Supporting Information

Hybrid antibiotic overcomes resistance in *P. aeruginosa* by enhancing outer membrane penetration and reducing efflux

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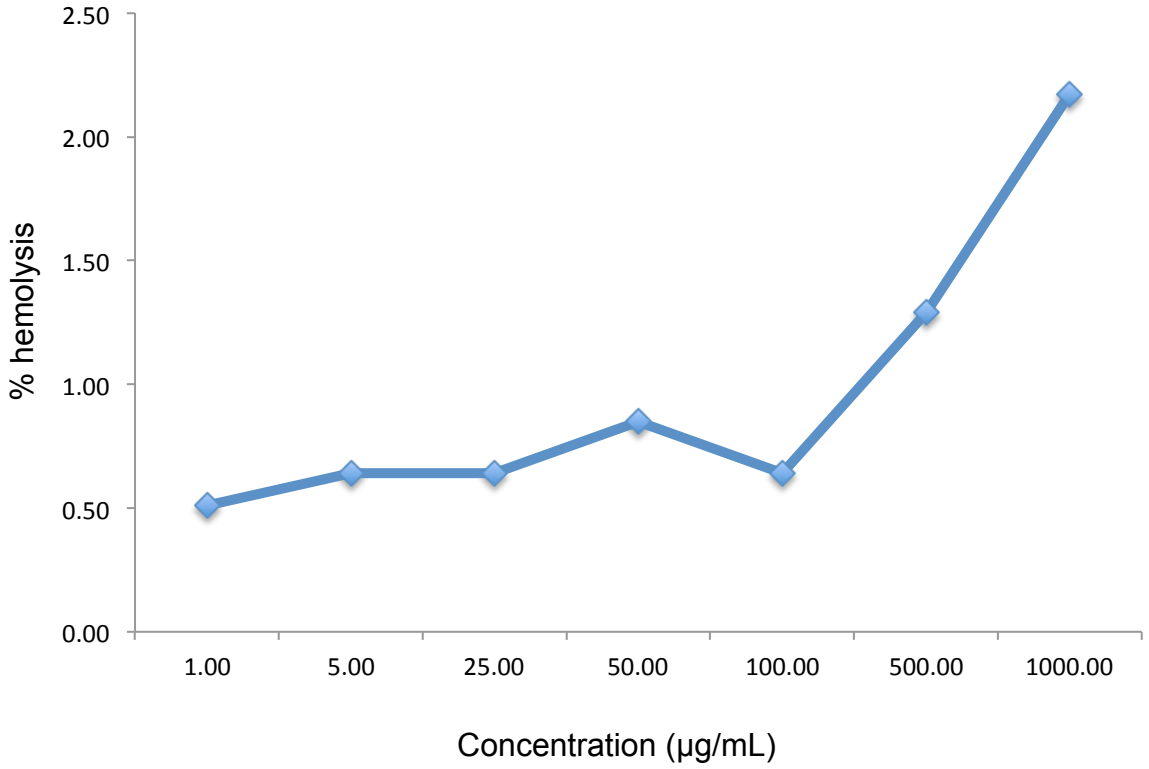
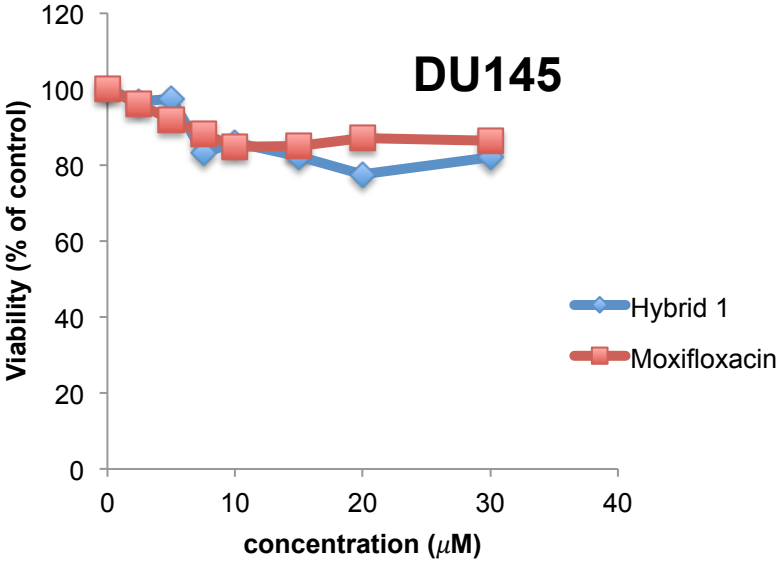


Fig. S2. Cytotoxicity of hybrid **1** in comparison with moxifloxacin against breast (JIMT1) and prostate cancer (DU145) cell lines determined by MTS assay



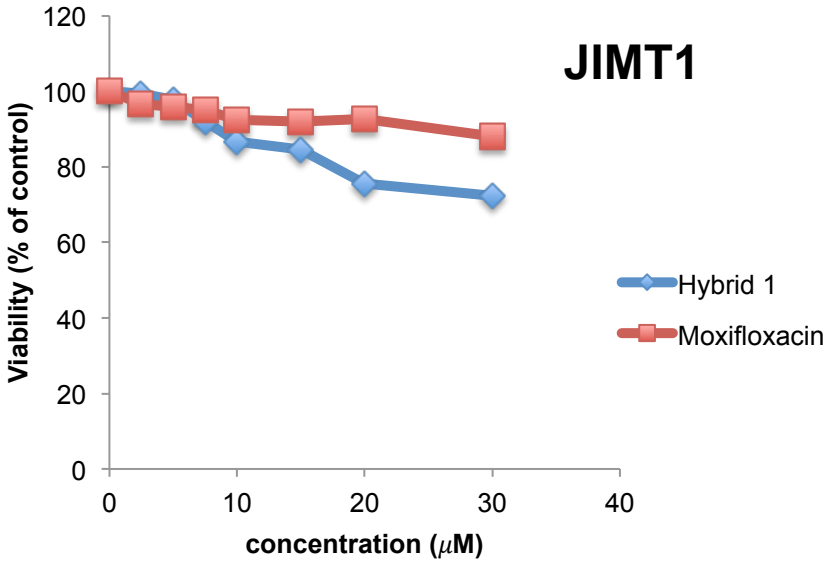


Fig. S3. Tolerability dosage of hybrid **1** on uninfected *Galleria* survival rate after 96 hours (n = 10 worms for each dosage)

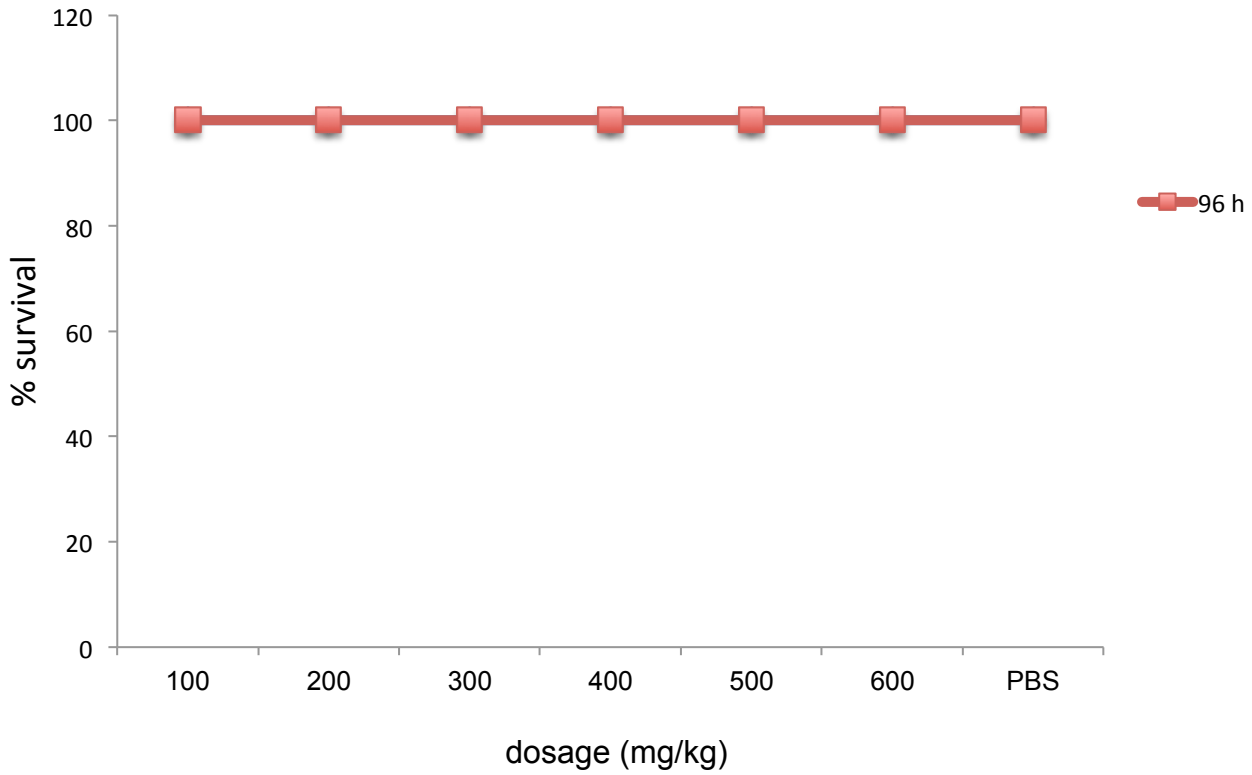


Fig. S4. Single dose efficacy (*Galleria mellonella*) of hybrid **1**, TOB (tobramycin) and MOX (moxifloxacin) using XDR clinical isolate #104354 (n = 30 worms)

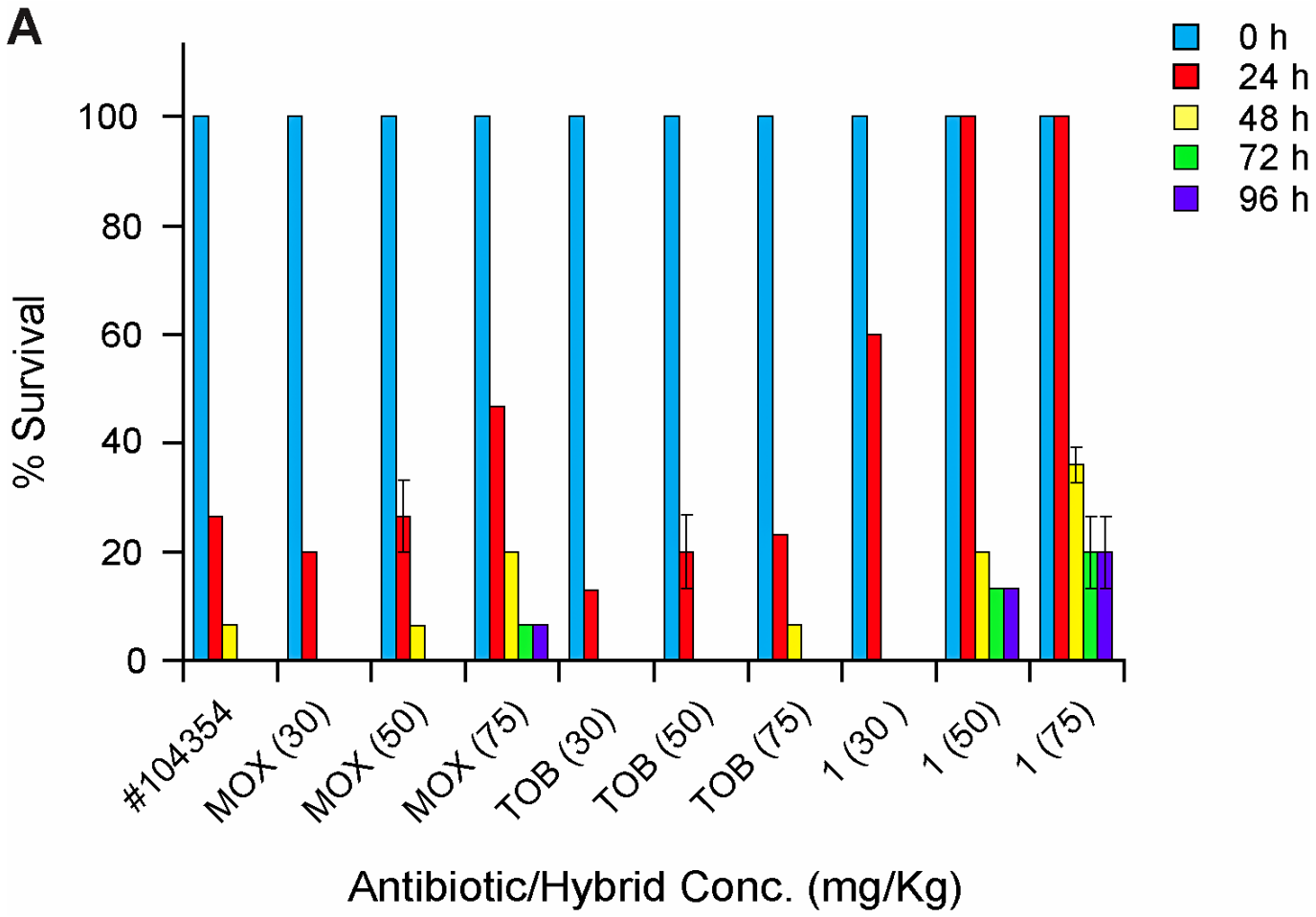


Fig. S5. Concentration-dependent motility assay of hybrid **1** (MIC = 4 μg mL^{-1}) against *P. aeruginosa* PAO1

