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

Design, Synthesis and Biological Evaluation of a Halogenated Phenazine-Erythromycin Conjugate Prodrug for Antibacterial Applications

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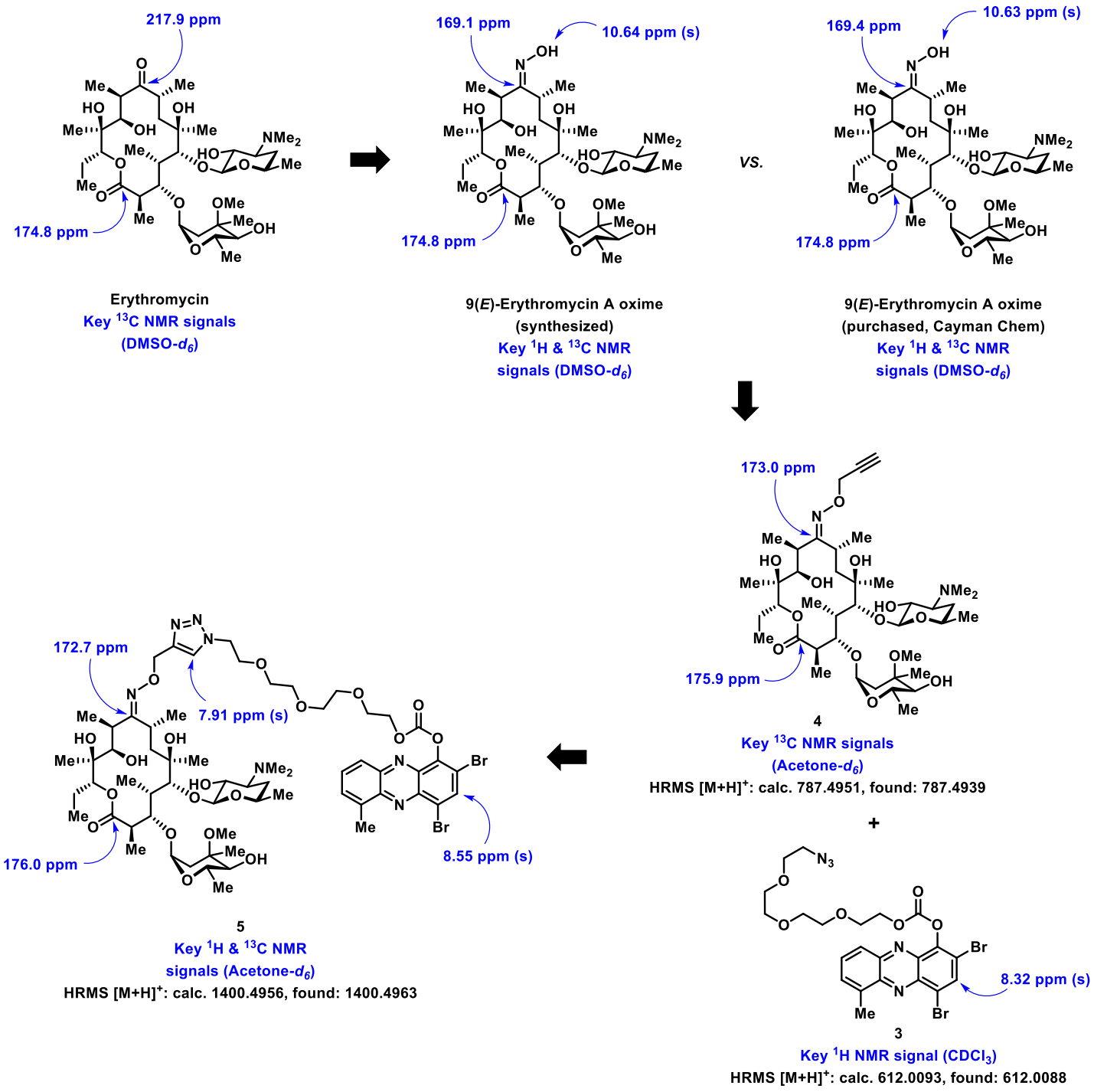
Supporting Information

1.) Supplemental Figure 1.



Supporting Figure 1. Diagnostic ^{1}H and ^{13}C NMR signals and HRMS tracked for erythromycin and all synthetic intermediates en route to erythromycin-HP conjugate **5**.

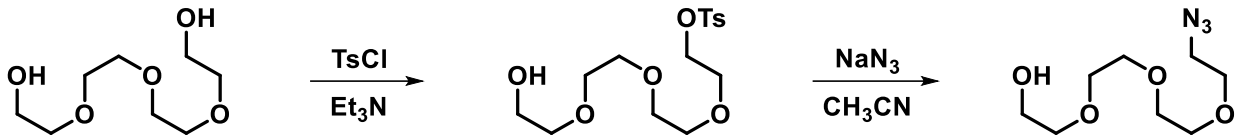
2.) General Information.

All reagents for chemical synthesis were purchased from commercial sources at ≥ 95% purity and used without further purification. The following key compounds were purchased from the commercial sources indicated: erythromycin (CAS: 114-07-8, Fisher Scientific; purity ≥ 98%) and 9-(*E*)-erythromycin A oxime (CAS No. 111321-02-9, Cayman Chemical Company; purity ≥ 95%). 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 carried out using broadband probes on a Bruker Avance II (600 MHz for ^{1}H; 150 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 the following solvents (reference peaks also included for ^{1}H and ^{13}C NMRs) at room temperature: $CDCl_3$ (^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77.23 ppm) and Acetone-d_6 (^{1}H NMR: 2.05 ppm; ^{13}C NMR: 29.84 ppm). Chemical shift values (δ) are reported in parts per million (ppm) for all ^{1}H NMR and ^{13}C NMR spectra. ^{1}H NMR signals and multiplicities are reported as: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. High-Resolution Mass Spectrometry (HRMS) were obtained from the Chemistry Department at the University of Florida.

Methicillin-resistant *Staphylococcus aureus* BAA-1707, methicillin-resistant *S. epidermidis* ATCC 35984 and vancomycin-resistant *Enterococcus faecium* ATCC 700221 were purchased from ATCC and used during these investigations. All compounds tested in biological assays were stored as DMSO stocks at room temperature in the absence of light. To ensure compound integrity of our DMSO stock solutions, we did not subject DMSO stocks of any test compound to freeze-thaw cycles.

3.) Synthetic Procedures & Characterization Data.



CAS: 77544-60-6 **CAS: 86770-67-4**

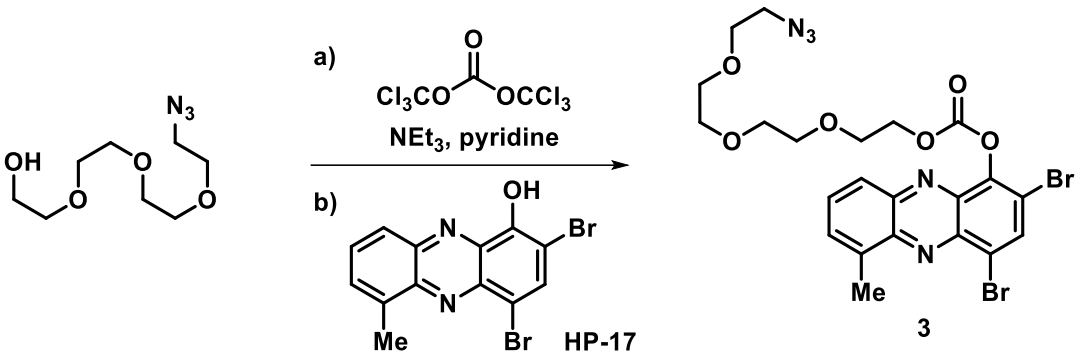
Chemical synthesis of PEG azide (CAS: 86770-67-4).

Step 1: Tetraethylene glycol (9.71 g/8.63 mL, 50 mmol) was dissolved in dichloromethane (50 mL) before triethylamine (8.43 mL, 60 mmol) was added. 4-Toluenesulfonyl chloride (9.53 g, 50 mmol) dissolved in dichloromethane (50 mL) was then added to the reaction mixture at 0 °C and allowed to stir, warming to room temperature on its own accord and reacting for 12 hours before being quenched with brine. The contents of the reaction were then transferred to a separatory funnel and the crude product was extracted with dichloromethane (3 x 30 mL). The organic layers were collected, dried with anhydrous sodium sulfate, filtered, and concentrated *in vacuo*. The crude mixture was then purified by column chromatography using hexanes:ethyl acetate 3:1 to isolate 5.27 grams of the desired tetraethylene glycol *p*-toluenesulfonate (30% yield) as a colorless oil. In addition to the desired tetraethylene glycol *p*-toluenesulfonate product, we isolated 7.79 grams of the undesired bis-tosylated tetraethylene compound (31% yield, structure not shown), which eluted from the column before the target compound.

Note: Our ^{1}H NMR tabulation matches those previously reported for tetraethylene glycol *p*-toluenesulfonate (CAS: 77544-60-6).[1]

Step 2: Sodium azide (757 mg, 11.65 mmol) was added to a stirring solution of tetraethylene glycol *p*-toluenesulfonate (701 mg, 2.01 mmol) in acetonitrile (10 mL) at room temperature. The resulting reaction mixture was then heated to 100 °C and allowed to react for 19 hours. Upon completion, water (10 mL) was added to quench the reaction, which was then concentrated to a third of the original volume via rotavap before being extracted with dichloromethane. The organic layers from the extraction were then dried with sodium sulfate, filtered and concentrated *in vacuo*. The resulting crude material was then purified using silica gel on column chromatography with a 5:1 dichloromethane:methanol solvent system to isolate 376 milligrams of the desired 1-azido-3,6,9-trioxaundecane-11-ol (85% yield) as colorless oil.

Note: Our ^{1}H NMR tabulation matches those previously reported for 1-azido-3,6,9-trioxaundecane-11-ol (CAS: 86770-67-4).[2]

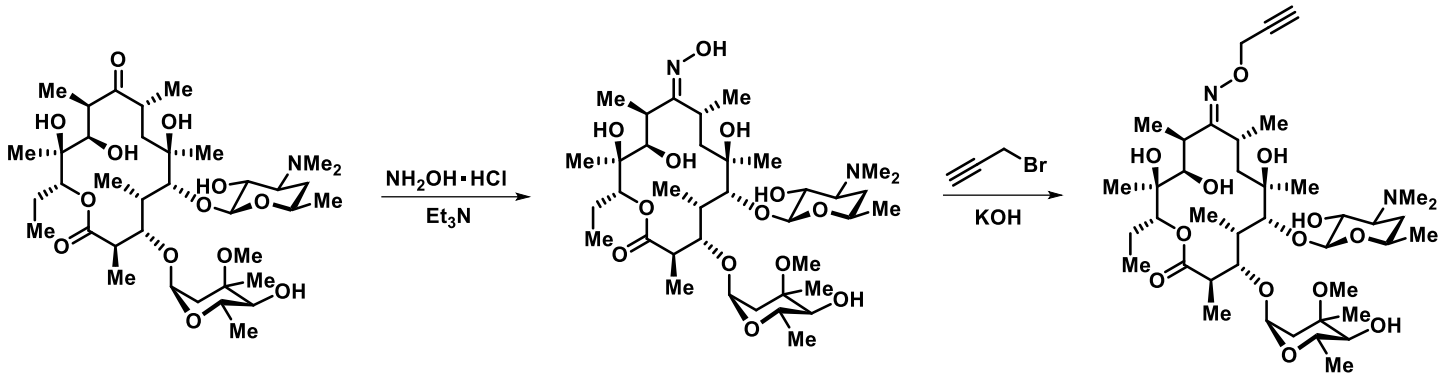


Chemical synthesis of HP-carbonate azide 3. 1-Azido-3,6,9-trioxaundecane-11-ol (54 mg, 0.25 mmol) was placed in an oven-dried round-bottomed flask and dissolved in anhydrous dichloromethane (1 mL). This solution was then cooled to 0 °C. Following this, a solution of dichloromethane (1 mL) containing pyridine (28.5 µL, 0.35 mmol), triethylamine (4.7 µL 0.034 mmol) and triphosgene (36 mg, 0.12 mmol) were then added to the starting material via syringe. The resulting mixture was stirred at 0 °C and allowed to warm to room temperature for 5 hours (generating a chloroformate intermediate *in situ*). After that time, the resulting mixture was cooled to 0 °C and a solution of **HP-17** (50 mg, 0.14 mmol) and triethylamine (28 µL, 0.20 mmol) in anhydrous dichloromethane (2 mL) was then added to the reaction mixture dropwise. The resulting mixture was then stirred at 0 °C for 5 minutes before being warmed to room temperature overnight. Upon completion, the resulting reaction mixture was transferred to a separatory funnel containing 1 M ammonium chloride and the biphasic mixture was shaken vigorously. Upon separation of organic and aqueous layers, dichloromethane was used to extract the crude product (2 x 30 mL). The organic layers were then collected, dried with sodium sulfate, filtered, and concentrated via rotavap. The resulting crude material was then purified using flash column chromatography with 3:1 hexanes:ethyl acetate to 100% ethyl acetate as eluent to isolate 21 milligrams of **3** as a yellow oil (25% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.32 (s, 1H), 8.10 (m, 1H), 7.79 (dd, *J* = 8.7, 6.8 Hz, 1H), 7.73 (m, 1H), 4.56 - 4.50 (m, 2H), 3.91 - 3.85 (m, 2H), 3.77 - 3.72 (m, 2H), 3.72 - 3.65 (m, 8H), 3.39 (t, *J* = 5.1 Hz, 2H), 2.96 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 152.4, 144.4, 143.7, 143.3, 139.3, 138.8, 137.2, 135.2, 132.5, 131.2, 127.7, 123.3, 116.6, 71.0, 70.9, 70.9, 70.3, 68.9 (2), 50.9, 17.6. Note: HSQC was used to determine two carbons at 68.9 ppm; 1 carbon signal is missing in the aliphatic region likely due to overlap of PEG carbon atoms ~ 71 ppm.

HRMS (ESI): calc. for $C_{22}H_{24}Br_2N_5O_6$ $[M+H]^+$: 612.0093, found: 612.0088.



Erythromycin

9(*E*)-Erythromycin A oxime
(CAS: 111321-02-9)

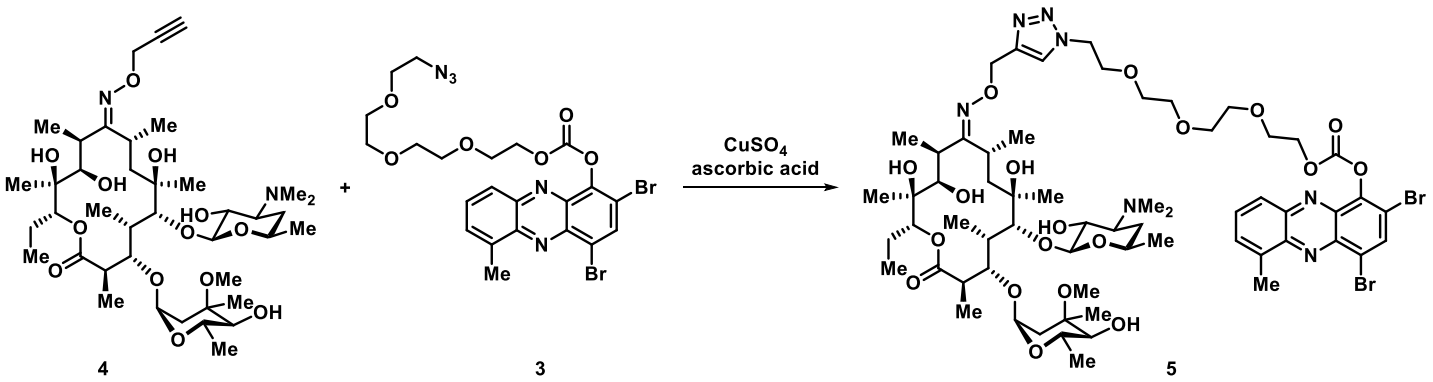
4
(CAS: 1832593-72-2)

Chemical synthesis of 4 (CAS: 1832593-72-2).

