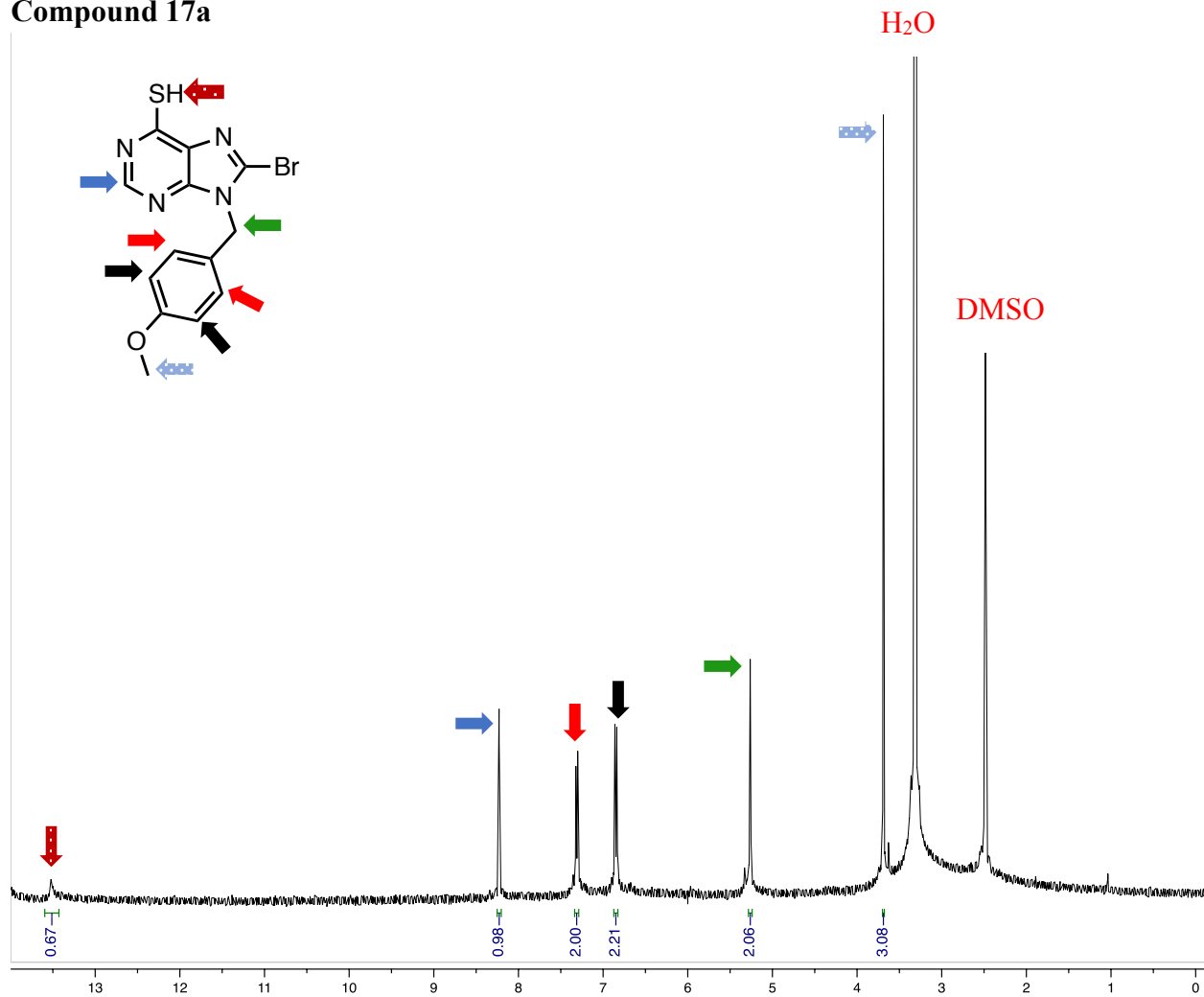
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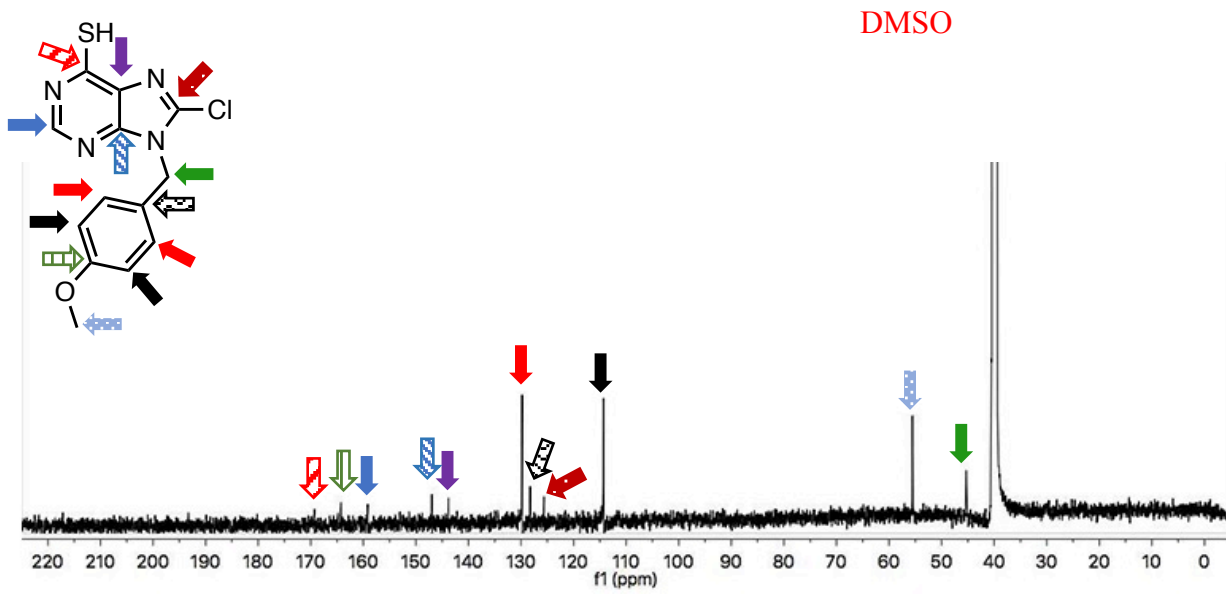
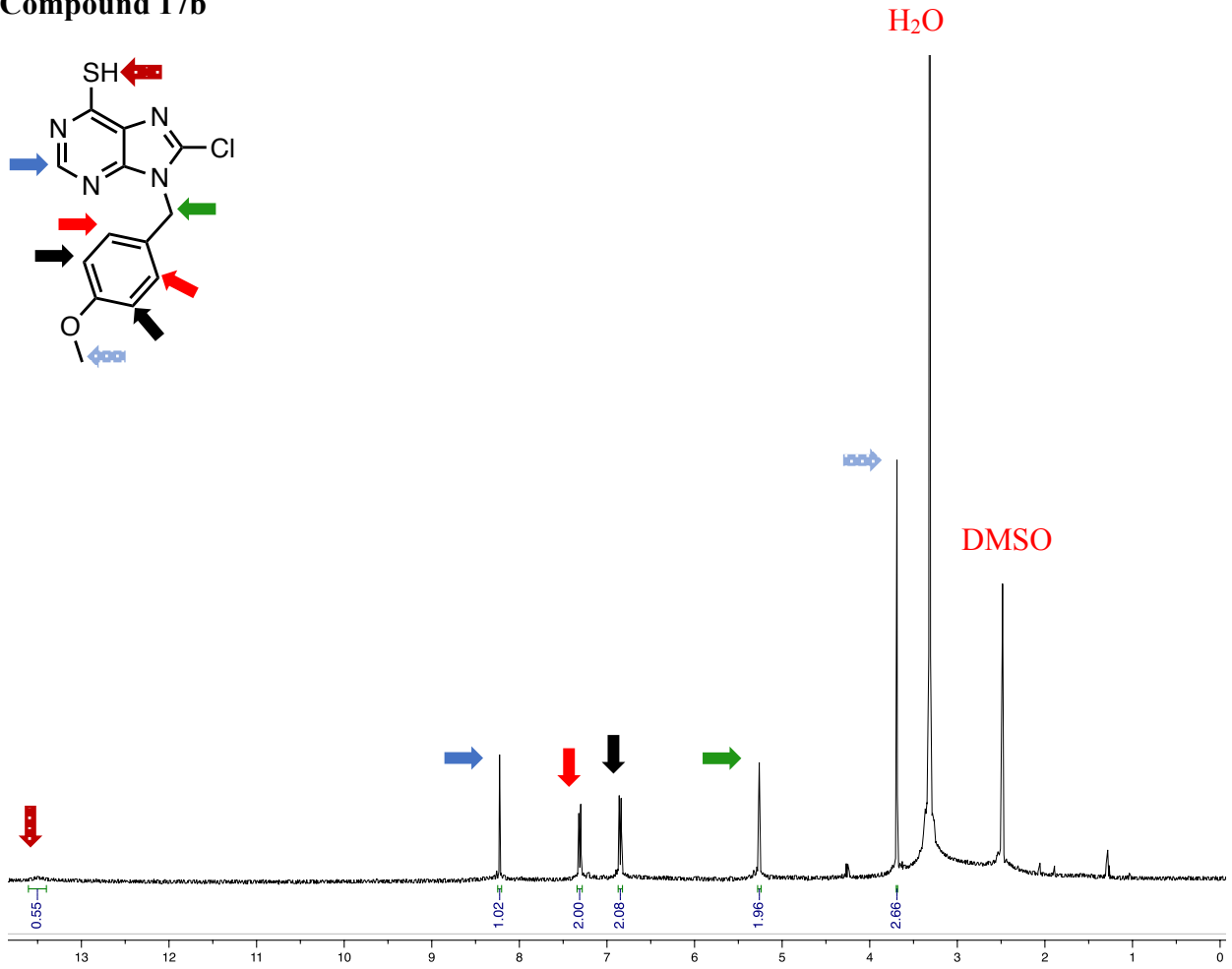
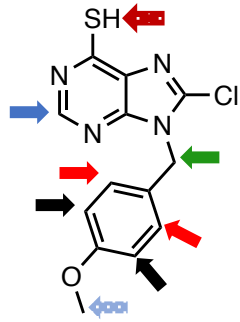