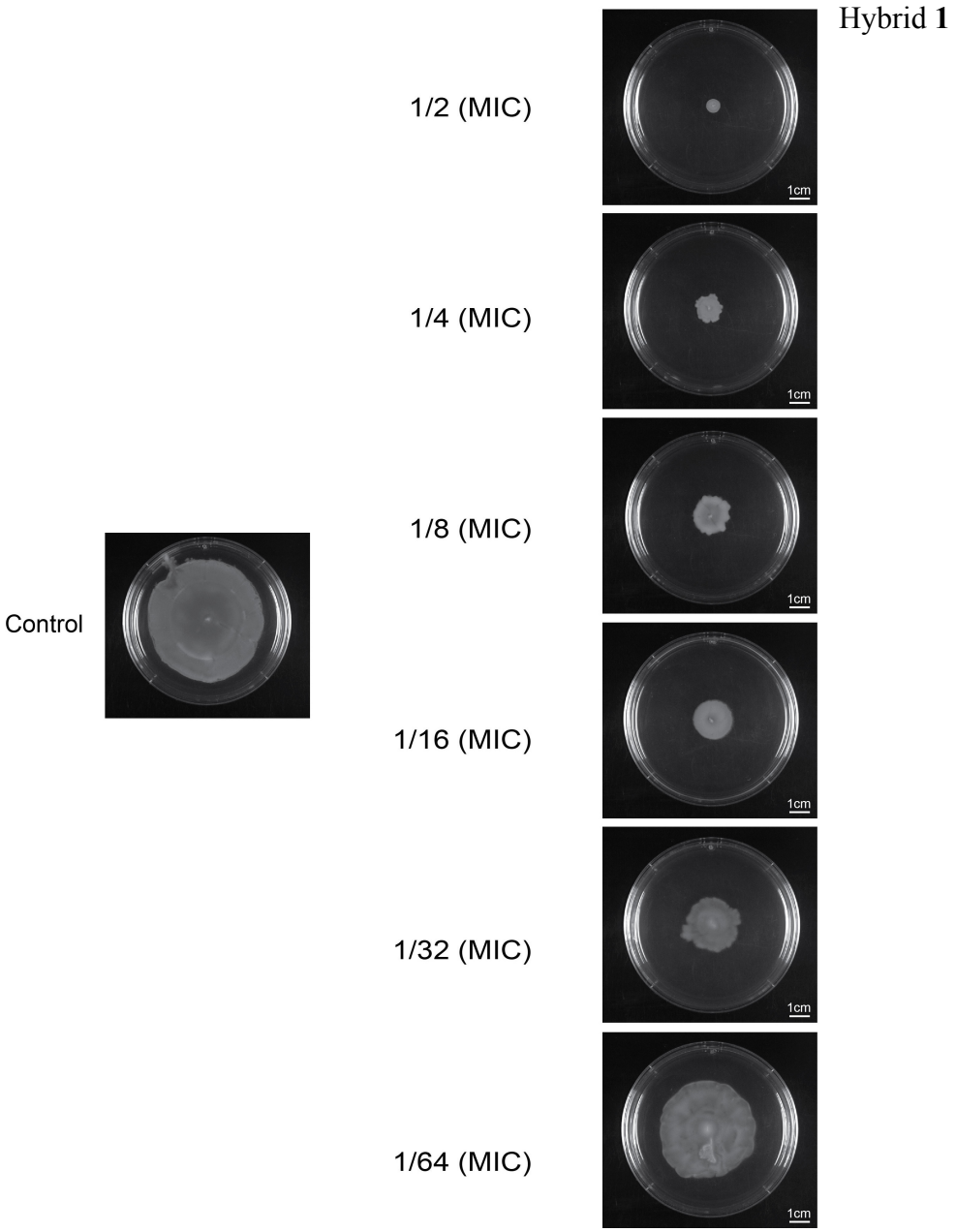


Fig. S6. TEM images of treated *P. aeruginosa* PAO1 with hybrid **1** Scale 100 nm

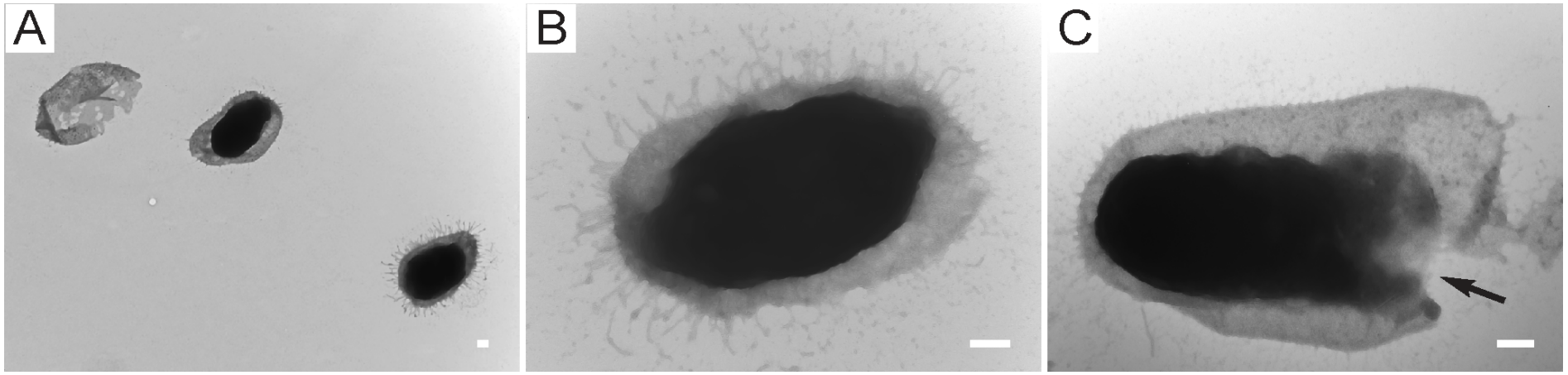


Table S1. Activity of hybrid **1** against FQ-resistant *E. coli and A. baumannii* clinical isolates MIC ((μg/mL)

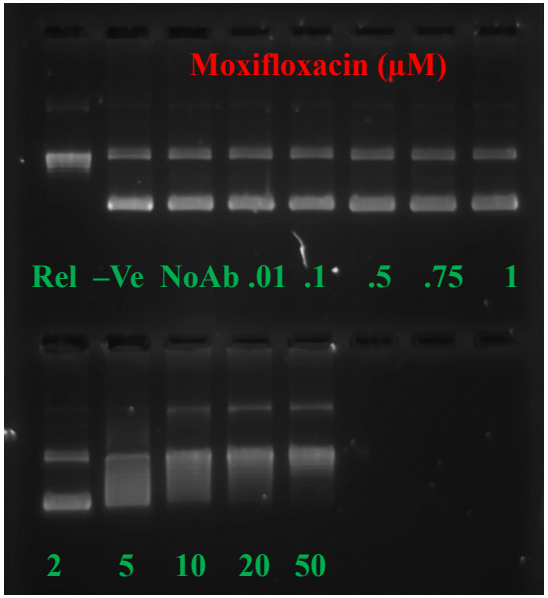
Stock #	*Organism*	Phenotype	CIP 0.06-16	MOX 0.06-16	TOB 0.5-64	COL 0.06-16	1 1-64
79768	*E.coli*	WILD TYPE	</=0.06	0.12	</=0.05	0.25	**2**
80083	*E.coli*	CTX-M-15,OXA-1	>16	>16	32	0.25	**>64**
80960	*E.coli*	CTX-M-15,TEM-1	>16	>16	1	0.5	**>64**
85332	*E.coli*	CTX-M-14,TEM-1	>16	>16	4	0.25	**>64**
87164	*E.coli*	CTX-M-15,TEM-1	>16	16	</=0.05	0.25	**>64**
88273	*E.coli*	CTX-M-15,TEM-1,OXA-1	>16	16	16	0.25	**>64**
89439	*E.coli*	CTX-M-15,OXA-1	>16	>16	64	0.25	**>64**
90087	*E.coli*	CTX-M-15,OXA-1	>16	>16	16	0.25	**>64**
90789	*E.coli*	KPC-3,TEM-1	>16	>16	64	0.5	**>64**
91191	*E.coli*	CTX-M-14,TEM-1	>16	>16	64	0.25	**>64**
92756	*E.coli*	CTX-M-14,TEM-1	>16	>16	64	0.25	**>64**
92969	*E.coli*	CTX-M-15,OXA-1	>16	8	32	0.25	**>64**
98550	*E.coli*	CTX-M-15,OXA-1	> 16	16	16	0.25	**>64**
95882	*E.coli*	KPC-3,TEM-1	> 16	16	2	0.25	**64**
N-10-1631	*E.coli*	CTX-M-15,OXA-1	>16	16	1	0.5	**>64**
ECMH01	*E.coli*	NDM-1	>16	>16	>64	0.25	**>64**
ABO27	*A. baumannii*	ND	>16	8	ND	1	**64**
ABO28	*A.Baumannii*	ND	>16	8	ND	0.5	**64**
ABO30	*A.baumannii*	ND	>16	2	ND	0.5	**4**

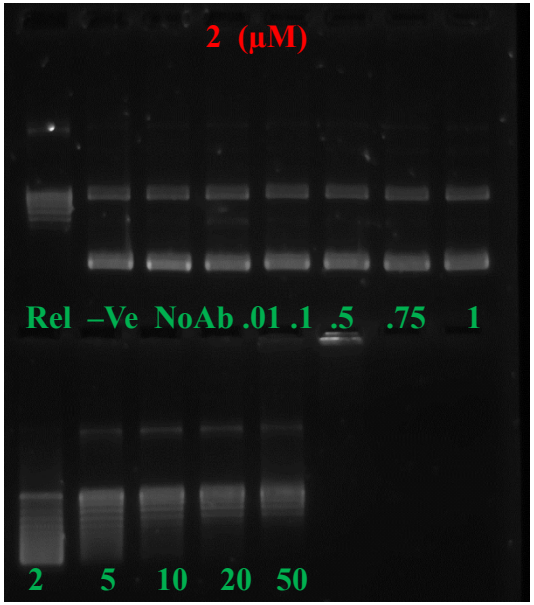
CIP = Ciprofloxacin; MOX = Moxifloxacin; TOB = Tobramycin; COL = Colistin; ND = Not determined

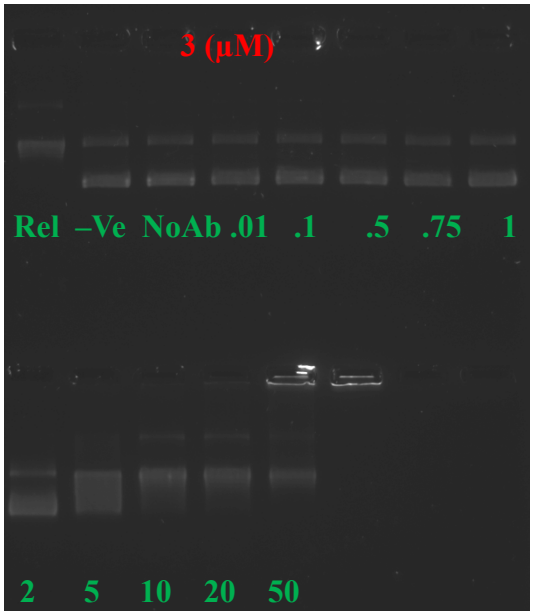
Table S2. Combination studies between hybrid **1** and various antibiotics with *P. aeruginosa* PAO1

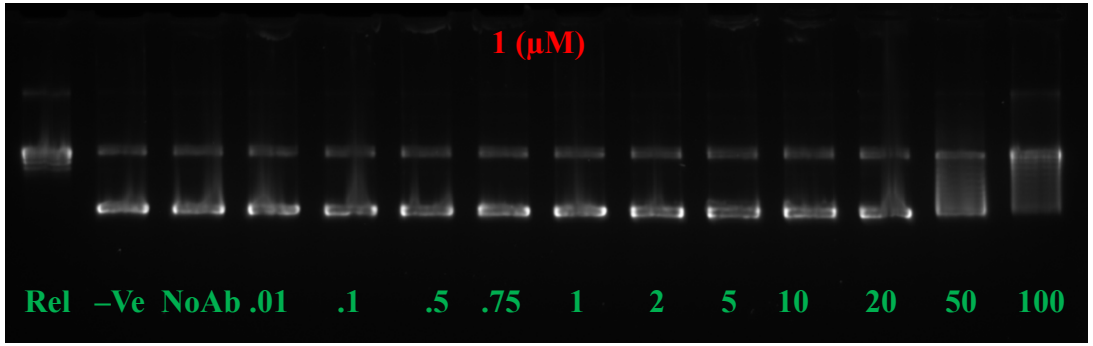
Entry	Strain	PAD	MIC	Synergistic MIC	FIC	Hybrid MIC	Synergistic MIC	FIC	FICI
1	PAO1	Novobiocin	1024	8	0.007	4	0.5	0.12	0.12
2	PAO1	Ceftazidime	2	0.25	0.12	4	1	0.25	0.37
3	PAO1	Minocycline	8	0.25	0.031	4	0.5	0.12	0.15
4	PA01	Erythromycin	256	4	0.015	4	1	0.25	0.26
5	PA01	Rifampicin	16	0.5	0.031	4	0.5	0.12	0.15
6	PA01	Chloramphenicol	64	2	0.031	4	0.5	0.12	0.15
7	PA01	Vancomycin	>1024	16	<0.015	4	1	0.25	<0.26
8	PA01	Colistin	0.5	0.125	0.25	4	0.5	0.12	0.37
9	PA01	Trimethoprim	128	1	0.007	2	0.5	0.25	0.25
10	PAO1	Tigecycline	16	2	0.12	2	0.25	0.12	0.24
11	PAO1	Tobramycin	0.5	>0.5	>1	4	4	1	>2
12	PAO1	Gentamicin	1	4	4	4	4	1	5
13	PAO1	Meropenem	0.5	0.5	1	4	4	1	2

Appendix: Comparative data of moxifloxacin, hybrids **1, 2** and **3** for inhibition of *P. aeruginosa* DNA Gyrase









