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Diaza-anthracene Antibiotics from a Freshwater-Derived Actinomycete with Selective Antibacterial Activity toward *Mycobacterium tuberculosis*

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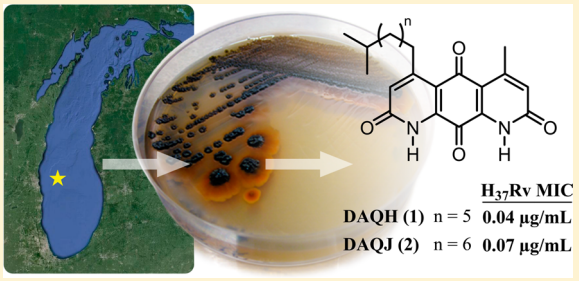
Supporting Information

ABSTRACT: Multidrug- and extensively drug-resistant strains of *Mycobacterium tuberculosis* are resistant to first- and second-line drug regimens and resulted in 210,000 fatalities in 2013. In the current study, we screened a library of aquatic bacterial natural product fractions for their ability to inhibit this pathogen. A fraction from a Lake Michigan bacterium exhibited significant inhibitory activity, from which we characterized novel diazaquinomycins H and J. This antibiotic class displayed an in vitro activity profile similar or superior to clinically used anti-tuberculosis agents and maintained this potency against a panel of drug-resistant *M. tuberculosis* strains. Importantly, these are among the only freshwater-derived actinomycete bacterial metabolites described to date. Further in vitro profiling against a broad panel of bacteria indicated that this antibiotic class selectively targets *M. tuberculosis*. Additionally, in the case of this pathogen we present evidence counter to previous reports that claim the diazaquinomycins target thymidylate synthase in Gram-positive bacteria. Thus, we establish freshwater environments as potential sources for novel antibiotic leads and present the diazaquinomycins as potent and selective inhibitors of *M. tuberculosis*.



KEYWORDS: *natural products, Great Lakes, actinobacteria, antibiotic, diazaquinomycin, drug discovery*

In 2013 the World Health Organization estimated that there were 1.5 million deaths as a result of tuberculosis (TB) infection, with 9.0 million new cases reported.[1] The most significant threat to our population is multidrug- and extensively drug-resistant strains of *Mycobacterium tuberculosis* (MDR- and XDR-TB), which are resistant to first- and second-line drug regimens and resulted in 210,000 fatalities in 2013 (of 480,000 reported infections).[1−3] A major deficiency of current TB treatment is its long duration, which is necessary to eliminate a persistent subpopulation of slow-growing or nonreplicating cells. Importantly, TB is a disease that affects predominantly underprivileged populations in the developing world, and the emergence of drug-resistant forms coupled with the ease with which TB spreads between humans has solidified it as a pathogen of global concern.[4]

Natural products have been essential components of antibacterial drug discovery; they serve both as a direct source of small molecule therapies and as an inspiration for biologically active synthetic derivatives.[5] In particular, actinomycete bacteria have been an abundant source of these bioactive secondary metabolites, providing us with greater than half of the

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antibiotics on the market today.[5,6] However, in previous decades extensive antibiotic screening efforts have exhausted the repertoire of unique terrestrial actinobacteria, resulting in the continuous re-isolation of known antibiotic scaffolds. As a result, researchers prospected new sources for drug lead discovery, such as the ocean. Libraries of organisms (including bacteria) and their resulting secondary metabolites were created that *were not* incorporated in the biological screening efforts of previous decades.[6,7] This investment had a considerable effect toward progress in natural product drug discovery, affording the development of several drugs from marine sources (Prialt, Yondelis, Halaven).[8−10]

The next logical step is to expand this paradigm to freshwater environments, which harbor distinct environmental selection pressures and growth conditions and to date are virtually unexplored for their capacity to afford unique actinomycete bacteria. Furthermore, despite several cultivation-independent studies aimed at characterizing lake actinomycete populations,[11] to the best of our knowledge few efforts (including one study from our laboratory) have identified secondary metabolites from freshwater-derived actinomycetes, and included in this gap is a notable absence of studies aimed specifically at generating anti-TB drug leads.[12−14] Thus, a major focus of our antibiotic discovery program is to study actinobacteria derived from the Great Lakes and other freshwater bodies. We have created an extensive library of these bacteria and their resulting secondary metabolite fractions. A preliminary in vitro growth inhibition screening of this fraction library against *M. tuberculosis* $H_{37}Rv$ led to the identification of a *Micromonospora* sp. isolated from Lake Michigan sediment, whose fraction exhibited submicromolar inhibitory activity. From this strain we isolated and characterized two novel secondary metabolites, diazaquinomycins H and J (DAQH and DAQJ), which to our knowledge are among the only freshwater-derived actinomycete metabolites described to date.[12,13] Further in vitro profiling suggested that this group of diaza-anthracene antibiotics selectively targets *M. tuberculosis* over other bacteria and is active against several forms of drug-resistant TB. Herein we present the identification and in vitro biological characterization of this unique antibiotic class.

RESULTS AND DISCUSSION

Isolation and Identification of DAQH (1) and DAQJ (2). Screening of our actinomycete secondary metabolite library against *M. tuberculosis* in the microplate Alamar Blue assay (MABA) and low-oxygen-recovery assay (LORA) led to the selection of Lake Michigan-derived strain B026 for further chemical investigation. A 28 L fermentation of B026 was performed, and following the extraction of secondary metabolites from the fermentation broth and several chromatographic steps using bioassay-guided fractionation, 0.3 mg each of **1** and **2** was purified using RP-C18 semipreparative HPLC (2.4 mL min^{-1}, gradient of 50% aqueous ACN to 100% ACN for 25 min, followed by an isocratic flow of 100% ACN for 15 min; t_R 18.6 and 22.0 min, respectively).

Detailed structure elucidation analysis for compounds **1** and **2**, including full ^1H and ^{13}C NMR assignments in addition to 2D NMR data and MS experiments, is located in the Supporting Information. Combined NMR and high-resolution IT-TOF mass spectrometry (MS) experiments of **1** established the molecular formula as $C_{22}H_{26}N_2O_4$, whereas the UV spectrum displayed a characteristic absorption profile of molecules from the diazaquinomycin class, thus helping to confirm the fused diaza-anthracene core skeleton. ^1H NMR resonances for aromatic (H3, H6) and aliphatic (H11, H12) hydrogens and their associated correlations from a heteronuclear multiple-bond correlation (HMBC) NMR experiment suggested that each lactam ring contained α-unsaturated, β-alkylated carbonyl moieties (Table S1). ^1H NMR data suggested there was a clear asymmetry to the ring system, and further exploration of the HMBC and MS data confirmed the presence of methyl and isononyl β-substituents on either lactam ring. The connectivity of each set of hydrogens in the isononyl group was determined using correlation NMR spectroscopy (COSY) and a series of one-dimensional selective total correlation spectroscopy (TOCSY) experiments (Figure 2). Therefore, the structure of **1** is as shown (Figure 1).

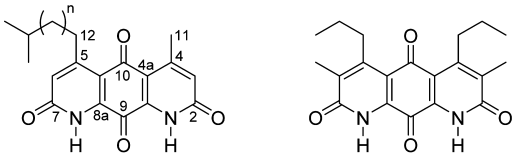

Figure 1. Structures of diazaquinomycins H (**1**), J (**2**), and A (**3**).

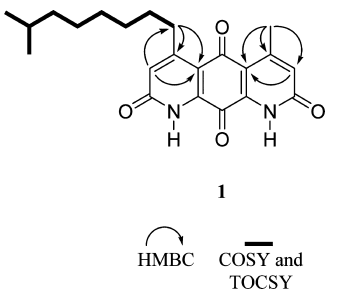

Figure 2. Key 2D NMR correlations of **1**.

Structure elucidation of DAQJ was executed in the same fashion as DAQH, using a similar series of MS and one- and two-dimensional NMR experiments to identify the molecule. A 14 Da increase in molecular weight and an extra resonance in the ^1H and ^{13}C spectra suggested an additional methylene was present in the aliphatic side chain.

In Vitro Evaluation of 1−3 in *M. tuberculosis* Whole Cell Assays. Once compounds **1** and **2** were identified, we tested their ability to inhibit replicating *M. tuberculosis* in vitro using the MABA. The MABA is a phenotypic whole cell-based microplate dilution assay that employs a fluorometric readout, relying on the correlation of resazurin dye reduction to bacterial proliferation.[15,16] Compounds **1** and **2** exhibited minimum inhibitory concentrations (MICs; defined as the lowest concentration resulting in ≥90% growth inhibition of $H_{37}Rv$ and averaged from triplicates) of 0.04 and 0.07 μg/mL, respectively. A luminescence reporter gene assay (LuxABCDE driven by hsp60) was used to confirm that the activities of **1** and **2** were not readout dependent. Further biological characterization of **1** and **2** was difficult, because a 28 L fermentation afforded only 0.3 mg of each. Fortunately, in a previous study, we isolated and identified four analogues of the diazaquinomycin antibiotic class from a marine-derived *Streptomyces* sp.[17] DAQs F and G were a coeluting isomeric mixture, but DAQA (**3**) and DAQE were purified and screened

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Table 1. In Vitro Activity of 3 against a Drug-Resistant *M. tuberculosis* Panel

		MIC (μg/mL)					
		3	RMP[a]	INH[b]	SM[c]	KM[d]	PA824[e]
M. tuberculosis $H_{37}Rv$	MABA	0.10	<0.08	0.05	0.12	0.65	0.01
	LORA	0.72	0.85	>13.7	1.10		
Drug-resistant *M. tuberculosis* strains	rRMP	0.27	>2.00	0.02	0.27	0.92	0.03
	rINH	0.13	0.01	>2.00	0.46	0.95	0.03
	rSM	0.17	0.03	0.02	>2.00	0.95	0.11
	rKM	0.06	<0.01	0.03	1.20	>2.00	0.19
	rCS	0.14	<0.01	0.01	0.41	0.98	0.12

[a]RMP, rifampicin. [b]INH, isoniazid. [c]SM, streptomycin. [d]KM, kanamycin. [e]PA824, experimental nitroimidazole antibiotic. rRMP (ATCC 35838); rINH (ATCC 35822); rSM (ATCC 35820); rCS (cycloserine; ATCC35826); rKM (ATCC 35827).

in the MABA, exhibiting MICs of 0.1 and 0.04 μg/mL, respectively. From this strain, compound **3** was produced in relatively large amounts; thus, all further biological experiments were carried out using this molecule. Moving forward, the solubility of DAQA was an important consideration in all biological testing. DAQA exhibited a maximum room temperature concentration of 0.15 μg/mL in water in a previous study and 600 μg/mL in DMSO in the current study.[18]

We also tested **3** for its ability to inhibit nonreplicating *M. tuberculosis* in the LORA.[19] Low-oxygen adapted *M. tuberculosis* carrying the luxABCDE plasmid was exposed for 10 days to serially diluted **3** in 96-well microplates in a low-oxygen environment created with an Anoxomat commercial system. After 28 h of normoxic "recovery," activity was assessed using the ability to block recovery of the production of a luminescent signal. The MIC value of **3** was 0.72 μg/mL.

As a confirmatory approach for its anti-TB activity, the minimum bactericidal concentration (MBC_{99}; defined as the lowest concentration that reduces cfu by 99% relative to the zero time inoculum) was determined for **3** by subculture onto 7H11 agar just prior to addition of the Alamar Blue and Tween 80 for MABA MBC_{99} and reading of luminescence on day 10 from a lux reporter strain for LORA MBC_{99}. Compound **3** exhibited an MBC_{99} of 0.37 μg/mL under normoxic conditions, but did not exhibit a significant MBC_{99} under hypoxic conditions.

Cytotoxicity Evaluation of 1−3. To assess the cytotoxicity of the DAQ class, compounds **1**−**3** were tested in vitro against Vero cells (ATCC CRL-1586). They did not exhibit cytotoxicity at 28 μM, the highest testing concentration. Insufficient yields of **1** and **2** prevented further cytotoxicity screening of these compounds; however, compound **3** exhibited a range of LC_{50} values when screened against a panel of human cancerous [MDA-MB-435 (0.09 μM), MDA-MB-231 (3.6 μM), HT-29 (5.7 μM), OVCAR3 (0.48 μM), OVACR4 (4.3 μM), Kuramochi (9.4 μM)] and noncancerous cell lines [MOSE (22 μM), MOE (>28 μM)]. Further details describing these experiments can be found in the Supporting Information. Compound **3** was also tested in a previous study against ovarian cancer cell line OVCAR5 and exhibited an LC_{50} value of 8.8 μM.[17] After further investigation, we determined that the moderate cytotoxicity in this cell line was due to DNA damage via the induction of apoptosis.[17] A previous paper indicated that compound **3** and DAQC exhibited no significant inhibition when screened for antifungal activity against *Mucor miehei* and *Candida albicans*.[20] We also assessed the ability of **3** to inhibit the growth of *C. albicans* (ATCC90028), but no significant activity was exhibited when tested at the highest concentration of 10 μg/mL.

We performed a tolerance assessment of **3** in vivo. It was previously published that **3** deposited solid residues and exhibited acute toxicity when dosed at 100 mg/kg intraperitoneally,[18,21] whereas in our study the compound exhibited no qualitatively detectable ill effect and was well tolerated when dosed daily by oral gavage at 100 mg/kg for 5 days in uninfected mice. Although it is possible this difference is due to limited oral bioavailability, our Caco-2 data predict otherwise.

Antibiotic Specificity of 3 toward *M. tuberculosis*. To assess the ability of DAQA to overcome antibiotic resistance in TB and to gain potential insight into the mechanism of action for this compound class, we screened **3** against a panel of mono-drug-resistant strains using the MABA (Table 1). Compound **3** maintained potency across the panel, which suggested the absence of cross-resistance with current anti-TB agents.