Step 1: Erythromycin (1.054 g, 1.44 mmol), hydroxylamine hydrochloride (519 mg, 7.47 mmol) and trimethylamine (786 µL, 5.64 mmol) were dissolved in methanol (10 mL), heated to reflux and allowed to stir for 16 hours. After this time, the reaction mixture was concentrated and a dilute aqueous ammonium hydroxide solution was added to the residue at 0 °C until the mixture reached a pH ~ 10. The resulting mixture was then filtered and a solid was collected, washed with water and dried to yield 828 milligrams of 9-(*E*)-erythromycin A oxime (77% yield) as tan solid.[3]

Step 2: 9-(*E*)-erythromycin A oxime (200 mg, 0.27 mmol) was dissolved in acetonitrile (2.4 mL). Then propargyl bromide (37 µL, 0.33 mmol; 80% by weight in toluene) was added to the stirring solution, followed by potassium hydroxide (24 mg, 0.43 mmol). The resulting reaction mixture was then allowed to stir at room temperature for 3 hours before methanol was added to quench. The resulting mixture was then concentrated via rotavap and the crude material was purified by column chromatography (silica gel) using a 30:1 dichloromethane:methanol solvent system to elute 61 milligrams of **4** (29% yield) as a white solid.[4]

Notes regarding this route: We modified literature protocols to synthesize 9-(*E*)-erythromycin A oxime[3] (CAS No. 111321-02-9) and 9-(*O*-2-propyn-1-yloxime) erythromycin[4] (CAS No. 1832593-72-2). Although these synthetic intermediates are known compounds[3-5], we were unable to locate ^{1}H and ^{13}C NMR spectra to directly compare with our synthetic samples. We purchased a sample of 9-(*E*)-erythromycin A oxime from Cayman Chemical Company to compare and confirm our synthetic sample by ^{1}H and ^{13}C NMR analysis. Our synthetic sample of 9-(*O*-2-propyn-1-yloxime) erythromycin was confirmed via HRMS (ESI; calc. for $C_{40}H_{71}N_2O_{13}$ $[M+H]^+$: 787.4951, found: 787.4939).



4 3 5

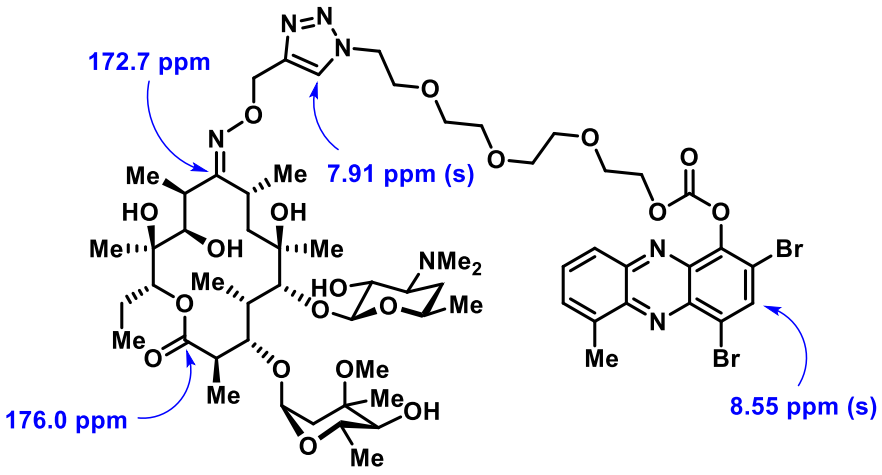
Synthesis of erythromycin-HP conjugate 5. HP-carbonate azide **3** (8 mg, 0.013 mmol) was added to a reaction vial and dissolved in a 2:1:1 water:*tert*-butanol:dichloromethane solution (1.24 mL). Then, 9-(*O*-2-propyn-1-yloxime) erythromycin **4** (20 mg, 0.026 mmol) was added to the reaction vial, followed by an aqueous solution of copper sulfate (77 µL of 100 mM solution, 0.008 mmol), and an aqueous solution of ascorbic acid (62 µL of 500 mM solution, 0.03 mmol). The reaction mixture continued to stir at room temperature for 3.5 hours until complete. After this time, the contents of the reaction were transferred to a separatory funnel containing brine and the organic materials were extracted with ethyl acetate. The organic layers were then combined, dried with sodium sulfate, filtered and concentrated *in vacuo*. The resulting crude residue was purified via column chromatography (silica gel) using 9:1 to 7:1 dichloromethane:methanol solvent system to elute 6 milligrams of **5** as yellow oil (34% yield). Note: This reaction was performed a second time and 9 milligrams of **5** was isolated for a 39% yield using a similar protocol. Preparatory TLC was required to purify **5** using 9:1 dichloromethane:methanol as the solvent system the second time synthesized.

Note: Our purchased samples of erythromycin and 9-(*E*)-erythromycin A oxime proved useful in the tabulation of **5** and we have provided some of these analyses in the spectra section of this supporting information document.

^{1}H NMR (600 MHz, Acetone-*d*$_6$): δ 8.55 (s, 1H), 8.14 (d, *J* = 8.7 Hz, 1H), 7.98 (dd, *J* = 8.7, 6.8 Hz, 1H), 7.95 - 7.90 (m, 2H), 5.21 - 5.14 (m, 2H), 5.12 (dd, *J* = 11.0, 2.3 Hz, 1H), 4.89 (d, *J* = 5.0 Hz, 1H), 4.64 - 4.58 (m, 3H), 4.53 - 4.50 (m, 2H), 4.13 (s, 1H), 4.08 (m, 1H), 3.97 - 3.89 (m, 3H), 3.87 - 3.83 (m, 2H), 3.77 (m, 1H), 3.74 - 3.68 (m, 2H), 3.68 - 3.55 (m, 9H), the remaining 60 protons fall between 3.50 - 0.76 ppm (including the diagnostic triplet at 0.81 ppm, 3H) and proved to be very challenging to tabulate in this region due to signal overlap, water and sample dilution. Note: Following several NMR experiments in multiple solvents, the data we report are the most accurate tabulation we were able to generate for the ^{1}H NMR spectra of **5**.

^{13}C NMR (150 MHz, Acetone-*d*$_6$): δ 175.9, 172.6, 152.9, 145.5, 144.9, 144.3, 143.8, 139.9, 139.2, 137.9, 136.1, 133.8, 132.4, 128.2, 124.8, 123.4, 117.0, 102.5, 96.9, 84.3, 80.1, 78.9, 77.5, 75.5, 74.9, 73.6, 71.4, 71.4, 71.3, 71.2, 71.1, 71.1, 70.2, 69.9, 69.4, 68.0, 67.6, 67.5, 66.4, 50.6, 49.8, 45.6, 40.4, 39.9, 38.5, 35.8, 32.1, 30.3, 27.3, 27.2, 22.0, 21.6, 21.5, 19.3, 19.0, 17.3, 17.0, 16.6, 15.3, 11.2, 9.9.

HRMS (ESI): calc. for $C_{62}H_{94}Br_2N_7O_{19}$ $[M+H]^+$: 1400.4956, found: 1400.4963.



5

Key ^{1}H & ^{13}C NMR signals (Acetone-d_6)

HRMS $[M+H]^+$: calc. 1400.4956, found: 1400.4963

4.) Biological Methods.

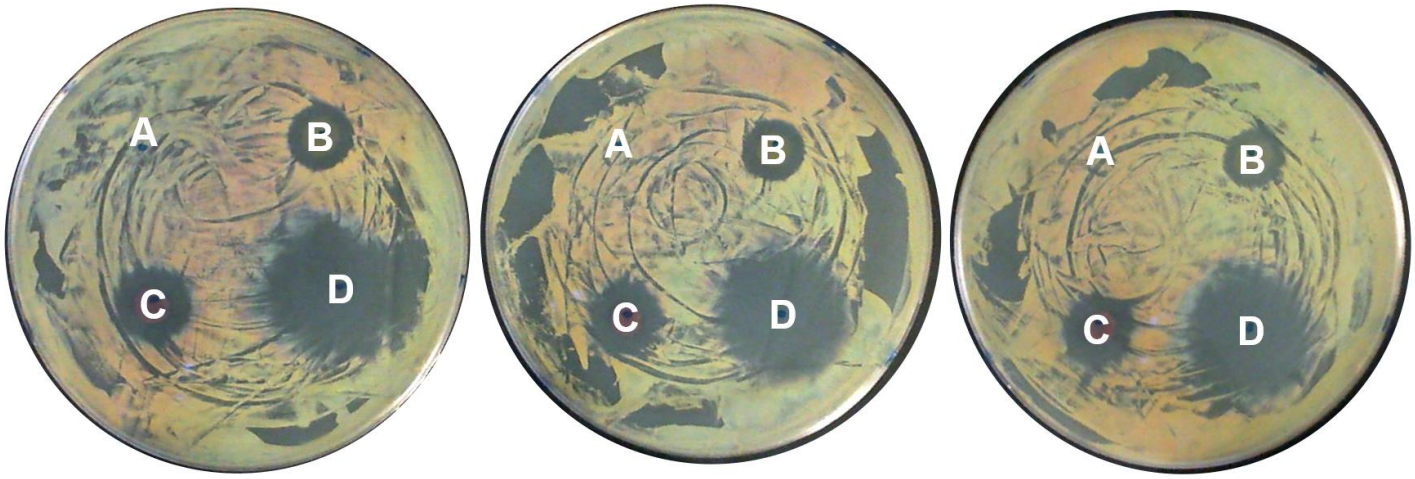
A.) Minimum Inhibitory Concentration (MIC) Susceptibility Assay (in 96-well plates).

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, two-fold serial dilutions of each test compound were made in a final volume of 100 µL Luria Broth. 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 for each test compound during this study was 0.05 to 50 µM. DMSO served as our vehicle and negative control in each microdilution MIC assay. DMSO was serially diluted with a top concentration of 0.5% v/v. All compounds were tested in three independent experiments.

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

MRSA-1707 (OD_{600} = 0.7, ~10^8 CFU) was spread on lysogeny broth (LB) agar plates in petri dishes, then allowed to dry for 30 minutes. After this time, 10 µL of test compound from a 10 mM DMSO stock, or DMSO (vehicle control), was gently pipetted onto the petri dishes containing a lawn of MRSA-1707 at specified locations. The petri dishes were then incubated at 37°C for 16 hours to allow visible growth of the MRSA-1707 lawn and clear zones of inhibition. After this time, images were taken of each experiment and zones of bacterial clearance (area) were measured and recorded in cm^2 using ImageJ software (NIH). The final data we report resulted from three independent experiments with DMSO (vehicle control), HP-17, HP-erythromycin conjugate **5** and erythromycin tested on the same petri dishes (1 replicate each per petri dish).

Images of petri dish experiments.

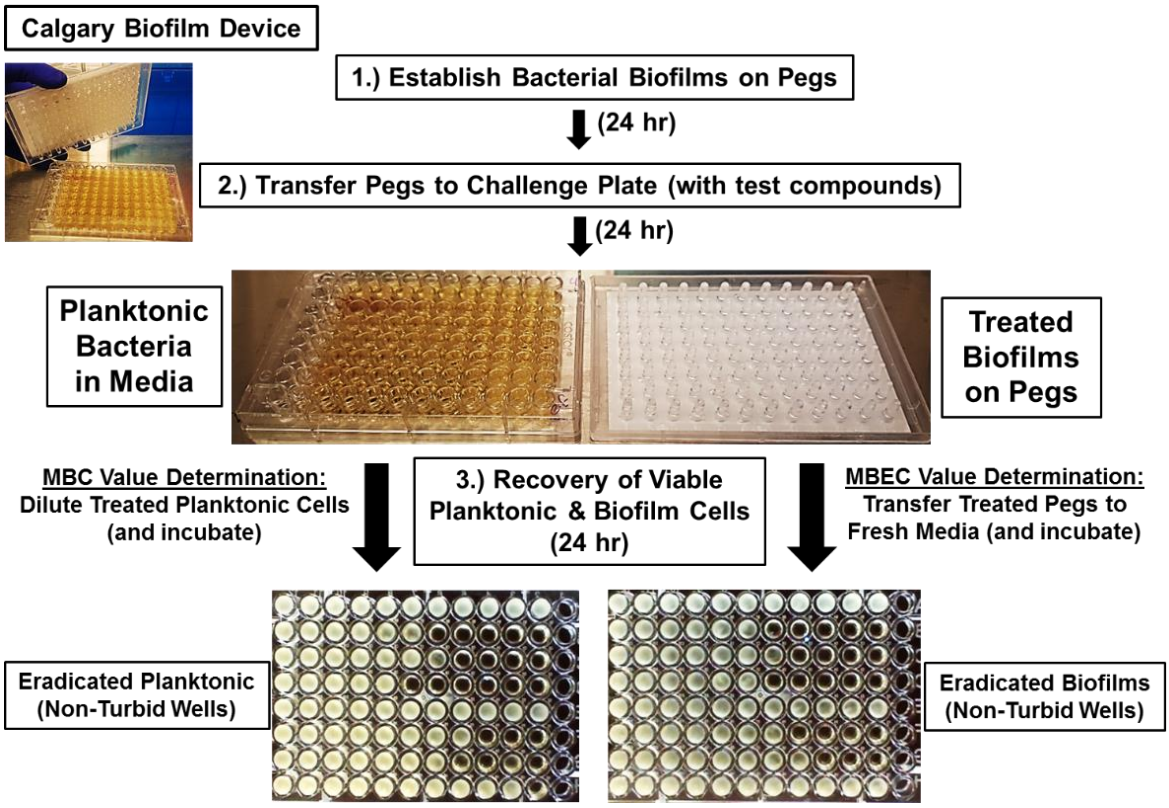


Agar diffusion assay results against MRSA-1707: (A) DMSO (vehicle control, zone of inhibition = 0 cm^2), (B) HP-17 (zone of inhibition = 1.63 ± 0.05 cm^2), (C) HP-erythromycin conjugate **5** (zone of inhibition = 2.77 ± 0.12 cm^2), (D) Erythromycin (zone of inhibition = 7.11 ± 0.38 cm^2)

C.) Calgary Biofilm Device (CBD) Experiments.

Minimum Bactericidal Concentrations (MBC) and Minimum Biofilm Eradication Concentrations (MBEC) Determination

Biofilm eradication experiments were performed using the Calgary Biofilm Device to determine MBC/MBEC values for various compounds of interest (Innovotech, product code: 19111). The Calgary device (96-well plate with lid containing pegs to establish biofilms on) was inoculated with 125 µL of a mid-log phase culture diluted 1,000-fold in tryptic soy broth with 0.5% glucose to establish bacterial biofilms after incubation at 37 °C for 24 hours. The lid of the Calgary device was then removed, washed and transferred to another 96-well plate containing 2-fold serial dilutions of the test compounds (the "challenge plate"). The total volume of media with compound in each well in the challenge plate is 150 µL. The Calgary device was then incubated at 37 °C for 24 hours. The lid was then removed from the challenge plate and MBC/MBEC values were determined using different experimental pathways. To determine **MBC values**, 20 µL of the challenge plate was transferred into a fresh 96-well plate containing 180 µL TSBG and incubated overnight at 37 °C. The MBC values were determined as the concentration giving a lack of visible bacterial growth (i.e., turbidity). For determination of **MBEC values**, the Calgary device lid (with attached pegs/treated biofilms) was transferred to a new 96-well plate containing 150 µL of fresh TSBG media in each well and incubated for 24 hours at 37 °C to allow viable biofilms to grow and disperse resulting in turbidity after the incubation period. MBEC values were determined as the lowest test concentration that resulted in eradicated biofilm (i.e., wells that had no turbidity after final incubation period). All MBC/MBEC data were obtained from a minimum of three independent experiments.



General Calgary Biofilm Device workflow to determine MBC and MBEC values of a test compound.

D.) Total RNA Extraction from MRSA Biofilms & RT-qPCR Experiments to Determine Transcript Levels.

Biofilm Formation: MRSA BAA-1707 (MRSA-1707, or *S. aureus* MW2) was grown in fresh tryptic soy broth with 0.5 % glucose (TSBG) to an optical density (OD_{600} ~ 0.8 - 1.0). Then 1 mL of this culture was added to 24-well plate coated with 0.1% gelatin. The plate was then incubated for 20 hours at 37 °C under static conditions to form biofilms. Following biofilm formation, the contents of the well was discarded leaving only the biofilm at the bottom. *Treating Established Biofilms with Test Compounds:* Compounds HP-17 and HP-erythromycin conjugate prodrug 5 was added to the established MRSA-1707 biofilm in TSBG at the desired concentration (1 µM). In addition, the same volume of DMSO (vehicle) was added as a negative control. The plate was then incubated under static conditions for 4 hours at 37 °C. After the incubation period, the liquid culture was discarded leaving only the surface-attached biofilm. *Extraction of Total RNA from MRSA-1707 Biofilms:* 0.5 mL of RNAprotect Bacteria Reagent (Qiagen) was added for 5 minutes to the plate then the biofilm suspension was scraped and transferred into 2 mL tubes. The bacterial cells were then centrifuged for 1 minute at 15,000 x*g*, then the supernatant was removed. Total RNA was extracted using the RiboPure RNA Purification Kit, Bacteria (Invitrogen, cat# AM1925) according to the manufacturer's protocols. Genomic DNA was digested using the materials supplied by the kit. Each experiment was performed in three replicates.