Appendix: *P. aeruginosa* clinical isolate sources

Isolate No	CANWARD	Isolate	Date	Location		Age	Sex	Source
79199	CANWARD 2008	Pseudomonas aeruginosa	08-01-27	London Health Sciences Centre	Surgery General	58	M	INT: Wound
79352	CANWARD 2008	Pseudomonas aeruginosa	08-02-09	London Health Sciences Centre	Surgery General	68	M	CVS: Blood
80621	CANWARD 2008	Pseudomonas aeruginosa	08-01-02	University of Alberta Hospital	General Unspecified ICU	9	M	Respiratory
83023	CANWARD 2008	Pseudomonas aeruginosa	08-09-11	London Health Sciences Centre	Medicine General	71	M	CVS: Blood
83182	CANWARD 2008	Pseudomonas aeruginosa	08-07-04	Royal University Hospital	General Unspecified ICU	89	M	CVS: Blood
84745	CANWARD 2009	Pseudomonas aeruginosa	09-01-21	University of Alberta Hospital	General Unspecified ICU	15	M	Respiratory
85322	CANWARD 2009	Pseudomonas aeruginosa	09-01-02	London Health Sciences Centre	Surgery General	69	M	INT: Wound
85559	CANWARD 2009	Pseudomonas aeruginosa	09-01-03	Royal Victoria Hospital	Clinic / Office	23	F	Respiratory
86052	CANWARD 2009	Pseudomonas aeruginosa	09-01-17	Mount Sinai Hospital	Medicine General	81	F	Respiratory
86053	CANWARD 2009	Pseudomonas aeruginosa	09-01-17	Mount Sinai Hospital	Medicine General	78	F	Respiratory
86067	CANWARD 2009	Pseudomonas aeruginosa	09-01-20	Mount Sinai Hospital	Clinic / Office	25	M	Respiratory
86079	CANWARD 2009	Pseudomonas aeruginosa	09-01-30	Mount Sinai Hospital	General Unspecified ICU	78	F	Respiratory
86141	CANWARD 2009	Pseudomonas aeruginosa	09-04-04	Mount Sinai Hospital	Medicine General	78	M	Respiratory
86182	CANWARD 2009	Pseudomonas aeruginosa	09-03-02	Mount Sinai Hospital	Medicine General	50	F	INT: Wound
88949	CANWARD 2009	Pseudomonas aeruginosa	09-08-08	London Health Sciences Centre	General Unspecified ICU	56	F	CVS: Blood
92014	CANWARD 2010	Pseudomonas aeruginosa	10-01-22	Royal University Hospital	Medicine General	30	M	INT: Wound
93605	CANWARD 2010	Pseudomonas aeruginosa	10-02-12	London Health Sciences Centre	Medicine General	56	M	Respiratory

Appendix: *P. aeruginosa* clinical isolate sources

Isolate No	CANWARD	Isolate	Date	Location		Age	Sex	Source
93654	CANWARD 2010	Pseudomonas aeruginosa	10-07-07	London Health Sciences Centre	General Unspecified ICU	63	M	CVS: Blood
104354	CANWARD 2013	Pseudomonas aeruginosa	13-02-07	Mount Sinai Hospital	Medicine General	67	M	Respiratory
105198	CANWARD 2013	Pseudomonas aeruginosa	13-02-07	London Health Sciences Centre	General Unspecified ICU	84	M	Respiratory
107092	CANWARD 2013	Pseudomonas aeruginosa	13-01-17	Mount Sinai Hospital	General Unspecified ICU	67	M	Respiratory
108590	CANWARD 2014	Pseudomonas aeruginosa	14-01-02	South East HealthCare Corporation	Emergency Room	79	F	Respiratory

10. Comparisons of chemical shifts of protons H-2'', and H-6'' of tobramycin, hybrids 1, 2 and 3

Compound	^{1}H-NMR Chemical shifts [ppm]		
	H-2''	H-6''	H-6''
Tobramycin (acidified)	3.95	3.92	3.87
1	3.93	3.87	3.73
2	3.76	3.94	3.81
3	3.96	3.94	3.76

11. Results of elemental analysis for Hybrids 1, 2 and 3

Hybrid	Carbon (%)		Hydrogen (%)		Nitrogen (%)		Fluorine		Chlorine (%)	
	Theoretical	Found	Theoretical	Found	Theoretical	Found	Theoretical	Found	Theoretical	Found
1	50.31	50.53	7.28	7.48	9.20	9.09	1.56	1.98	14.56	13.95
2	50.31	50.24	7.28	7.38	9.20	9.26	1.56	1.40	14.56	14.81
3	50.31	50.15	7.28	7.14	9.20	9.43	1.56	1.33	14.56	15.02

12. Gradient used

Time duration (min)	Buffer A	Buffer B
0	85	15
3	85	15
4	80	20
6	80	20
7	70	30
9	70	30
10	60	40
13	60	40
14	50	50
15	50	50

18	85	15
20	85	15

13. HPLC analysis for the purity determination of hybrids 1 and 2

Hybrid	Method	Retention time (min)	Purity %
1	II	9.45	99.7
2	II	9.39	98.3

Method II. Column Kinetex 5u EVO 100 Å, LC Column 150 x 4.6 mm; flow, 1mL/min; buffer A, water 0.1% TFA; buffer B, MeCN 0.1% TFA; gradient used, run time, 20 min; injection, 30 µL of ~0.1 mg/mL in water; UV detection at 299 nm

14. Biochemical methods

Antibacterial MIC testing

Bacterial Isolates

Study isolates were obtained as part of the Canadian National Intensive Care Unit (CAN-ICU) study[1] and CANWARD studies.[2, 3] The CAN-ICU study included 19 medical centers from all regions of Canada with active ICUs. From September 2005 to June 2006, inclusive, each center collected a maximum of 300 consecutive isolates recovered from clinical specimens including from blood, urine, wound/tissue, and respiratory specimens (one pathogen per cultured site per patient) of ICU patients. The 4180 isolates obtained represented 2580 patients (or 1.62 isolates⁄patient). Participating study sites were requested to only obtain "clinically significant" specimens from patients with a presumed infectious disease. Isolates were shipped to the reference laboratory (Health Sciences Centre, Winnipeg, Canada) on Amies charcoal swabs, subcultured S10 onto appropriate media, and stocked in skim milk at -80°C until minimum inhibitory concentration (MIC) testing was carried out.

Antimicrobial Susceptibilities

Following two subcultures from frozen stock, the *in vitro* activities of antimicrobials were determined by microtitre broth dilution in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines. The MICs of the antimicrobial agents for the isolates were determined using glass test tubes (2 ml/tube) containing doubling antimicrobial dilutions of cation adjusted Mueller-Hinton broth and inoculated to achieve a final concentration of approximately 5 x 10^5 CFU/ml then incubated in ambient air for 24 hours prior to reading. Reference strains including *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* (MRSA) ATCC 33592, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 27270, *Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 13883 were acquired from the American Type Culture Collection (ATCC) and were used as a quality control strains. The clinical strains methicillin-resistant *Staphylococcus epidermidis* (MRSE cefazolin MIC >32 μg/mL) CAN-ICU 61589, gentamicin resistant *E. coli* CAN-ICU 61714, Amikacin-resistant (MIC = 32 μg/mL) *E. coli* CAN-ICU 63074, gentamicin resistant *P. aeruginosa* CAN-ICU 62584, *Strenotrophomonas maltophilia* CAN-ICU 62584 and *Acinetobacter baumannii* CAN-ICU 63169 were obtained from hospitals across Canada as a part of the Canadian National Intensive Care Unit (CAN-ICU) study. Methicillin-susceptible *S. epidermidis* (MSSE) CANWARD-2008 81388 was obtained from the 2008 Canadian Ward Surveillance (CANWARD) study while gentamicin-resistant tobramycin-resistant ciprofloxacin-resistant [aminoglycoside modifying enzyme aac(3)-IIa present] *E. coli* CANWARD-2011 97615 and gentamicin-resistant tobramycin-resistant *P. aeruginosa* CANWARD-2011 96846 were obtained from the 2011 CANWARD study.

Antibacterial combination screening

FIC index determination

FICs were determined by setting up checkerboards with 8 concentrations each of drug and selected hybrid. At least 2 replicates were done for each combination and the means used for calculation. The MIC for each drug was the lowest showing no growth. The FIC for each drug was calculated as the [drug in the presence of hybrid] for a well showing no growth divided by the MIC for that drug. The FIC for each hybrid was calculated as the [hybrid in the presence drug] for a well showing no growth divided by the MIC for that hybrid. The FIC index is the sum of the two FICs.

Combination checkerboard studies: Checkerboard assays were carried out using previously described methods.[4] Fifty microliter of cation-adjusted Mueller-Hinton broth (CAMHB) was dispensed into each well of a 96-well plate. Antibiotic to be tested was diluted along the abscissa while hybrid **1** was diluted along the ordinate. Overnight bacterial culture was standardized in saline using the 0.5 McFarland turbidity standard and diluted 1:50 in CAMHB. Fifty microliter of standardized culture was added to each of the wells and plates incubated at 37 ^{o}C for 18 hours. MIC was recorded as wells with the lowest concentrations of drugs with no visible growth.

Haemolytic assay

In vitro toxicity was determined using a human red blood cell (erythrocytes) haemolytic assay.[5] Erythrocytes were washed and resuspended in tris-buffered saline. The cell suspension was combined with varying concentrations (low to very high) of test hybrid. The samples were centrifuged and the absorbance of the supernatants measured at 540 nm. Tris-buffered saline and Triton X were used as negative and positive controls, respectively. The toxicity was assessed by percentage haemolysis.

Cytotoxicity Assay

Cell culture

DU145 cell lines were grown from frozen stocks of cell lines that were originally obtained from ATCC (Manassas, VA, USA). JIMT-1 cells were grown from frozen stocks of cells obtained from DSMZ (Braunschweig, Germany). JIMT1 and DU145 cells were grown in Dulbecco's modified Eagle's References medium. The cells were grown in media supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and 0.1 mg/mL streptomycin in a humidified 5% CO_2 atmospheric incubator at 37 ^{o}C.

Toxicity assay

Cell viability was determined with the Cell Titer 96 Aqueous One solution (MTS assay; Promega). Equal numbers of the cancer cell lines (7500e9000) in media (100 mL) were dispersed into 96-well

plates. As blanks, media without cells (100 mL) were also placed in some wells and treated similarly to the cell-containing wells. After an incubation period of 24 h, a solution of test compound (100 mL) in medium at twice the desired concentration was added to each well.[6] The treated cells were further incubated for 48 h, after which methanethiosulfonate (MTS) reagent (20% v/v) was added to each well. The plates were incubated for 14 h on a Nutating mixer in a CO_2 incubator, and then the optical density (OD) was read at 490 nm by using a SpectraMax M2 plate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The blank values were subtracted from each value, and the viability values of the treated samples relative to the controls with vehicle were calculated. The values for the plots are the means standard deviation. Values of zero indicate there are no viable cells in the wells.

Galleria mellonella model of *P. aeruginosa* infection

Batches of last instar *G. mellonella* waxworms were obtained from a commercial source and used within 7 days of delivery. Worms on average were 250 mg and used to determine treatment dosage as previously described.[7] Single colonies of XDR *P. aeruginsoa* #104354 were used to inoculate 3mL of LB broth and grown overnight at 37°C with 250rpm shaking. Next day overnight culture was standardized in 2mL PBS to 1.0 x 10^8 CFU/mL using a 0.5 McFarland Standard (Remel, Lenexa, USA) and diluted to 1.0 x 10^6 CFU/mL and 10µL injected into worms. This CFU/mL concentration was optimized by previous infections of decreasing concentrations of #104354. Monotherapy experiment used 15 larvae and these experiments were repeated two times using larvae from different batches (n = 30). Survival data curves were plotted using the Kaplan Mier method. Monotherapies were assayed 2 hours post bacterial infection hybrid **1**, tobramycin and moxifloxacin were individually tested at 30, 50, and 75 mg/kg. The larvae were incubated at 37 °C in petri dishes lined with filter paper and scored for survivability every 24 hours. Larvae considered dead if they do not respond to touch.

Outer membrane permeabilization assay

The non-polar dye 1-*N*-phenylnaphthylamine (NPN) was used to ascertain possible mechanism of uptake and action of hybrid **1** on the bacterial outer cell membrane.[8] Single colonies were picked and inoculated into 3mL of LB media and grown overnight at 37 °C shaking at 250rpm. After 16-18 hours of incubation bacteria were sub-cultured 1% into fresh BM2-glucose media (62mM potassium

phosphate buffer pH 7, 7mM $(NH_4)_2SO_4$, 10μM $FeSO_4$, 0.4% (wt/vol) glucose and 2mM $MgSO_4$). Cultures were grown to mid-logarithmic phase and 1mL of culture was spun down and washed with 5mM HEPES pH 7.0, 5mM glucose before final resuspension in 1mL of 5mM HEPES pH 7.0, 5mM glucose. The absorbance (600 nm) of samples was standardized to 0.5 in same buffer. To a 96-well plate, 200μL of standardized cells were added followed by a final concentration of 10μM NPN. Plates were read on a FlexStation 3 (Molecular Devices, Sunnyvale, USA) microplate reader at excitation 350nm and 420 nm emissions measured every 30 seconds for 2 minutes. This was followed by addition of hybrid **1** respective concentrations (8, 16, 32 and 50 μg/mL) or colistin 5 μg/mL and measured every 30 seconds for an additional 4.5 minutes. NPN and compound were added without cells as a negative control to account for any background fluorescence. Data was corrected for background fluorescence and plotted with standard deviation.

Membrane depolarization assay

The ability of hybrid **1** to depolarize the bacterial transmembrane potential was determined by a fluorescence-based assay using a membrane potential sensitive dye 3,3'-dipropylthiadicarbocyanine iodide $DiSC_3(5)$.[9] Cells accumulate this cationic probe owing to the electrical gradient of the cytoplasmic membrane. Once concentrated inside the cells, it self-quenches its fluorescence. The dye gets released into the medium and regains its fluorescence following addition of a compound that disrupts transmembrane potential. Briefly, *P. aeruginosa* PAO1 cells were grown till mid-logarithmic phase (A_{600} = 0.4-0.5). The cells were harvested by centrifugation, washed and resuspended in HEPES buffer (5 mM HEPES, 20 mM glucose, pH 7.2) added with 0.2 mM EDTA to achieve A_{600} of 0.05. The cell suspensions were incubated with 0.4 μM $DiSC_3(5)$ for 30 min at 37 ^oC with constant stirring followed by the addition of 0.1 M KCl and further incubated for 15 min for maximum dye uptake (stable fluorescence quenching was observed). The cell suspension was then treated with the desired concentration of the test compound (hybrid **1**) and the fluorescence was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm (FlexStation 3, Molecular Devices, Sunnyvale, USA). A blank with only cells and the dye was used as background. Cells treated with colistin were used as positive control. Fluorescence measurements were recorded for three independent experimental samples.