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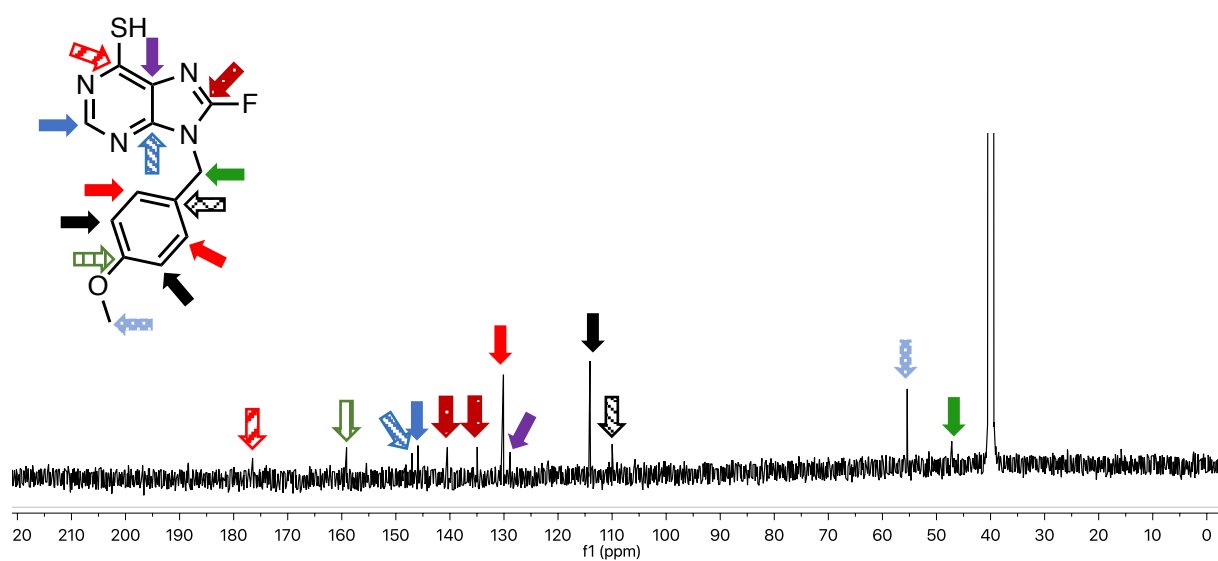
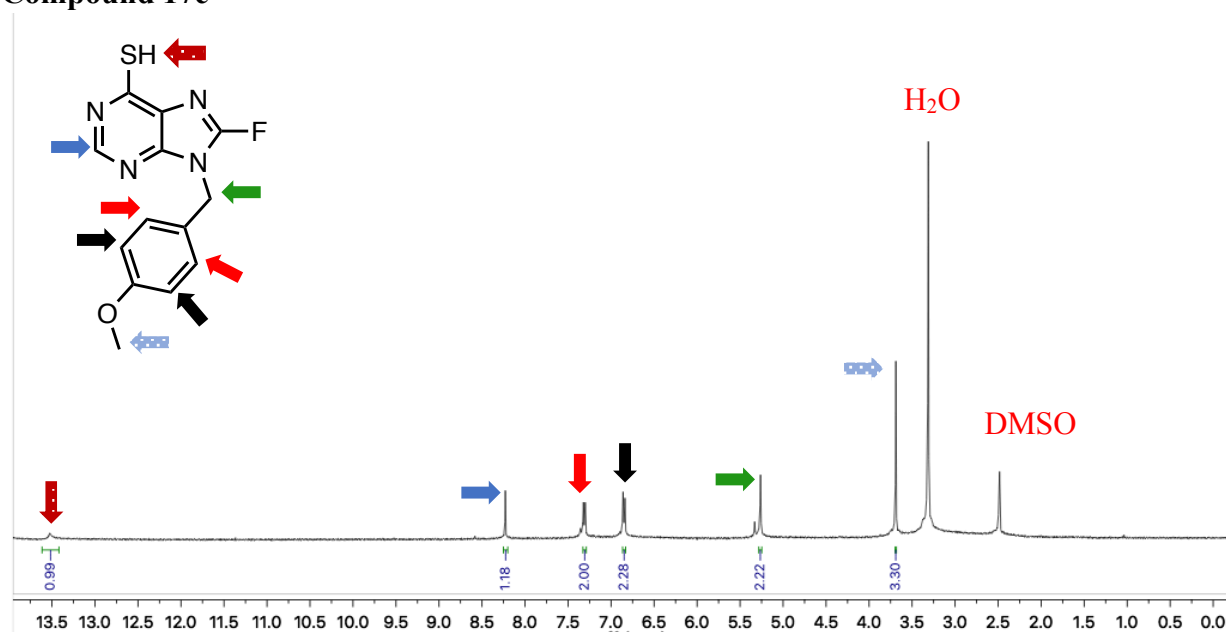
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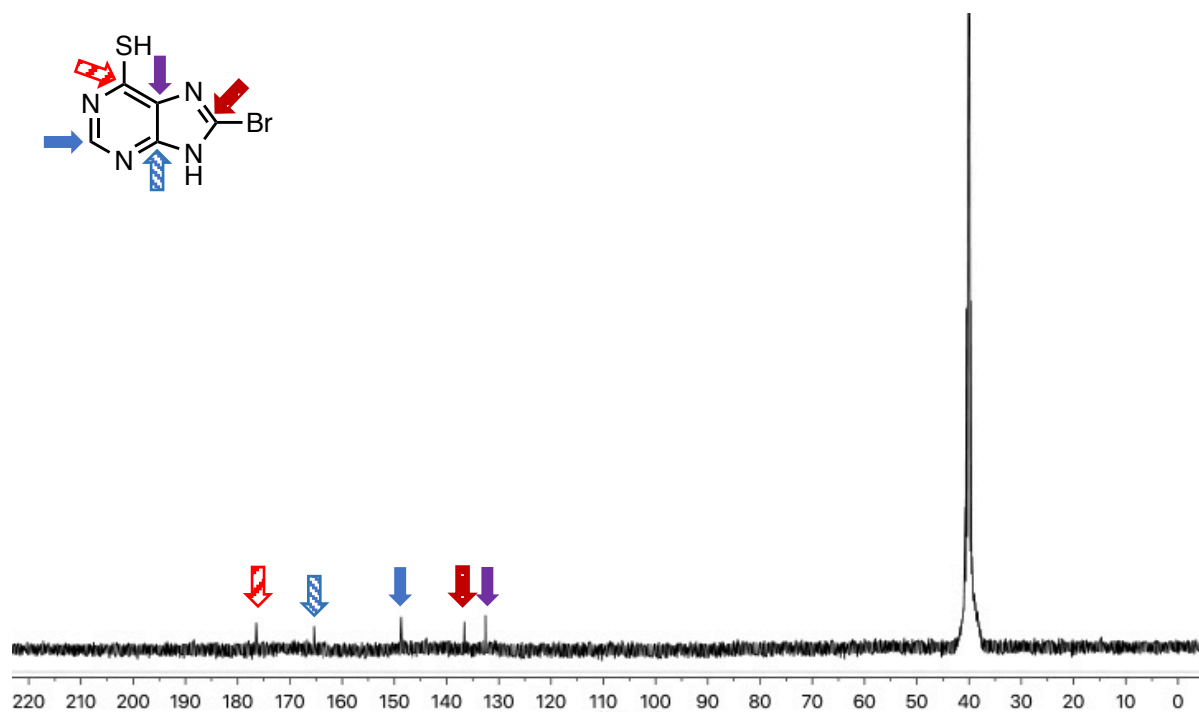
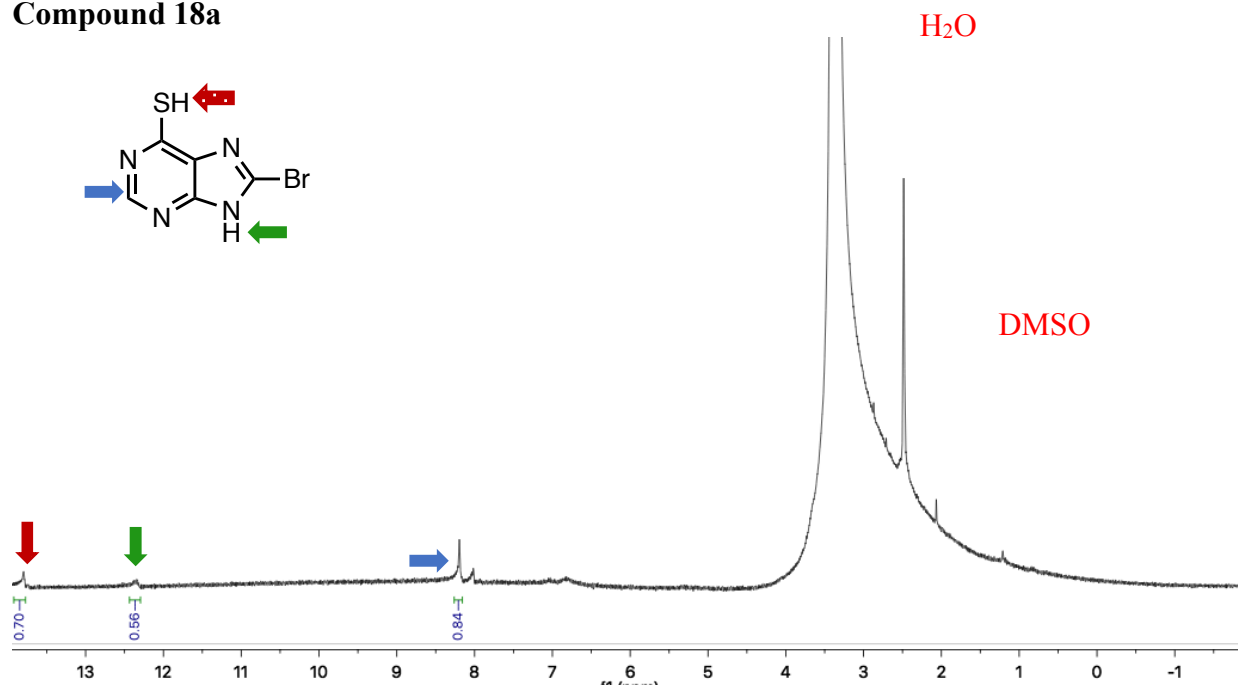