RiboPure RNA Purification kit, Bacteria protocol as provided by the manufacturer

https://www.thermofisher.com/order/catalog/product/AM1925

1. For each sample, pour ~ 250 µL of ice-cold Zirconia Beads into a 0.5 mL screw cap tube.
2. Add 350 µL RNAwiz to the cell pellet and re-suspend by vortexing vigorously for 10 to 15 seconds.
3. Transfer the cells in RNAwiz to a tube containing 250 µL Zirconia Beads and securely fasten the lid.
4. Vortex the tubes for 10 minutes then centrifuge for 5 min at 4 °C.
5. Transfer the bacterial lysate to a fresh 1.5 mL tube and discard the Zirconia Beads. Estimate the lysate volume while transferring the lysate (note: typically 200 to 250 µL of lysate is recovered at this step).
6. Add 0.2 volumes chloroform to the lysate.
7. Shake vigorously for 30 seconds, then incubate 10 minutes at room temperature. Adding chloroform and incubating at room temperature will allow the aqueous and organic phases to be separated by centrifugation.
8. Centrifuge for 5 minutes at 4 °C.
9. Transfer the aqueous phase (top), containing the partially purified RNA, to a fresh 1.5 mL Tube. Estimate the lysate volume while transferring the lysate (note: typically 200 to 250 µL of aqueous phase is recovered at this step).
10. Add 0.5 volumes of 100% ethanol to the aqueous phase recovered and mix thoroughly.
11. Transfer the sample to the Filter Cartridge, close the lid, and centrifuge for ~1 minute, or until all the liquid is through the filter.
12. Discard the flow-through and return the Filter Cartridge to the same Collection Tube.

13. Wash the filter by adding 700 µL Wash Solution 1 to the Filter Cartridge, and centrifuge for ~ 1 minute or until all of the liquid is through the filter.
14. Discard the flow-through and return the Filter Cartridge to the same Collection Tube.
15. Wash the filter by adding 500 µL Wash Solution 2/3 to the Filter Cartridge and centrifuge for ~1 minute, or until all of the liquid is through the filter.
16. Discard the flow-through and return the Filter Cartridge to the same Collection Tube.
17. Repeat with a second 500 µL aliquot of Wash Solution 2/3.
18. Spin the Filter Cartridge for 1 minute to remove excess wash.
19. Transfer the Filter Cartridge to a fresh 2 mL Collection Tube.
20. Elute RNA by applying 25 to 50 µL Elution Solution, preheated to 95 to 100 °C, to the center of the filter.
21. Centrifuge for 1 minute.
22. Repeating the elution step with a second 25 to 50 µL aliquot of preheated Elution Solution will maximize total RNA yields.
23. DNase treatment: Add 10X DNase buffer equal to 1/9 volume of the RNA and add 4 µL of DNase 1 (2 U/µL)
24. Incubate 30 minutes at 37 °C so that the DNase 1 can digest the genomic DNA.
25. Add a volume of DNase Inactivation Reagent equal to 20% of the volume of RNA treated to each sample. For example, if 100 µL of RNA is treated with DNase, then add 20 µL of DNase Inactivation Reagent.
26. Vortex the tube of RNA after adding the DNase Inactivation Reagent to mix well.
27. Store at room temperature for 2 minutes flicking the tube once or twice during this period to re-suspend the DNase Inactivation Reagent.
28. Centrifuge the sample for ~1 minute at maximum speed to pellet the DNase Inactivation Reagent, then transfer the RNA solution to a new RNase-free tube.

Real-Time PCR Experiments to Determine Relative Transcript Levels of isdB

Total RNA was isolated from MRSA-1707 biofilms treated and untreated with compounds. Real-time PCR reactions were performed using the Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems cat# 4389986) using the manufacturer's guidelines. The materials in the table below were added to a 1.5 mL Eppendorf tube on ice.

Material	**Amount / Reaction (20 µL)**	**Triplicate + Excess (88 µL)**
SYBR	10 µL	44 µL
Primer (5 nM)_forward	1.5 µL	6.5 µL
Primer (5 nM)_reverse	1.5 µL	6.5 µL
Rt enzyme	0.16 µL	0.704 µL
RNA	30 ng	132 ng
Water	20 µL	88 µL

After all contents were added to Eppendorf tubes, they were mixed by centrifugation for 1 minute at 10,000 x*g*. 20 µL were then removed from the reaction tubes and were added to each well of a MicroAmp Optical 96-Well Reaction Plate with Barcode (Applied Biosystems 4306737) on ice. The plate was then sealed with MicoAmp

Optical Adhesive Film (Applied Biosystems 4311971). The plate was centrifuged for 2 minutes at 1200 x*g*. qPCR was carried out on an ABI 7300 sequence detection system using the thermocycler program: 30 minutes at 50 °C, 10 minutes at 95 °C, 15 seconds at 95 °C (40 cycles) and 1 minute at 60 °C. Relative gene expression changes were calculated using the ΔΔCT method. For each experiment, the CT values of gene tested were normalized to the CT values of the housekeeping gene *ptaA.* Graph and data analysis were performed using the GraphPad Prism 6. All qPCR data were generated from three independent experiments.

RT-qPCR Results of isdB in MRSA-1707 Biofilms Treated with HP-17 and 5 at 1 μM for 4 h

Gene	DMSO	HP-17	5	Gene Information / Function
	Mean ± SD	Mean ± SD	Mean ± SD	
isdB	1.0 ± 0.02	11.4 ± 2.93	2.9 ± 0.54	hemoglobin receptor required for heme iron utilization
P-value	--	0.0077	0.0090	

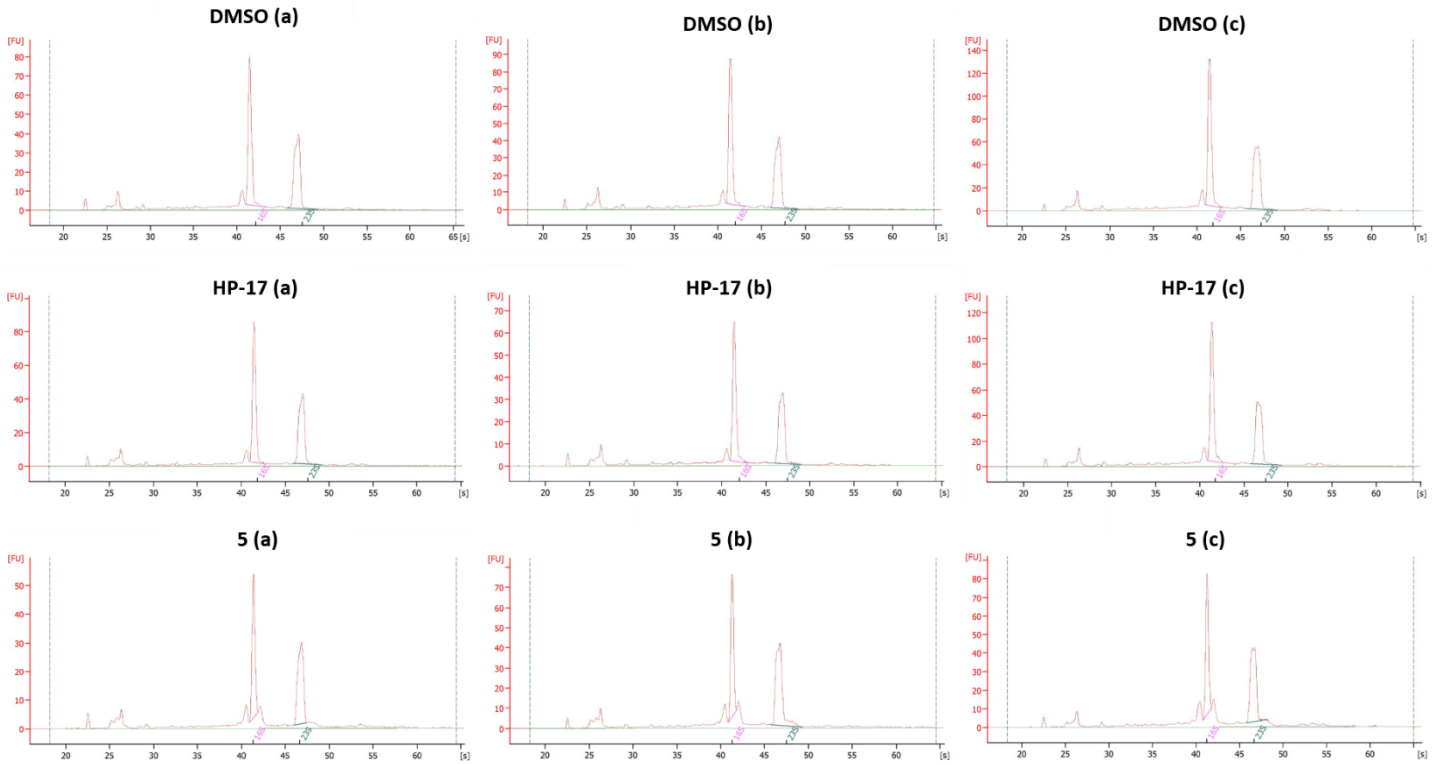
Note: P-values were calculated using Student's t test.

Primers used for qPCR Experiments

Gene	Symbol	Forward primer	Reverse primer
MW1011	*isdB*	CCAGCAGCAAAAGCCACTAA	CGAGAGTTTGGTGCGCTATG
MW1668	*ptaA*	TCCTAGCGAGTTCAGTTGCA	CCAATGGAATGTAGCTGCGA

Notes: Primers were designed using OligoPerfect Primer Designer (Thermofisher). During these studies, *ptaA* was used as the housekeeping gene for MRSA-1707 (*S. aureus* MW2) biofilms.

RNA Quality (RIN data for each sample on following page)



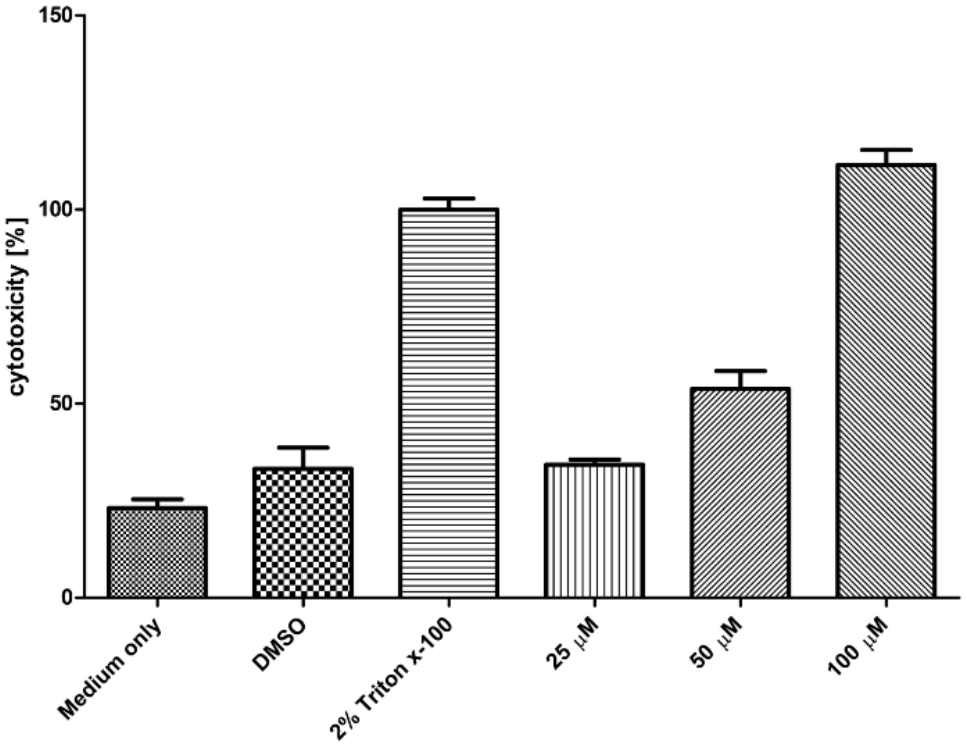
Sample	Concentration (ng/µL)	RIN
DMSO (a)	481.129	8.6
DMSO (b)	408.953	8.6
DMSO (c)	693.937	8.5
HP-17 (a)	586.208	9.0
HP-17 (b)	462.751	8.7
HP-17 (c)	829.616	8.5
5 (a)	360.674	8.6
5 (b)	336.693	8.6
5 (c)	541.827	8.3

Data obtained from the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida.

E.) 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, the test erythromycin-HP conjugate prodrug **5** was then diluted by DMEM (10% FBS) at concentrations of 25, 50 and 100 µM and added to HeLa cells. 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 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 were corded from three independent experiments.

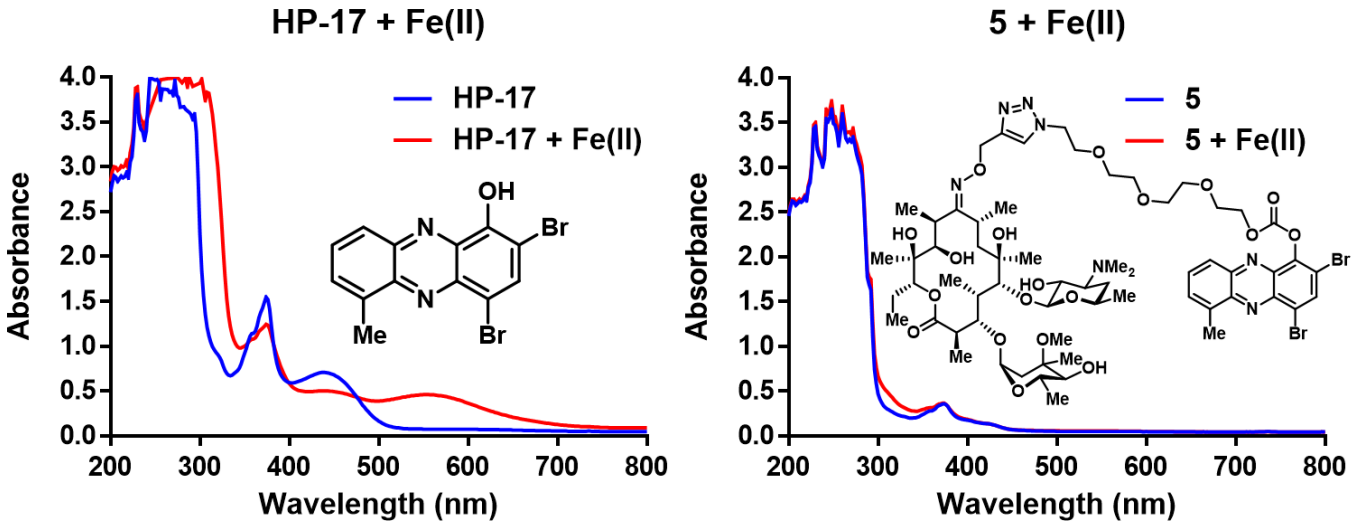
Halogenated Phenazine cytotoxicity results (Triton-X = 100% cell death; Medium Only: 0% cell death):



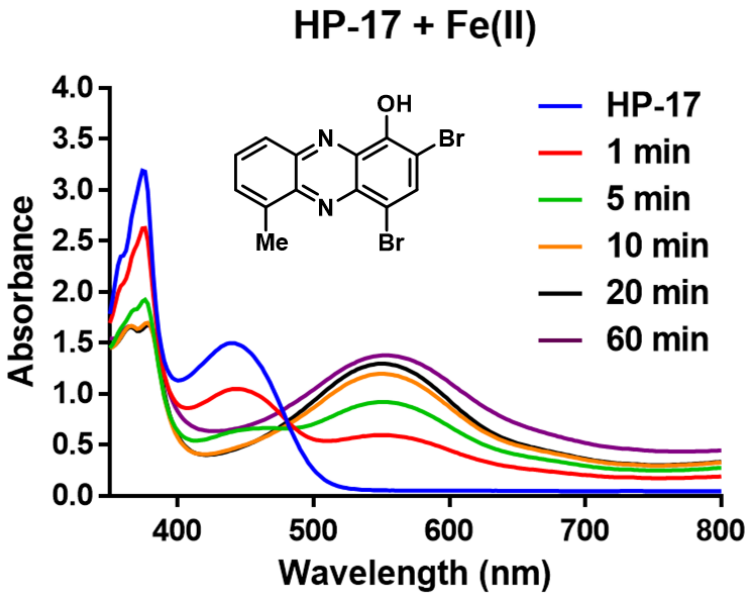
5.) UV-Vis Spectroscopy.

HP-erythromycin conjugate **5** (30 µL of a 10 mM DMSO stock solution) was added to 970 µL DMSO in a 1.5 mL cuvette to obtain its UV-vis spectrum in the absence of iron(II). In a separate cuvette, 955 µL DMSO, 30 µL of HP-erythromycin **5** (10 mM DMSO stock), and 15 µL of ammonium iron(II) sulfate hexahydrate (10 mM solution in water) were thoroughly mixed together for 30 seconds to determine if **5** could bind iron(II) directly. After a total of one minute, spectral scanning was performed from 200 to 800 nm in 2 nm increments. Results were plotted using GraphPad Prism. An analogous experiment was done with HP-17, which showed rapid binding to iron(II) via UV-vis spectroscopy.

Results from above protocol (premade iron(II) solution gives rapid binding) used during these studies.