Protein Translation Assay

To study the prokaryotic protein translation inhibition effect of different compounds (Colistin, Moxifloxacin, Tobramycin, hybrid **1, 2** and **3**), the *E. coli* S30 Extract for Circular DNA translation (#L2010, Promega) was utilized. Translation reactions (25 µL) were performed as per manufacturer's protocol. The inhibitory effect of the compounds were tested over a wide spectrum of concentations and the reaction was incubated at 37 °C for 60 min, cooled on ice for 5 minutes, and diluted with a dilution reagent (25 mM tris-phosphate buffer at pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexanetetraacetate, 10% glycerol, 1% Triton X-100 and 1 mg/mL BSA. The luminescence was measured in a 96 well opaque bottom plate after the addition of the luciferase assay reagent (50 µL; Promega), and the light emitted was recorded with a FlexStation 3 (Molecular Devices, Sunnyvale, USA) microplate reader. The concentration of half-maximal inhibition (IC50) was obtained from concentration-response curves fitted to the data of at least two independent experiments by using Graphpad prism software.

Gyrase A Inhibition Assay

DNA supercoiling activity was assayed with relaxed pBR322 DNA as a substrate (#PAS001, Inspiralis Ltd.). Supercoiling activity at different concentrations of the antibiotics were studied as per manufacturer's protocol. The reaction mixture (30µL) was incubated at 37 °C for 30 minutes and then was terminated by addition of 30µL of the 2X stop buffer/loading dye (40% sucrose, 100 mM Tris.HCl at pH 7.5, 1 mM EDTA, 0.5 mg/ml bromophenol blue. This was followed by addition of 30µL of chloroform/isoamyl alcohol (24:1) mixture. The contents were vortexed briefly and spun for a 2 second pulse on a tabletop centrifuge and the aqueous phase (top phase will stop buffer/load dye) was loaded on a 1% agarose gel. The agarose gel electrophoresis in TAE (40mM Tris, 20mM acetic acid, and 1mM EDTA) was performed for an hour and the gels were stained with SYBR®Gold (Life technologies, Thermo Scientific) for 30 minutes in TAE buffer follwed by destaining in water for 30 minutes. The gels were pictured in FluroChem®Q (Cell biosciences) and analyzed using Alphaview software. The One unit of supercoiling activity here was defined as the amount of *P aeruginosa* DNA gyrase required to supercoil 0.5 µg of plasmid in 30 minutes. The IC50 was defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%.

Emergence of Resistance

The induction of *in vitro* resistance in *P. aeruginosa* PAO1 against hybrid **1**, tobramycin and moxifloxacin was determined in a multistep experiment following a standard method described earlier.[10] Briefly, MIC values of the tested compounds ascertained by microtitre broth dilution method. Visually bacterial turbidity ceases to appear at MIC. Bacterial suspension from sub-MIC of the compounds (at 0.5 x MIC) was used to prepare the inoculum for the next day MIC experiment. The process of determining MIC using the bacterial suspension from sub-MIC (at 0.5 x MIC) was repeated for next 25 passages and the fold increase in MIC was established. The results were expressed as the fold of increase in MIC with each passage or day.

Motility assay

Plates for motility assay were prepared using trypticase peptone [5 mg/mL], NaCl [2.5 mg/mL] and 0.3% (w/v) agar. Hybrid **1** was added in the molten motility media to achieve the desired concentration Plates were allowed to dry for 60 min and inoculated with 2.5 µl of overnight grown culture of *P. aeruginosa* PAO1 diluted in sterile PBS to OD_{600} 1.0.[11] Plates were incubated for 20 hours at 37 °C.

Transmission Electron Microscope (TEM) Analysis

P. aeruginosa PAO1 cells were grown to early log growth phase (OD_{600} = 0.3), washed twice with 5mM HEPES buffer (pH 7.2) and resuspended in the same buffer. Cells were treated with the desired concentration of hybrid **1** for 3-4 h at 37 ^{o}C. Control samples consisted of untreated cells suspended in buffer. Treated and control cells were thoroughly washed and resuspended in the same buffer. An aliquot of each sample (2.0 µl) was spotted on carbon coated TEM grid (CF-400-Cu carbon film on 400 square mesh). Grids were then stained for 20 to 30 s with a freshly prepared 1% solution of uranyl acetate. Samples were air-dried in laminar hood and examined in a transmission electron microscope (Hitachi Scanning/ Transmission Electron Microscope (STEM). Model H-7000 equipped with Advanced Microscopy Techniques (AMT) CCD Camera, Model 1600 M Woburn, Massachusetts, United States of America) operating at 75 kV. Software: AMT Image Capture Engine V601.

Fluorescence microscope analysis

P. aeruginosa PAO1 cells were grown in LB media at 37 °C with vigorous shaking to an early log growth phase (OD_{600} = 0.3). Cells were harvested, wasted and resuspended in 5mM HEPES buffer (pH 7.2). Cells were treated with the desired concentration of hybrid **1** for 3-4 h at 37 ^{o}C. Control (untreated) and treated samples were washed with buffer and labeled in separate sets with 30 μM PI for 15 mins. Then, cells were washed thoroughly with buffer to remove excess dye and images of the stained cells were captured using fluorescence microscope (Zeiss Axio Imager Z1 eqipped with Zeiss Axio Cam MRm) with a filter that allowed green light excitation at 495-570 nm.

Kinetics of bactericidal activity of hybrid 1 (Time-kill Curve)

Target *P. aeruginosa* PAO1 cells (approximately 1 x 10^6 CFU/mL) were grown in freshly prepared MH broth at 37 °C and 250 rpm incorporated with the desired concentrations of hybrid 1 (0.5x MIC, 1x MIC and 2x MIC) for 24 h. Untreated cells in media and cell treated with 1xMIC colistin were used as positive and negative controls respectively. The kinetics of bacterial cell death were determined by calculating viable cell numbers (Log_{10} CFU mL^{-1}) at regular intervals (1h, 2h, 3h, 6h, 9h and 24h) by serial dilution and plating.

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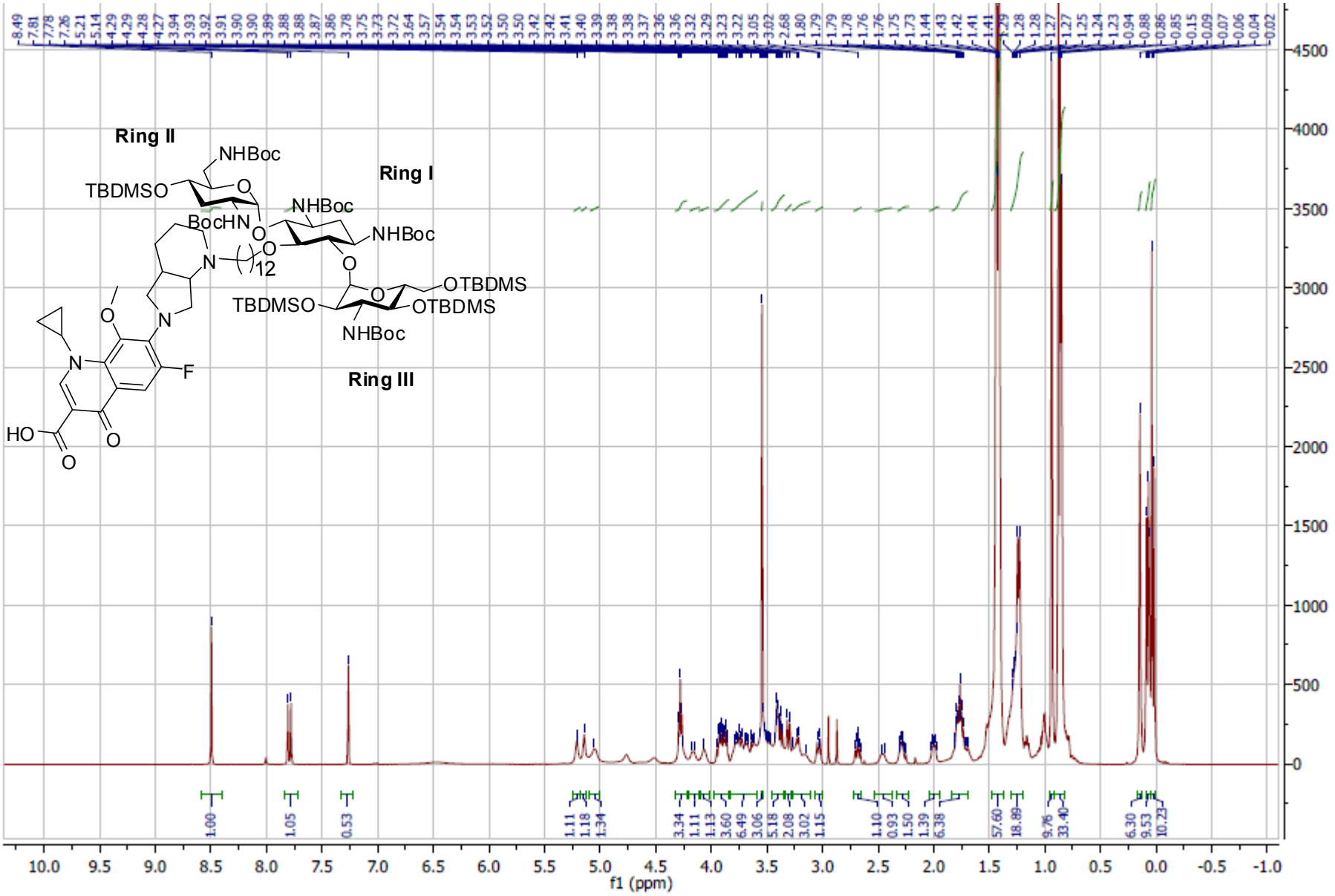
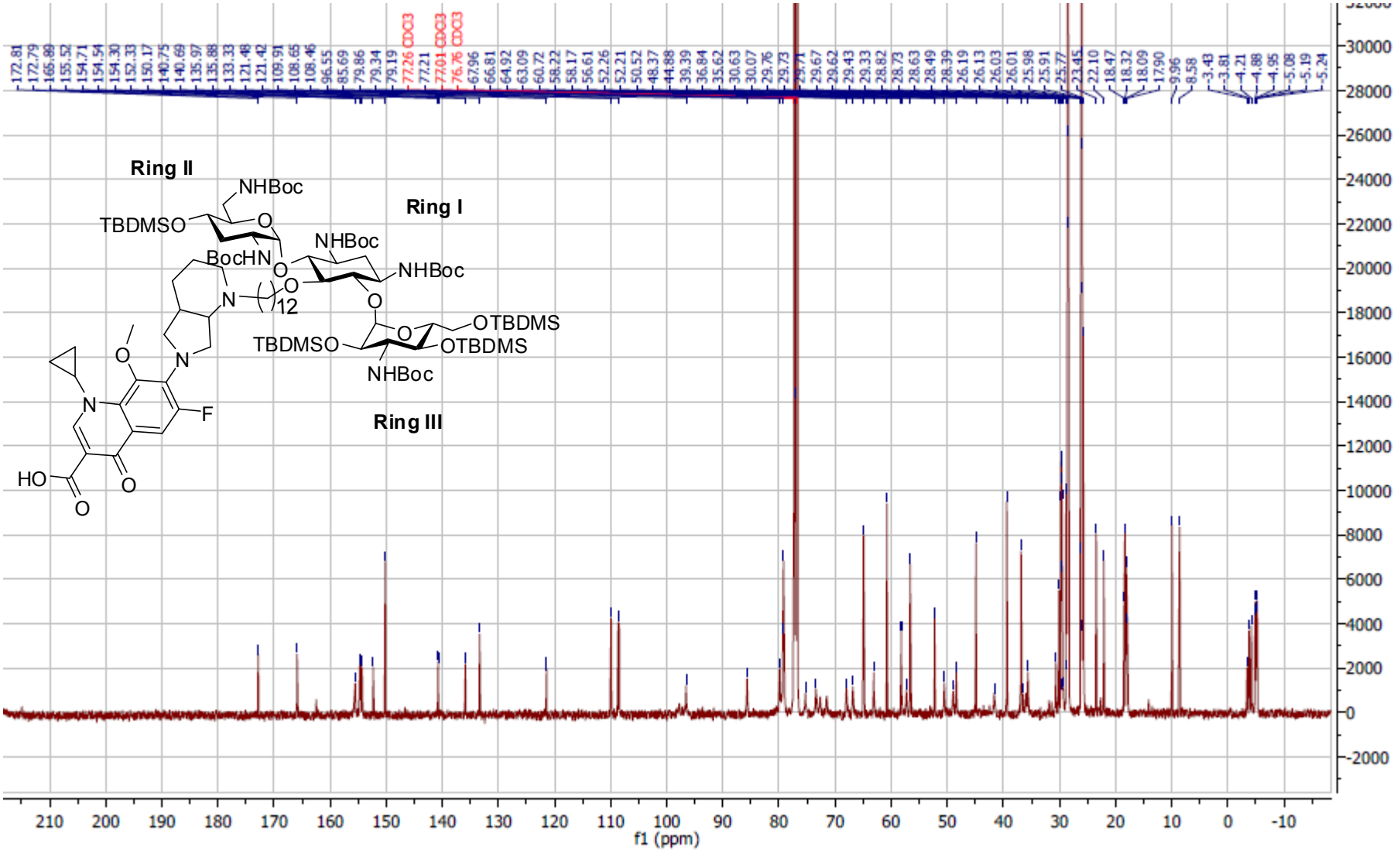