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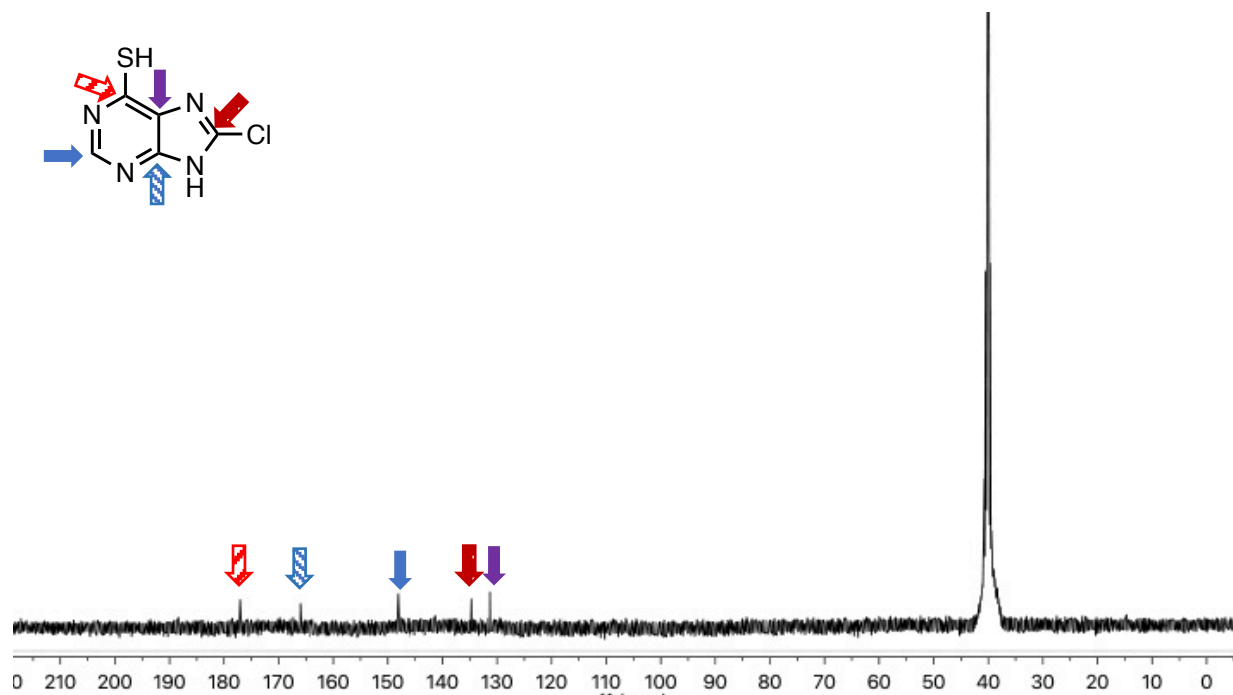
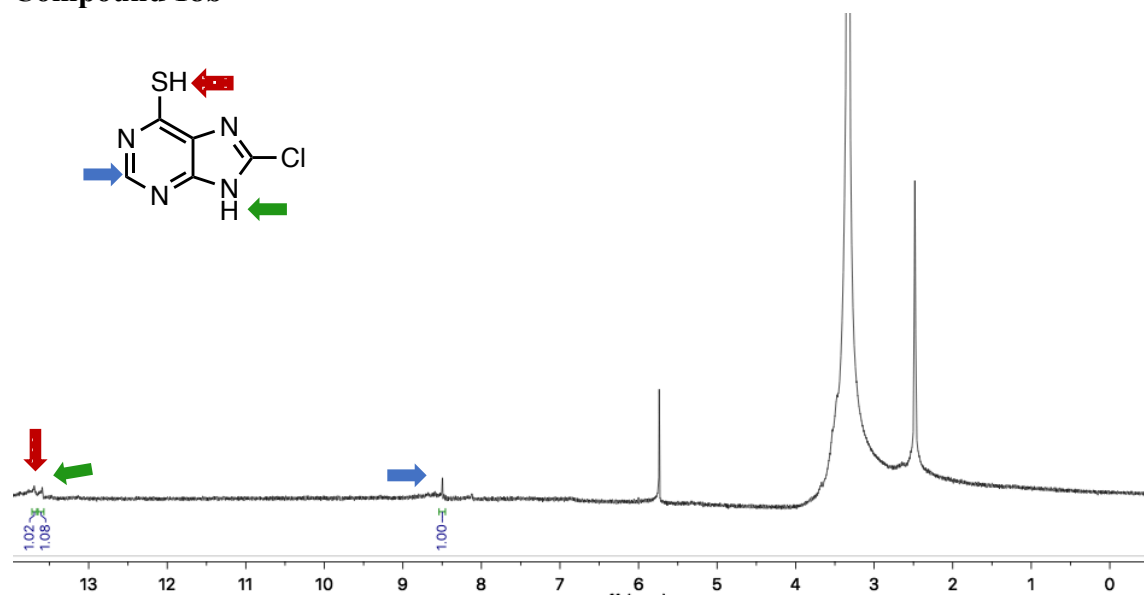
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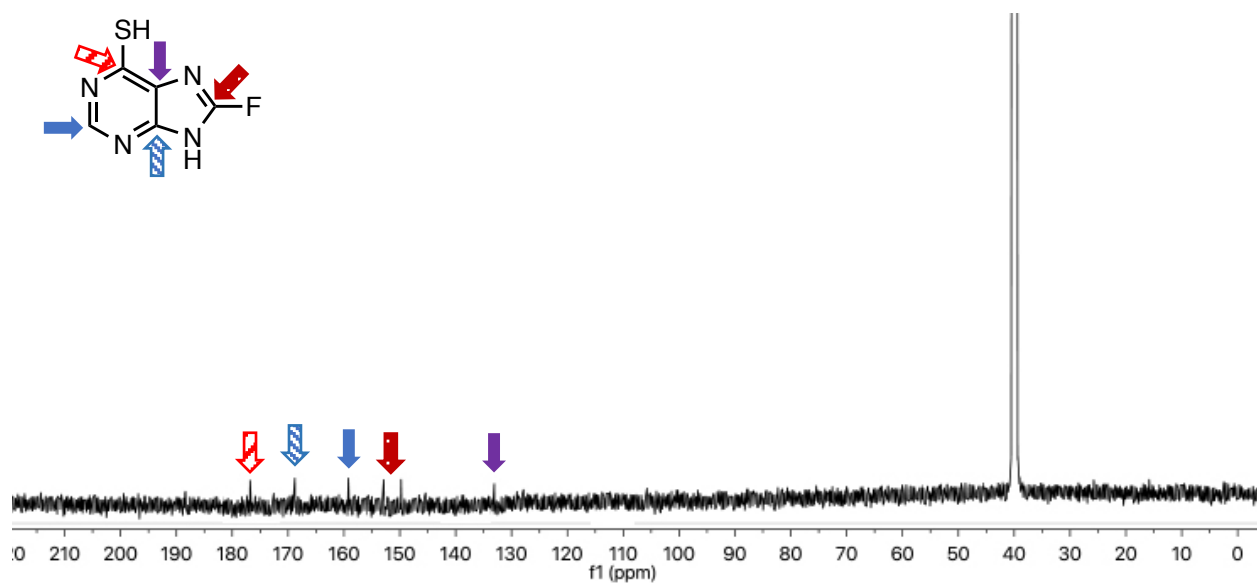