To assess the specificity of **3** toward *M. tuberculosis*, we screened **3** against a panel of non-tuberculosis mycobacteria using broth microdilution with a spectrophotometric readout at A_{570} (Table 2). Interestingly, **3** exhibited selectivity toward *M. tuberculosis* and the closely related *M. bovis* BCG (MIC = 0.12 μg/mL).

In the first reports of the diazaquinomycin class, screening of **3** and its 9,10-dihydro derivative, DAQB, against a panel of bacteria revealed a relatively weak MIC of 6.25 μg/mL against three *Staphylococcus aureus* strains (FDA209P, ATCC6538P, KB199) and *Streptococcus faecium* IFO3181 while exhibiting a MIC of 1.13 μg/mL against *Micrococcus luteus*.[21,22] In the current study, we determined the MICs of **3** against a diverse panel of Gram-negative and Gram-positive pathogens.[23] Compound **3** did not exhibit significant inhibitory activity toward Gram-negative bacteria and showed weak inhibitory activity against the Gram-positive bacteria tested. The results of this and previous studies indicate that **3** possesses a narrow spectrum of activity and shows greatest inhibition toward the pathogen *M. tuberculosis*.

Caco-2 Permeability and Liver Microsome Stability of 3. Compound **3** was incubated at 1 μM for 30 min with 0.5 mg/mL of human and mouse liver microsomes and resulted in 96 and 98% of the parent compound remaining, respectively. In addition, to predict absorption via oral administration, Caco-2 bidirectional permeability was performed by incubating 5 μM **3** (with vinblastine and propranolol controls) and measuring apical and basolateral concentrations using tandem LC-MS. Compared to control compounds, **3** exhibited moderate absorption showing permeabilities of 44 nm/s with 82%

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Table 2. Antimicrobial Spectrum of 3[a]

		MIC (μg/mL)
mycobacteria	*M. abscessus*	>7.5
	M. chelonae	>7.5
	M. marinum	>7.5
	M. kansasii	>7.5
	M. avium	3.85
	M. smegmatis	4.56
	M. bovis	0.12
G+[e]	MSSA[b]	4
	MRSA[c]	16
	E. faecalis	8
	E. faecium	8
	VRE[d]	32
	S. pyogenes	>25
	S. pneumoniae	>10
	B. thuringiensis	25
	B. cereus 14579	25
	B. cereus 10987	12.5
	B. anthracis	6.25
	B. subtilis	100
G−[f]	*A. baumannii*	128
	E. coli	128
	E. cloacae	64
	K. pneumoniae	128
	P. aeruginosa	128
	P. mirabilis	64

[a]ATCC designations are listed in the Supporting Information. [b]Methicillin-sensitive *S. aureus*. [c]Methicillin-resistant *S. aureus*. [d]Vancomycin-resistant *E. faecium*. [e]Gram-positive bacteria. [f]Gram-negative bacteria.

recovery (from apical to basolateral) and 9 nm/s with 98% recovery (from basolateral to apical) and no efflux substrate potential (0.2 efflux ratio).

Studies toward Mechanism of Action of 3. Given its mild reported antibiotic activity, observation of potency against *M. tuberculosis* provided sufficient motivation for us to further explore its biological mechanism of action. It was reported that the antibacterial activity of **3** was reversed when folate, dihydrofolate, leucovorin, and thymidine were added to the growth medium, suggesting that the target was within the folate pathway.[24] Shortly after its discovery, previous studies claimed that **3** inhibited thymidylate synthase competitively with its coenzyme 5,10-methylenetetrahydrofolate in both *E. faecium* and Ehrlich ascites carcinoma, with K_i values of 36 and 14 μM, respectively.[25] Thymidylate synthase is an attractive target as it plays a crucial role in DNA replication and repair by synthesizing de novo deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP). *M. tuberculosis* encodes for two structurally and mechanistically unrelated thymidylate synthase enzymes (ThyA and ThyX); therefore, we attempted to determine whether either was the target of **3**.[26,27]

Using a cell-free enzyme assay, we found that **3** did not significantly inhibit the activity of purified human ThyA (HsThyA), *M. tuberculosis* ThyA (MtThyA), or MtThyX when compared to the positive control, folate analogue and known ThyA inhibitor 1843U89 (Figures S1 and S2).[28] To rule out the possibility of **3** being metabolized to a biologically active species within the cell, we performed in vitro testing against *M. tuberculosis thyA* and *thyX* overexpression mutants. We did not observe a significant differential in the MIC when **3** was screened against *M. tuberculosis thyA* and *thyX* overexpression mutants under induced (+ anhydrotetracycline) and uninduced (− anhydrotetracycline) conditions, compared to the H_{37}Rv wild-type strain (Figure S3). Additionally, we observed no reversal of the antibiotic properties of **3** when the culture medium was supplemented with thymidine, indicating that there was likely no disruption in the thymidylate synthase related enzymatic processes of the folate pathway.

Despite decades of research into actinomycete small molecules from terrestrial and marine environments, to the best of our knowledge identification of secondary metabolites from freshwater actinobacteria has been virtually absent from the peer-reviewed literature. The unique structures and activity of the diazaquinomycins provide first evidence that the Great Lakes and, more broadly, freshwater environments are a relatively unexplored resource for novel biologically active molecules.

From a *Micromonospora* sp. in Lake Michigan sediment, we isolated novel antibiotic molecules of the diazaquinomycin class. An analogue, compound **3**, displayed an in vitro activity profile similar or superior to those of clinically used TB agents and maintained potent inhibitory activity against a panel of drug-resistant TB strains. This compound displayed a selectivity profile targeted toward *M. tuberculosis*, even within the genus *Mycobacterium*. Since the 1980s, other research groups have reported that members of the diazaquinomycin class exhibited weak antibacterial activity by targeting thymidylate synthase, although no reports of their anti-TB activity existed and our studies have suggested an alternate mechanism of action in *M. tuberculosis*. Preliminary in vitro analysis of **3** predicts that DAQA will exhibit high metabolic stability and, therefore, a long predicted serum half-life in vivo. Further studies are underway in our laboratories including mutant generation experiments, screening of **3** against a large overexpression library of essential *M. tuberculosis* genes, and determination of the pharmacokinetic properties of **3** in mice. These studies aim to further assess the potential of **3** as a TB drug lead and identify the molecular target of the diazaquinomycins in *M. tuberculosis*.

METHODS

Collection and Identification of Actinomycete Strain B026. Strain B026 was isolated from a sediment sample collected by PONAR at a depth of 56 m, from ca. 16.5 miles off the coast north of Milwaukee, WI, USA, in Lake Michigan (43°13′27″ N, 87°34′12″ W) on August 23, 2010. Strain B026 (GenBank accession no. KP009553) shared 100% 16S rRNA gene sequence identity with the type strain *Micromonospora maritima* strain D10-9-5 (GenBank accession no. NR109311).[29]

Isolation and Characterization of DAQH (1) and DAQJ (2) from Strain B026 Fermentation Broth. Strain B026 was grown in 28 L for 5 days at 21 °C while shaking at 220 rpm. The extracellular secondary metabolites were absorbed from the fermentation broth using Amberlite XAD-16 resin, followed by extraction with acetone and partitioning between water and ethyl acetate. The organic layer was dried under vacuum to afford 1.1 g of extract.

Using a series of bioassay-guided steps involving preparative and semipreparative normal phase (NP) and reversed phase

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(RP) high-performance liquid chromatography (HPLC), the organic layer from the liquid−liquid partition was fractionated and purified to afford diazaquinomycin H (**1**, 0.3 mg, 0.00026% yield) and diazaquinomycin J (**2**, 0.3 mg, 0.00026% yield). See the Supporting Information for more detailed descriptions of fermentation and extraction methods for B026 and subsequent isolation and purification methods for **1** and **2**.

*Diazaquinomycin H (**1**):* red solid (0.3 mg); UV (MeOH) λ_{max} (log ε) = 278 (3.83), 301 (3.72), 357 (3.26), and a broad absorption with maximum at 472 (2.59) nm; ^{1}H NMR (900 MHz, $CDCl_3$−1% CF_3CO_2D) and ^{13}C NMR (226.2 MHz, $CDCl_3$−1% CF_3CO_2D), see Table S1; HRESI-ITTOF MS *m*/*z* 383.1993 $[M + H]^+$ (calcd for $C_{22}H_{27}N_2O_4$, 383.1971), *m*/*z* 381.1771 $[M - H]^-$ (calcd for $C_{22}H_{25}N_2O_4$, 381.1820), *m*/*z* 765.3778 $[2M + H]^+$ (calcd for $C_{44}H_{53}N_4O_8$, 765.3863), and *m*/*z* 763.3639 $[2M - H]^-$ (calcd for $C_{44}H_{51}N_4O_8$, 763.3712).

*Diazaquinomycin J (**2**):* red solid (0.3 mg); UV (MeOH) λ_{max} (log ε) = 278 (3.37), 300 (3.26), 356 (2.90), and a broad absorption with maximum at 472 (2.44) nm; ^{1}H NMR (900 MHz, $CDCl_3$−1% CF_3CO_2D) and ^{13}C NMR (226.2 MHz, $CDCl_3$−1% CF_3CO_2D), see Table S1; HRESI-ITTOF MS *m*/*z* 397.2162 $[M + H]^+$ (calcd for $C_{23}H_{29}N_2O_4$, 397.2127), *m*/*z* 395.1924 $[M - H]^-$ (calcd for $C_{23}H_{27}N_2O_4$, 395.1976), *m*/*z* 793.4129 $[2M + H]^+$ (calcd for $C_{46}H_{57}N_4O_8$, 793.4176), and *m*/*z* 791.3825 $[2M - H]^-$ (calcd for $C_{46}H_{55}N_4O_8$, 791.4025).

In Vivo Tolerance Assessment of 3. Compound **3** was prepared in a dosing vehicle of 10% polyethylene glycol 400 (PEG 400), 10% polyoxyl 35 castor oil (Kolliphor EL, BASF), and 80% oleic acid. The formulation was then administered by oral gavage at 100 mg/kg of **3** to a pair of female BALB/c mice once daily for 5 days followed by observation for qualitative indicators of toxicity (e.g., weight loss, ragged fur, huddling). Approval for animal studies was provided by the Office of Animal Care and Institutional Biosafety (OACIB) Institutional Biosafety Committee (IBC), case 12-183.

Cell-free ThyA and ThyX Enzyme Inhibition Assay. Overexpression and purification of *M. tuberculosis* ThyA and ThyX were performed as detailed in Hunter et al.[28] Human thymidylate synthase A gene (commonly referred to as TYMS) in pET17xb was transformed into BL21(DE3)pLysS. The enzyme was overexpressed in LB media supplemented with 40 μg/mL chloramphenicol and 100 μg/mL ampicillin at 37 °C until OD_{600} 0.6, when the culture was inoculated with 1 mM IPTG and incubated further at 18 °C for 18 h. Cultures were spun down, and the cell-pellet from a 1.5 L culture was lysed in 30 mL of 20 mM potassium phosphate, 500 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM 2-mercaptoethanol at pH 7.9 on ice by sonication (5 cycles, 1 min, 90 W). The crude extract was spun down at 4 °C and 30597 RCF. A total of 27.5 mL of clarified lysate was obtained; 5.4 g of ammonium sulfate (around 35% saturation) was added in three parts and allowed to dissolve at 4 °C. The precipitate obtained was spun-down at 4 °C and 11952 RCF. Thirty milliliters of supernatant was obtained, to which 10.75 g of ammonium sulfate (approximately 80% saturation) was added slowly at 4 °C and allowed to dissolve. The precipitate obtained was again spun-down at 4 °C and 11952 RCF. It was redissolved in 15 mL of 20 mM Tris, 100 mM NaCl, 2 mM 2-mercaptoethanol, and 10% glycerol at pH 7.8, concentrated to 7 mL, and buffer exchanged into 20 mM Tris, 100 mM NaCl, 2 mM 2-mercaptoethanol, and 10% glycerol at pH 7.8. The sample was purified on a 5 mL HiTrap Q column (low-salt buffer, 20 mM Tris, 100 mM NaCl, 2 mM 2-mercaptoethanol, and 10% glycerol at pH 7.8; high-salt buffer, 20 mM Tris, 1 M NaCl, 2 mM 2-mercaptoethanol, and 10% glycerol at pH 7.8). The protein eluted out before 40% high-salt buffer. Further purification was carried out using a Hiload 16/200 Superdex 200 pg size exclusion column using 20 mM Tris, 100 mM NaCl, 2 mM 2-mercaptoethanol, and 10% glycerol at pH 7.8. HsThyA-containing fractions were pooled and buffer exchanged into 50 mM Tris, 150 mM NaCl, 2 mM TCEP, and 30% glycerol at pH 7.8 and stored at −80 °C until further use.

MtThyA (562.8 ng), MtThyX (8.4 μg), and HsThyA (638.4 ng) were pre-incubated with inhibitors (13 μM) for 15 min at room temperature in 125 mM TES, 60 mM $MgCl_2$, 2.5 mM EDTA, and 2 mM 2-mercaptoethanol at pH 7.9. The control contained DMSO without inhibitors. FAD (10.5 μM) was included in the pre-incubation in the case of MtThyX. The total reaction volume was 151 μL for MtThyA and HsThyA and 160 μL for MtThyX.