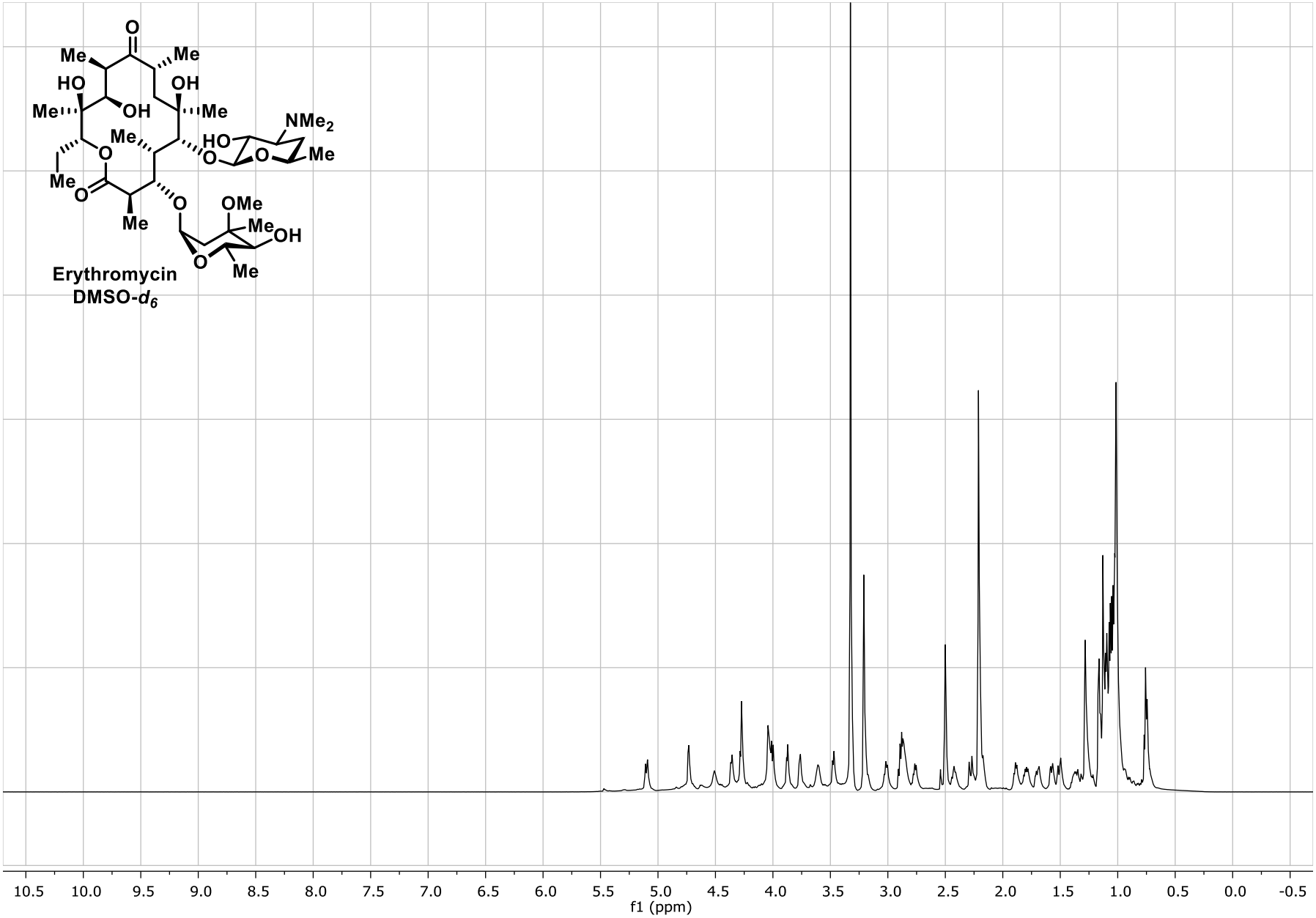
Results from previous studies (iron(II) dissolved as solid gives less rapid binding) utilized time course.[6]



6.) Literature References.

1. H. Wagner, M. K. Brinks, M. Hirtz, A. Schäfer, L. Chi and A. Studer, Chemical Surface Modification of Self-Assembled Monolayers by Radical Nitroxide Exchange Reactions. *Chem. Eur. J.*, 2011, **17**, 9107.
2. B. C. Sanders, F. Friscourt, P. A. Ledin, N. E. Mbua, S. Arumugam, J. Guo, T. J. Boltje, V. V. Popik and G. J. Boons, Metal-Free Sequential [3+2]-Dipolar Cycloadditions using Cyclooctynes and 1,3-Dipoles of Different Reactivity. *J. Am. Chem. Soc.*, 2011, **133**, 949957.
3. D. E. Pereira, Hydrogen Bond Forming Fluoro Ketolides for Treating Disease. PCT Int. Appl. 2012034058, 2012.
4. S. A. Cochrane, X. Li, S. He, M. Yu, M. Wu and J. C. Vederas, Synthesis of Tridecaptin-Antibiotic Conjugates with in Vivo Activity against Gram-Negative Bacteria. *J. Med. Chem.,* 2015, **58**, 9779.
5. J. C. Tian, X. Han, W. Lv, Y. X. Li, H. Wang, B. Z. Fan, M. Cushman and J. H. Liang, Design, Synthesis and Structure-Bactericidal Activity Relationships of Novel 9-Oxime Ketolides and Reductive Epimers of Acylides. *Bioorg. Med. Chem. Lett.*, 2017, **27**, 1513.
6. H. Yang, Y. Abouelhassan, G. M. Burch, D. Kallifdas, G. Huang, H. Yousaf, S. Jin, H. Luesch and R. W. Huigens III, A Highly Potent Class of Halogenated Phenazine Antibacterial and Biofilm-Eradicating Agents Accessed Through a Modular Wohl-Aue Synthesis. *Sci. Rep.*, 2017, **7**, 2003.

7.) NMR Spectra.


Erythromycin
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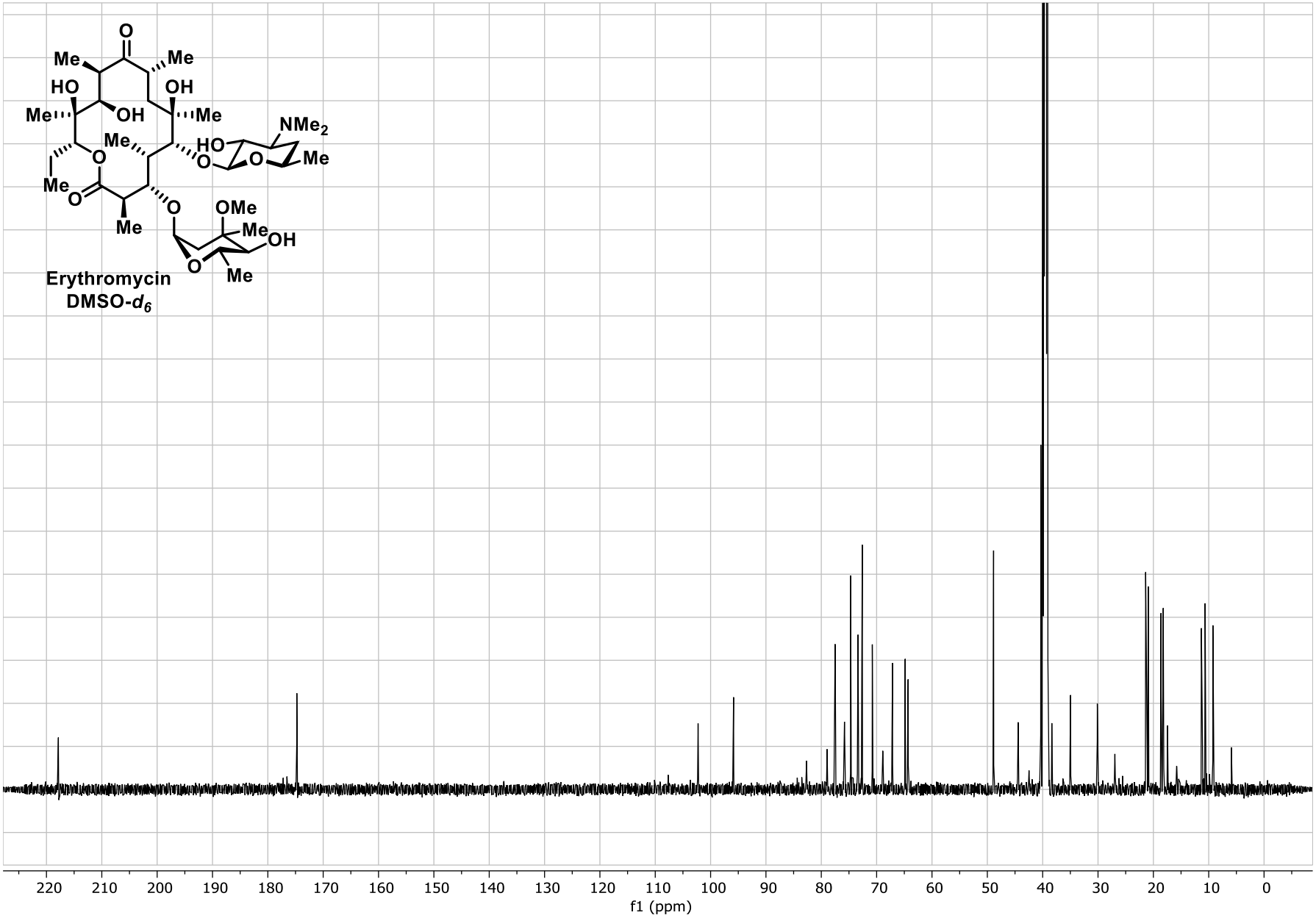
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5
f1 (ppm)


Erythromycin
DMSO-d_6
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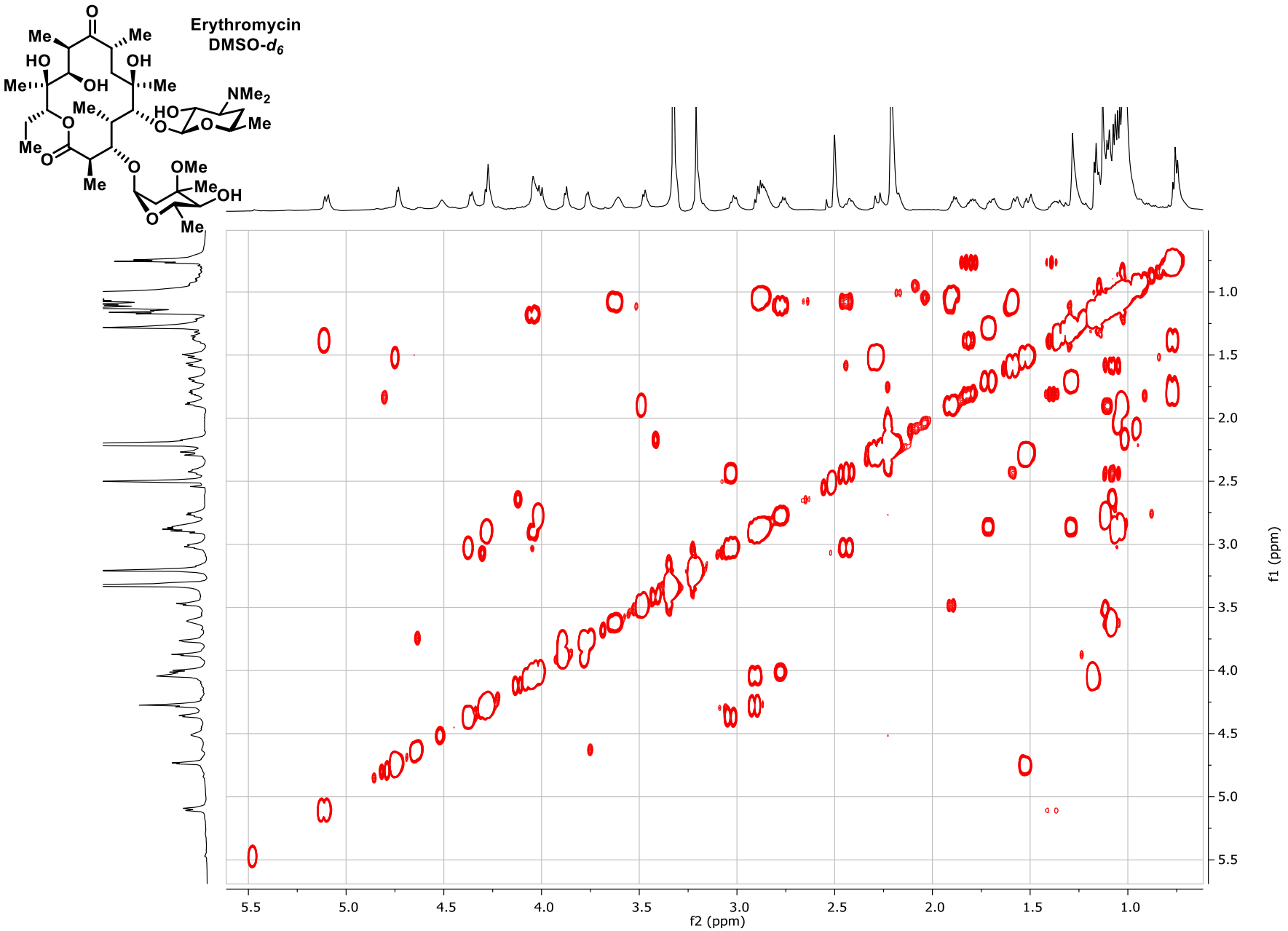
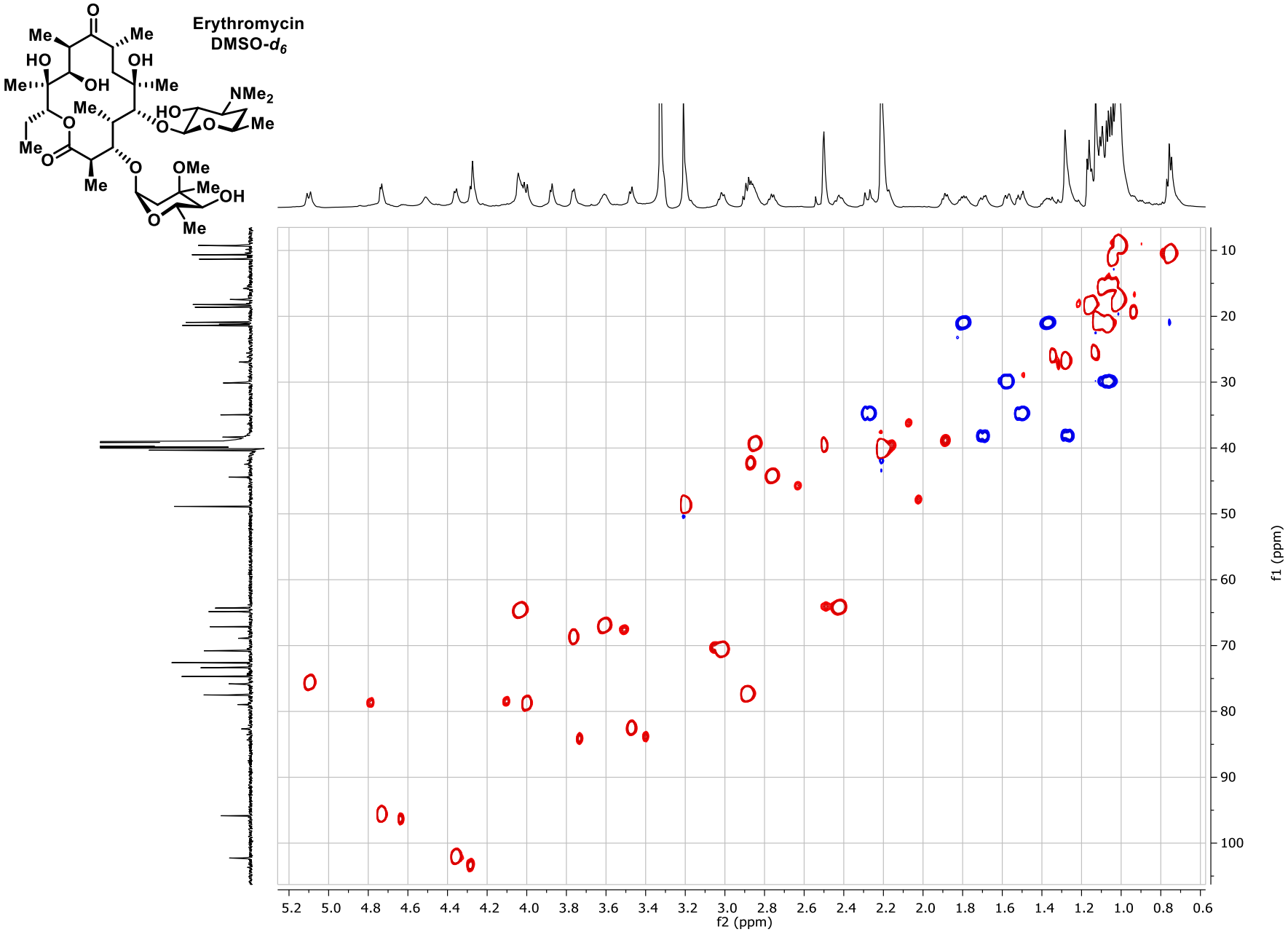
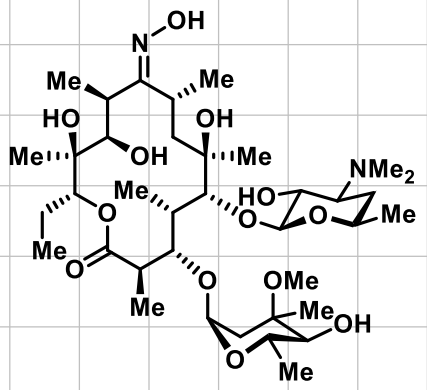
f1 (ppm)