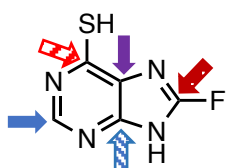
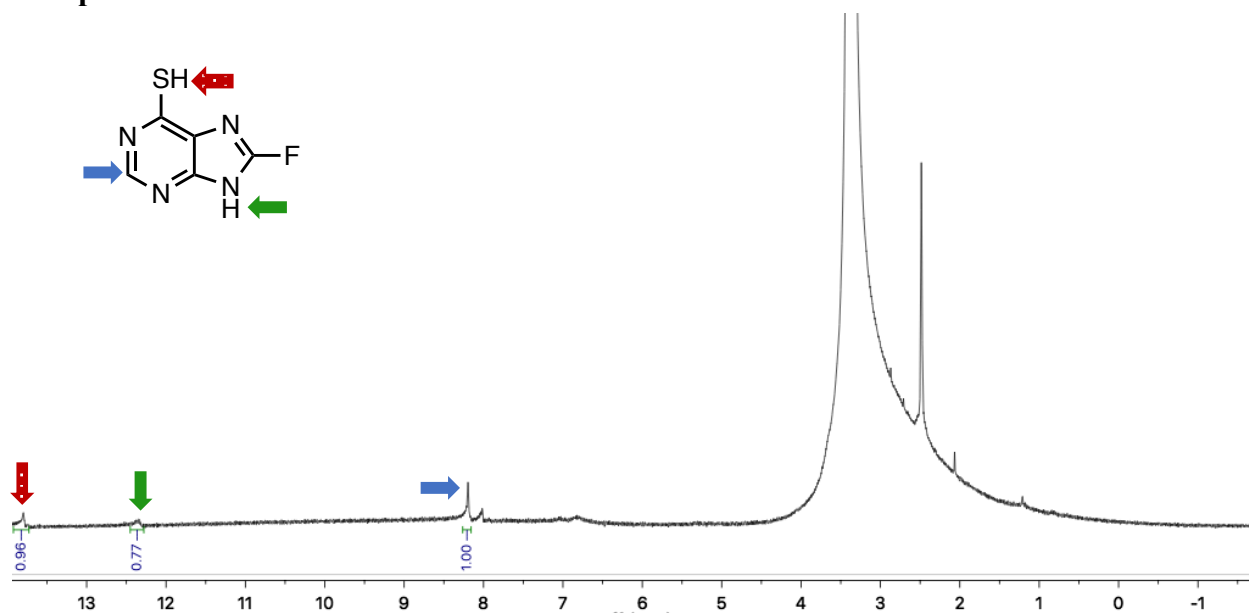
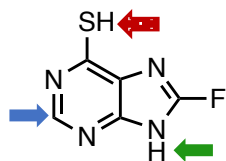
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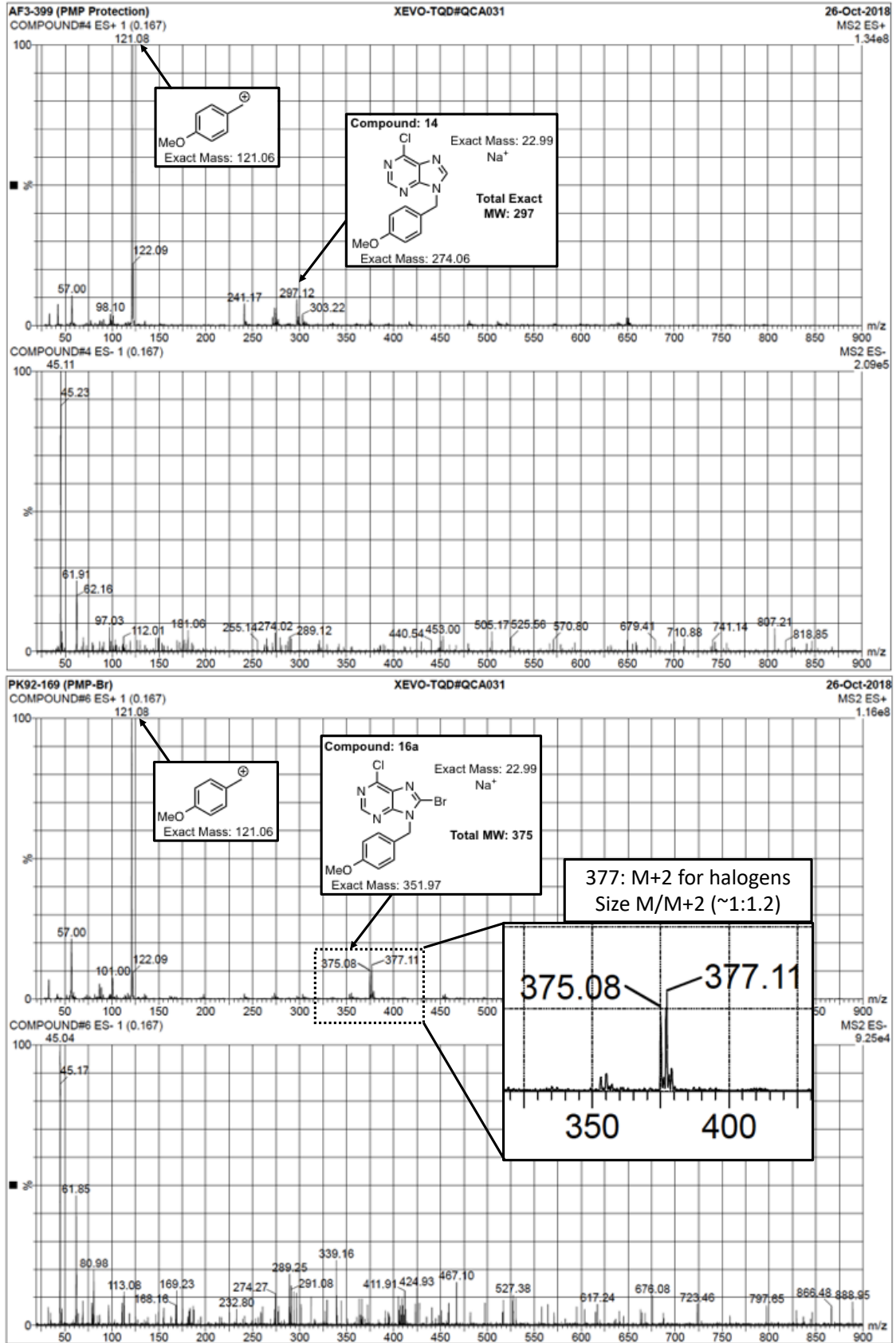