Radiolabeled dUMP solution was made by mixing 20 μL of 5′-^{3}H-dUMP and 5 μL of 10 mM dUMP in 475 μL of water. After 15 min, mTHF (67 μM for MtThyA, 5.1 μM for MtThyX, and 5.5 μM for HsThyA) and radiolabeled dUMP (5 μL) were added to 18 μL of the enzyme/inhibitor samples. NADPH at 145 μM was also added to the MtThyX samples. All samples were incubated at room temperature for 30 min. Total reaction volume in all cases was 26 μL. Reaction was stopped after 30 min by the addition of 20 μL of stop solution (1.5 N TCA, 1.1 mM nonradioactive dUMP). A volume of 200 μL of 10% charcoal (w/v) was added, and samples were incubated on ice for 15 min before being spun-down at 4 °C and 14100 RCF for 15 min. A 100 μL aliquot of the supernatant was assayed by liquid scintillation counting to determine the amount of tritium-containing water produced by the reaction.

In Vitro Screening of 3 against *M. tuberculosis thyA* and *thyX* Overexpression Mutants. Recombinant strains were constructed in which each gene (*thyA* or *thyX*) was under the control of a tetracycline inducible promoter.[30] Plasmids were transformed into *M. tuberculosis* by electroporation.[31] Recombinant and wild-type strains were grown to late log phase in roller bottles with the presence of inducer (150 ng/mL anhydrotetracycline). The MIC of compound **3** was determined by measuring bacterial growth after 5 days in the presence of the test compound and inducer.[32] Compounds were prepared as a 10-point 2-fold serial dilution in DMSO and diluted into 7H9-Tw-OADC medium in 96-well plates with a final DMSO concentration of 2%. Each plate included assay controls for background (medium/DMSO only, no bacterial cells), zero growth (2% 100 μM rifampicin), and maximum growth (DMSO only), as well as a rifampicin dose response curve. Cultures were filtered through a 5 μm filter and inoculated to a starting OD_{590} of 0.02. Plates were incubated for 5 days, and growth was measured by OD_{590}. The percent growth was calculated and fitted to the Gompertz model.[33] MIC was defined as the minimum concentration at which growth was completely inhibited and was calculated from the inflection point of the fitted curve to the lower asymptote (zero growth).

■ ASSOCIATED CONTENT

Supporting Information

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Complete NMR assignments, 1D and 2D NMR spectra, HRMS data, and UV data for **1** and **2**, in addition to bioassay methods and figures from investigations of the bioactivity and mechanism of action of **3** (PDF)

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Notes

The authors declare no competing financial interest.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on March 11, 2015, with a minor text error. The concentration of the MOSE cell line in the description of cytotoxicity of compound 3 was corrected, and the paper was reposted on March 12, 2015.

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Supporting Information for:

Diaza-anthracene antibiotics from a freshwater-derived actinomycete with selective antibacterial activity toward *M. tuberculosis.*

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Methods

General Experimental Procedures for the Isolation and Characterization of DAQH (1) and DAQJ (2).

UV spectra were measured on a Shimadzu Pharma Spec UV-1700 spectrophotometer. NMR spectra were obtained on a Bruker 600 MHz DRX NMR spectrometer equipped with an inverse 5 mm TXI cryogenic probe with z-axis pfg and XWINNMR version 3.5 operating software, and a 900 (226.2) MHz Bruker AVANCE NMR spectrometer equipped with an inverse 5 mm TCI cryogenic probe with z-axis pfg and TopSpin version 1.3 operating software at the University of Illinois at Chicago Center for Structural Biology. Chemical shifts (δ) are given in ppm and coupling constants (*J*) are reported in Hz. ^{1}H and ^{13}C NMR resonances of **1** and **2** are reported in Table S1. ^{1}H and ^{13}C NMR chemical shifts were referenced to the $CDCl_3$ with vapor TFA solvent signals (δ_H 7.26 ppm and δ_C 77.0 ppm, respectively). High resolution mass spectra (HRMS) were obtained on a Shimadzu IT-TOF LC-MS spectrometer at the University of Illinois at Chicago Research Resources Center. High-performance liquid chromatography (HPLC-UV) data were obtained using a Hewlett-Packard series 1100 system controller and pumps with a Model G1315A diode array detector (DAD) equipped with a reversed-phase C18 column (Phenomenex Luna, 100 × 4.6 mm, 5 μm) at a flow rate of 0.5 mL • min^{-1}. Semi-preparative scale HPLC separations were performed using a Hewlett Packard Series 1050 system with a Phenomenex Luna semi-preparative C18 column (250 × 10 mm, 5 μm) at a flow rate of 2.4 mL • min^{-1}. Preparative scale HPLC separations were performed using a Waters LC4000 System equipped with a Phenomenex Luna preparative C18 column (250 × 21.2 mm, 5 μm) at a flow rate of 16 mL • min^{-1}. Silica gel column chromatography was conducted using Bonna-Angela Technologies Cleanert® silica gel with an average particle size of 40-60 μm and an average pore size of 60 Å.

Fermentation and Extraction.

Strain B026 was grown in 28 × 1 L portions in Fernbach flasks containing high nutrient A1 medium (0.5 L of filtered Lake Michigan water, 0.5 L DI water, 10 g starch, 4 g yeast extract, 2 g peptone, 1 g calcium carbonate, 100 mg potassium bromide, and 40 mg iron sulfate) for 5 days at 21 °C while shaking at 220 rpm. Sterilized Amberlite XAD-16 resin (15 g·L−1) was added to each flask to absorb the extracellular secondary metabolites. The culture medium and resin were shaken for 8 h and filtered using cheesecloth to remove the resin. The resin, cell mass, and cheesecloth were extracted with acetone overnight, concentrated under vacuum, and partitioned between water and ethyl acetate. The organic layer was dried under vacuum to afford 1.1 g of extract.

Isolation and Characterization of DAQH (1) and DAQJ (2).

The organic layer from the liquid-liquid partition was fractionated using silica gel flash column chromatography (100 g of silica) eluting with an isocratic 95% chloroform ($CHCl_3$):5% methanol (MeOH) solvent system to afford 15 fractions. Using bioassay-guided fractionation, it was determined that fraction 2 contained the bioactive constituents, thus it was separated using RP-C18 preparative HPLC (16 mL • min^{-1}, gradient of 50% aqueous acetonitrile (ACN) to 100% ACN for 20 min, followed by an isocratic flow of 100% ACN for 10 min) to afford five fractions. Compounds **1** and **2** were observed in fractions 3 and 4, respectively.

To further isolate **1**, fraction 3 was separated using RP-C18 semi-preparative HPLC (2.4 mL • min^{-1}, gradient of 50% aqueous ACN to 100% ACN for 25 min, followed by an isocratic flow of 100% ACN for 15 min) to afford 4 fractions. Fraction 4 was determined to contain **1** and was further purified using RP-C18 semi-preparative HPLC (2.4 mL • min^{-1}, gradient of 50% aqueous ACN to 100% ACN for 25 min, followed by an isocratic flow of 100% ACN for 15 min) to afford diazaquinomycin H (**1**, t_R 18.6 min, 0.3 mg, 0.00026% yield).

To further isolate **2**, fraction 4 was separated further using RP-C18 semi-preparative HPLC (2.4 mL • min^{-1}, gradient of 50% aqueous ACN to 100% ACN for 25 min, followed by an isocratic flow of 100% ACN for 15 min) to afford 5 fractions. Fraction 3 was separated further using RP-C18 semi-preparative HPLC (2.4 mL • min^{-1}, gradient of 50% aqueous ACN to 100% ACN for 25 min, followed by an isocratic flow of 100% ACN for 15 min) to afford 5 fractions. Fraction 4 was determined to contain **2** and was further purified using RP-C18 semi-preparative HPLC (2.4 mL • min^{-1}, gradient of 50% aqueous ACN to 100% ACN for 25 min, followed by an isocratic flow of 100% ACN for 15 min) to afford diazaquinomycin J (**2**, t_R 22.0 min, 0.3 mg, 0.00026% yield).

***M. tuberculosis* Fermentation for Determination of Minimum Inhibitory Concentration (MIC).**

M. tuberculosis H_{37}Rv ATCC 27294 was purchased from American Type Culture Collection (ATCC) and cultured to late log phase in the 7H12 media, Middlebrook 7H9 broth supplemented with 0.2% (vol/vol) glycerol, 0.05% Tween80, and 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC). The culture was harvested and resuspended in phosphate-buffered saline. Suspensions were then filtered through 8 μm filter membranes and frozen at 80 °C. Prior to use of bacterial stocks for the anti-TB assay, CFUs were determined by plating on 7H11 agar media. The MIC is defined here as the lowest concentration resulting in ≥90% growth inhibition of the bacteria relative to untreated controls. MIC against replicating *M. tuberculosis* was measured by the Microplate Alamar Blue Assay (MABA).[1-2]

Low Oxygen Recovery Assay (LORA).

The luciferase reporter gene luxABCDE-recombinant *M. tuberculosis* was prepared as previously reported.[3] The bacteria were adapted to low oxygen conditions during culture in a BioStatQ fermenter. The low oxygen-adapted culture was exposed to test samples in a 96-well microplate for 10 days at 37 °C in a hypoxic environment created with an Anoxomat (WS-8080, MART Microbiology). The cultures were then transferred to a normoxic environment at 37 °C for 28 hours. Viability was assessed by the measurement of luciferase-mediated luminescence. The LORA MIC was defined as the lowest concentration effecting a reduction of luminescence of ≥90% relative to untreated cultures.

Determination of Cytotoxicity.

Vero (ATCC CRL-1586) cells were cultivated in 10% fetal bovine serum (FBS) in Eagle minimum essential medium.[4-5] The culture was incubated at 37 °C under 5% CO_2 in air and then diluted with phosphate-buffered saline to 106 cells/mL. Two-fold serial dilutions of testing samples with a final volume of 200 µL cell culture suspension were prepared in a transparent 96-well plate (Falcon Microtest 96). After 72 h incubation at 37 °C, the medium was removed and monolayers were washed twice with 100 µL of warm Hanks balanced salt solution (HBSS). 100 µL of medium and 20 µL of MTS-PMS (Promega) were added to each well. Plates were then incubated for 3 hours and cytotoxicity was determined by the measurement of absorbance at 490 nm.

Human melanoma MDA-MB-435 cancer cells, human breast MDA-MB-231 cancer cells, human ovarian OVCAR3 cancer cells and human colon HT-29 cancer cells were purchased from the American Type Culture Collection (Manassas, VA). The cell line was propagated at 37 °C in 5% CO_2 in RPMI 1640 medium, supplemented with fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells in log phase growth were harvested by trypsinization followed by two washings to remove all traces of enzyme. A total of 5,000 cells were seeded per well of a 96-well clear, flat-bottom plate (Microtest 96®, Falcon) and incubated overnight (37 °C in 5% CO_2). Samples dissolved in DMSO were then diluted and added to the appropriate wells (concentrations: 20 µg/mL, 4 µg/mL, 0.8 µg/mL, 0.16 µg/mL, 0.032 µg/mL; total volume: 100 µL; DMSO: 0.5%). The cells were incubated in the presence of test substance for 72 h at 37 °C and evaluated for viability with a commercial absorbance assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega Corp, Madison, WI) that measured viable cells. LC_{50} values are expressed in µg/mL relative to the solvent (DMSO) control.

Human ovarian OVCAR4 and Kuramochi cancer cells were maintained in RPMI 1640 (11875-093, Life-technologies) supplemented with 10% FBS (16000-044, Life-technologies) and 1% penicillin/streptomycin. Non-cancerous murine ovarian surface

epithelium (MOSE) and murine oviductal epithelium (MOE) cells were maintained as previously reported.[6] Concentration-response experiments were performed as previously described for 72 hours.[7]

MIC Determination against Drug-Resistant *M. tuberculosis* Isolates.

M. tuberculosis strains individually resistant to rifampin (RMP, ATCC 35838), isoniazid (INH, ATCC 35822), streptomycin (SM, ATCC 35820), cycloserine (CS, ATCC 35826) and kanamycin (KM, ATCC 35827) were obtained from the American Type Culture Collection (ATCC). Cultures were prepared and MICs against drug-resistant *M. tuberculosis* isolates were determined by the MABA as described above for *M. tuberculosis* H_{37}Rv.

MIC Determination against Non-Tuberculous Mycobacteria (NTM).

Mycobacterium smegmatis (mc^2155), *Mycobacterium abscessus* (ATCC19977), *Mycobacterium chelonae* (ATCC35752), *Mycobacterium avium* (ATCC15769), *Mycobacterium marinum* (ATCC927), *Mycobacterium kansasii* (ATCC12478), and *Mycobacterium bovis* BCG (ATCC35734) were obtained from the American Type Culture Collection (ATCC). Cultures were prepared and MICs against these mycobacteria were determined by the MABA as described above for *M. tuberculosis* H_{37}Rv. *M. abscessus* was incubated with 7H12 medium at 37 °C for 3 days and for an additional 4 hours after adding 12 μL of 20% Tween80 and 20 μL of Alamar blue dye. *M. bovis* was incubated with 7H12 medium at 37 °C for 7 days, and an additional 1 day of incubation after adding 12 μL of 20% Tween80 and 20 μL of Alamar blue dye. *M. chelonae* was incubated with 7H9 medium at 30 °C for 3 days, plus an additional 6 days of incubation after adding 12 μL of 20% Tween80 and 20 μL of Alamar blue dye. *M. marinum* was incubated with 7H9 medium at 30 °C for 5 days, and an additional 1 day of incubation after adding 12 μL of 20% Tween80 and 20 μL of Alamar blue dye. *M. avium* and *M. kansasii* were incubated with 7H9 media at 37 °C for 6 days, plus an additional day of incubation after adding 12 μL of 20% Tween80 and 20 μL of Alamar blue dye. Viability was assessed by measuring fluorescence at 530 nm excitation/590 nm emission with a Victor^3 multilabel reader (PerkinElmer).