^{1}H spectrum of compound **7** in $CDCl_3$



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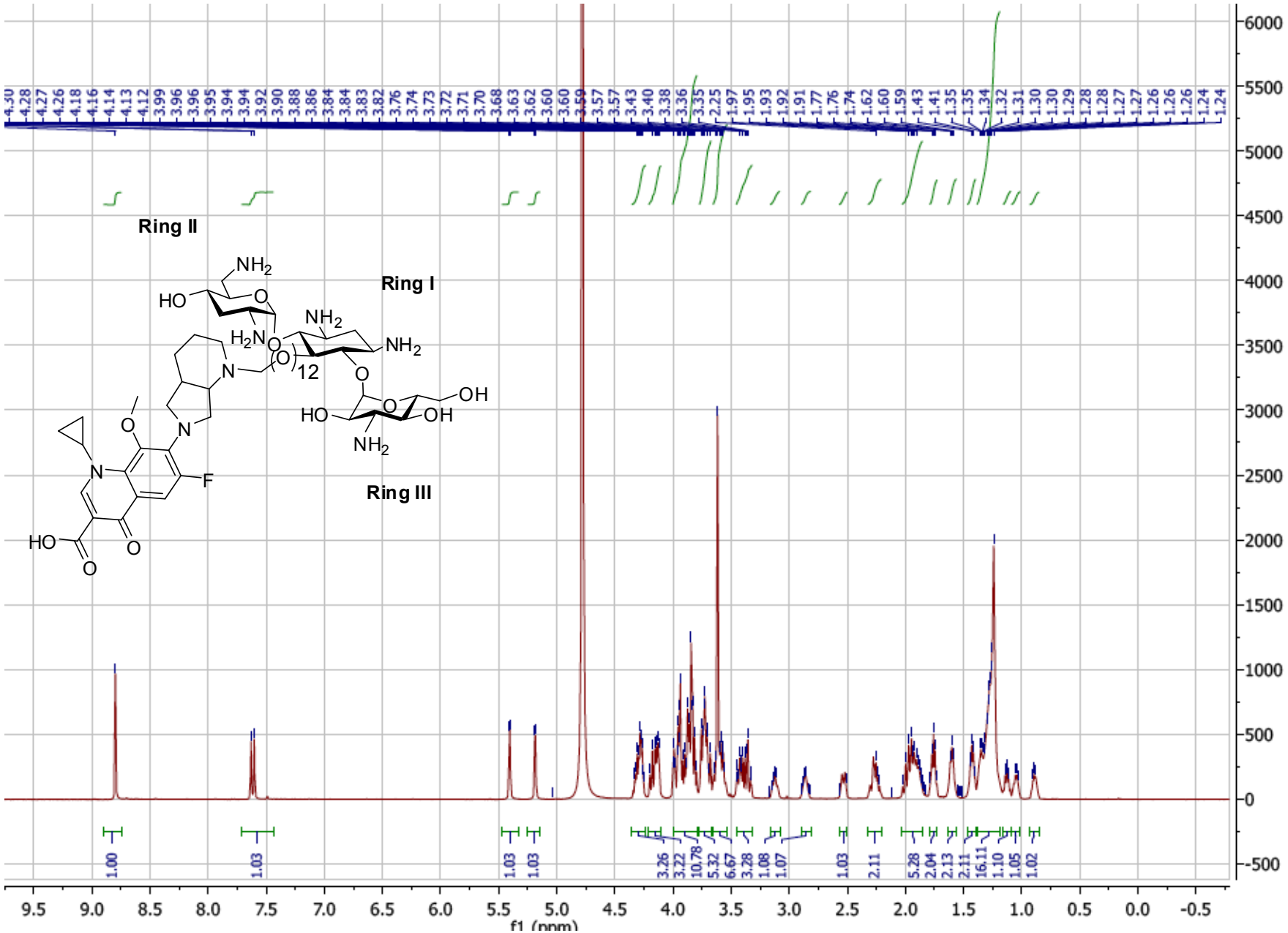

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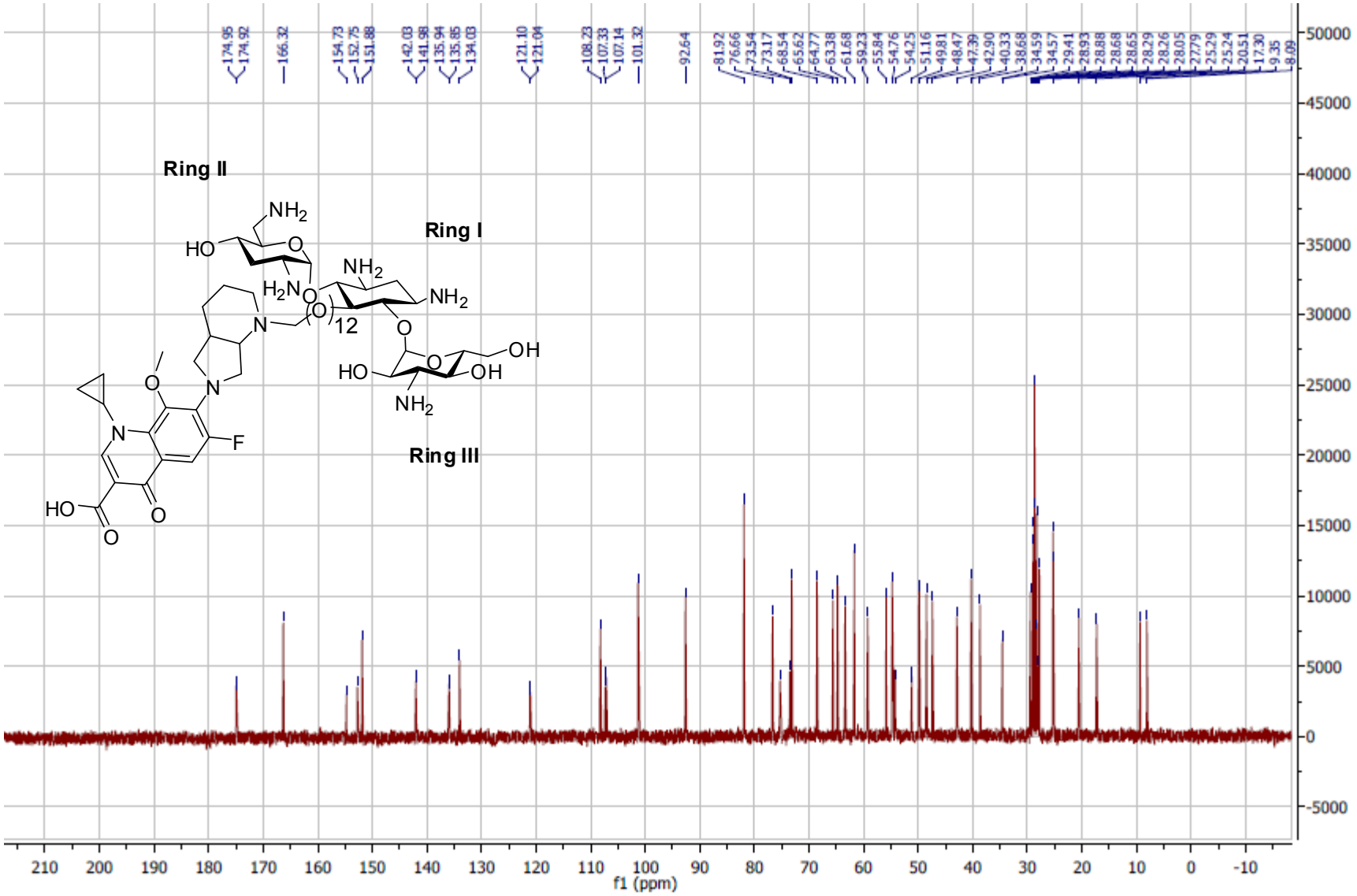

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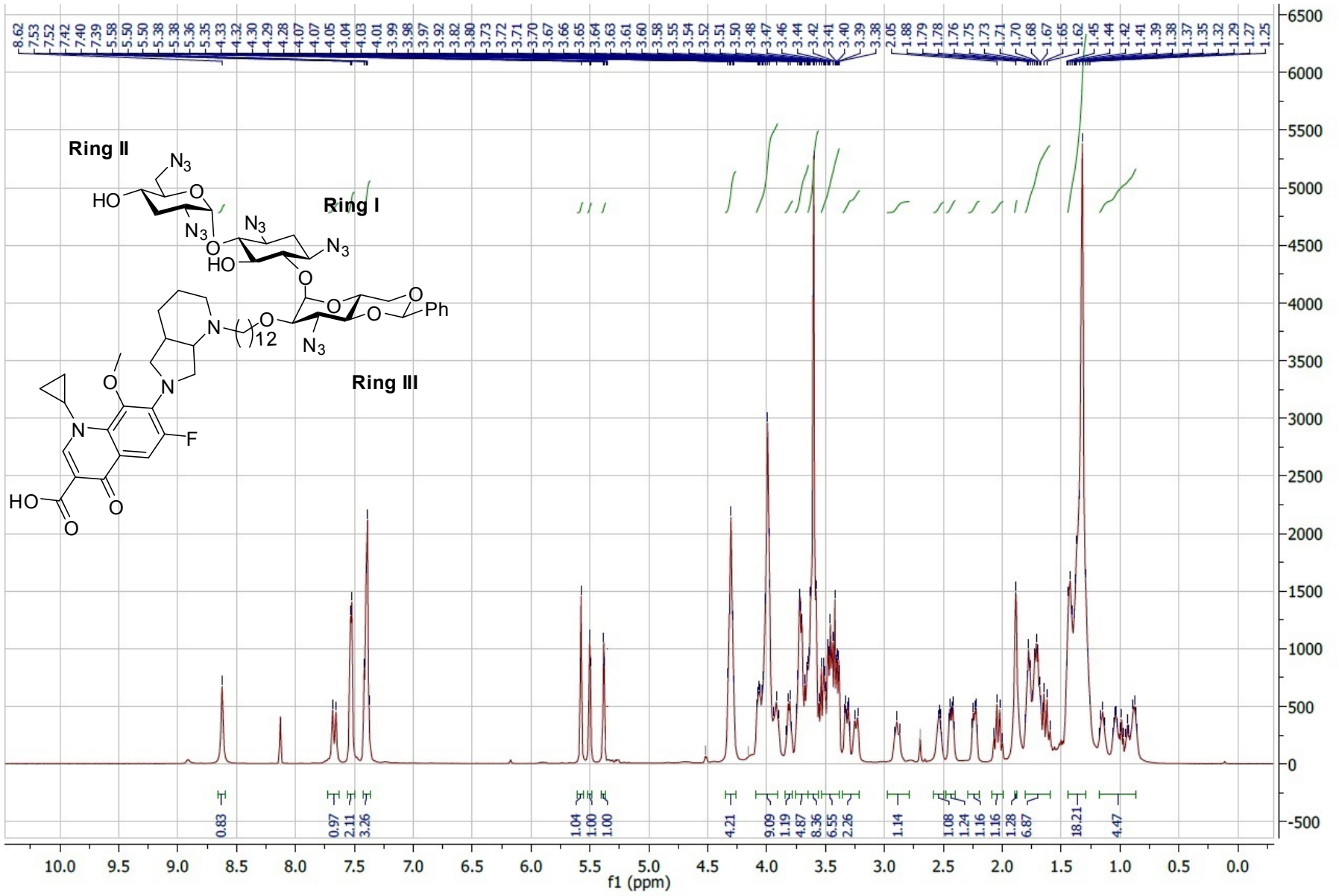

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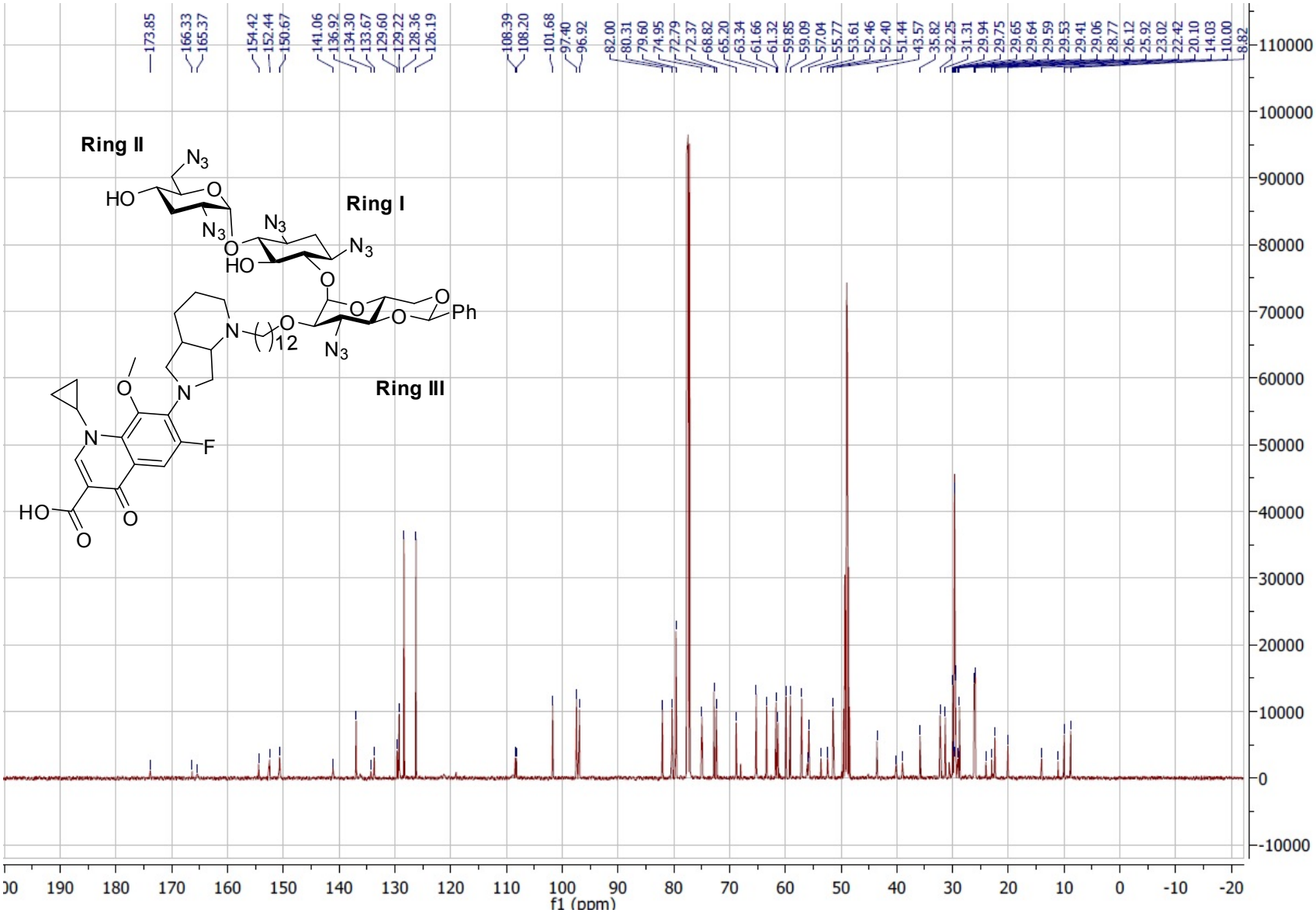