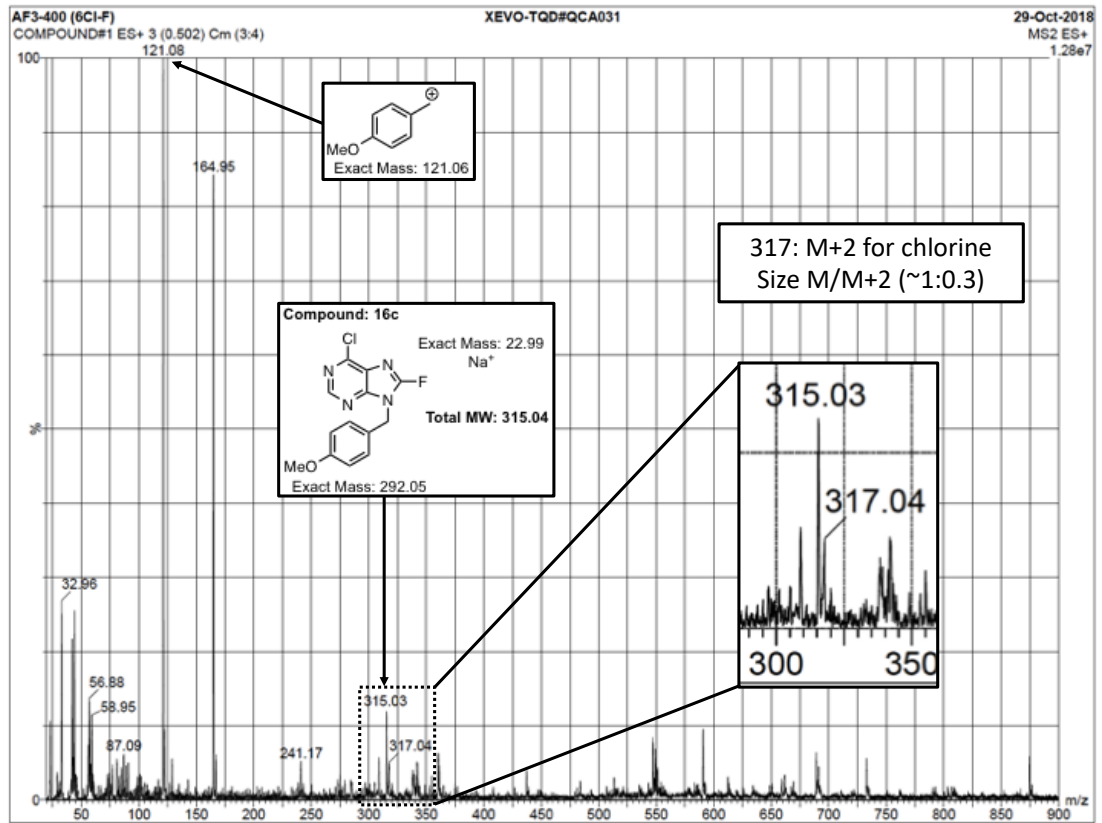
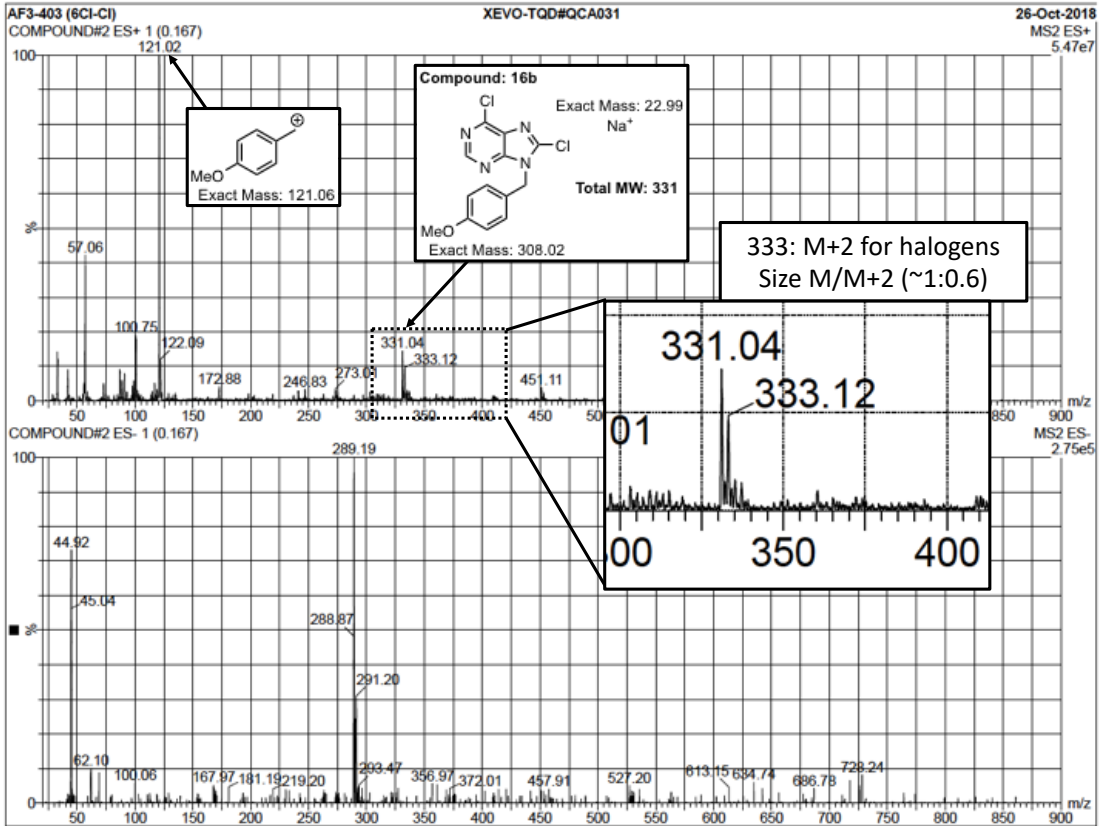
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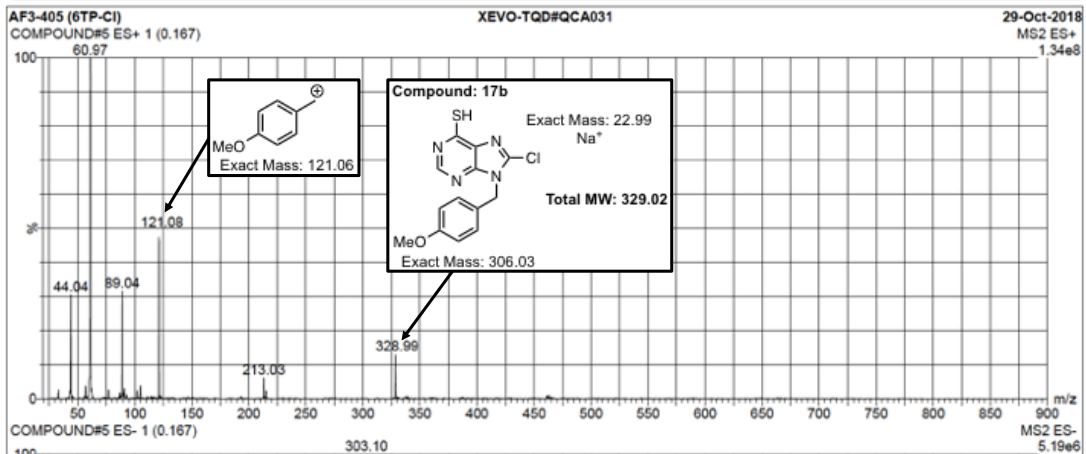
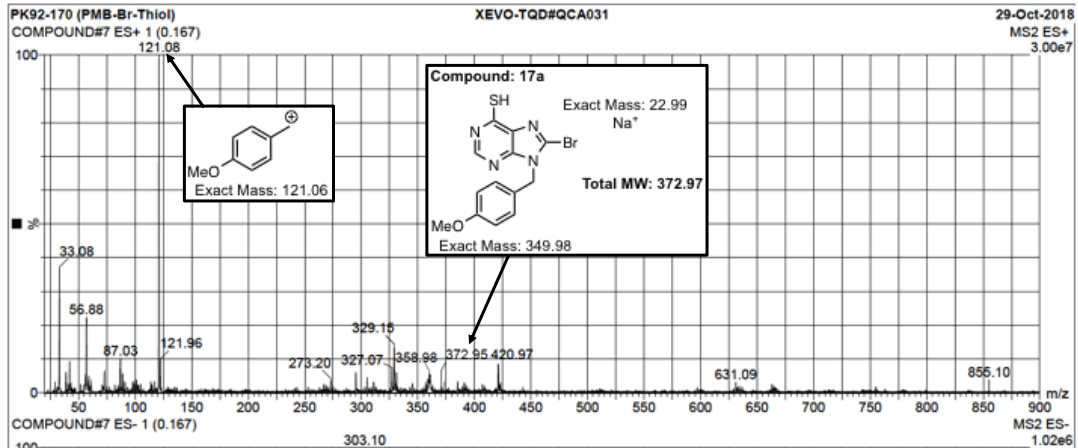