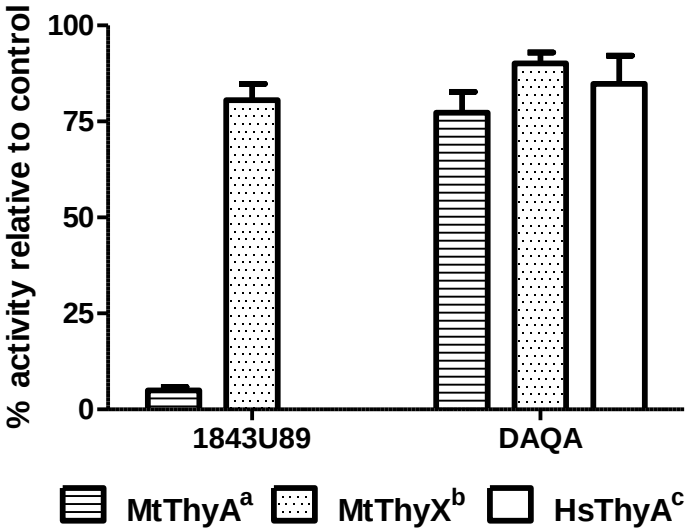
MIC Determination against Gram-Positive and Gram-Negative Bacteria.

The following Gram-positive and Gram-negative reference organisms were obtained from the ATCC: Methicillin-sensitive *Staphylococcus aureus* (ATCC29213), methicillin-resistant *Staphylococcus aureus* (ATCC33591), *Enterococcus faecalis*

(ATCC29212), *Enterococcus faecium* (ATCC19434), vancomycin-resistant *Enterococcus faecium* (ATCC51559), *Streptococcus pneumoniae* (ATCC49619), *Streptococcus pyogenes* Rosenbach (ATCCBAA-1633), *Bacillus cereus* (ATCC14579), *Bacillus cereus* (ATCC10987), *Bacillus thuringiensis* serovar konkukian str. 97-27, *Acinetobacter baumannii* (ATCC19606), *Escherichia coli* (ATCC25922), *Enterobacter cloacae* (ATCC13047), *Klebsiella pneumoniae* (ATCC13883), *Pseudomonas aeruginosa* (ATCC27853), and *Proteus mirabilis* (ATCC29906). *Bacillus subtilis* subsp. subtilis 168 is a laboratory strain and *Bacillus anthracis* Sterne was obtained from Philip C. Hanna at the University of Michigan, Ann Arbor. Positive controls piperacillin, ampicillin, vancomycin and gentamicin were obtained from Sigma-Aldrich, while daptomycin was obtained from Fisher Scientific; each was used to prepare a stock solution of at least 1280 μg/mL on the same day of each experiment. Inocula for MSSA, MRSA, *E. faecalis*, *E. faecium*, VRE, *A. baumannii*, *E. coli*, *E. cloacae*, *K. pneumoniae*, *P. aeruginosa*, and *P. mirabilis* consisted of 5 x 10^5 CFU/mL of each bacterial species with determination of MICs by broth microdilution in duplicate according to Clinical Laboratory Standards Institute (CLSI) guidelines with growth scored by visual observation of turbidity.[8] The MIC against *S. pneumoniae* (ATCC49619) was determined by broth microdilution method using a 4 x 10^5 CFU/mL inoculum as described in the National Committee on Clinical Laboratory Standards[9-10] with viability assessed by spectrophotometric readout at 490 nm using a Victor^3 multilabel reader (PerkinElmer). Inocula for *S. pyogenes* consisted of approximately 5 x 10^6 CFU/mL with determination of MICs by broth microdilution with growth scored by optical density (OD) at 625 nm using a Biotek Synergy 2 Microplate Reader. Each *Bacillus* inoculum consisted of 1 x 10^5 to 2 x 10^5 CFU/mL, with determination of MICs by broth microdilution and growth scored by visual observation of turbidity.

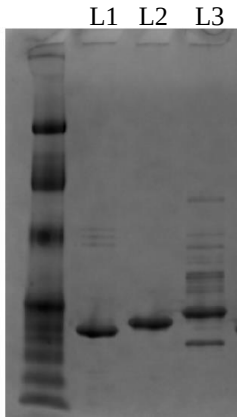
Studies toward Mechanism of Action of DAQA (3)

Figure S1. Activity of MtThyA, MtThyX, and HsThyA Enzymes in the Presence of 9 µM **3** and 9 µM 1843U89 (Positive Control).



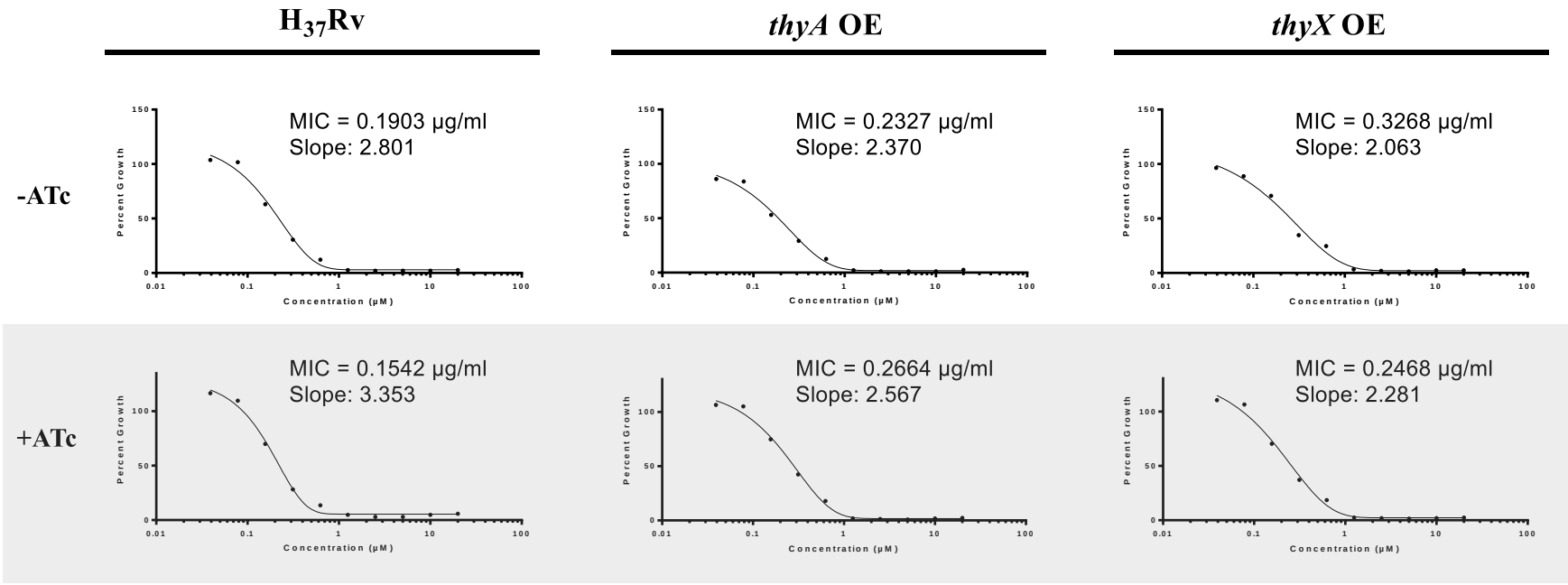
[a] *M. tuberculosis* thymidylate synthase A; [b] *M. tuberculosis* thymidylate synthase X; [c] human thymidylate synthase A

Figure S2. Purity of MtThyA, MtThyX, and HsThyA Enzymes.



Lane 1: MtThyA
Lane 2: MtThyX
Lane 3: HsThyA

Figure S3. Concentration-Response Curves of **3** against *M. tuberculosis* Anhydrotetracycline-Inducible *thyA* and *thyX* Overexpression Mutants.



Structure Elucidation of DAQH (1) and DAQJ (2).

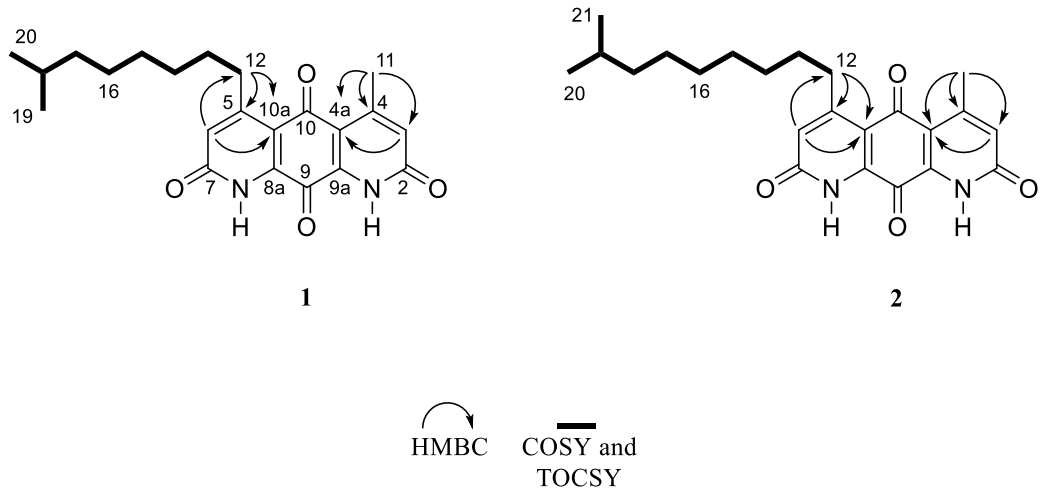
Following a series of chromatographic steps, **1** was obtained as red powder. Combined HRMS and NMR experiments allowed for the assignment of the molecular formula as $C_{22}H_{27}N_2O_4$. The UV absorption profile of **1** displayed characteristics of the diazaquinomycins as previously reported.[7] Combined NMR and high resolution IT-TOF MS experiments of **1** established the molecular formula as $C_{22}H_{27}N_2O_4$, which was indicative of eleven degrees of unsaturation. Analysis revealed that **1** lacked the symmetry of the previously reported DAQA (**3**) or DAQC.[11-13] A resonance at δ_H 7.00 (2H, H-3, H-6) observed in the ^{1}H NMR spectrum, determined by integration to represent two hydrogens, indicated a less substituted anthraquinone compound when compared to known DAQs. A triplet signal at δ_H 3.13 (2H, H-12) indicated one β-substituted methylene group and a singlet at δ_H 2.77 (3H, H-11) revealed one aromate-bound, β-substituted methyl group. A doublet with an integration of six at δ_H 0.87 (6H, H-19, H-20) and a methine multiplet resonance at δ_H 1.53 (1H, H-18) provided evidence of the isononyl group. Multiplet signals at δ_H 1.60 (2H, H-13), δ_H 1.47 (2H, H-14), δ_H 1.35 (2H, H-15), δ_H 1.31 (2H, H-16) and δ_H 1.19 (2H, H-17) were indicative of the methylene groups that constituted the remainder of the aliphatic side chain.

Analysis of ^{13}C NMR data suggested the presence of two quinone carbonyls (δ_C 180.1, C-10; 172.9, C-9), two near overlapping lactam carbonyls (δ_C 163.2 and 163.0; C-2 and C-7), two methine alkene carbons (δ_C 127.6, C-6; 128.6, C-3), six quaternary carbons, two of which were interchangeable (δ_C 160.1, C-5; 155.6, C-4; 136.3 and 136.8, C-8a and C-9a; 118.0, C-4a; 117.5, C-10a), six methylene carbons, three of which were overlapping (δ_C 39.1, C-17; 35.0, C-12; 29.7, C-15; 29.7, C-14; 29.7, C-13; 27.4, C-16), and three methyl carbons (δ_C 23.1, C-11; 22.8, C-19 and C-20) (Table S1). Given that the molecular formula afforded 11 degrees of unsaturation and the molecule contained 4 carbonyls, 6 quaternary alkene carbons, and 2 methine alkene carbons, the remaining degrees were satisfied by the fused ring system. Key HMBC, COSY, and 1D-TOCSY correlations are given in Figure S4. Interpretation of COSY and 1D-TOCSY data defined one distinct aliphatic spin system (H_2-12 to H_3-20). Connectivity of the aliphatic side chain and the methyl group to opposing β-substituted positions of the core ring system was established using HMBC correlations (Figures S4 and S8).

HMBC correlations between H_3-11 and C-3, C-4 and C-4a, as well as correlations between the H_2-12 and C-5, C-6, C-10a gave evidence for the connectivity of the alkyl groups to opposing sides of the anthracene core skeleton of **1**. Two lactam carbonyl resonances, C-2 and C-7, were observed in the ^{13}C DEPTQ spectrum; due to overlap it was not possible to distinguish between them in an HMBC experiment (Table S1). Similarly, two quaternary carbon resonances, C-8a and C-9a, were observed in the ^{13}C DEPTQ spectrum but were indistinguishable by an HMBC experiment.

Following a series of chromatographic steps, **2** was obtained as red powder. Structure elucidation of **2** was executed in the same fashion as **1**, using a similar series of 1D-TOCSY experiments to identify the 14 Da increase in molecular weight as an additional methylene in the aliphatic side chain (Figure S16).

Figure S4. Key 2D NMR Correlations of **1** and **2**.



1 **2**

HMBC COSY and TOCSY

Table S1. ^{1}H and ^{13}C NMR Chemical Shift Data ($CDCl_3$–1% CF_3CO_2D) of **1** and **2**.