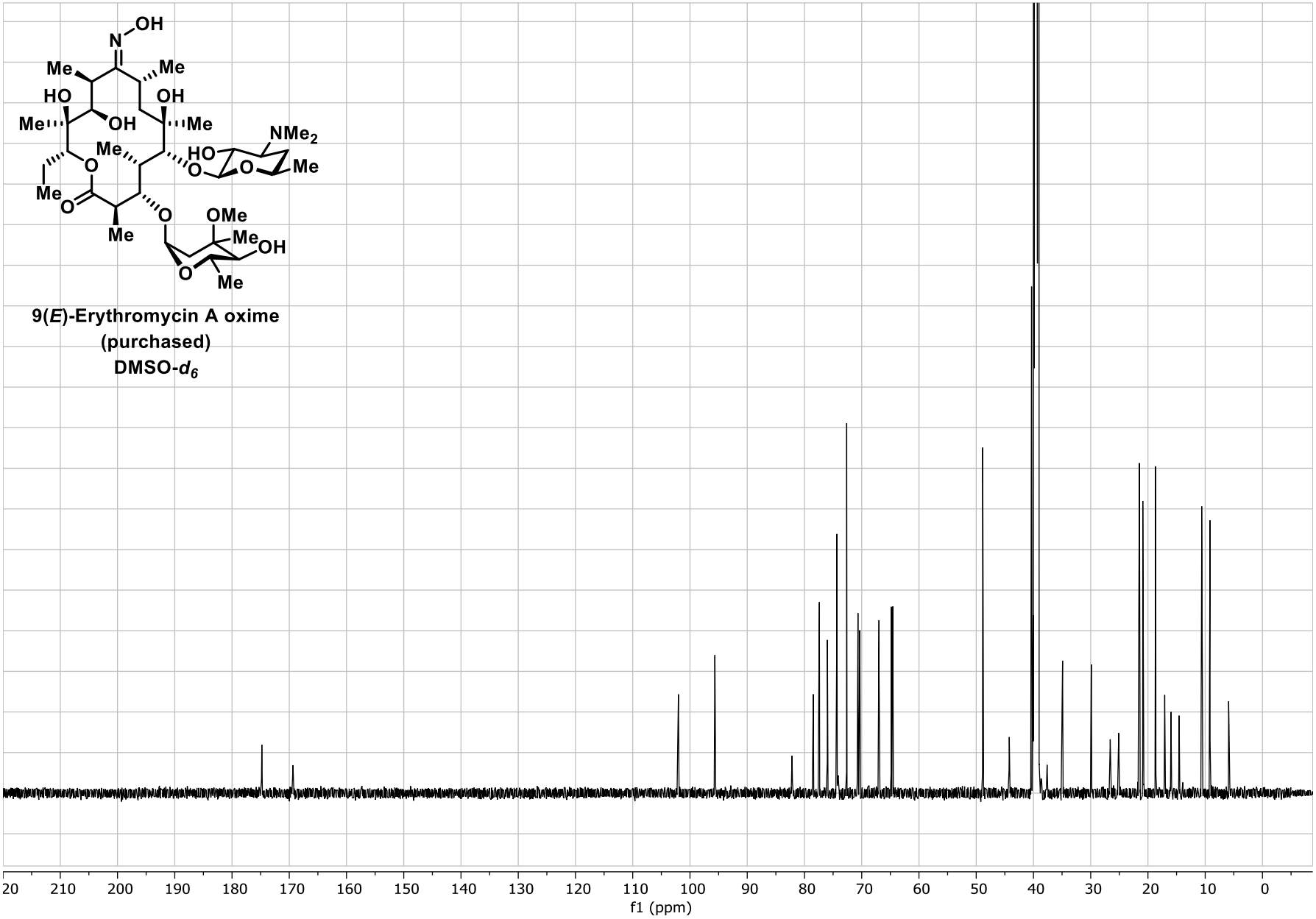
COSY



HSQC



9(*E*)-Erythromycin A oxime
(purchased)
DMSO-d_6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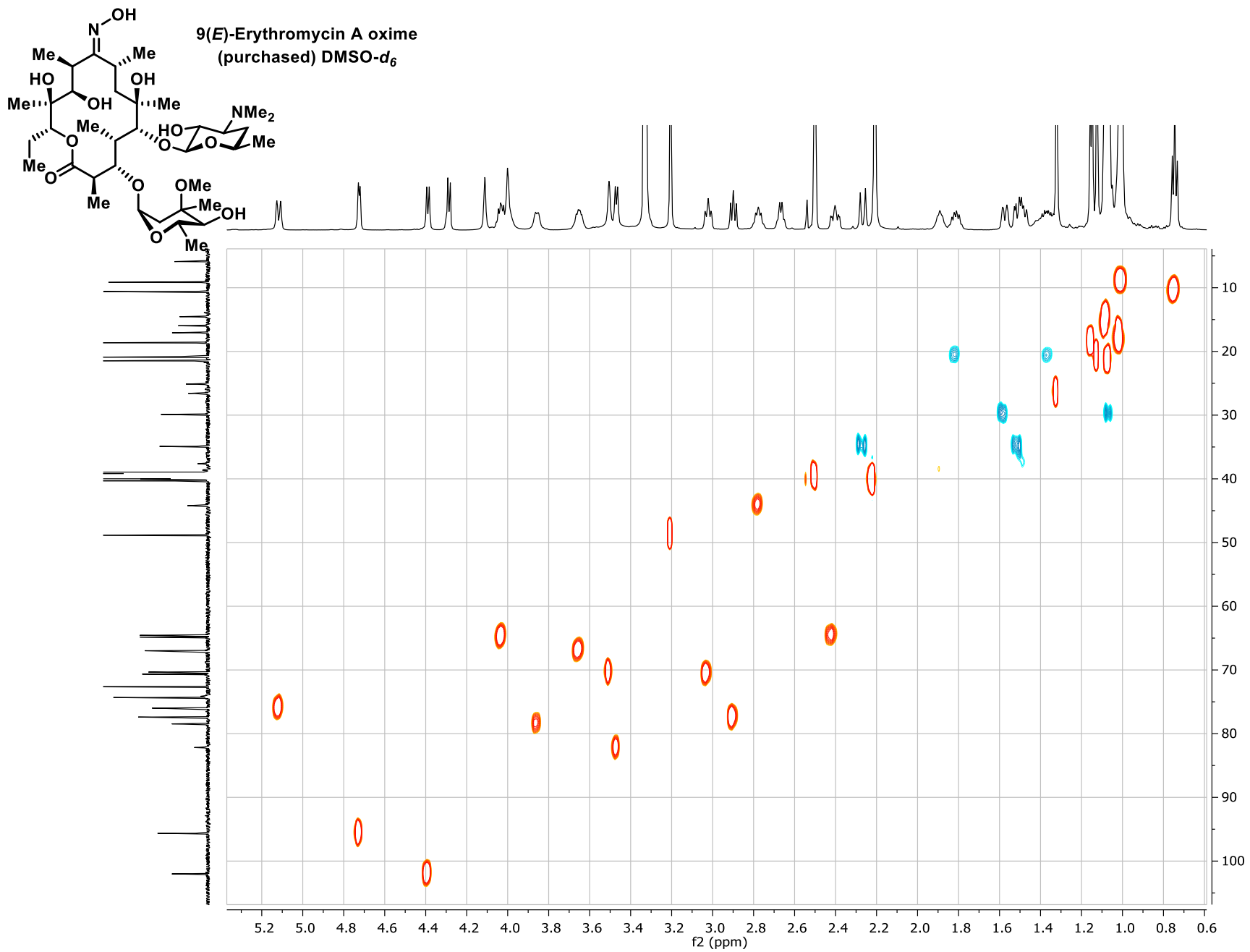
Me
Me
HO
OH
Me
OH
Me
Me
NMe2
HO
O
O
Me
Me
O
O
Me
O
OMe
Me
Me
OH
O
Me
9(E)-Erythromycin A oxime
(purchased)
DMSO-d6
20
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



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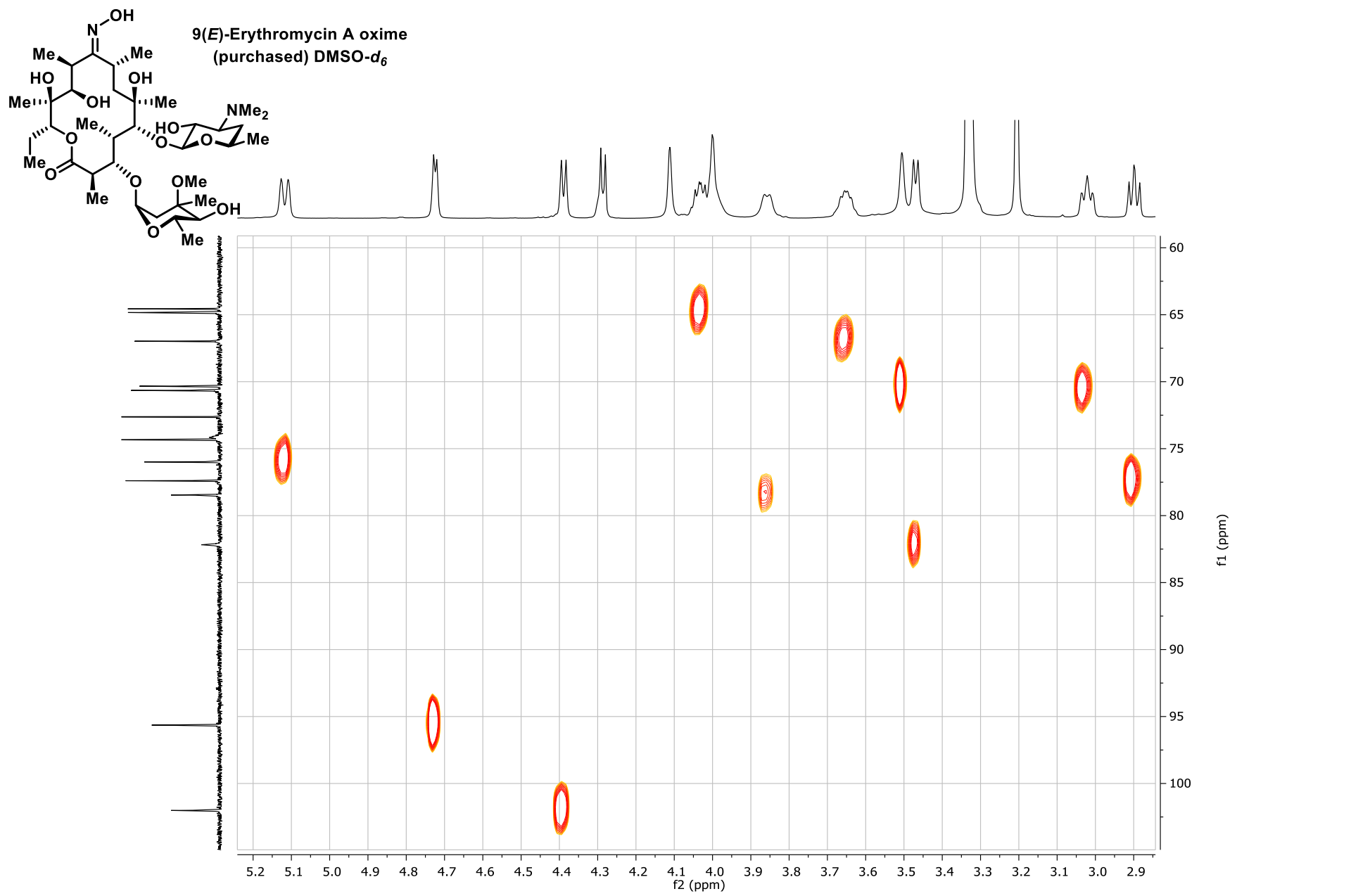
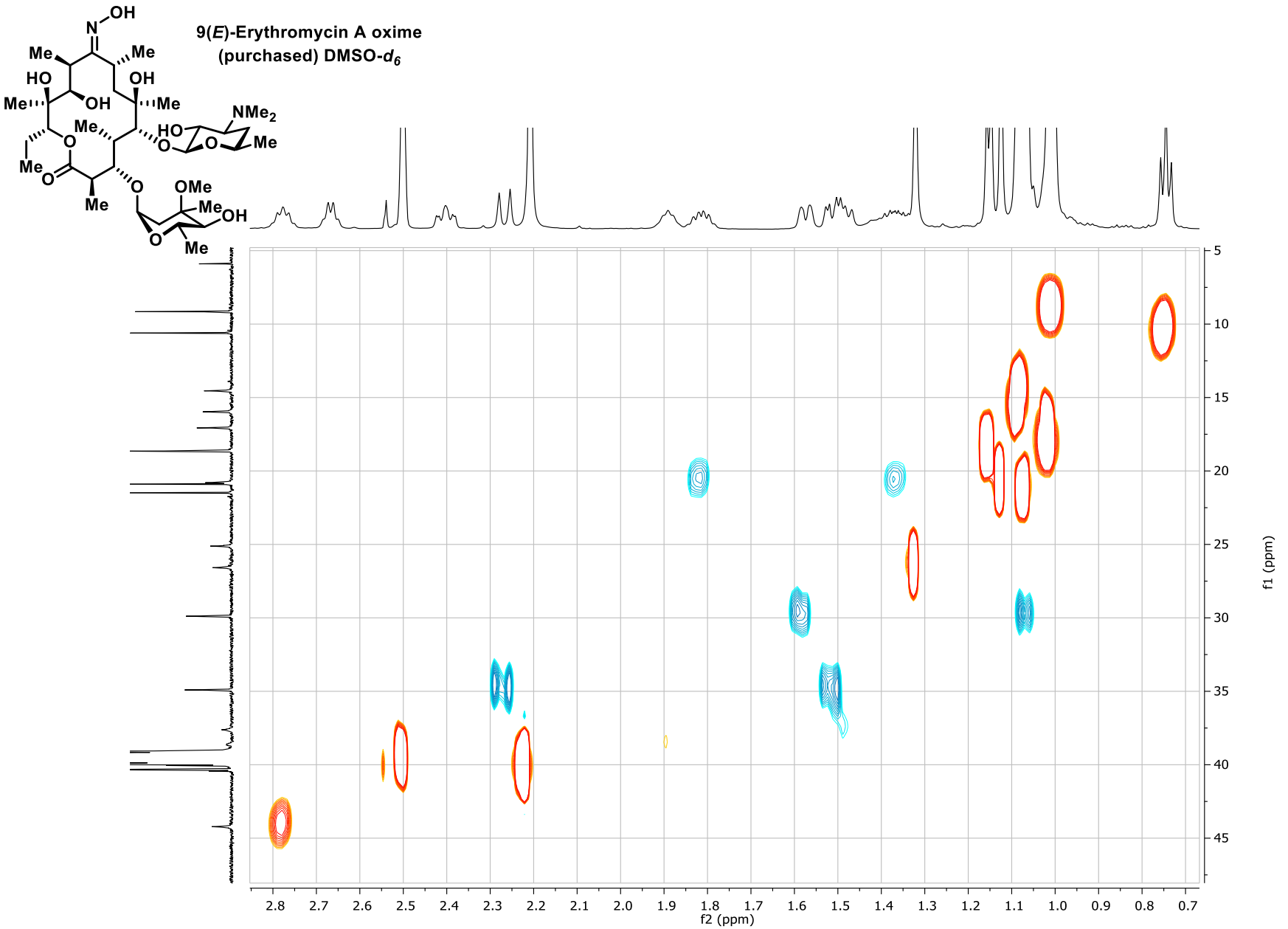
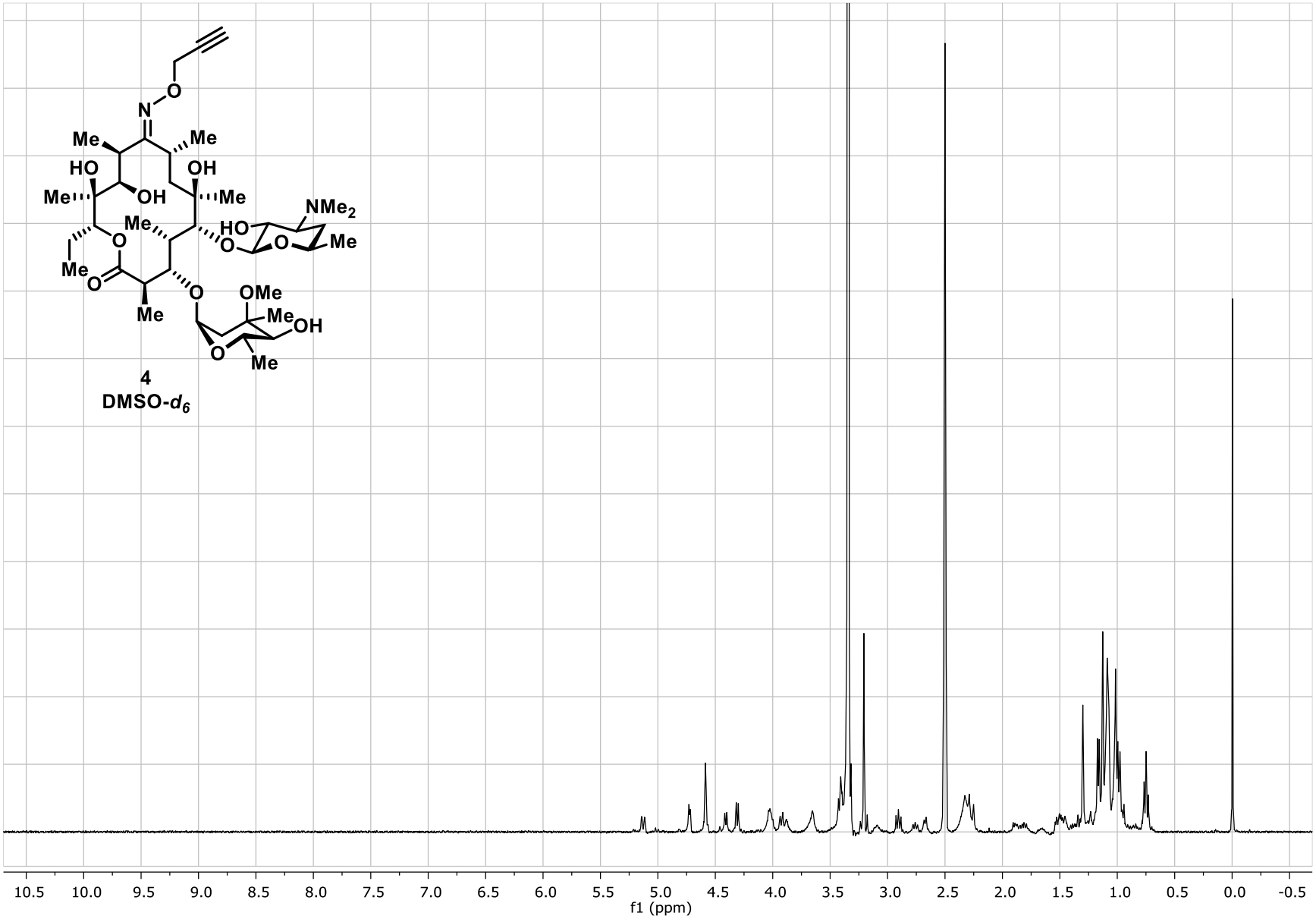