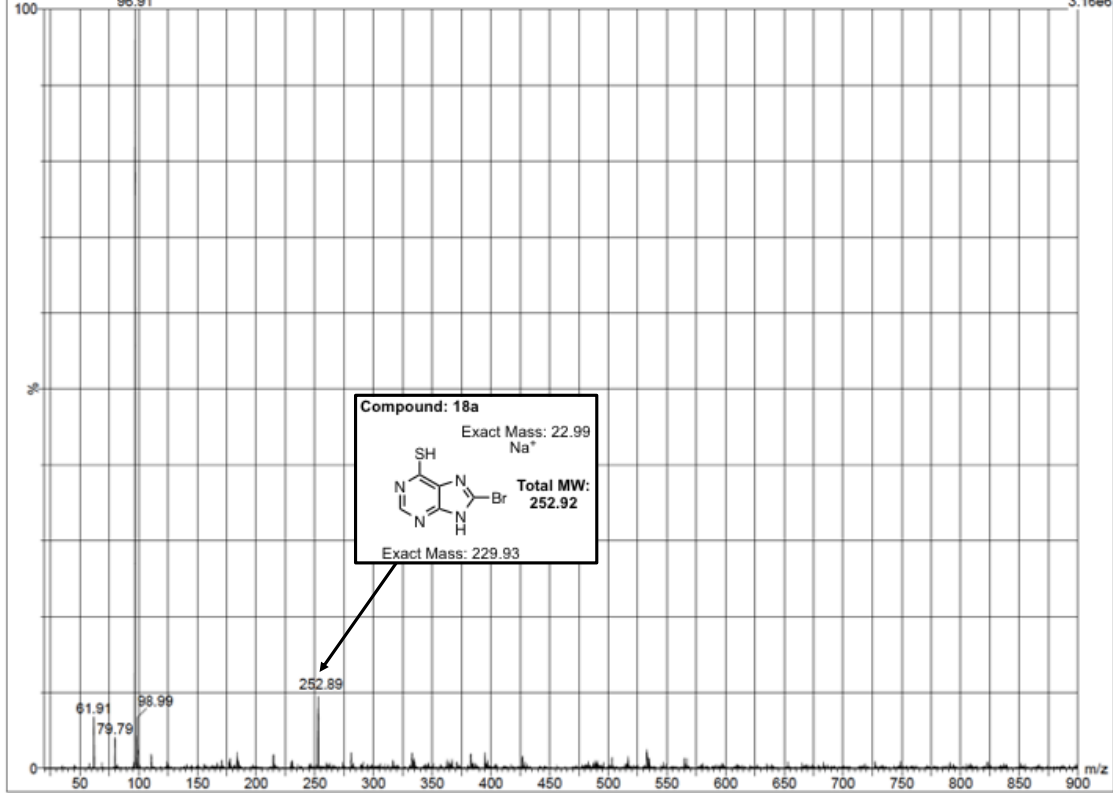
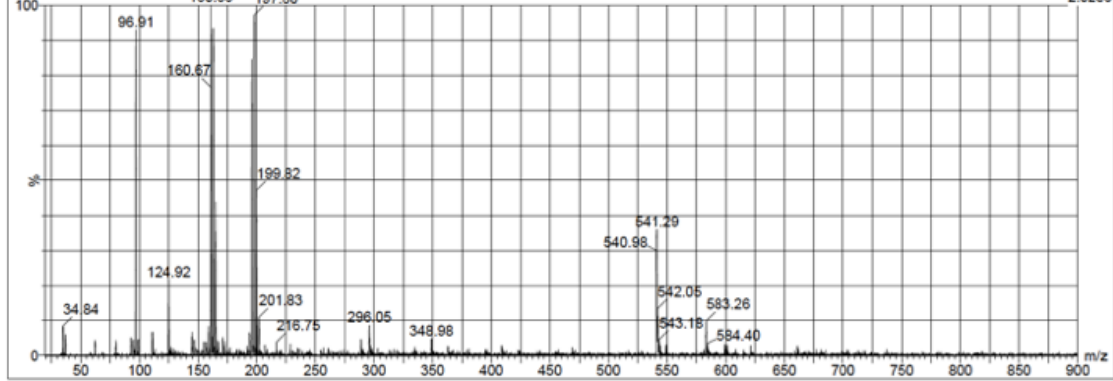
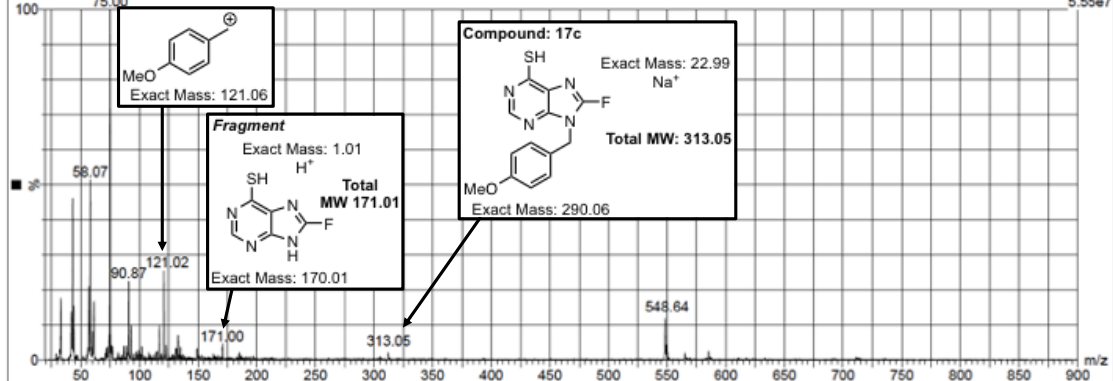
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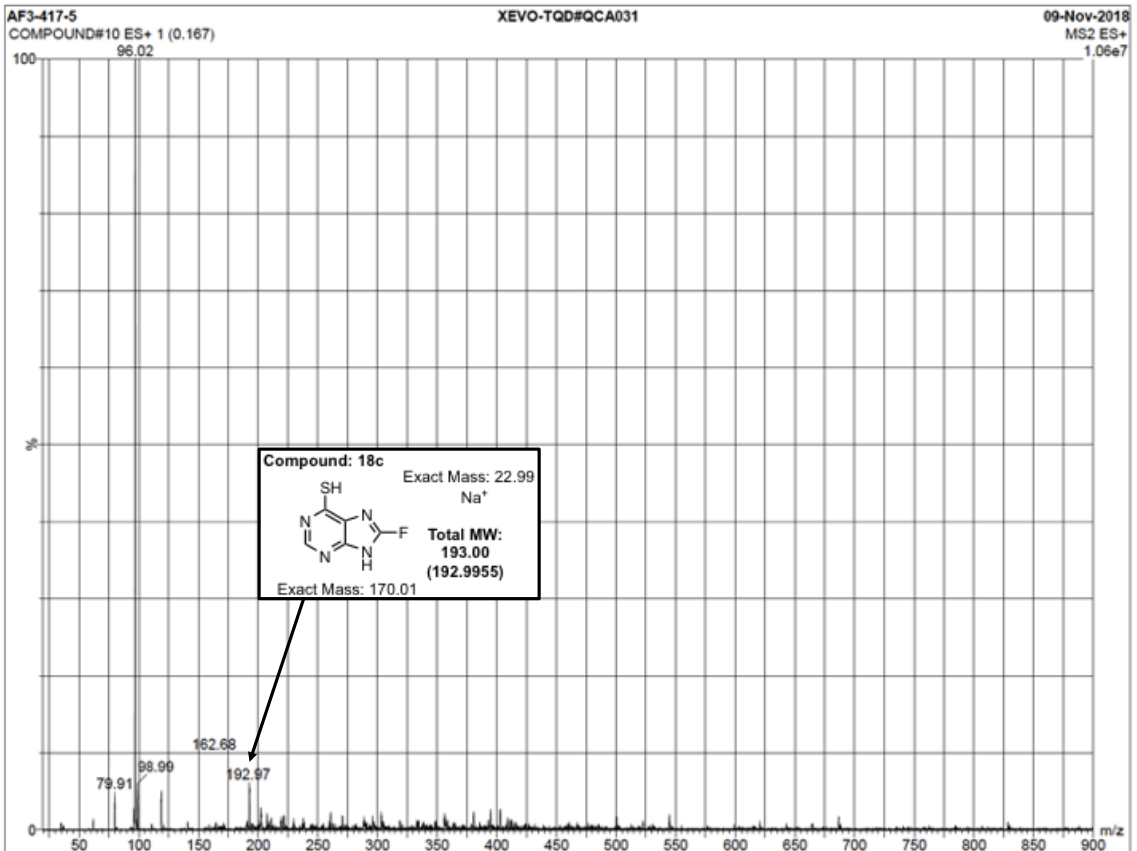
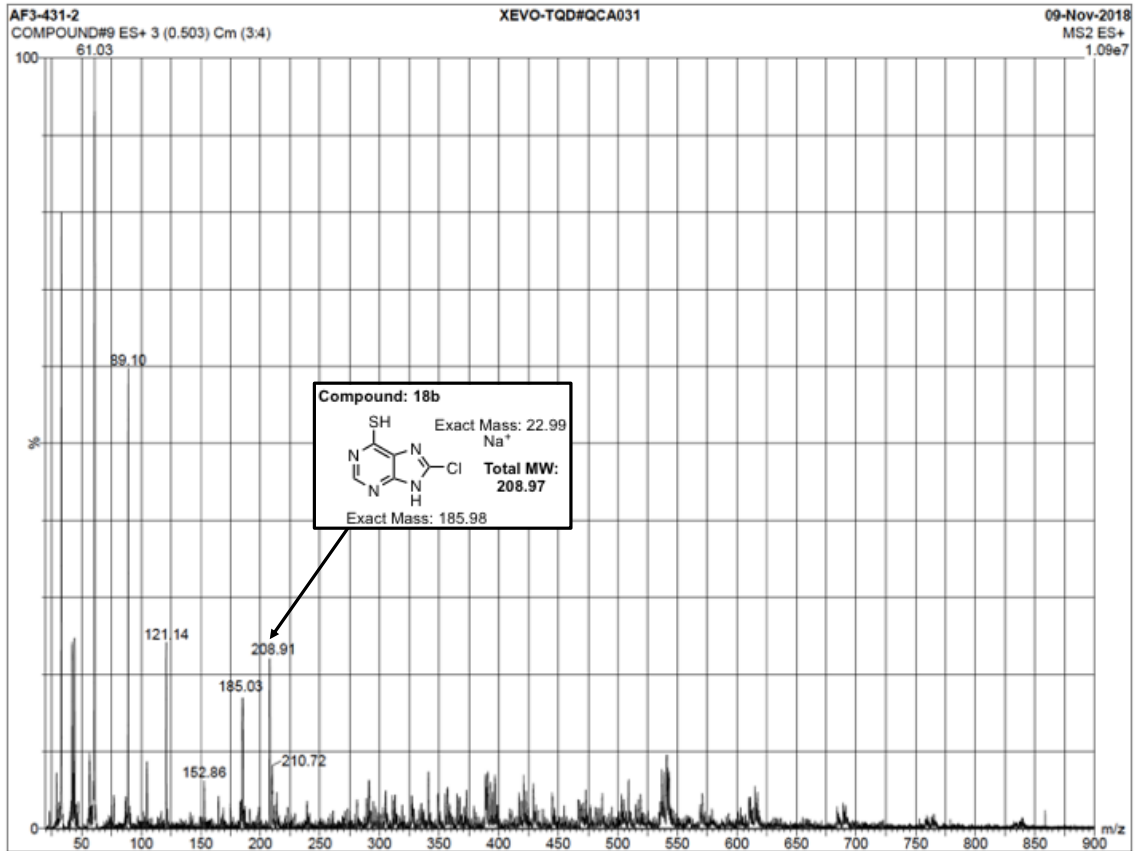