Position	**1**		**2**	
	^{13}C [a]	^{1}H mult. (J, Hz) [b]	^{13}C [a]	^{1}H mult. (J, Hz) [b]
1		8.05 s		8.03 s
2	163.2 [d]		161.0	
3	128.6	7.00 s	128.9 [c]	6.93 s
4	155.6		154.6	
4a	118.0		118.3	
5	160.1		160.2 [c]	
6	127.6	7.00 s	128.0 [c]	6.93 s
7	163.0 [d]		160.9 [c]	
8		8.05 s		8.03 s
8a	136.3 [e]		136.2	
9	172.9		173.0	
9a	136.8 [e]		136.8	
10	180.1		180.0	
10a	117.5		117.8	
11	23.1	2.77 s	23.0	2.74 s
12	35.0	3.13 t (7.7)	35.0	3.10 t (7.0)
13	29.7 [f]	1.60 p (7.5)	29.9	1.59 m
14	29.7 [f]	1.47 p (7.4)	29.8	1.45 m
15	29.7 [f]	1.35 m	29.7	1.35 m
16	27.4	1.31 m	29.6	1.31 m
17	39.1	1.19 q (6.9)	27.5	1.28 m
18	28.1	1.53 m	39.1	1.17 m
19	22.8	0.87 d (6.6)	28.1	1.52 m
20	22.8	0.87 d (6.6)	22.8	0.86 d (6.6)
21			22.8	0.86 d (6.6)

[a] 226.2 MHz; [b] 900 MHz; [c] shifts obtained through HMBC and/or HSQC NMR experiments; [d, e, f] shifts indistinguishable by HMBC and HSQC NMR experiments.
s = singlet; t = triplet; q = quartet; p = pentet; m = multiplet.

Figure S5. ^{1}H NMR Spectrum (900 MHz) of **1** in $CDCl_3$–1% CF_3CO_2D.

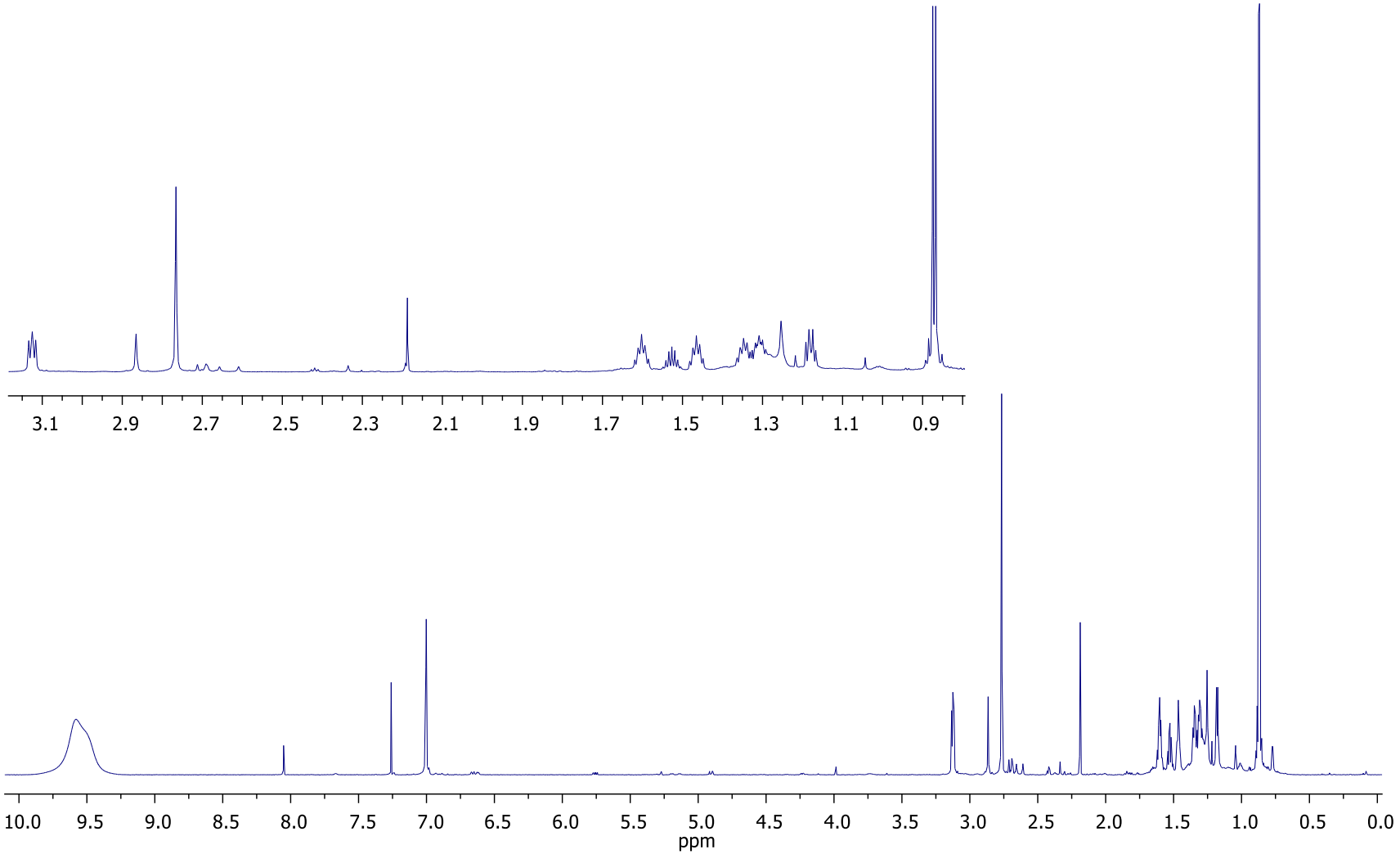


Figure S6. ^{13}C DEPTQ Spectrum (226.2 MHz) of **1** in $CDCl_3$–1% CF_3CO_2D.

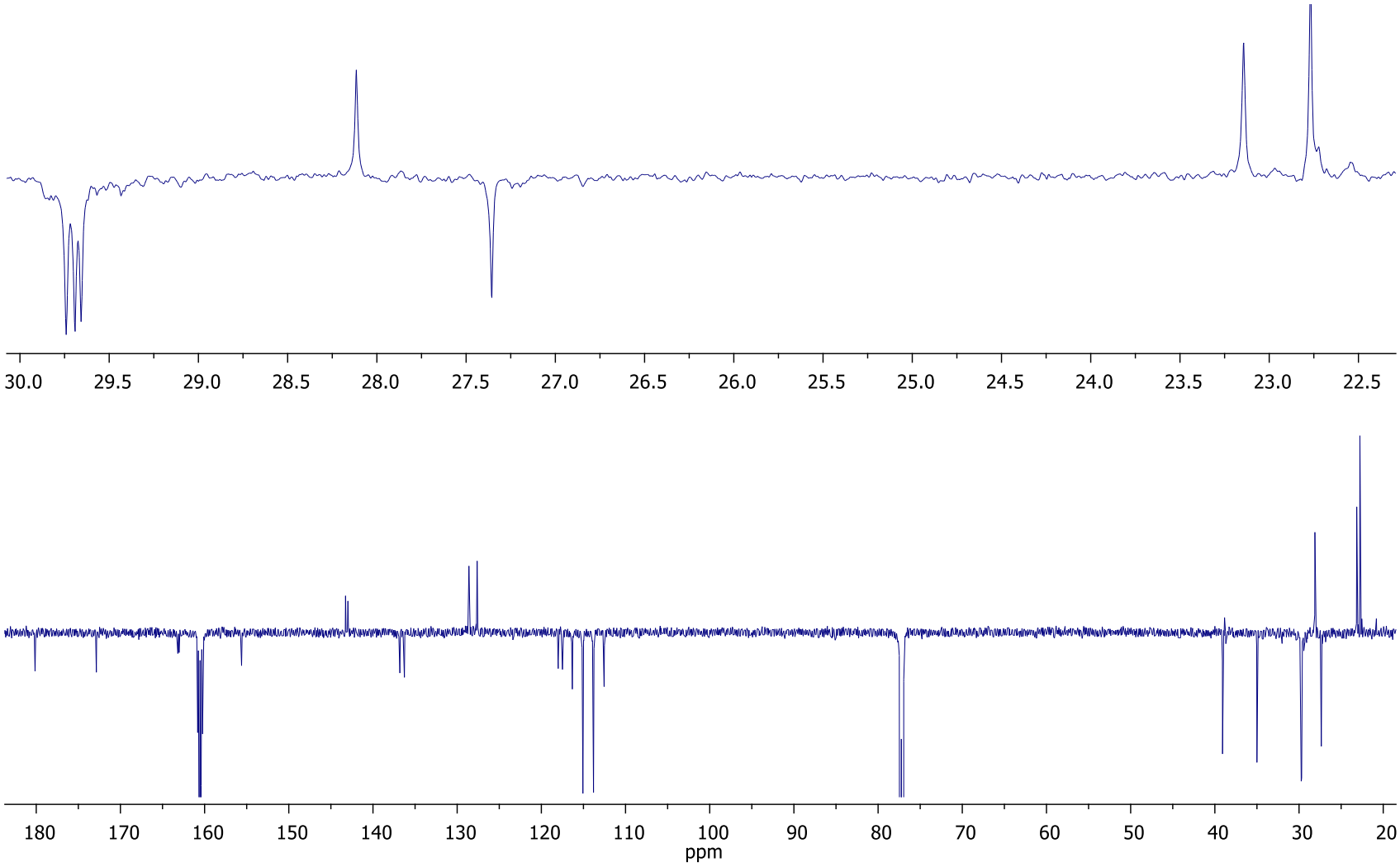


Figure S7. COSY Spectrum (600 MHz) of **1** in $CDCl_3$–1% CF_3CO_2D.

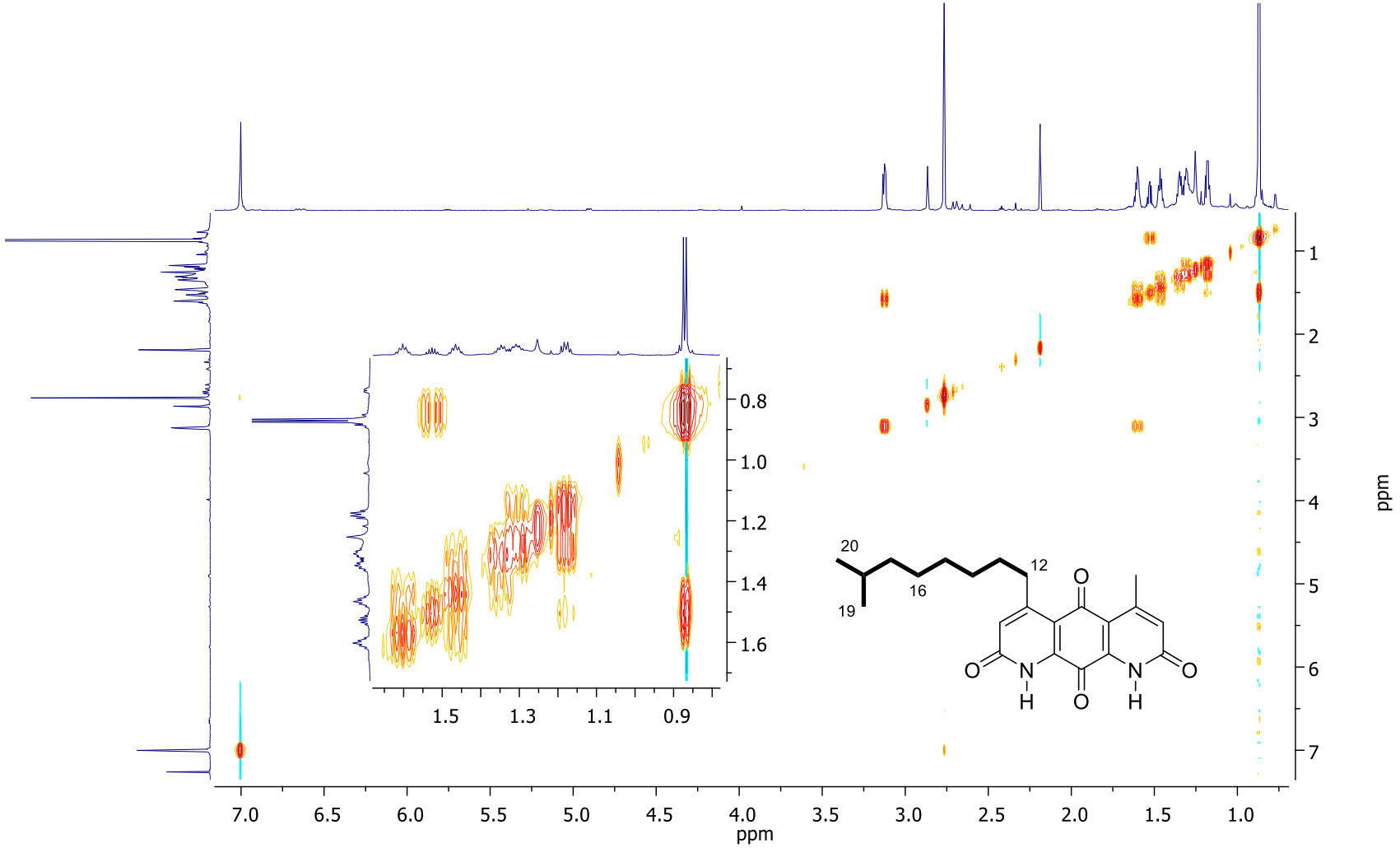


Figure S8. HMBC spectrum (600 MHz) of **1** in $CDCl_3$–1% CF_3CO_2D.