HSQC (zoomed in version, 5.2 - 2.9 ppm)



HSQC (zoomed in version, 2.8 - 0.7 ppm)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DMSO-d_6
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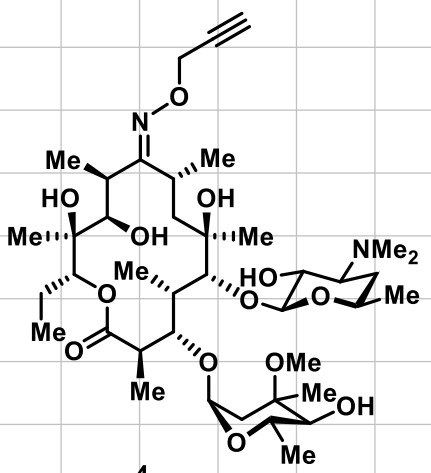
f1 (ppm)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Me
Me
HO
OH
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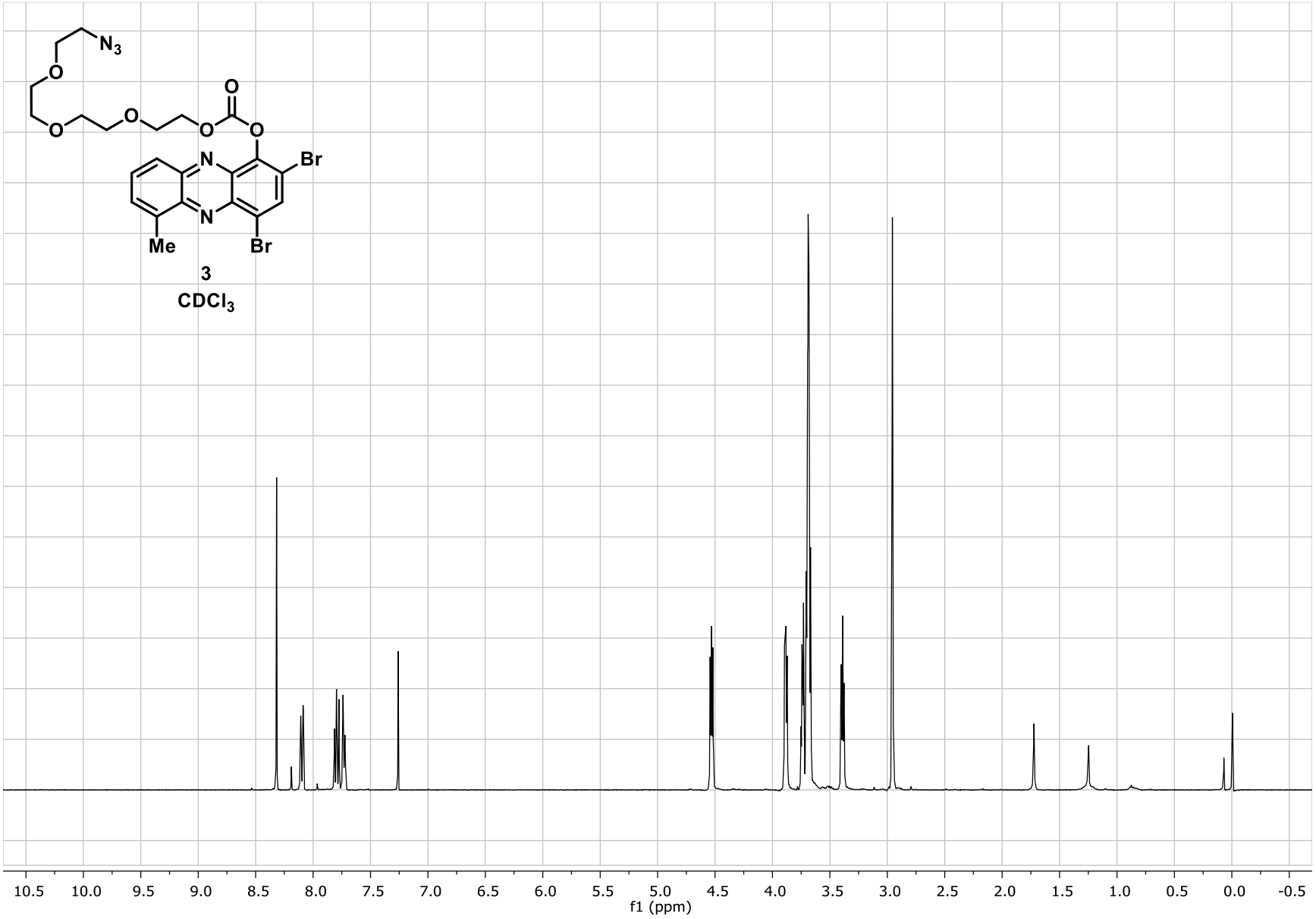
OH
Me
Me
NMe2
HO
O
O
Me
Me
O
O
Me
OMe
Me
OH
O
Me
Me

4

Acetone-*d*$_6$

210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)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O
O
O
O
O
O
N
N
Br
Br
Me
3
CDCl3
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
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4.0
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3.0
2.5
2.0
1.5
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0.5
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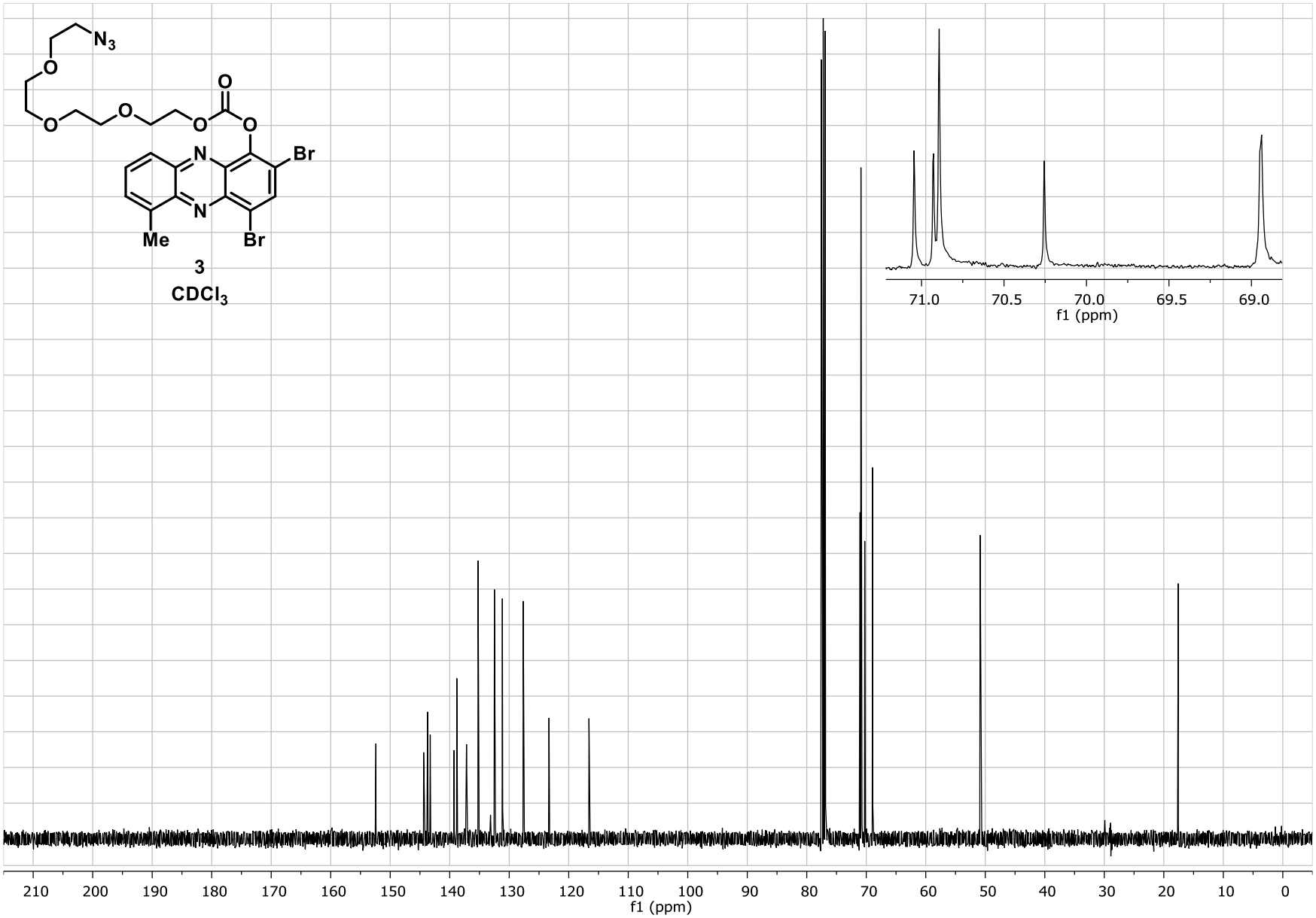
-0.5
f1 (ppm)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O
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Br
Br
Me
3
CDCl3
71.0
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70.0
69.5
69.0
f1 (ppm)
210
200
190
180
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140
130
120
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90
80
70
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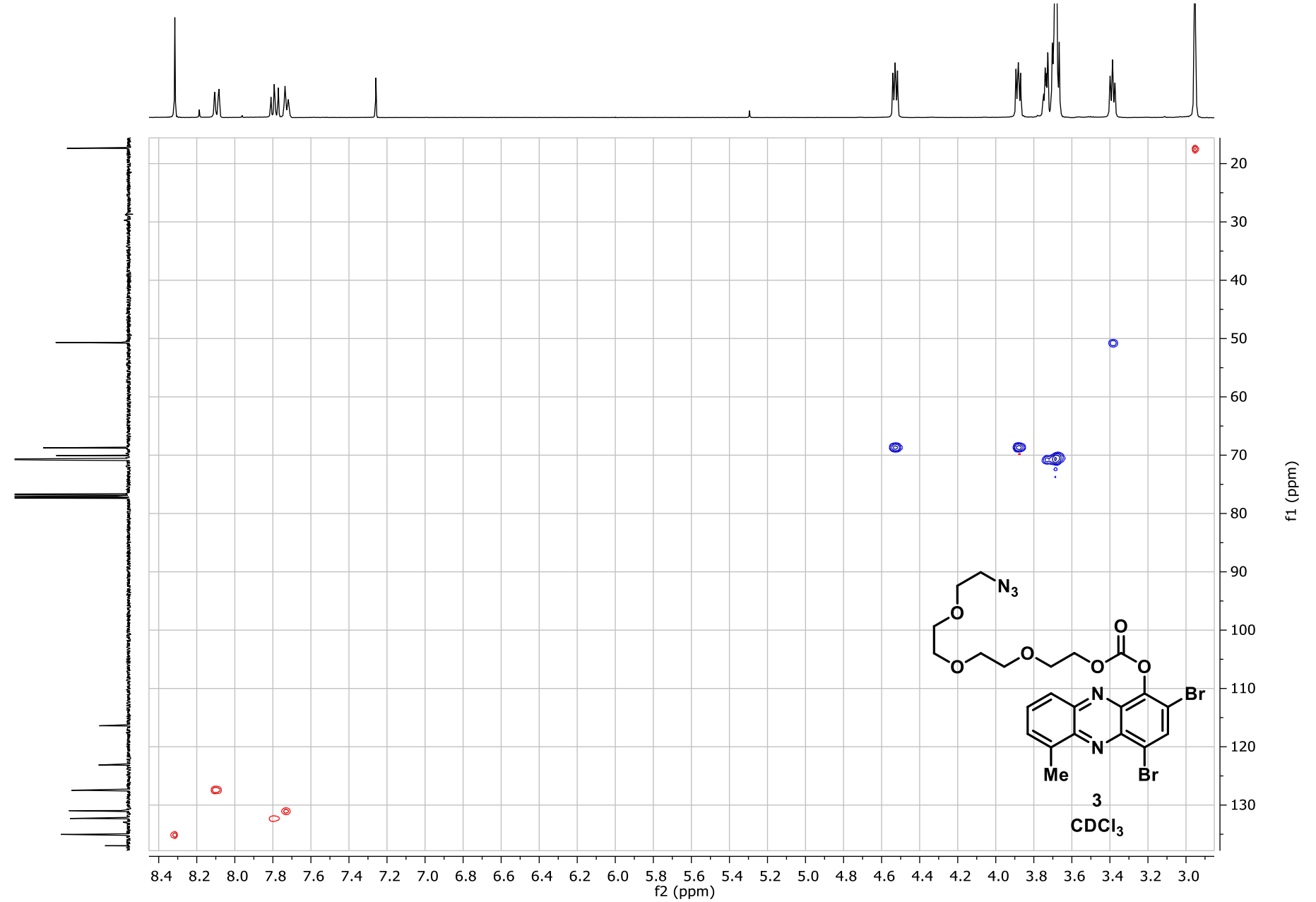
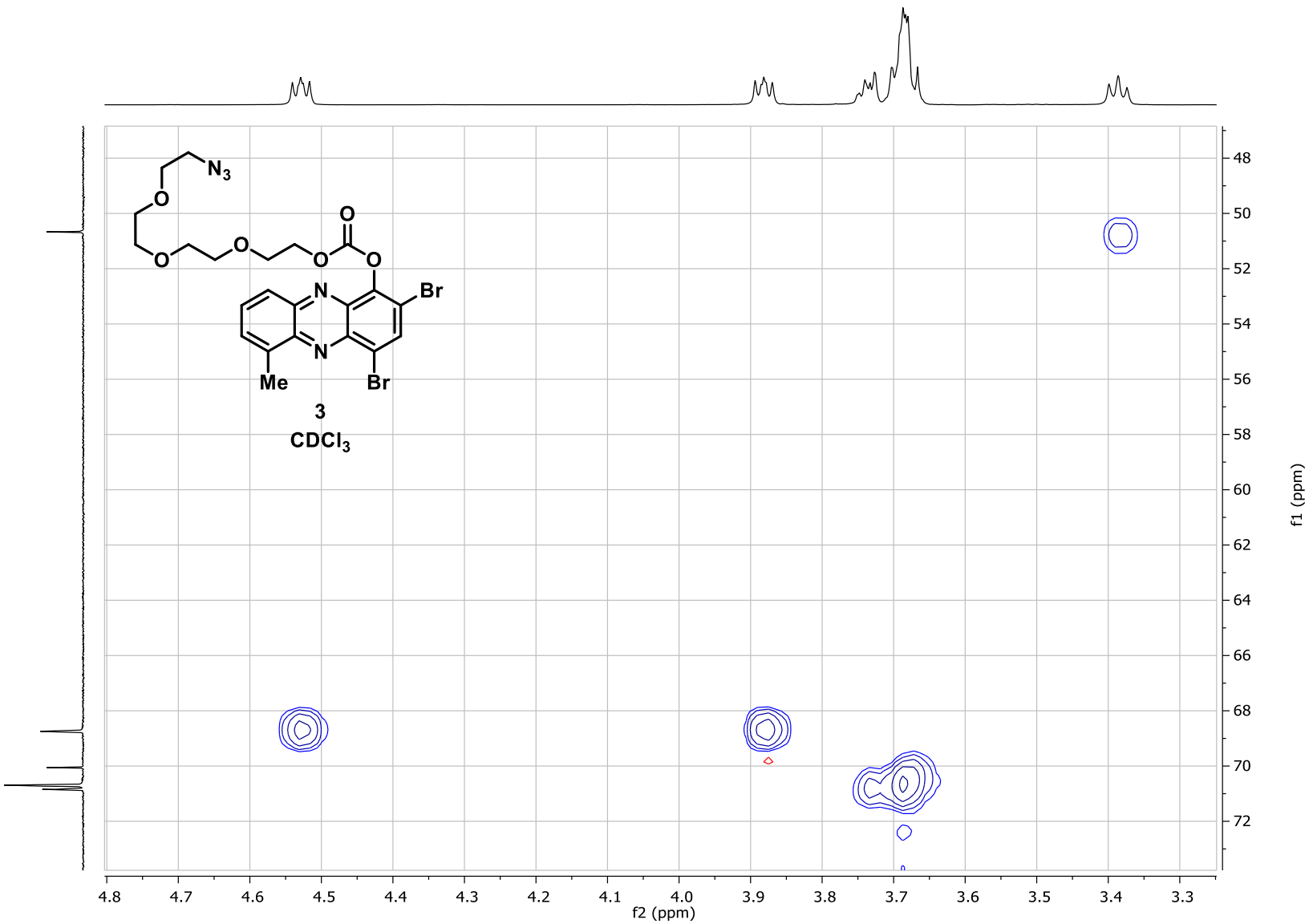
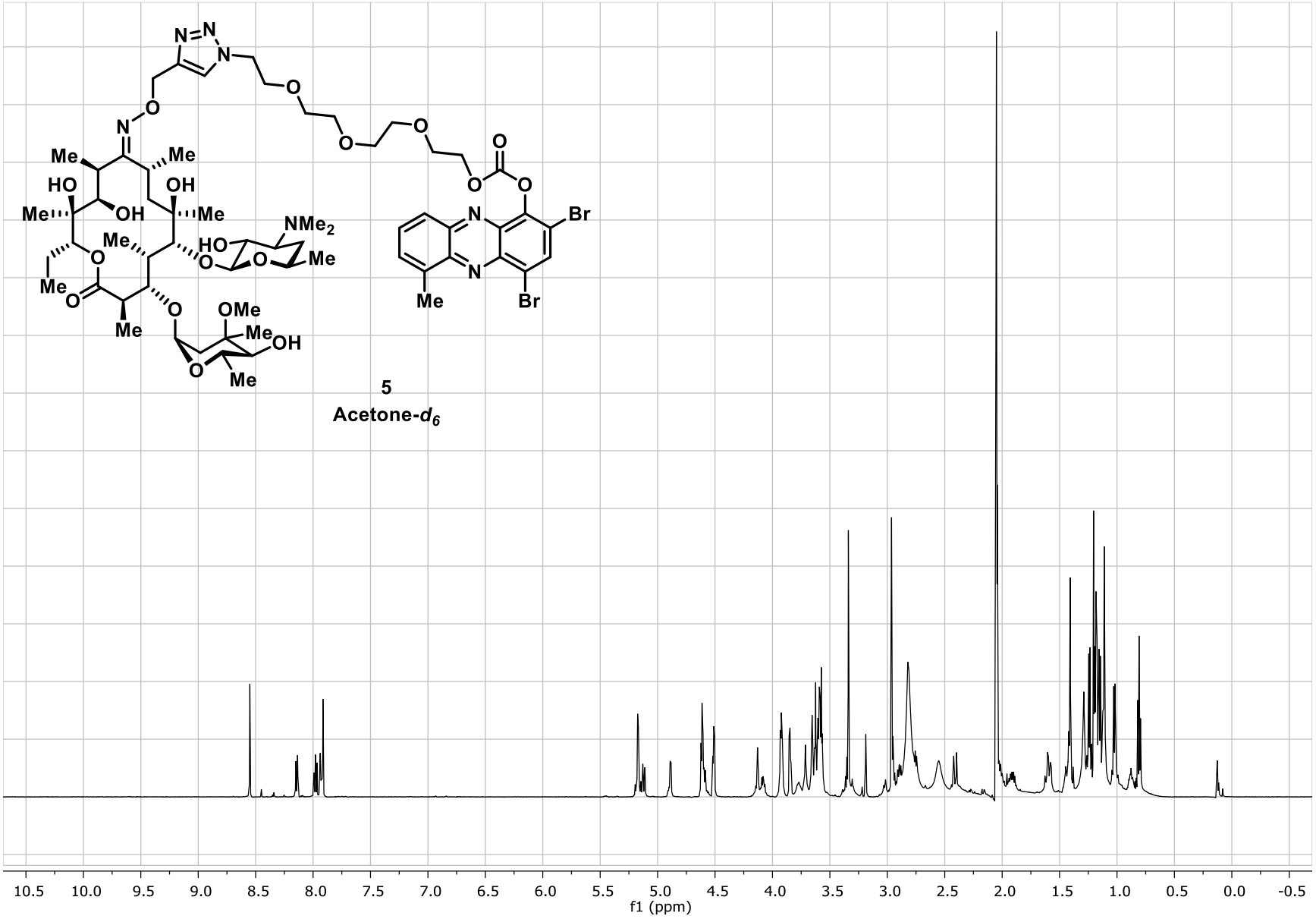
50
40
30
20
10
0
f1 (ppm)