Biological

All standard chemicals used this study were the highest grades available and were purchased through Sigma Aldrich (Saint Louis, MO, USA), VWR (Radnor, PA, USA), Fisher Scientific (Denver, CO, USA), or AK Scientific (Union City, CA, USA). Specialized reagents were purchased through specific vendors. Glycylglycine (gly-gly: G1127), β -nicotinamide adenine dinucleotide (NAD⁺: N1636), uridine 5'-diphosphoglucose (UDPG disodium salt: U4625), uridine 5'-diphosphoglucuronic acid (UDPGA: U6751), uridine 5'-diphosphoglucose dehydrogenase (UDPGDH: U6885), 6-thiopurine monohydrate (6TP), bilirubin (including three mixed isomer, B4126), alamethicin (A5361), pooled rat liver microsomes, Williams' Medium E Formulation (WME: W1878), AND (M9066) were purchased from Sigma Aldrich. 6-Thiouric acid (6TU: SC-213040), and doxorubicin (SC-280681) was purchased from Santa Cruz Biotechnology. HPLC-grade water was obtained by passing distilled water through a reverse osmosis system followed by treatment with a Thermo Scientific Barnstead Smart2Pure 3UV purification system (Fisher: 10-451-045), herein referred to as nanopure water. Trypsin (30-2101), RPMI-1640 media (30-2001), 100 μ g/mL penicillin & 100 U/mL streptomycin (30-2300), and Fetal Bovine Serum (FBS: 30-2020) was purchased from American Tissue Culture Collection (ATCC). Sprague-Dawley male rats, 8-10 weeks old, were purchased from Taconic.

All standard consumable supplies used in this study were purchased from VWR or Fisher Scientific. All materials used in biological evaluation was purchased in sterile packaging. Specific equipment utilized in this work are: 1) Hewlett-Packard 8452 Diode Array UV/Vis spectrophotometer (Palo Alto, CA, USA) equipped with a Lauda Brinkman Ecoline RE 106 E100 circulating water bath (VWR), 2) HPLC system consisting of an CBM-20A/20Alite system controller, SIL-20AHT Autosampler, SPD-20A SPD-20AVUV-Vis detector, LC-20AT Solvent

delivery module, CTO-20A column oven, DGU-20A3R Degassing unit and LC-20AD/20AT Gradient Value Kit purchased from Shimadzu Scientific Instruments (Kyoto, Japan), 3) all incubated reactions were performed with a Labcare American PRECISION water bath model 25 purchased from Fisher Scientific, and 4) BioTek Synergy H1 Hybrid Multi-Mode reader purchased from Fisher Scientific. All HPLC separations were performed on a Discovery C18 analytical column, 4.6 mm x 100 mm, 5 μ m particle size (504955-30) along with the respective guard column (59576) purchased from Sigma Aldrich. Data was processed and all figures and tables constructed via the program Prism 7.02 for Mac, GraphPad Software (La Jolla, CA, USA). All chemical structures were prepared with ChemDraw Professional 16.0 by Perkin Elmer (Waltham, MA). All statistical calculations within this body of work was performed by the treatment of two-way factorials (positive and negative controls, designed structure of RCBD, and T-tests).

UDP-Glucose Dehydrogenase Inhibition Assay

Standard preparation: A 0.50 M Gly-Gly buffer (pH 8.7 at 25 °C) was prepared by dissolving 1.62 g of Gly-Gly in 22 mL nanopure water, pH adjusted to 8.7 with 9 M potassium hydroxide and then diluted to 25 mL with nanopure water all while being shaken at 25 °C. Solutions of UDPG and NAD⁺ were prepared by dissolving 1.7 mg and 4.5 mg, respectively, into nanopure water resulting in 2 mM and 10 mM solutions, respectively. Preparation of the 0.1 mM 6TU solution was done by dissolving 1.8 mg into 95.5 mL of nanopure water, pH was adjusted to 9.5 with a 2 M sodium hydroxide solution (for solubility), pH adjusted to 7.5 with a 0.5 M hydrochloric acid solution (a dilute solution of HCl is required to prevent thiol oxidation), and then diluted to 100 mL. All other purine samples were prepared in an analogous fashion to desired concentrations. A 5 unit/mL UDPGDH solution was prepared by dissolving 0.11 mg of UDPGDH in 0.8 mL of 0.5 M Gly-Gly while stirring; once dissolved, the solution was diluted to volume with gly-gly in a 1 mL volumetric flask. All solutions were kept at 4 °C when not in use.

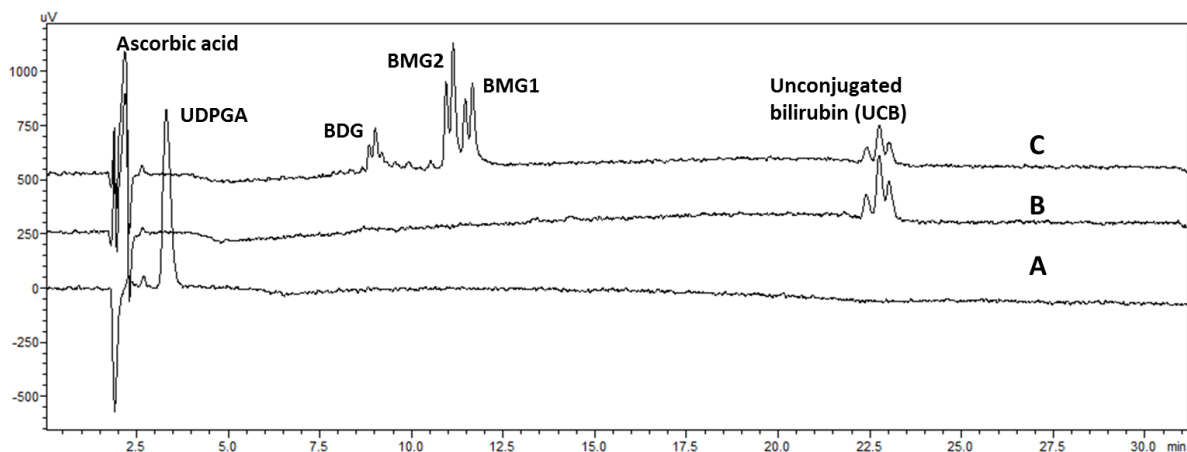
UDP-glucuronosyltransferase activity assay

Standard preparation: A bilirubin stock solution was prepared by dissolving bilirubin in 100% dimethyl sulfoxide to yield a concentration of 2 mM, the stock solution was aliquoted, and stored at -70 °C until use. A 25 mM UDPGA stock solution was prepared by diluting 8 mg to 0.5 mL with nanopure water, and a 10 mg/mL alamethicin solution was prepared by taking 5 mg and diluting to 500 µL with methanol. Preparation of the 100 mM potassium dihydrogen phosphate buffer was done by dissolving 2.3 g of KH₂PO₄ into 80 mL of nanopure water, pH adjusted to 7.4 with 1 M HCl and diluted to volume in a 100-mL volumetric flask.