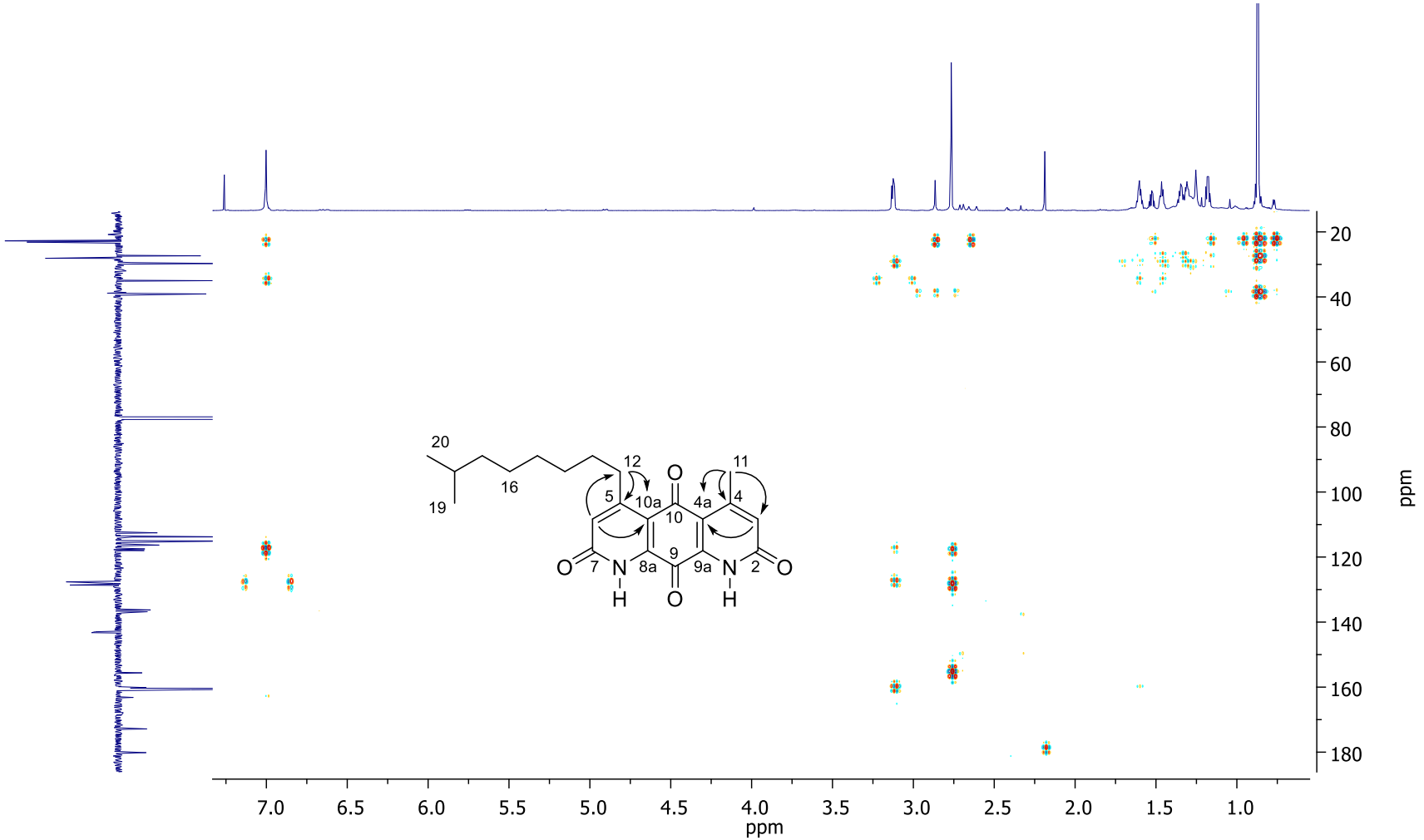
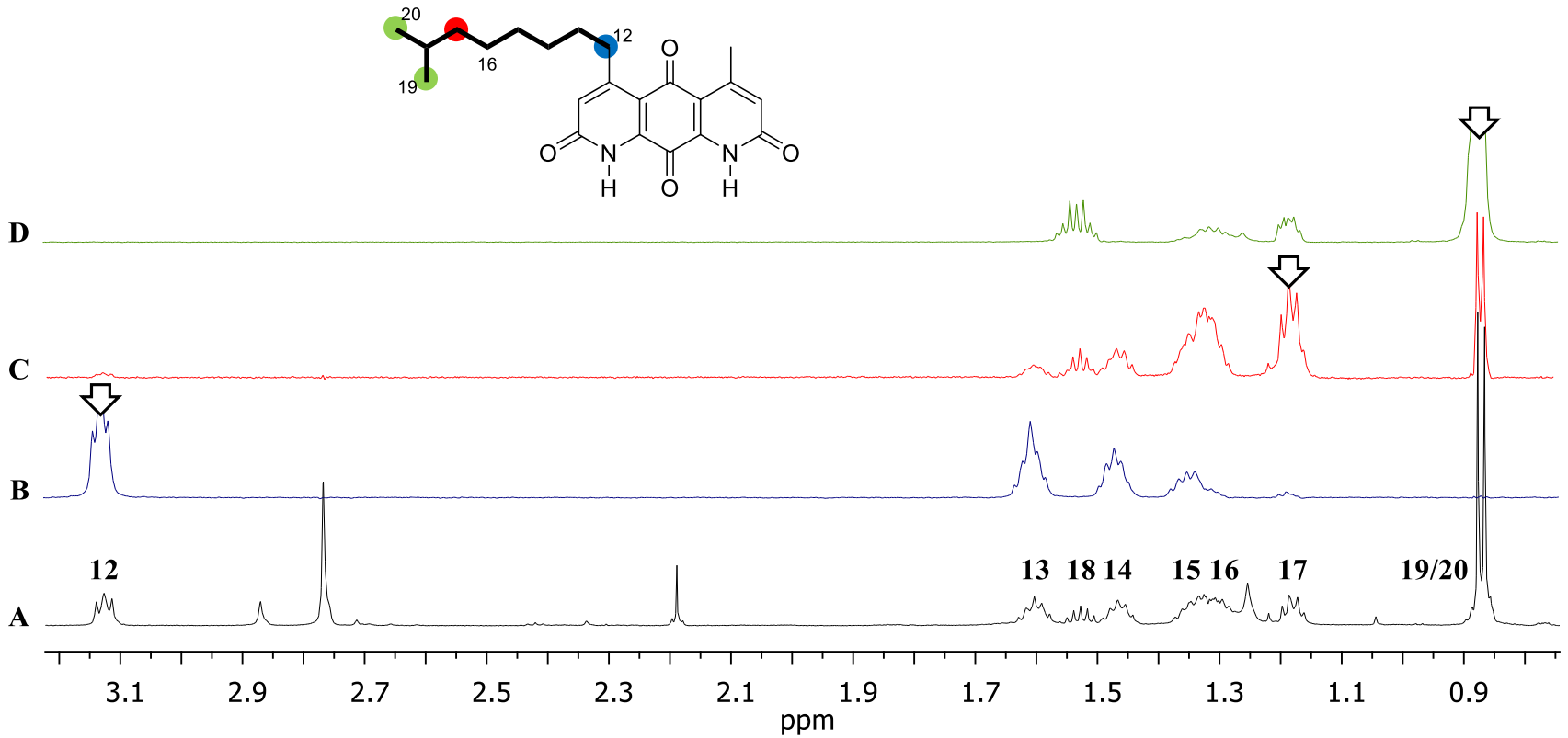
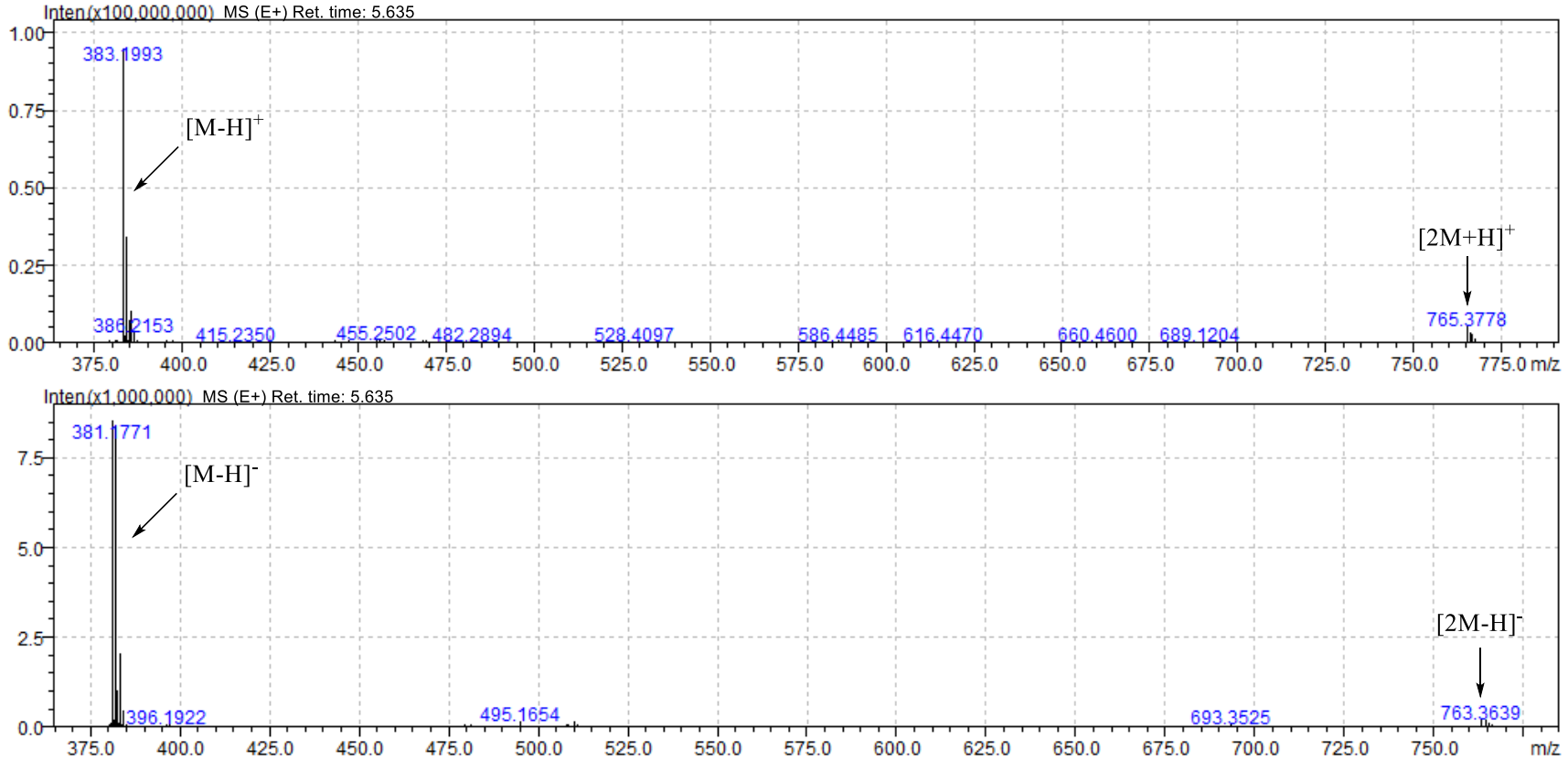


Figure S9. 1D-TOCSY Spectra (600 MHz) of **1** in $CDCl_3$–1% CF_3CO_2D.



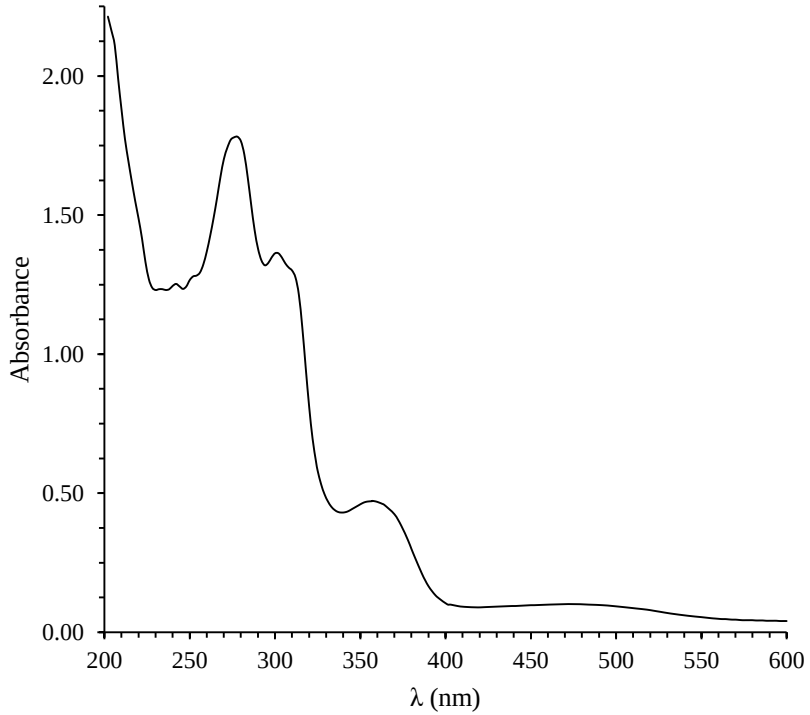
Arrows indicate irradiated resonances. (A) Expansion of ^{1}H NMR spectrum (600 MHz) of **1**; (B) Expansion of 1D TOCSY spectrum of **1** (irradiation of δ_H 3.13); (C) Expansion of 1D TOCSY spectrum of **1** (irradiation of δ_H 1.19); (D) Expansion of 1D TOCSY spectrum of **1** (irradiation of δ_H 0.87).

Figure S10. Expanded HR-ESI-ITTOF Mass Spectrum of **1**.