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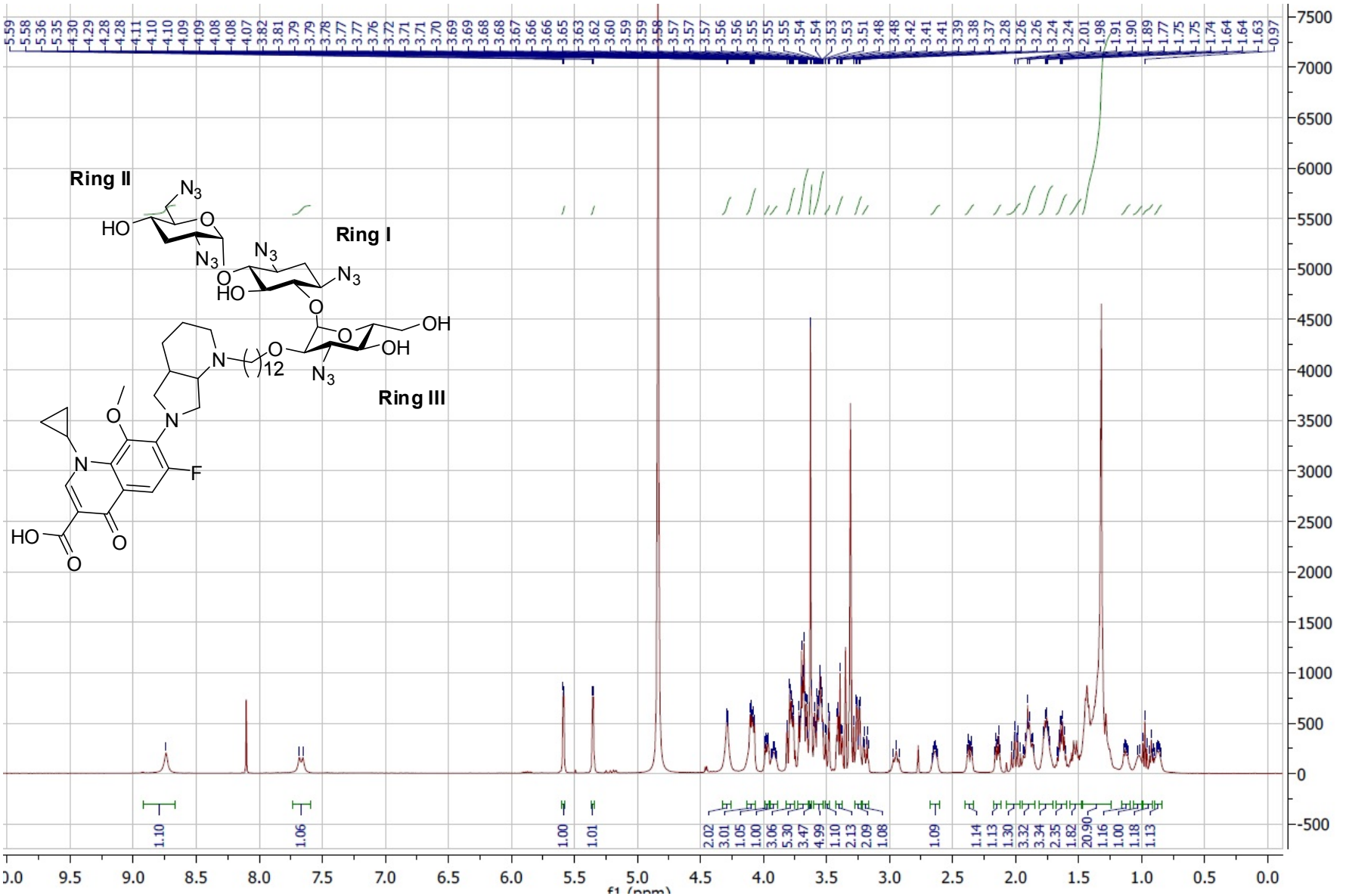

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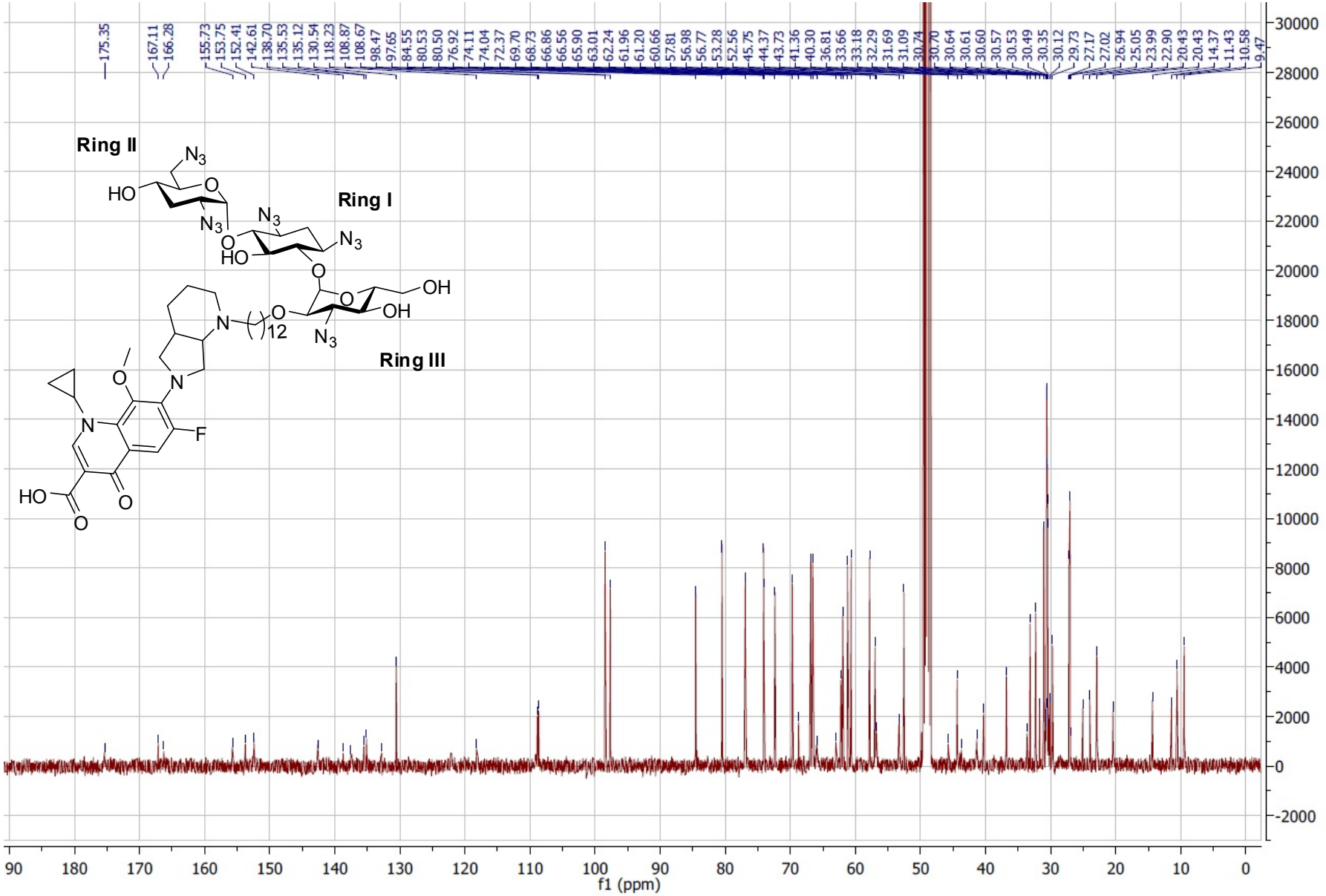

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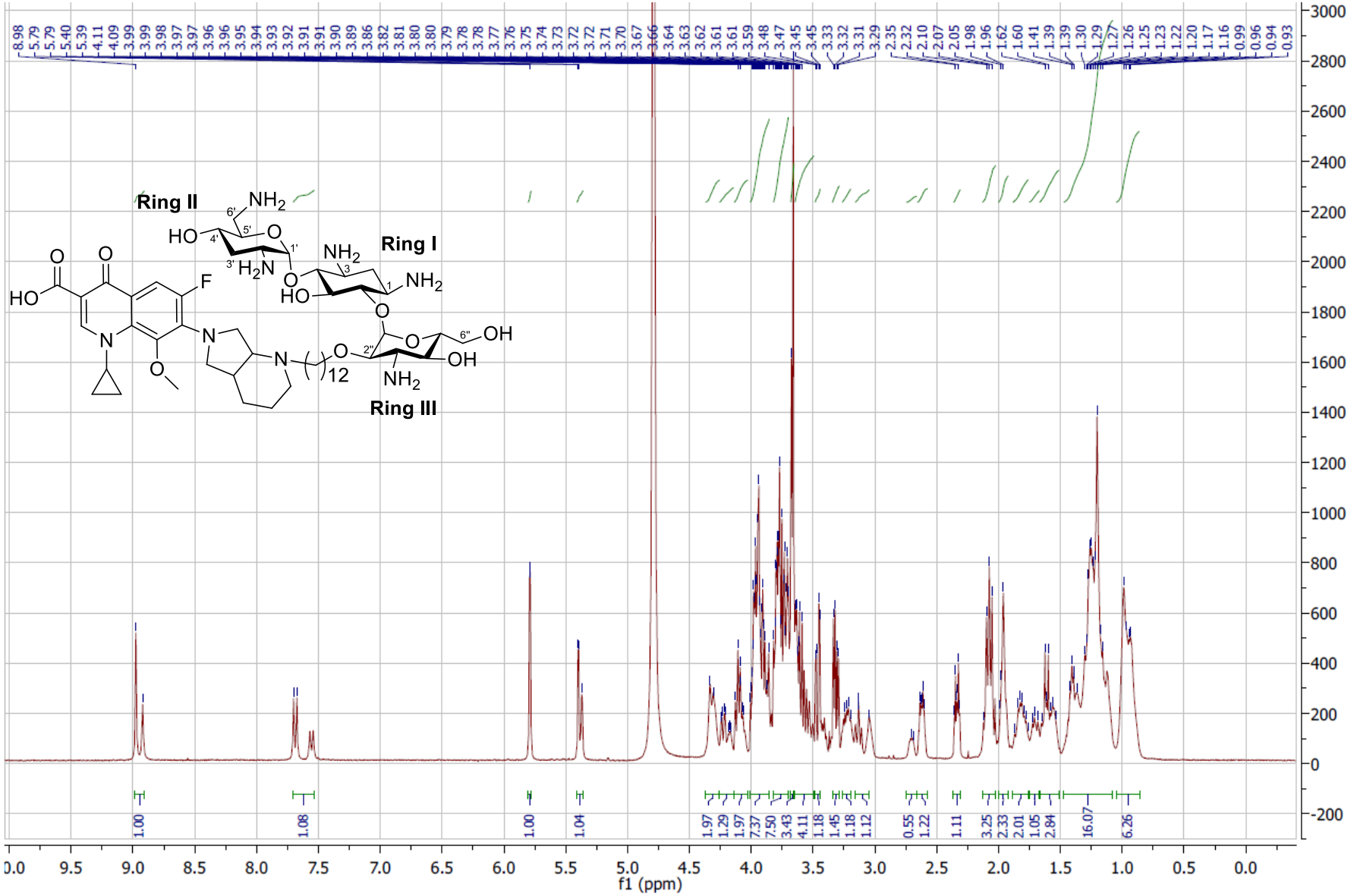

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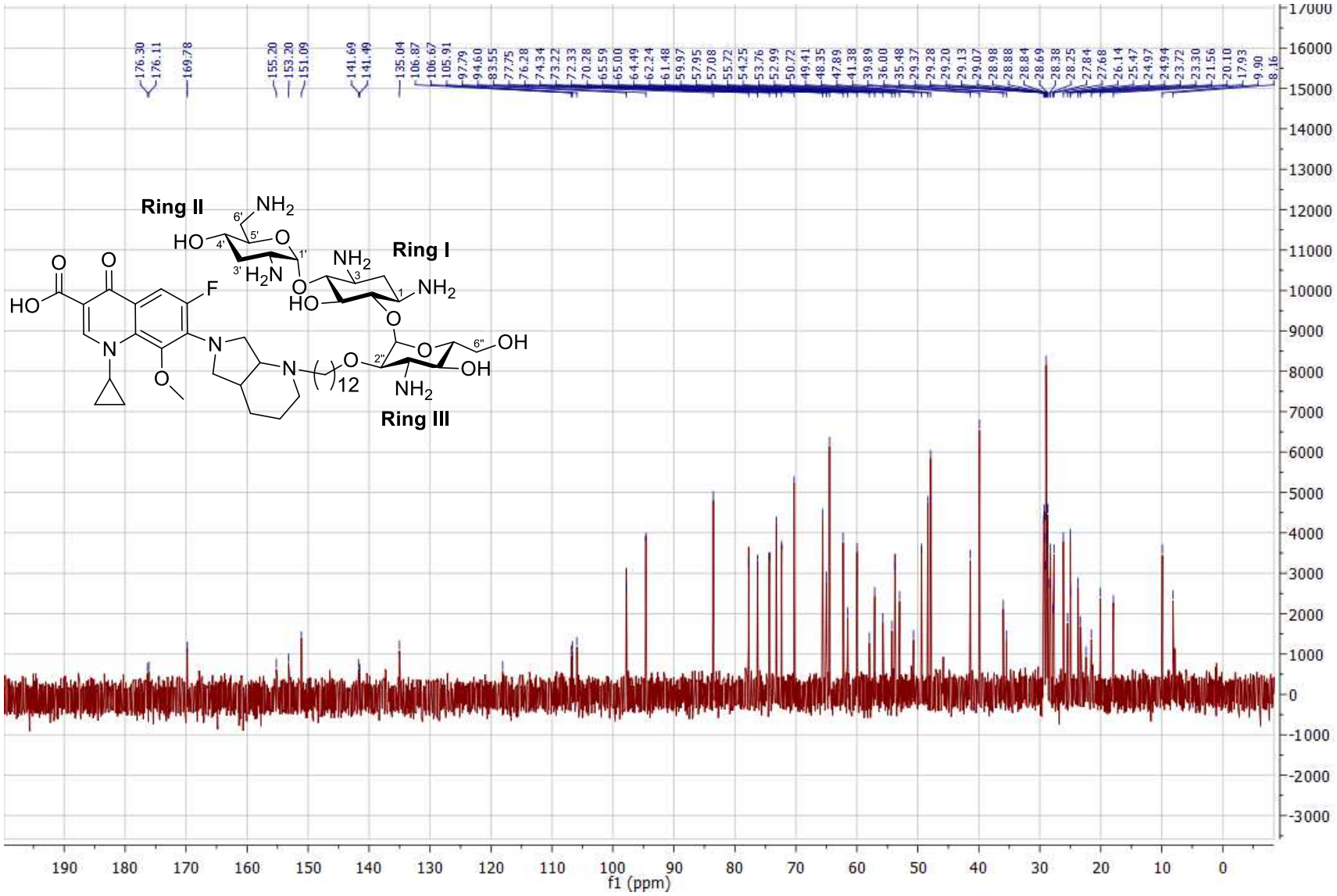