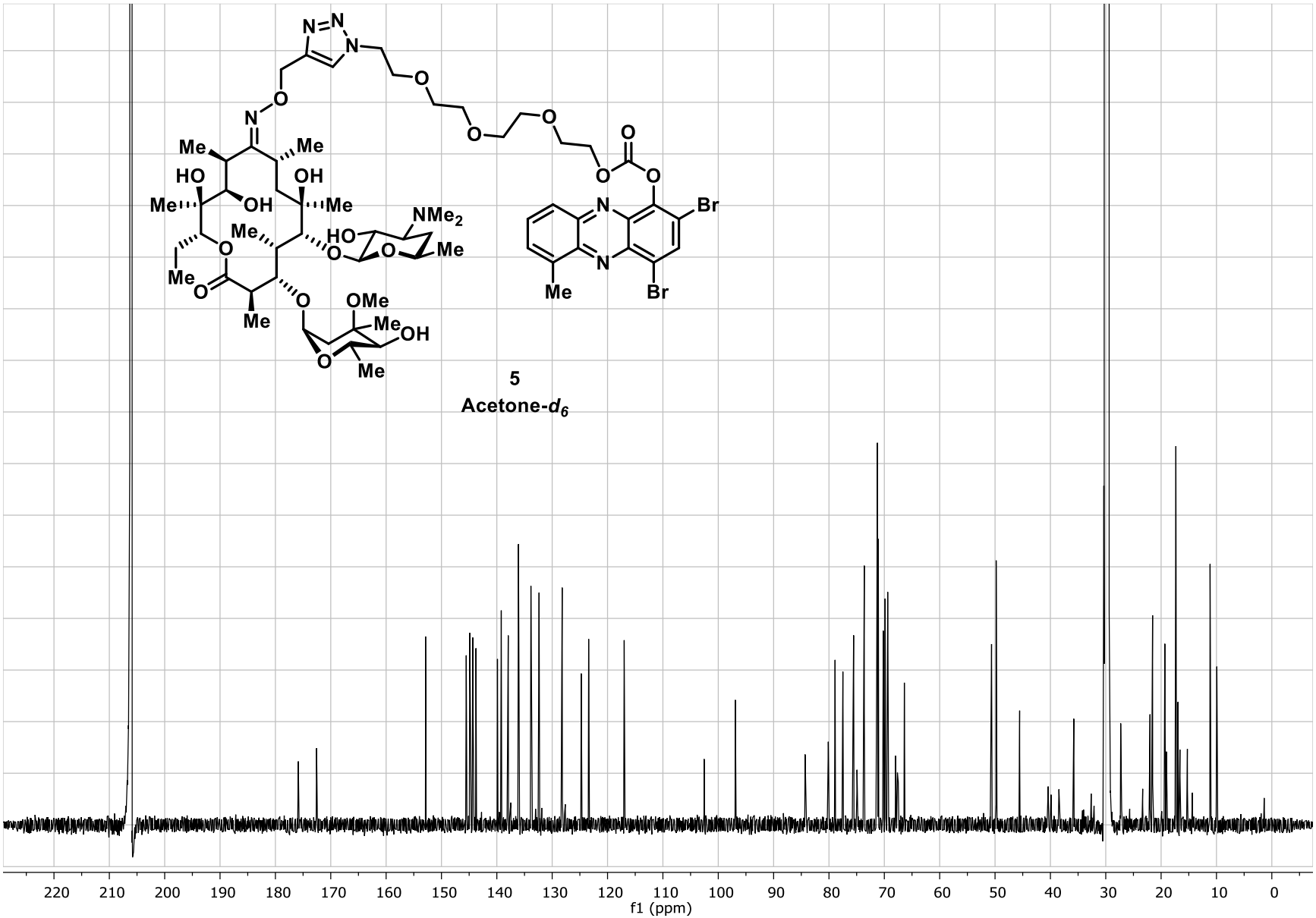


HSQC



HSQC (zoomed in version)


5
Acetone-d_6
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)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Acetone-d_6
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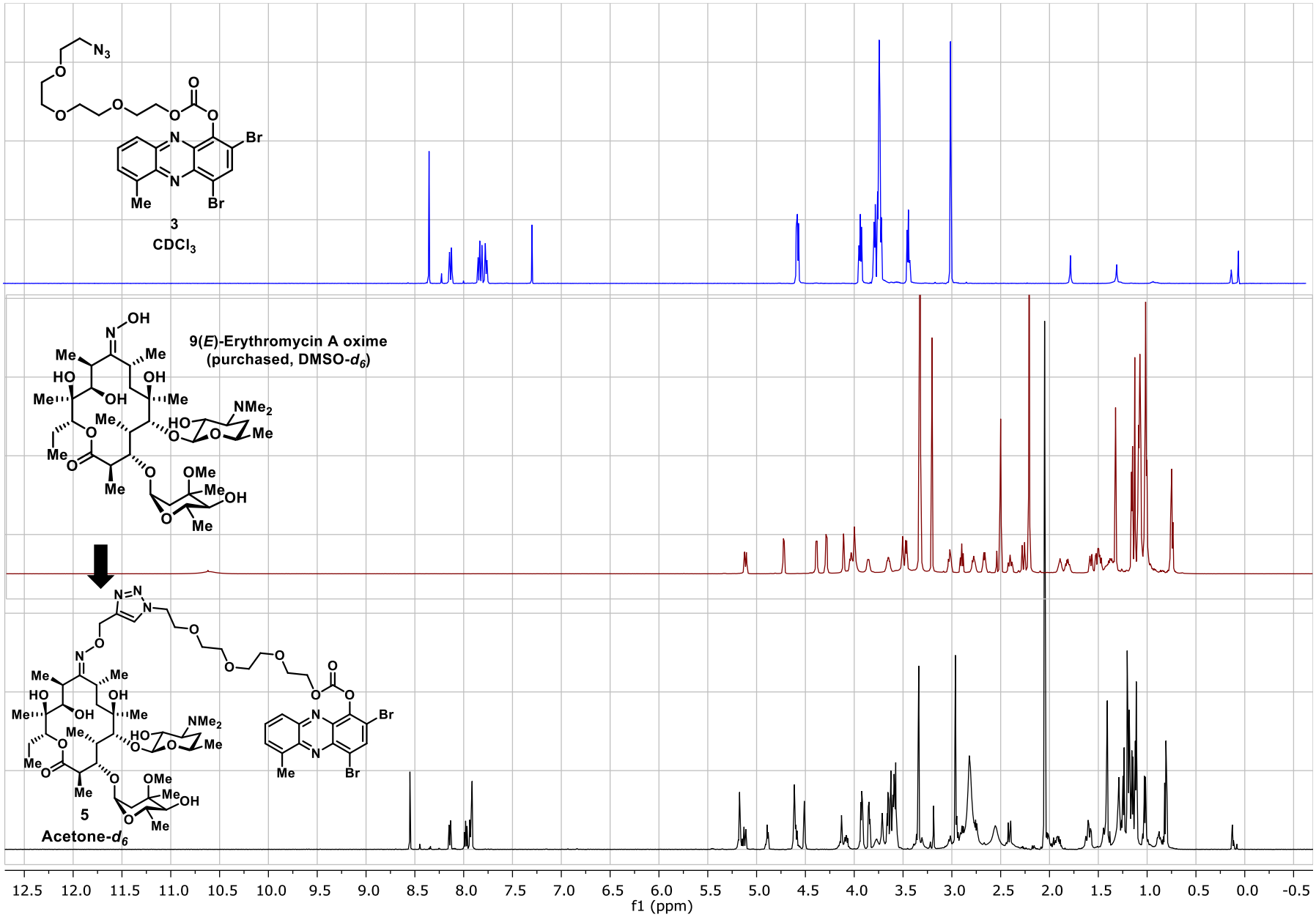
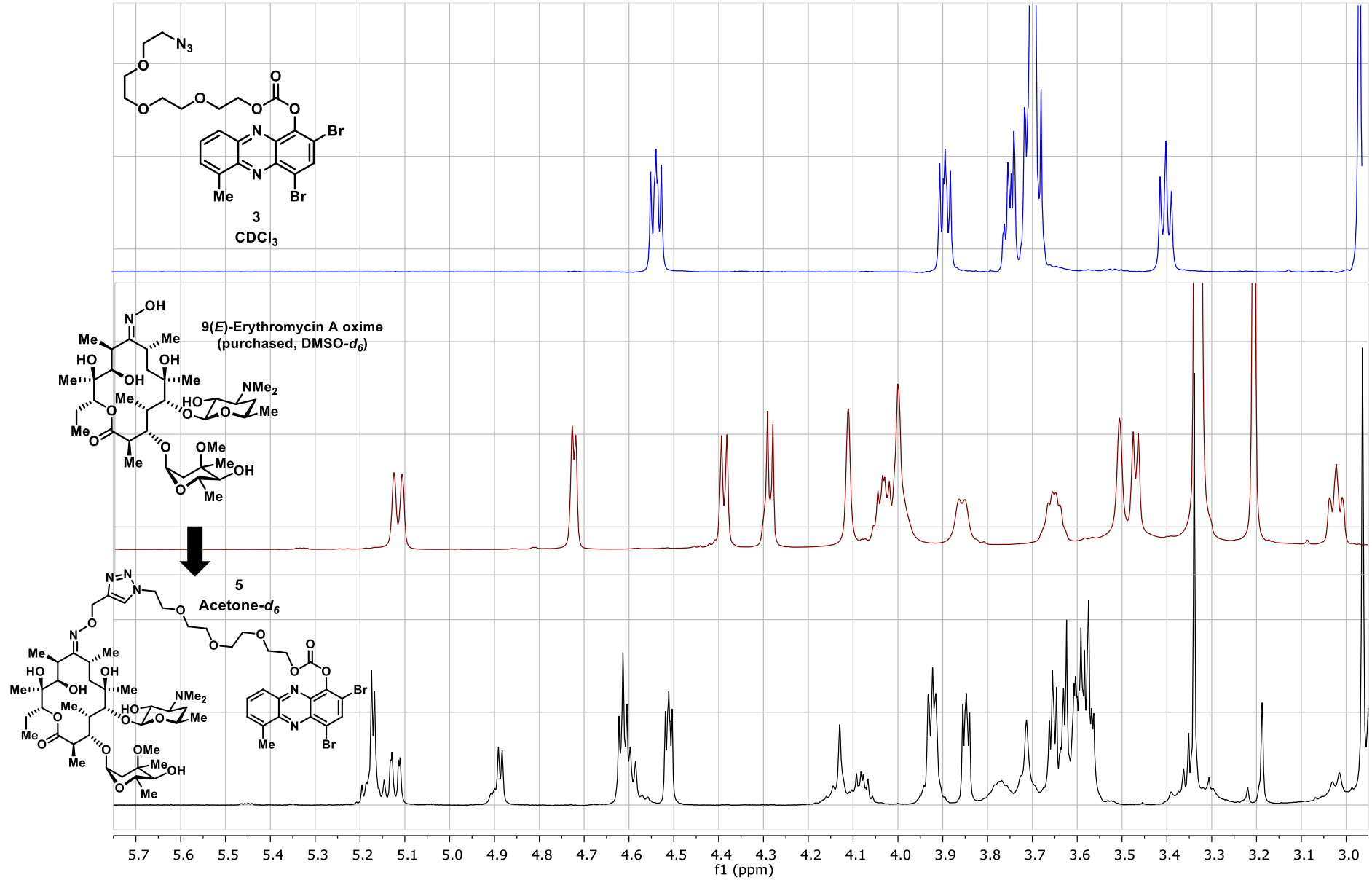
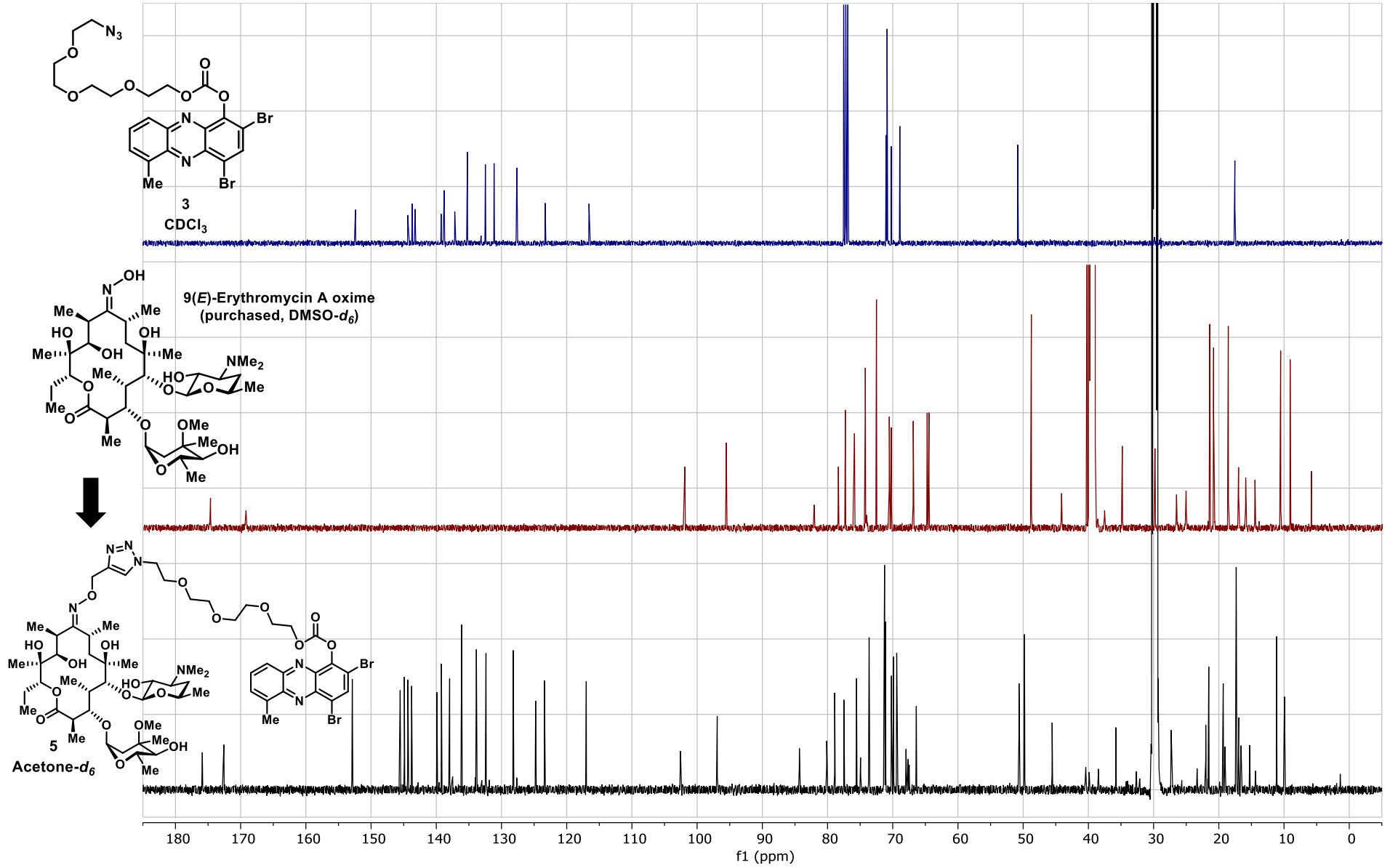
f1 (ppm)