HRESI-ITTOF MS *m/z* 383.1993 $[M + H]^+$ (calcd. for $C_{22}H_{27}N_2O_4$: 383.1971), *m/z* 381.1771 $[M - H]^-$ (calcd. for $C_{22}H_{25}N_2O_4$: 381.1820), *m/z* 765.3778 $[2M + H]^+$ (calcd. for $C_{44}H_{53}N_4O_8$: 765.3863), and *m/z* 763.3639 $[2M - H]^-$ (calcd. for $C_{44}H_{51}N_4O_8$: 763.3712).

Figure S11. UV Spectrum of **1** in Methanol.



UV (MeOH) λ_{max} (log ε) = 278 (3.83), 301 (3.72), 357 (3.26) and a broad absorption with maximum at 472 (2.59) nm.

Figure S12. ^{1}H NMR Spectrum (900 MHz) of **2** in $CDCl_3$–1% CF_3CO_2D.

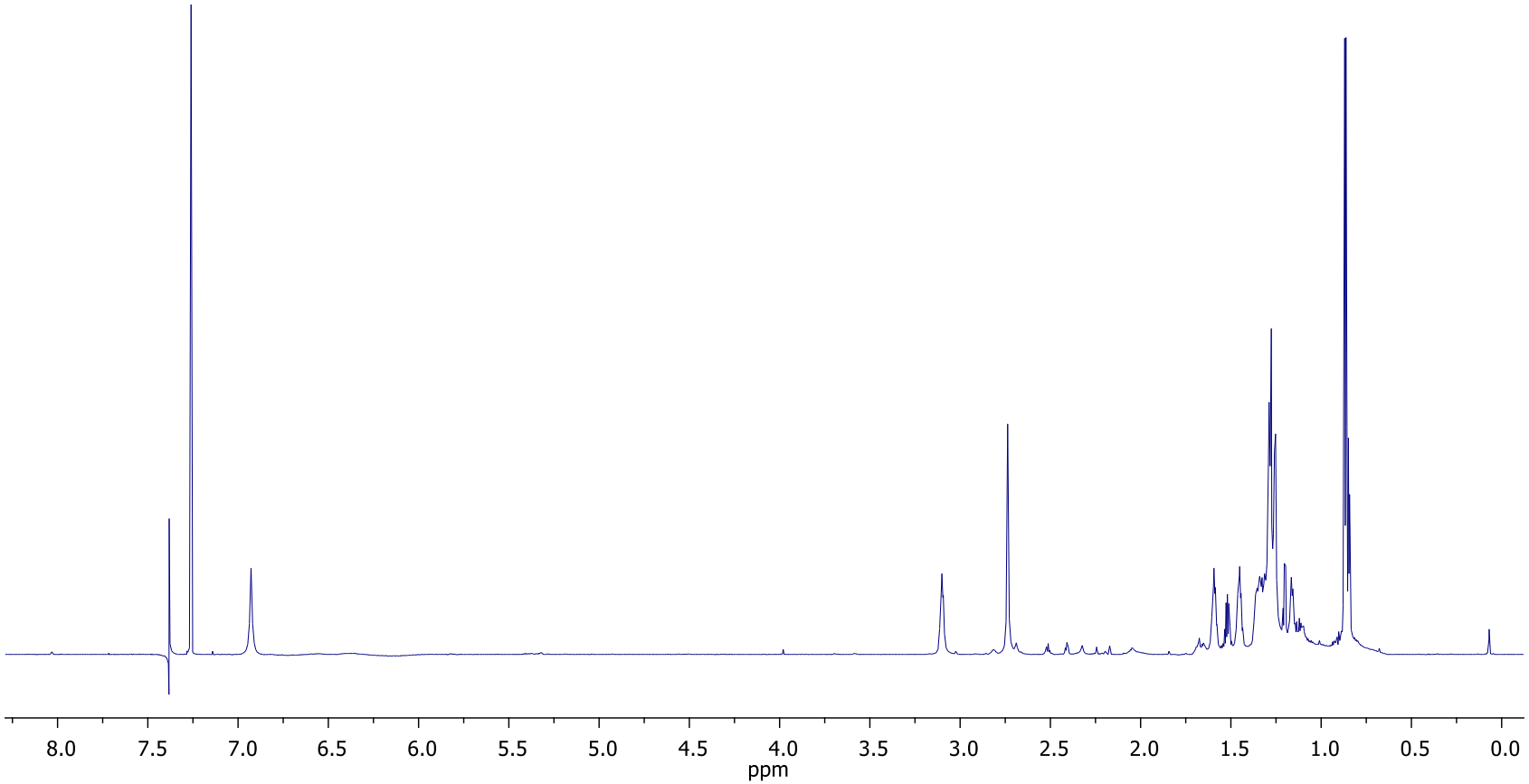


Figure S13. ^{13}C DEPTQ Spectrum (226.2 MHz) of **2** in $CDCl_3$–1% CF_3CO_2D.

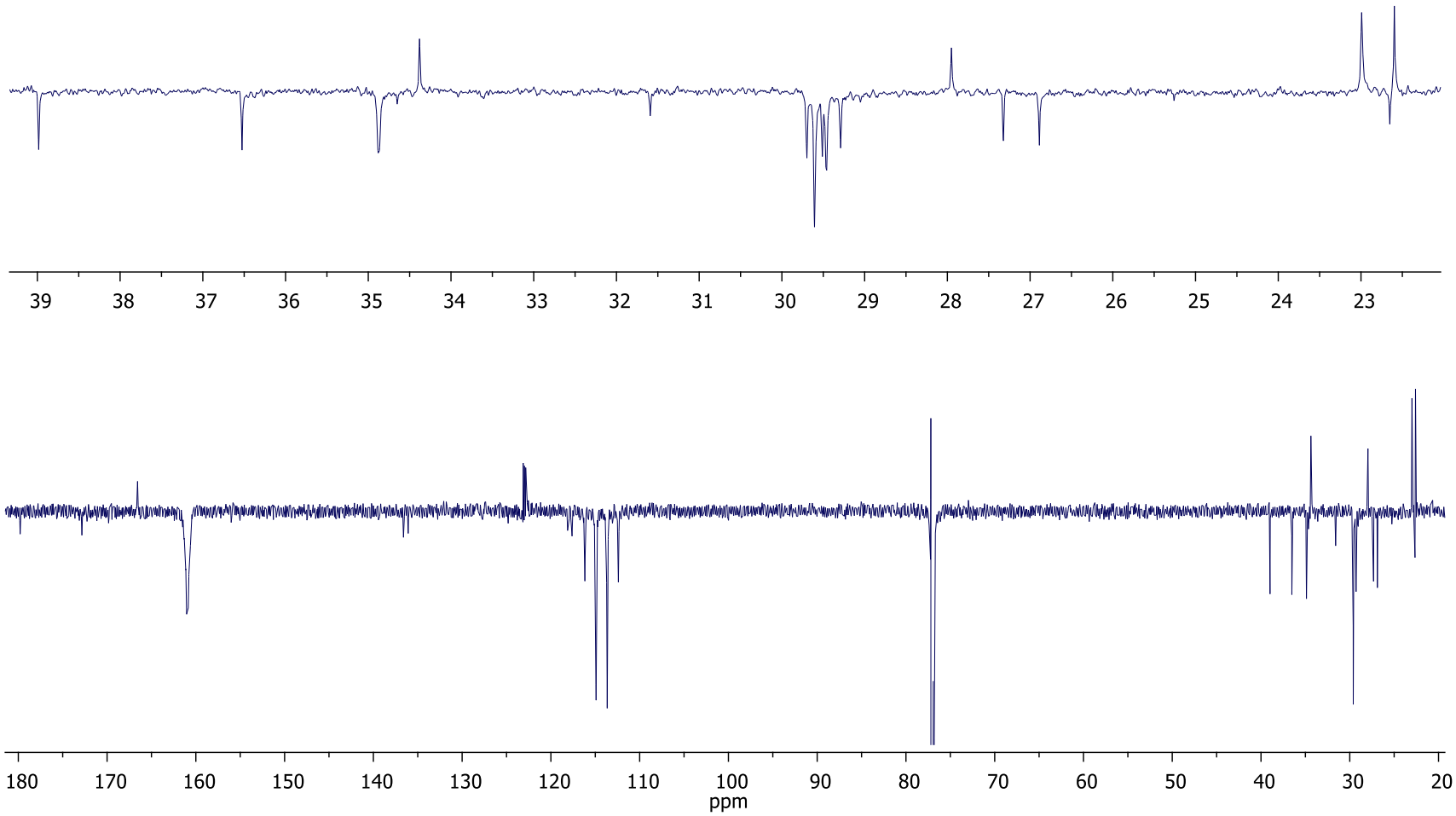


Figure S14. COSY Spectrum (600 MHz) of **2** in $CDCl_3$–1% CF_3CO_2D.

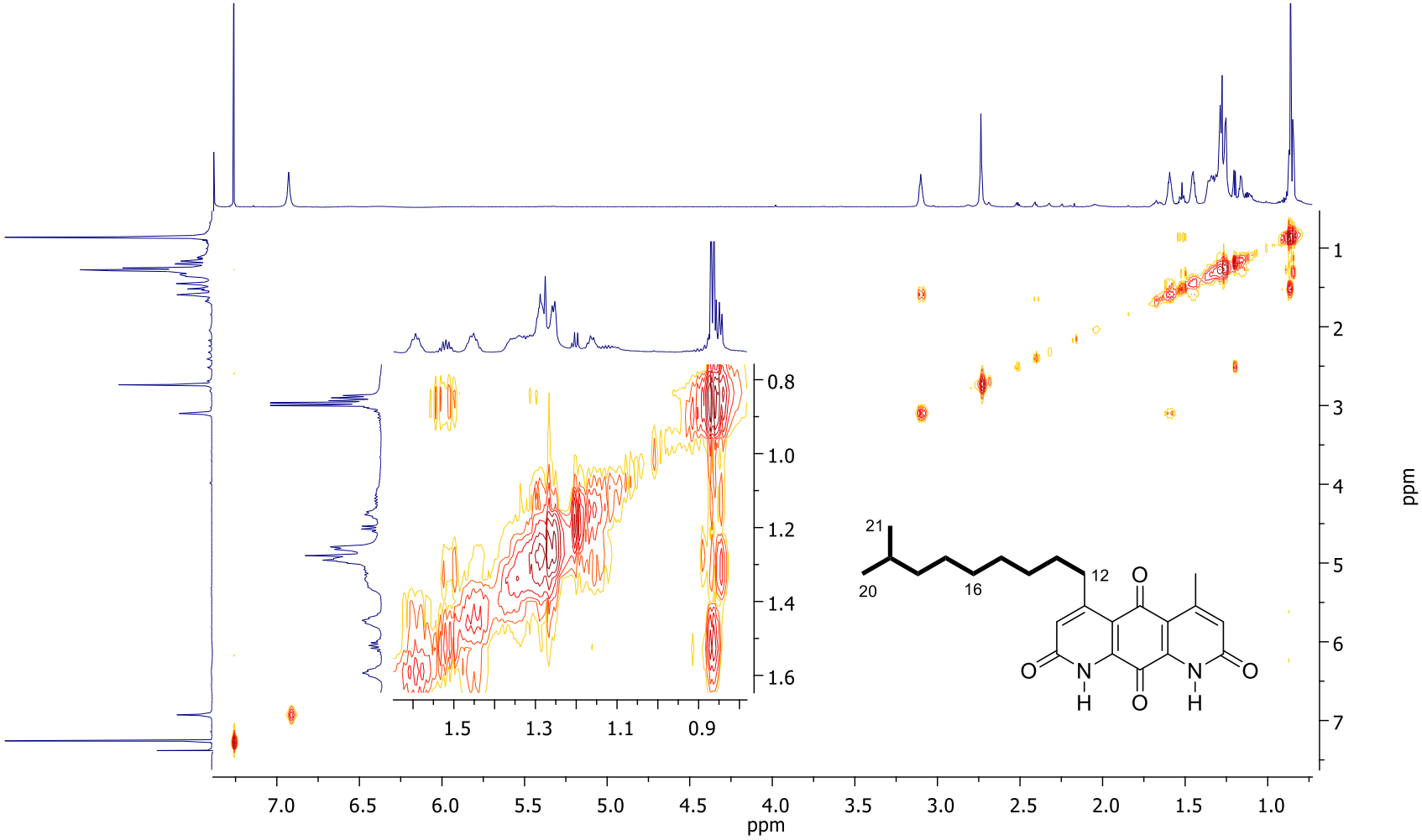


Figure S15. HMBC Spectrum (600 MHz) of **2** $CDCl_3$–1% CF_3CO_2D.