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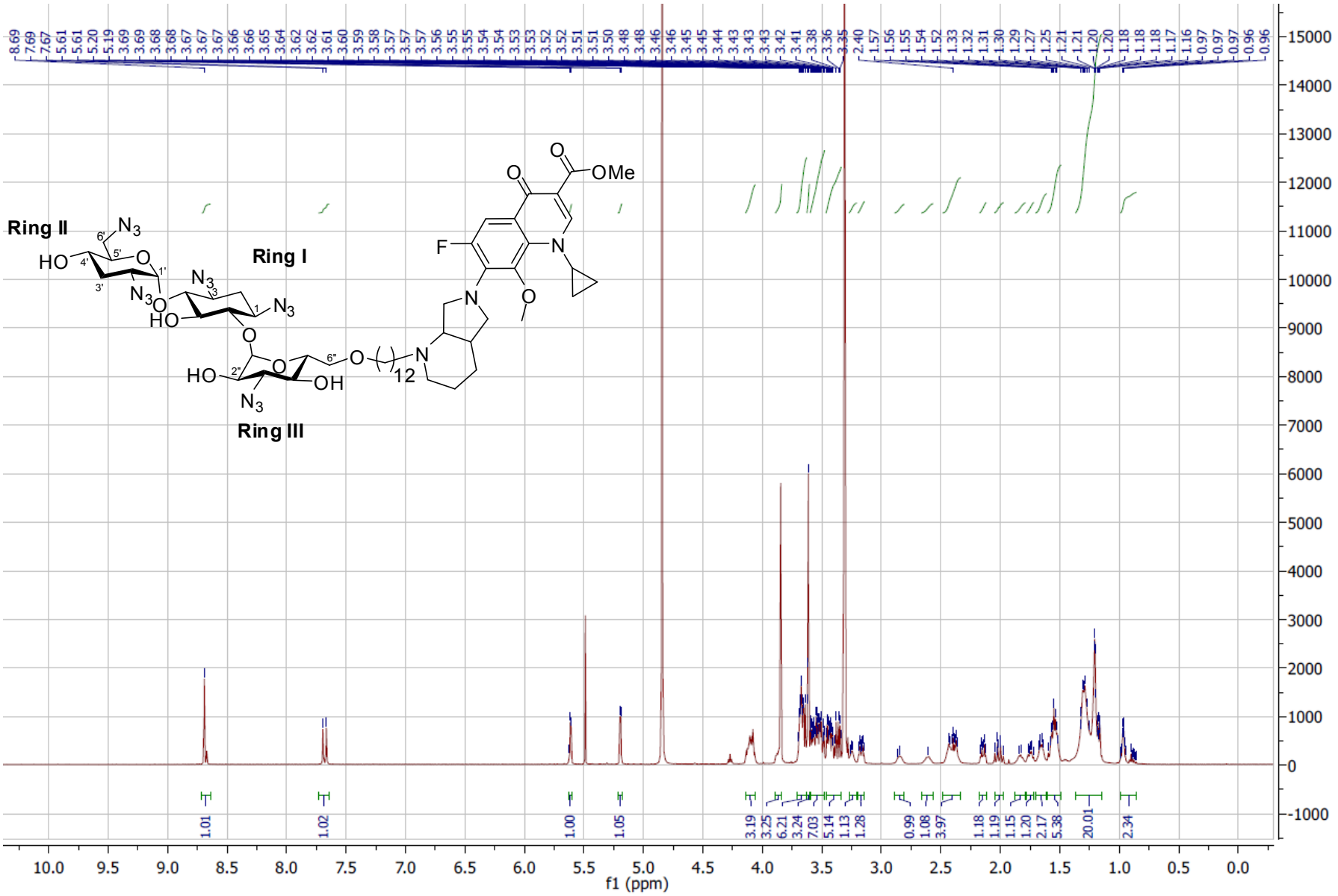
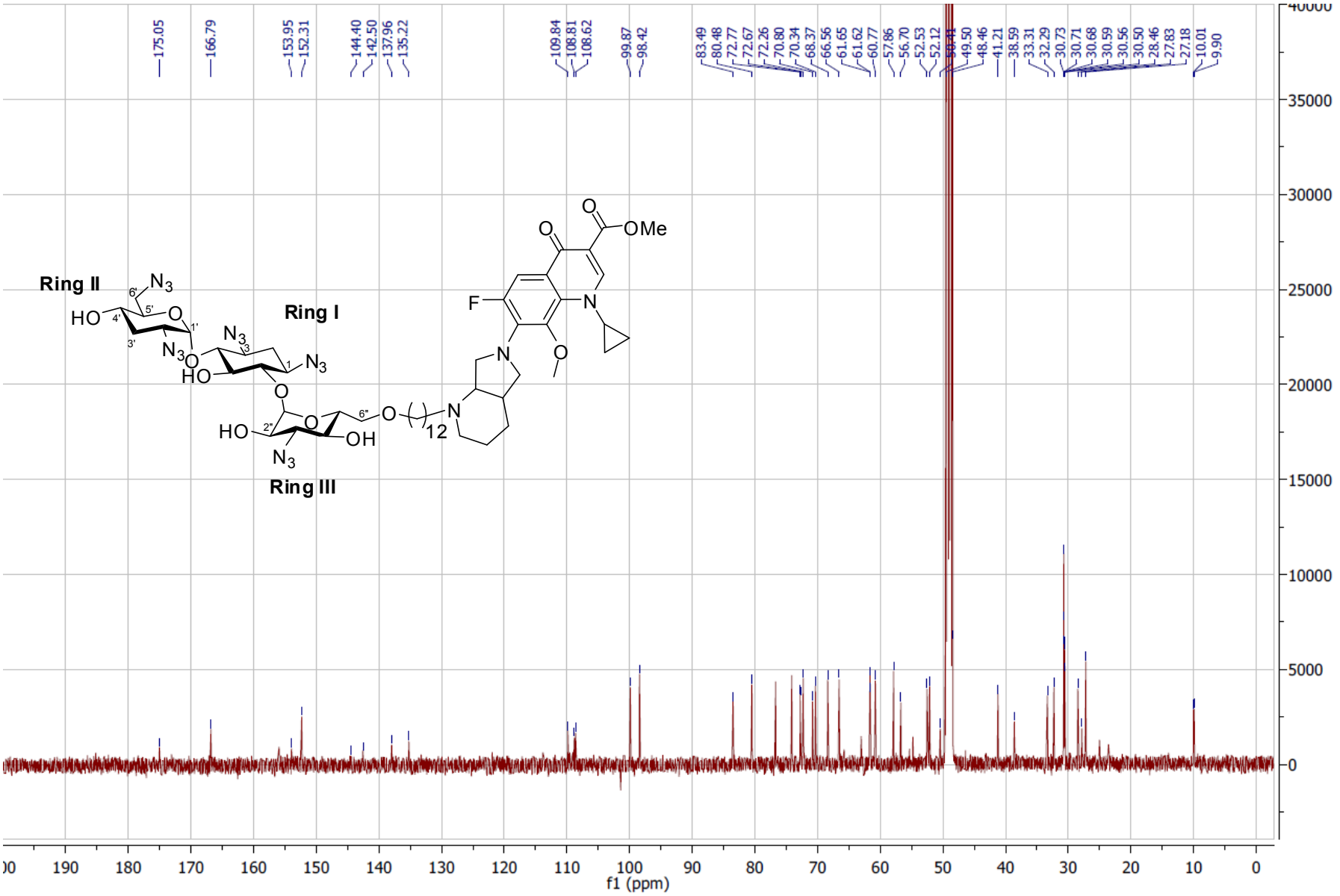


^{1}H spectrum of compound **18** in CD_3OD



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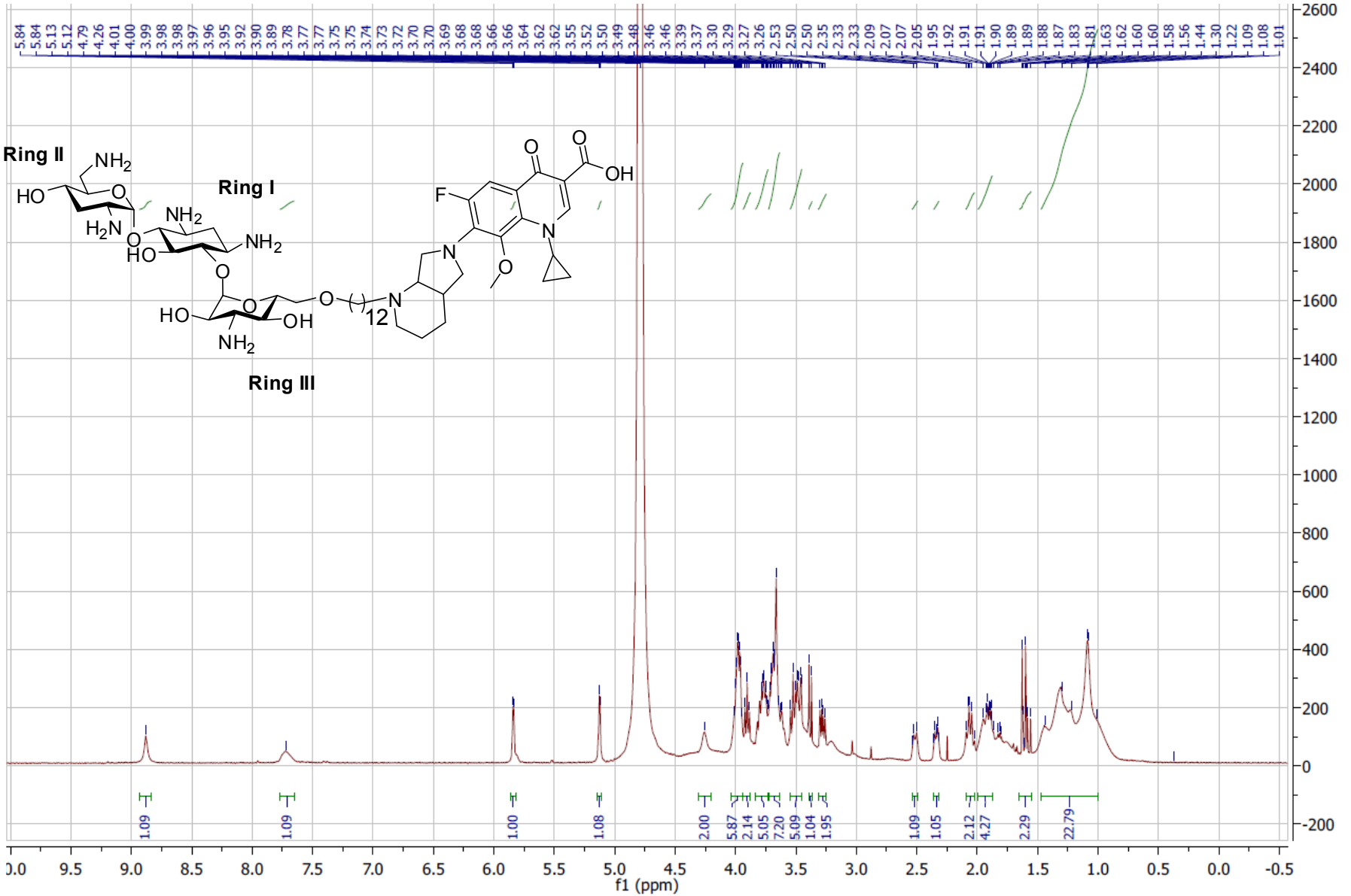