Chromatographic Conditions – Bilirubin and Bilirubin Glucuronide: Bilirubin and its glucuronide were separated on a Discovery C18 analytical column, 4.5 mm x 100 mm, 5 µM

particle size with guard column. A dual mobile phase was employed; the aqueous phase consisted of an 8 mM imidazole & 2.5 mM tetrabutylammonium hydrogen sulfate (TBAHS) buffer at a pH of 6.5 in nanopure water and acetonitrile as the organic phase. A gradient elution profile was employed for full separation at a flow rate of 0.5 mL/min, the method begins at 10% acetonitrile and increases to 50% over 8 minutes, held for 5.5 minutes, increased to 95% over 4.5 minutes, held for 10 minutes, returned to 10% over 4 minutes and held at 10% for 2 minutes to allow for column regeneration. The detection wavelength was 450 nm with a sample injection volume of 5 μ L. The combined peak area for bilirubin (sum of the three isomers) was plotted relative to the concentration prepared for the generation of a working standard curve.

Figure S1: Chromatograms of UDPGA (at 262 nm) line A, unconjugated bilirubin (at 450 nm) line B, and BMG1&2 and BDG (at 450 nm) line C.



Quantification of Bilirubin, and Mono/Di-glucuronide Levels: A total of ten peaks for the glucuronide species, including their isomers were detected in the incubation samples. Peak assignment and identification of UCB, BMG1, BMG2, BDG and their isomers were based on their lipophilicity and polarity, as well as the elution pattern, chromatographic peak position and relative

retention time from previous reports.¹⁻⁴ The calibration curves for bilirubin were used to determine the concentration of the mono- and di-glucuronide species employing the gradient HPLC bilirubin method described above.

Figure S2: Reaction progressive curve for bilirubin: Bilirubin consumption was measured in a UGT1A1 catalyzed reaction every 15 minutes for two-hour period and bilirubin level went down linearly for 1 hour. Therefore, glucuronidation reaction was carried out for 45 minutes for inhibition assay.

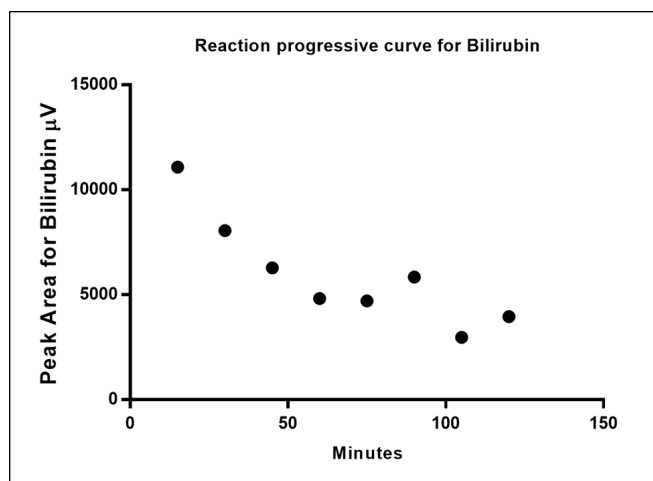
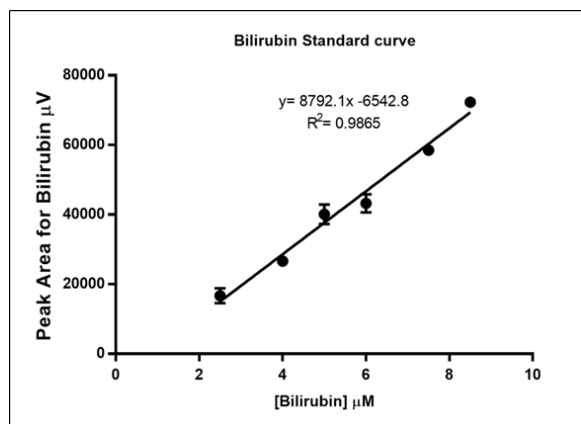


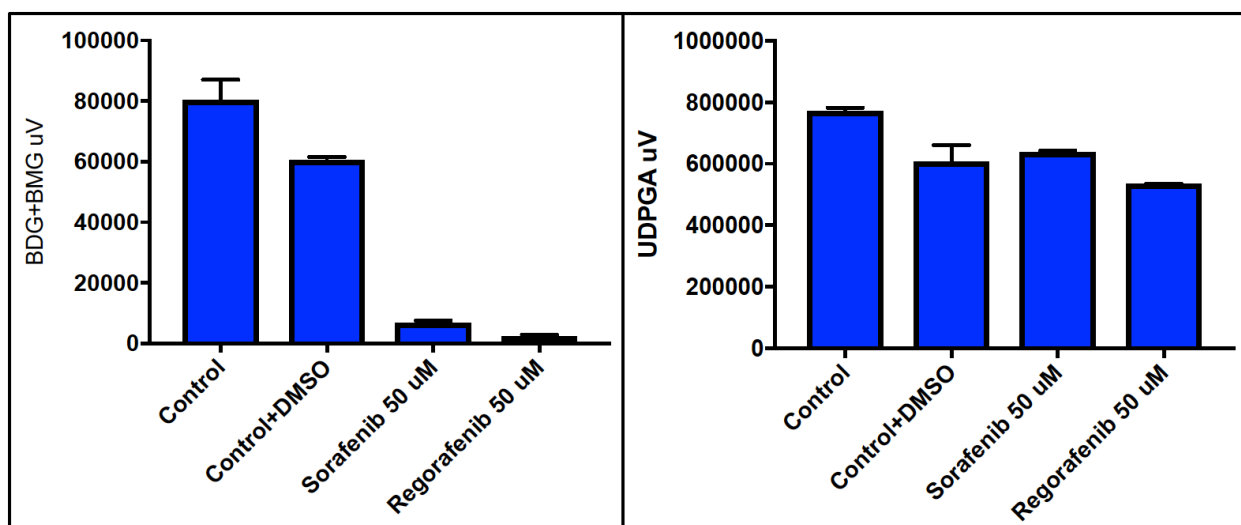
Figure S3: Standard curve for bilirubin: The sum of the peak areas of three isomers of bilirubin was plotted against the standard concentrations to establish the standard curve (n=3).



Bilirubin Glucuronide Formation: Bilirubin glucuronidation was performed at 37 °C in a shaking water bath. All steps taken were performed in the lowest light conditions possible; the glucuronide formed was found to be unstable to ambient lighting. The following was added to an Eppendorf tube to achieve the final concentrations indicated, final volume 200 μL : potassium phosphate buffer (50 mM, pH 7.4), bilirubin (10 μM), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.88 mM), rat liver microsomes (RLM, 100 μg of protein/mL), alamethicin (22 $\mu\text{g}/\text{mL}$), and allowed to pre-incubated

for 2 min. Addition of UDPGA (3.5 mM), referred to as the zero-time point, initiated the reaction. The mixture was allowed to shake at 37 °C for each of the time course experiments. To each reaction 600 µL of ice-cold methanol containing 200 mM ascorbic acid was added to terminate the enzymatic reaction, vortexed for 2 min, and then centrifuged at 12,000 rpm for 10 min. The supernatant was then analyzed by the developed gradient HPLC protocol for separation and quantification of UCB, BMG1, BMG2, and BDGs.

Regorafenib and Sorafenib Inhibition Assessment Towards UGT-1A and UDPGDH



Supplemental Table S1. Inhibitor assessment of known UGT inhibitors sorafenib and regorafenib towards UGT (left) and UDPGDH (right). Inhibitors were dissolved in DMSO, as such controls with no inhibitors used had the same volume of DMSO added for direct comparison of the inhibition assessments. Each analyte is an average of three independent runs with standard error bars (SEBs). Many SEBs are not visible due to small value of the error. (n=3)

References

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