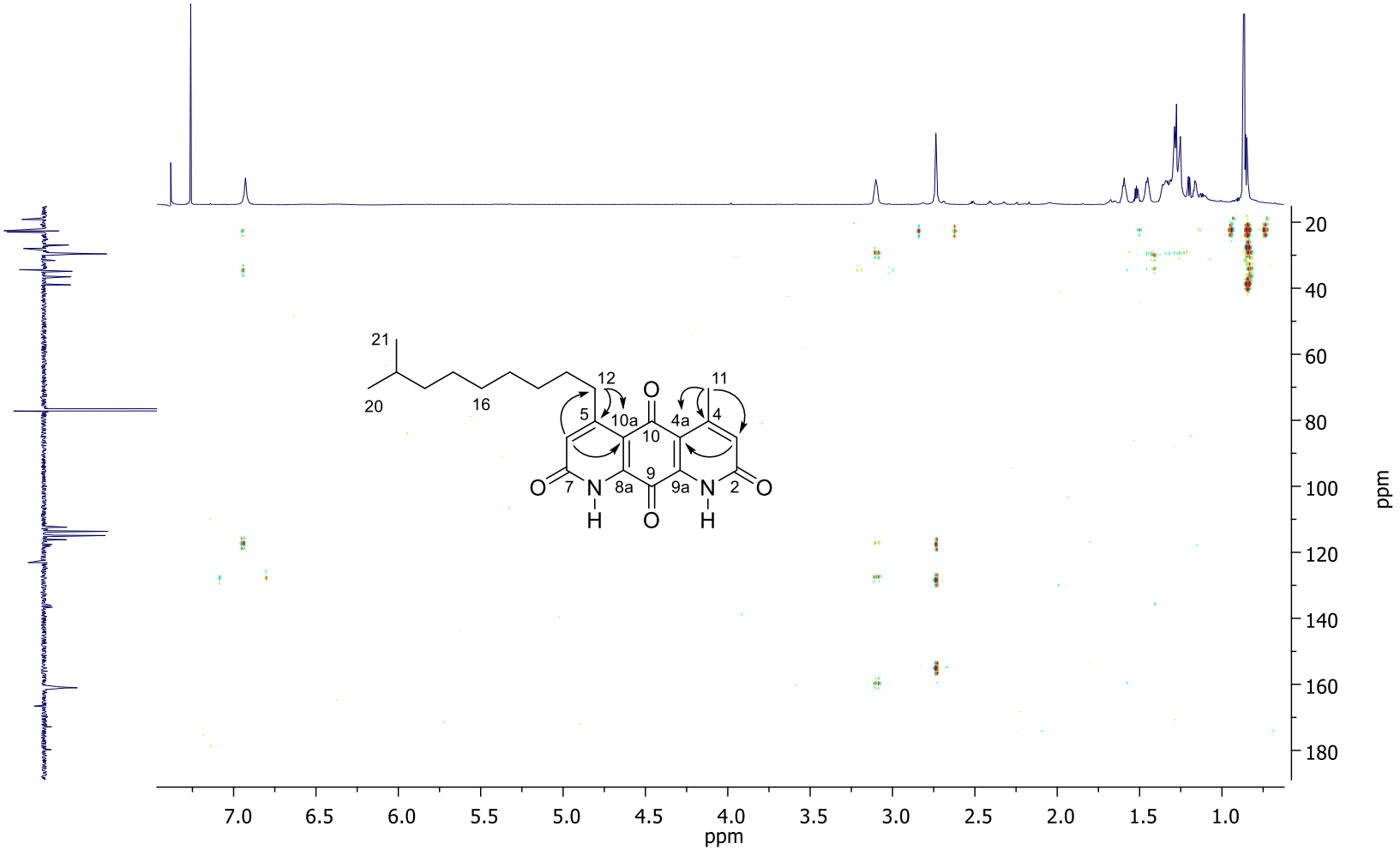
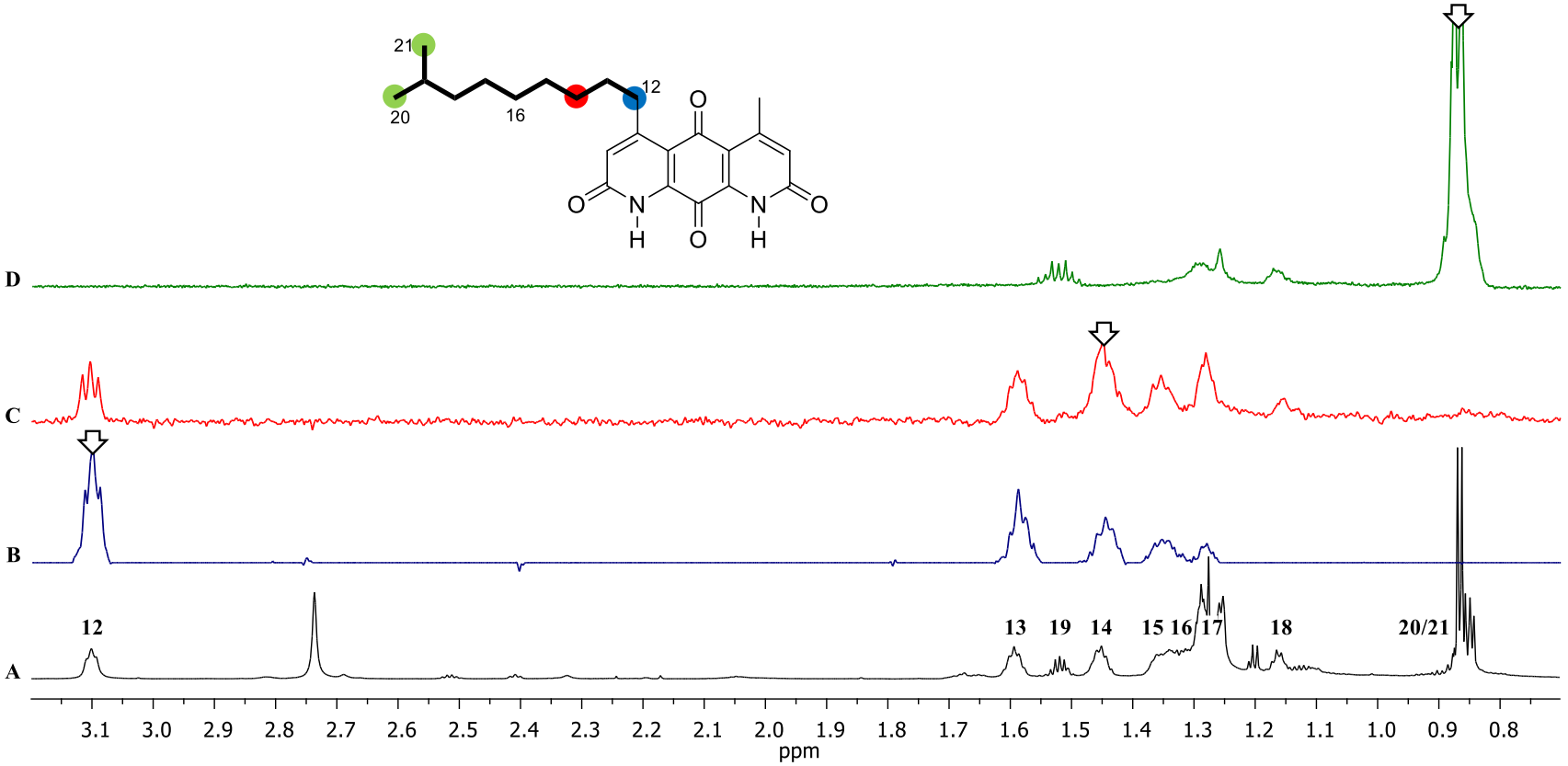
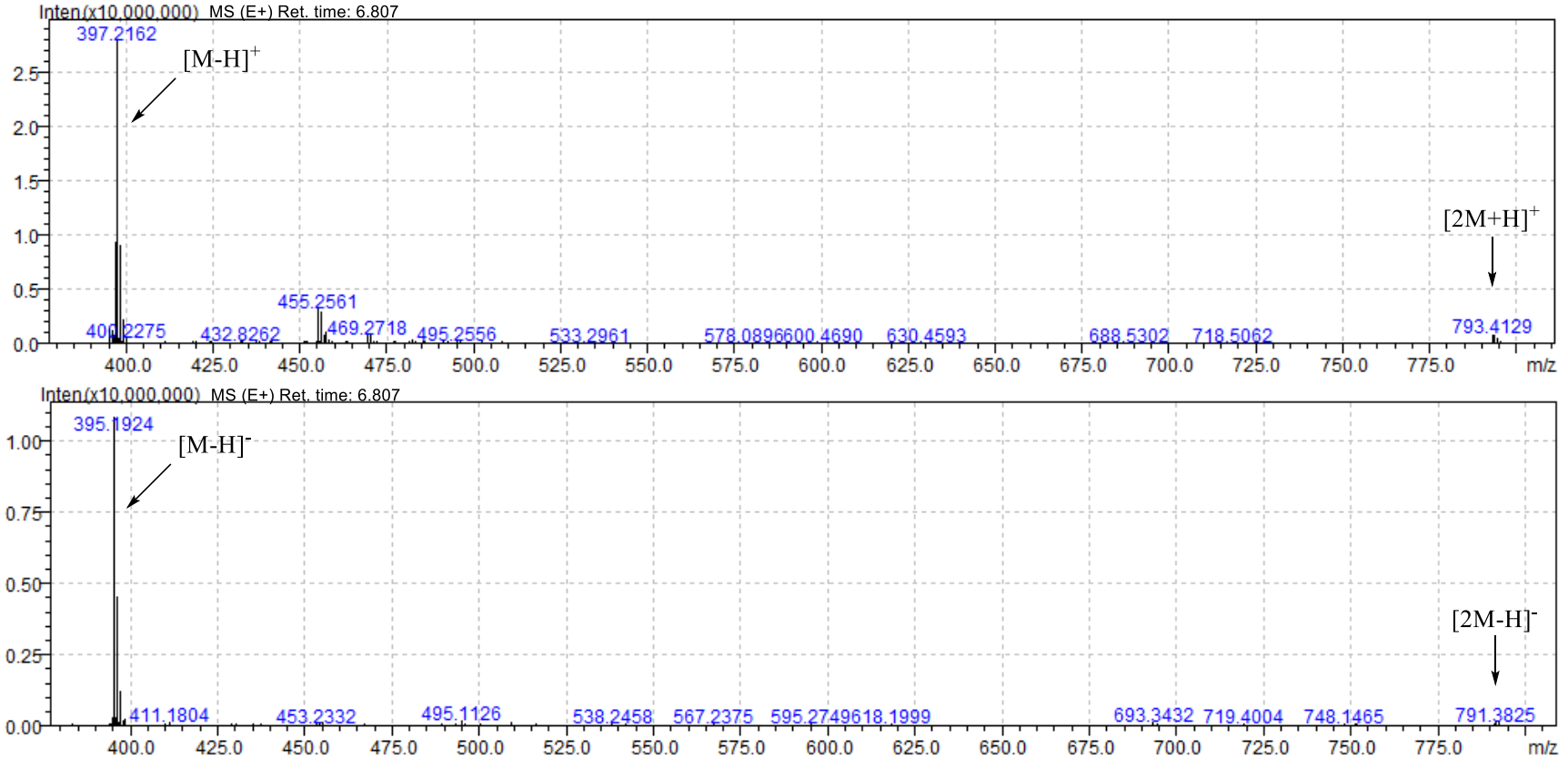


Figure S16. 1D-TOCSY Spectra (600 MHz) of **2** in $CDCl_3$–1% CF_3CO_2D.



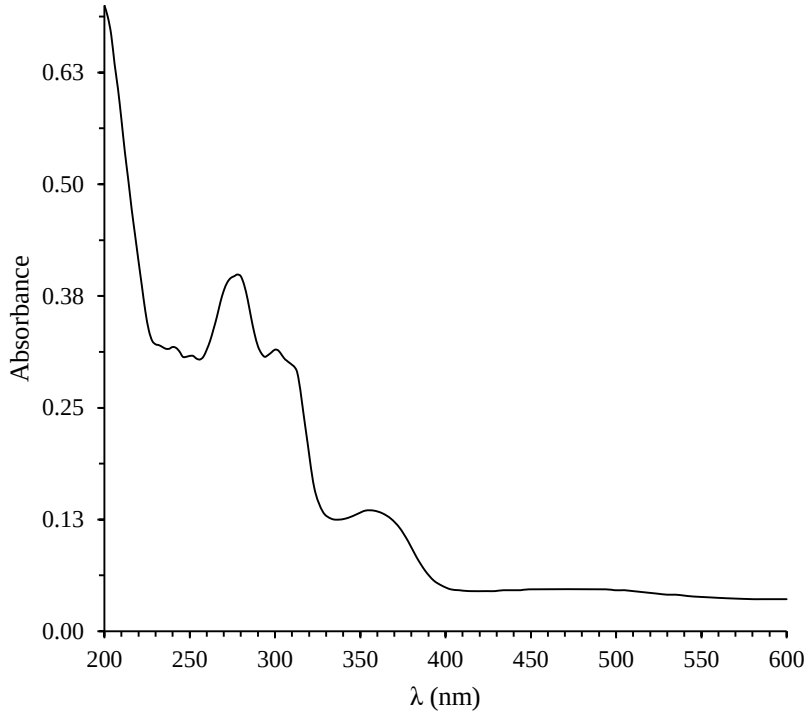
Arrows indicate irradiated resonances. (A) Expansion of ^{1}H NMR spectrum (600 MHz) of **2**; (B) Expansion of 1D TOCSY spectrum of **2** (irradiation of δ_H 3.10); (C) Expansion of 1D TOCSY spectrum of **2** (irradiation of δ_H 1.45); (D) Expansion of 1D TOCSY spectrum of **2** (irradiation of δ_H 0.86).

Figure S17. Expanded HR-ESI-ITTOF Mass Spectrum of **2**.



HRESI-ITTOF MS *m/z* 397.2162 $[M + H]^+$ (calcd. for $C_{23}H_{29}N_2O_4$: 397.2127), *m/z* 395.1924 $[M - H]^-$ (calcd. for $C_{23}H_{27}N_2O_4$: 395.1976), *m/z* 793.4129 $[2M + H]^+$ (calcd. for $C_{46}H_{57}N_4O_8$: 793.4176), and *m/z* 791.3825 $[2M - H]^-$ (calcd. for $C_{46}H_{55}N_4O_8$: 791.4025).

Figure S18. UV Spectrum of **2** in Methanol.



UV (MeOH) λ_{max} (log ε) = 278 (3.37), 300 (3.26), 356 (2.90) and a broad absorption with maximum at 472 (2.44) nm.

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