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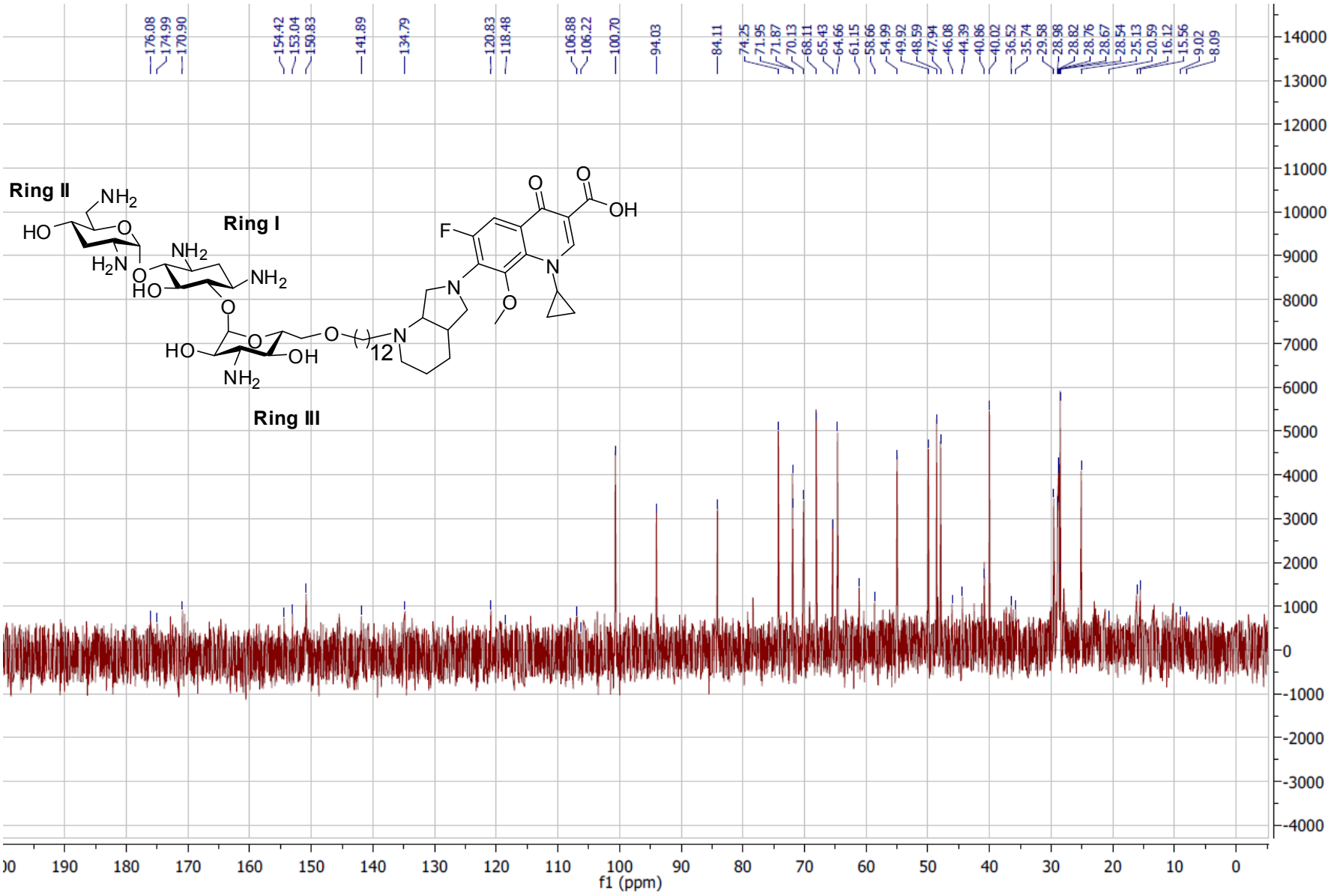
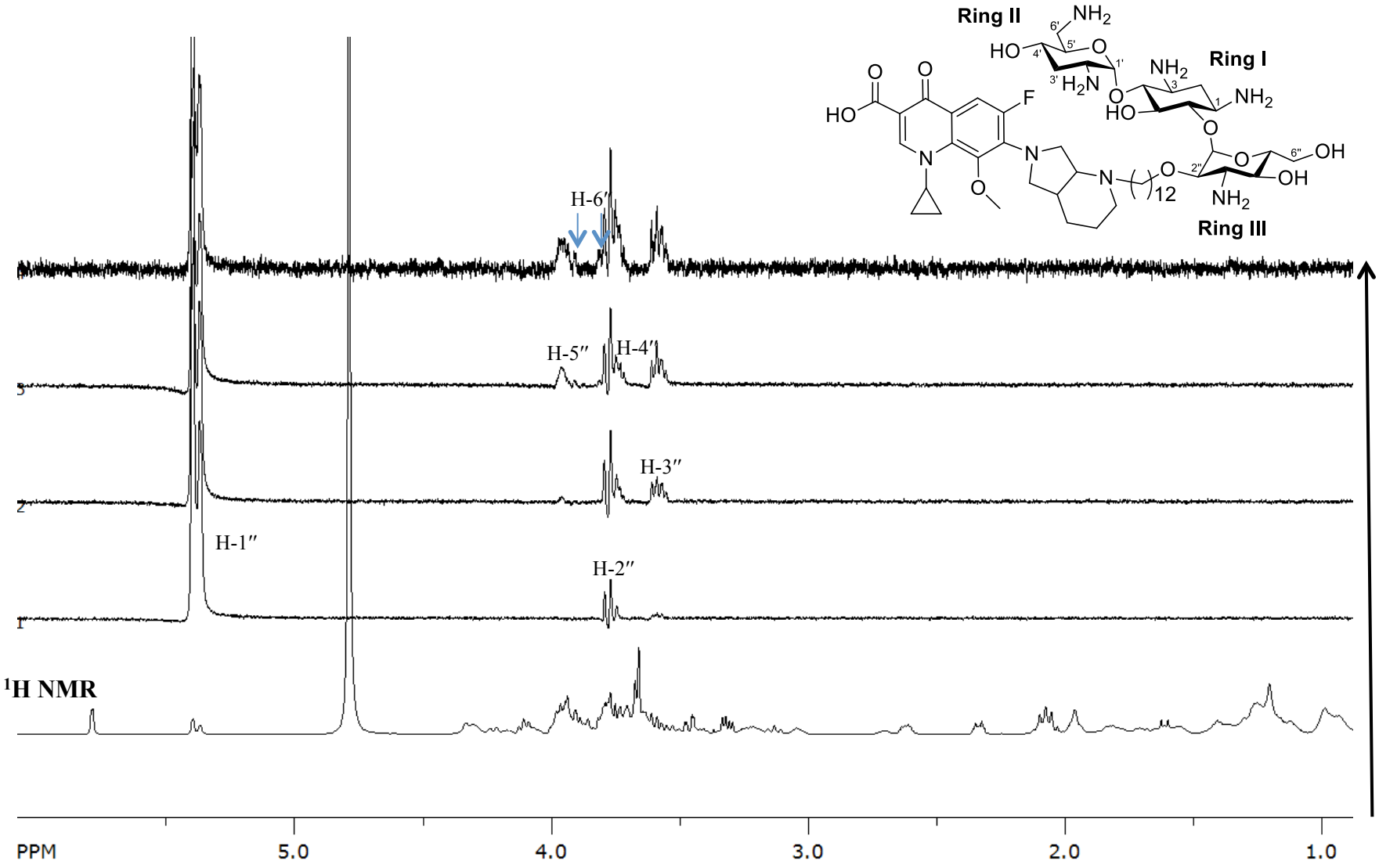
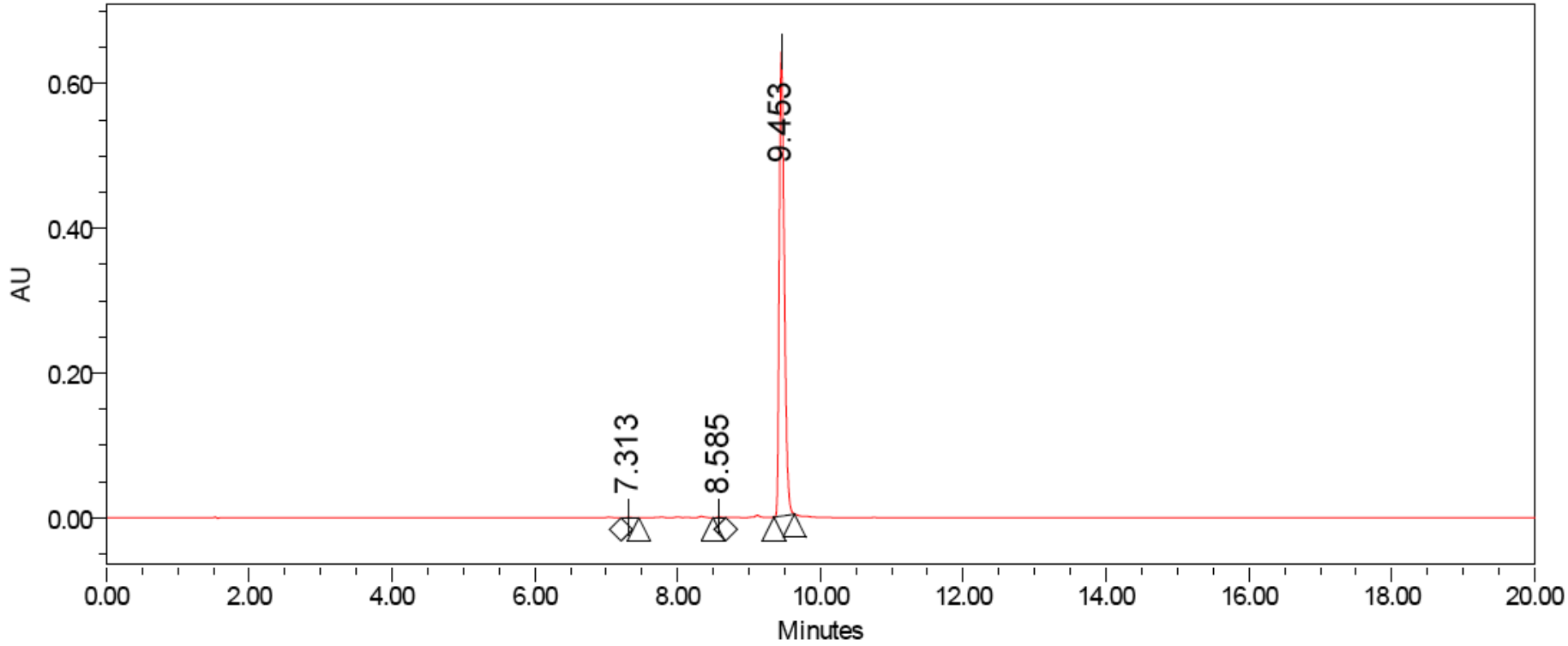


^{13}C spectrum of compound **3** in D_2O



1D-TOCSY and 1H-NMR-based structural analysis **Ring III** of compound **2**

HPLC data of Hybrid **1**



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Processed Channel: PDA 296.0 nm

	Processed Channel	Retention Time (min)	Area	% Area	Height
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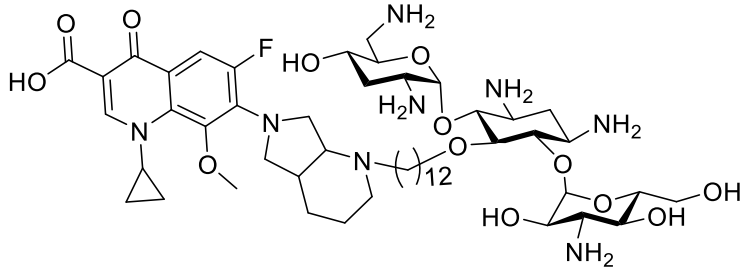
3	PDA 296.0 nm	9.453	3131854	99.74	642119



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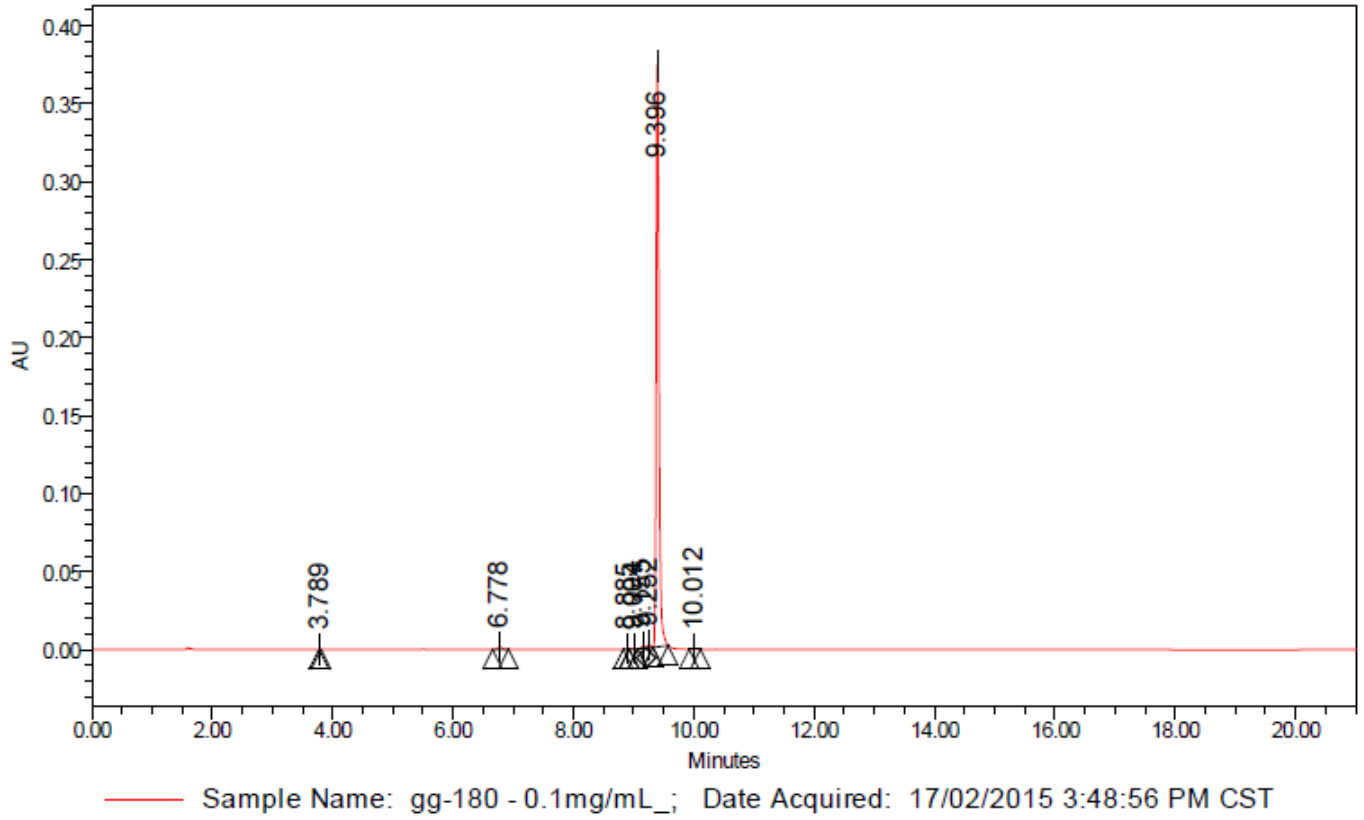

Processed Channel: 2998 Ch4 296nm@1.2nm

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2	2998 Ch4 296nm@1.2nm	6.778	7651	0.62	1480
3	2998 Ch4 296nm@1.2nm	8.885	170	0.01	58
4	2998 Ch4 296nm@1.2nm	9.004	903	0.07	264
5	2998 Ch4 296nm@1.2nm	9.155	4013	0.32	1175
6	2998 Ch4 296nm@1.2nm	9.252	6292	0.51	1897
7	2998 Ch4 296nm@1.2nm	9.396	1222058	98.38	373403
8	2998 Ch4 296nm@1.2nm	10.012	1121	0.09	234