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Deuteromethylactin B from a Freshwater-derived *Streptomyces* sp.

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Abstract – Compared to their terrestrial and marine counterparts, little is known about the capacity of freshwater-derived actinomycete bacteria to produce novel secondary metabolites. In the current study, we highlight the disparities that exist between cultivation-independent and -dependent analyses of actinomycete communities from four locations in Lake Michigan sediment. Furthermore, through phylogenetic analysis of strains isolated from these locations, we identified a *Streptomyces* sp., strain B025, as being distinct from other *Streptomyces* spp. isolated from sediment. Upon fermentation this strain produced a rare class of eight-membered lactone secondary metabolites, which have been for their antitumor properties. We used spectroscopic and chemical derivitization techniques to characterize octalactin B (**1**) in addition to its corresponding novel, unnatural degradation product (**2**).
Keywords – Actinomycete, Freshwater, *Streptomyces*, Octalactin

Introduction

Natural products have been a major driving force in the discovery of new drugs over the last several decades. Since the 1940s, nearly 50% of antitumor drugs approved worldwide were either natural products or were directly derived from such sources.[1] Among a diverse array of natural product sources, actinomycete bacteria produce an abundance of structurally unique and novel biologically active secondary metabolites, as they account for more than half of the marine microbial-derived small molecules reported to date.[2] However, as time passed, focus on terrestrial actinomycetes began to decline as researchers continuously isolated known bioactive chemical scaffolds. As a result, there was a movement in the past two decades to explore the capacity of marine bacteria to produce novel secondary metabolites, and these efforts have been met with great successes.[3] Our research program extends this drug discovery platform to freshwater systems, which have been virtually unexplored for their capacity to yield novel actinomycete natural products. We previously reported among the only freshwater-derived bacterial secondary metabolites in peer-reviewed literature.[4,5] In the current study, we present a brief analysis of the bacterial community that exists in four locations of Lake Michigan sediment, and assess the corresponding cultivatable actinomycete populations. From one isolate (strain B025), we used spectroscopic and chemical derivitization techniques to characterize a class of cytotoxic lactones and its corresponding novel, unnatural degradation product.

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Experimental

General experimental procedures – UV spectra were obtained on a Shimadzu Pharma Spec UV-1700 spectrophotometer. One- and two-dimensional NMR spectra were acquired at 600 MHz in CD_3OD solvent 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, while ^{13}C NMR spectra were recorded at 226.2 MHz on a 900 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. ^{1}H and ^{13}C NMR resonances of **2** are listed in Table 1 with chemical shift values referenced according to the residual

Table 1. ^{1}H and ^{13}C NMR data of **2**.

Position	^{13}C [a,b]	^{13}C [a,c]	^{1}H mult. (*J*, Hz) [b,d]
-OCD_3	49.0	52.0	
1	173.9	174.2	
2	39.8	38.1	2.36 dd (15.1, 9.4)
			2.51 dd (15.1, 3.4)
3	73.4	72.7	3.88 ddd (9.4, 5.2, 3.4)
4	39.9	38.3	1.55 m
5	28.9	28.6	1.32 m, 1.54 m
6	32.5	32.7	1.32 m, 1.48 m
7	74.7	74.6	3.74 ddd (3.0, 7.3, 8.0)
8	46.2	43.4	3.46 p (7.3)
9	207.7	207.6	
10	139.4	138.5	
11	142.5	141.2	6.91 t (7.2)
12	35.2	34.1	2.48 m
			2.40 dd (16.0, 7.2)
13	76.7	76.0	3.49 m
14	35.0	34.1	1.70 m
15	19.3	18.9	0.96 d (7.0)
16	15.4	15.3	0.90 d (6.7)
17	14.8	16.3	1.02 d (7.3)
18	11.9	11.7	1.79 s
19	18.0	17.6	0.97 d (7.0)

[a]226.2 MHz; [b]experiment performed in CD_3OD; [c]experiment performed in $CDCl_3$; [d]600 MHz. s = singlet; d = doublet; t = triplet; p = pentet; dd = doublet of doublets; ddd = doublet of doublet of doublets; m = multiplet.

CD_3OD solvent signal at 3.31 ppm for ^{1}H resonances and 49.0 ppm for ^{13}C resonances. High- resolution mass spectra were obtained on a Shimadzu IT-TOF spectrometer at the University of Illinois at Chicago Research Resources Center. High-performance liquid chromatography (HPLC)-ESI MS data were obtained from an Agilent 1100 series LCMS system controller and pumps with a Model G1315A diode array detector equipped with a reversed-phase C_{18} column (Phenomenex Luna, 100 × 4.6 mm, 5 μm) at a 0.7 mL/min flow rate. Semi-preparative scale HPLC chromatographic separations were conducted on an Agilent 1050 series system with a reversed-phase C_{18} column (Phenomenex Luna, 250 × 10 mm, 5 μm) at a 2.0 mL/min flow rate.