3
$CDCl_3$
9(*E*)-Erythromycin A oxime
(purchased, DMSO-d_6)
5
Acetone-d_6
12.5 12.0 11.5 11.0 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)


3
CDCl3
9(E)-Erythromycin A oxime
(purchased, DMSO-d6)
5
Acetone-d6
5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 3.0
f1 (ppm)


3
CDCl3
9(E)-Erythromycin A oxime
(purchased, DMSO-d6)
5
Acetone-d6
180
170
160
150
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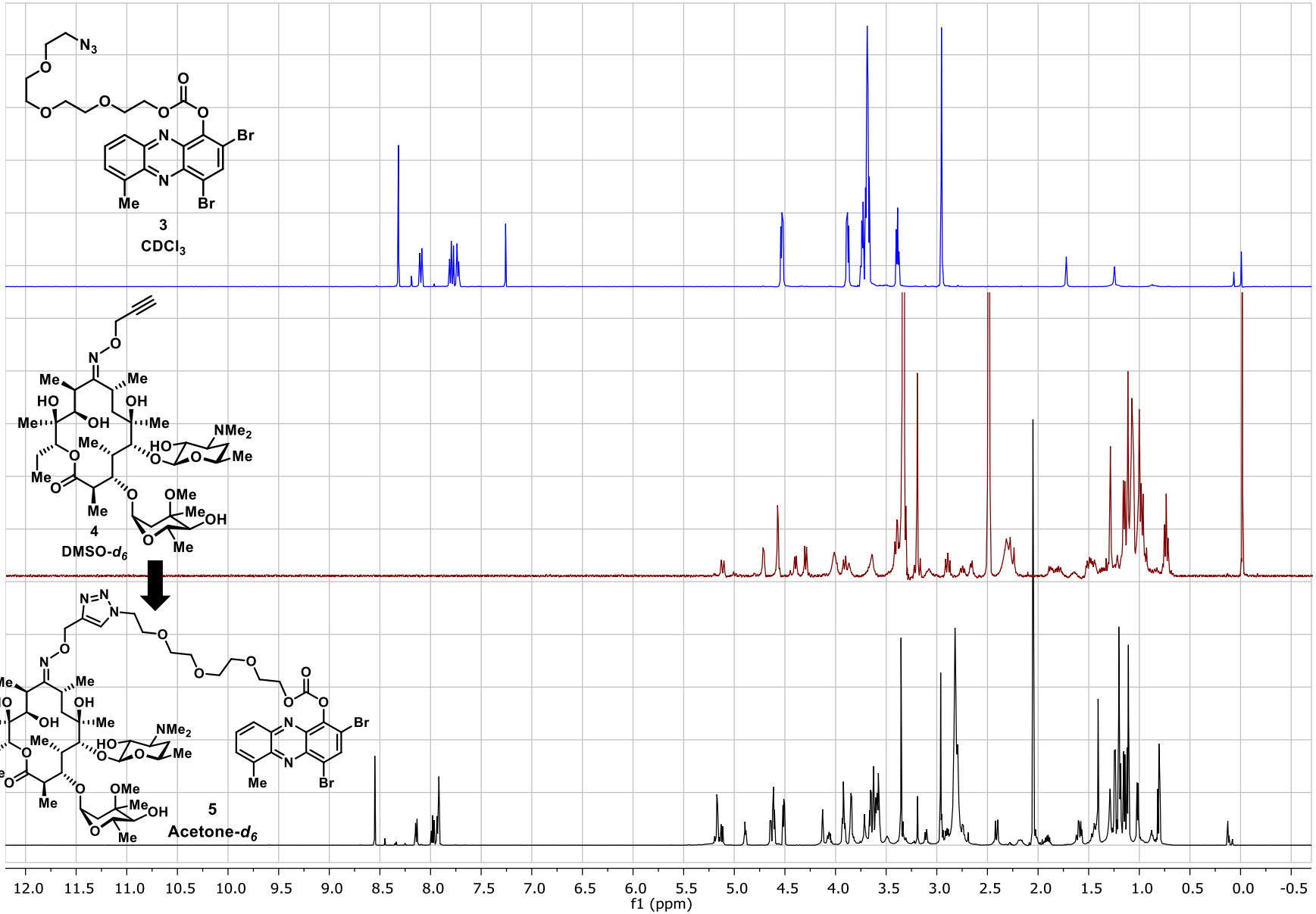
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)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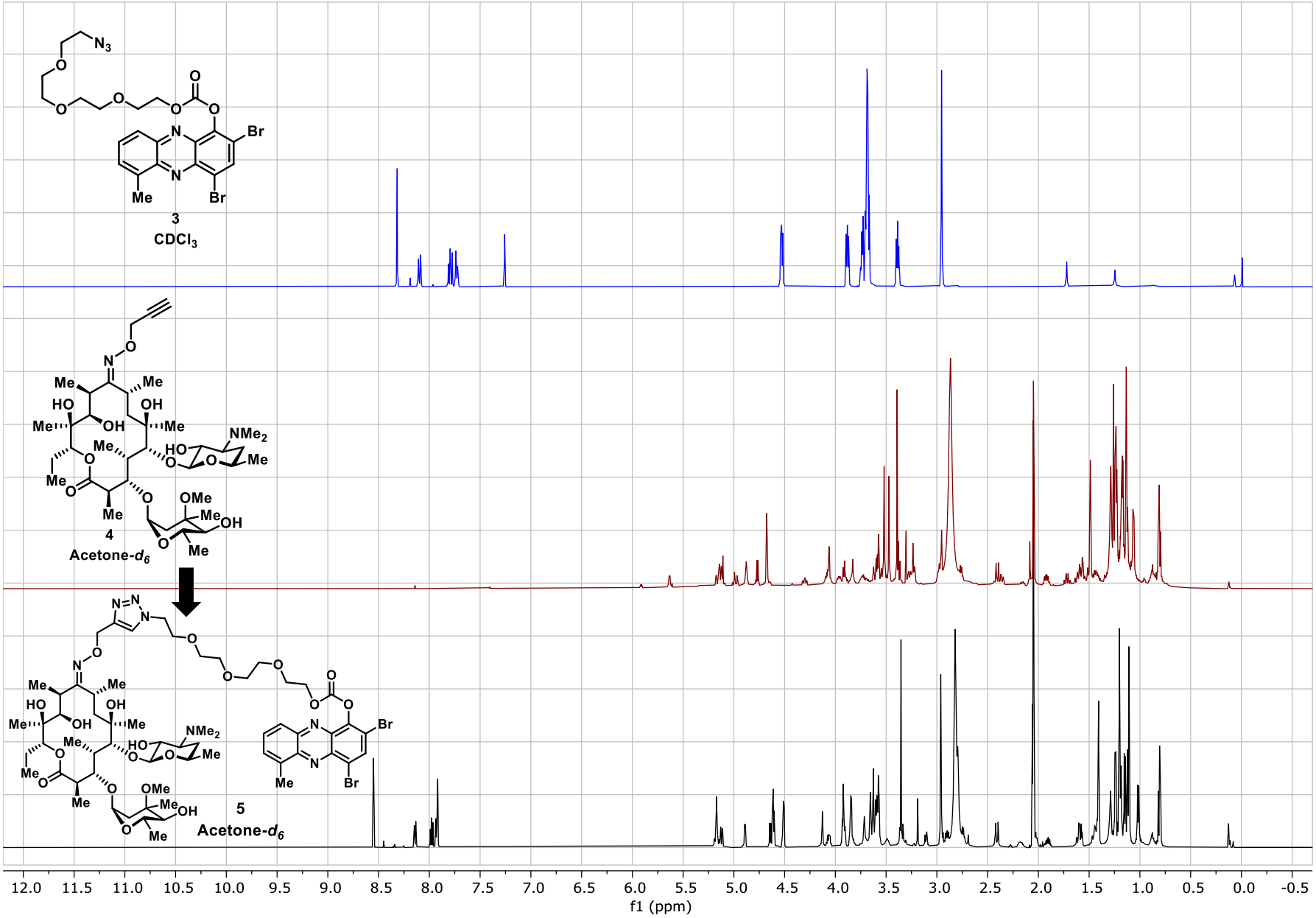
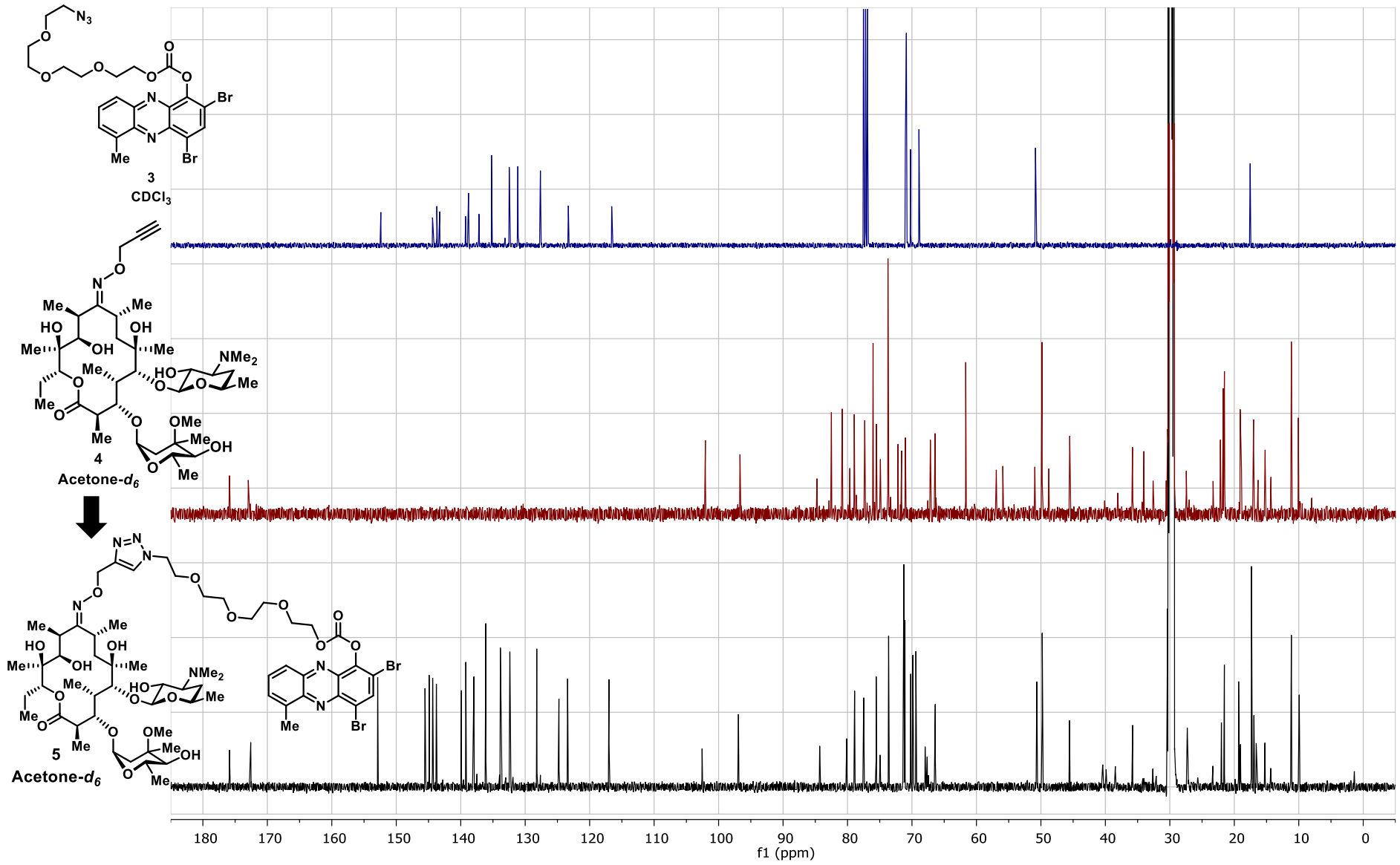
CDCl3
4
DMSO-d6
5
Acetone-d6
f1 (ppm)


3
CDCl3
4
Acetone-d6
5
Acetone-d6
12.0 11.5 11.0 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)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CDCl3
4
Acetone-d6
5
Acetone-d6
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)