DNA extraction and cultivation-independent analysis of bacterial communities from Lake Michigan sediment samples – Each of the four sediment samples used in these analyses was collected using PONAR, from a small research vessel. The location of each sediment sample is as follows: 1) 43°21'3.16"N, 87°34'11.63"W, 145 m; 2) 43°16'48.00"N, 87°34'12.55"W, 80 m; 3) 43°16'12.76"N, 87°34'12.22"W, 56 m; 4) 43°13'27.63"N, 87°34'10.62"W, 56 m. The top 1 cm of sediment was used for analysis. Genomic DNA was extracted from all sediment samples in duplicate using the PowerSoil® DNA Isolation Kit. Fragments of microbial small subunit ribosomal RNA (SSU or 16S rRNA) genes were PCR amplified from genomic DNA using primers 515F and 806R, as described previously.[6] Following pooling and cleanup of samples, the final pool was loaded onto an Illumina MiSeq sequencer, employing V2, 2x250 read chemistry.[6,7]

Analysis of microbial community composition – Sequence data were initially processed using the software package CLC Genomics Workbench (CLC Bio, Qiagen) to merge forward and reverse reads, trim poor quality data, and remove primer sequences. Sequences were then processed using the software package QIIME to remove chimeric sequences, perform clustering and annotation. Briefly, sequences were screened for chimeras using the Usearch61 algorithm and putative chimeric sequences were removed from the dataset.[7,8] Subsequently, each sample sequence set was sub-sampled to 9,000 sequences per sample to avoid analytical issues associated with variable library size.[9] Sub-sampled data were pooled and renamed, and clustered into operational taxonomic units (OTU) at 97% similarity. Representative sequences from each OTU were extracted, and these sequences were classified using the "assign_taxonomy" algorithm implementing the uclust consensus taxonomy assigner, with the Greengenes reference OTU build.[10,11] A biological observation matrix (BIOM) was generated at taxonomic levels from phylum to genus using the "make_OTU_table" algorithm. The BIOMs were imported into the software package Primer6 for analysis and visualization.[12] Figures were generated using the software package OriginPro8.5 (OriginLab Corporation, Northampton, MA) and in the software packages Excel and Powerpoint (Microsoft, Redmond, Washington).

Isolation of actinomycete strains from Lake Michigan sediment – Sediment samples were placed in glass vials and treated with heat (55 °C for 6 minutes), vortexed, and inoculated onto agar plates (50 μL) on five different types of solid media (A1, M1, ISP1, ISP2, and LWA – filtered Lake Michigan water and agar; each containing 28 μM of the antifungal agent cycloheximide). Actinomycete colonies appeared between two and four weeks and upon observation of branched hyphae or sporulation, individual strains were isolated with sterile toothpicks and re-plated

on A1 media to assess their purity.

Phylogenetic analysis of cultivatable Lake Michigan strains – Genomic DNA was extracted from individual colonies using the MasterPure Gram Positive DNA Purification (epicentre) kit. Near-full length 16S rRNA genes were PCR amplified from gDNA extracts using the primers 27F-1492R, as described previously.[13] Sequencing was performed on an ABI 3730XL DNA Analyzer Sequencer in the DNA Services Facility at the University of Illinois at Chicago. SSU rRNA gene sequences of isolates recovered in this study, and those of the most similar sequences were aligned using the software package Greengenes.[14] This alignment was imported into the software package ARB, and filtered using the Actinobacterial conservation filter, removing from the analysis positions where fewer than 50% of the sequences shared the same base.[15] This filtered alignment was imported into the phylogenetic software package MEGA5 and into the software package MrBayes v3.1.2 for phylogenetic tree construction.[16,17] Neighbor-joining phylogenetic trees were constructed with aligned sequences using the maximum composite likelihood substitution model with complete deletion of gapped positions. The robustness of inferred tree topologies was evaluated by 1000 bootstrap re-samplings of the data. For maximum likelihood trees, the general time reversible substitution model was employed, with complete deletion of gapped positions, and 1000 bootstrap re-samplings of the data. Additionally, Bayesian analyses were performed on the aligned sequence data by running five simultaneous chains (four heated, one cold) for ten million generations, sampling every 1000 generations. The selected model was the general time reversible (GTR) using empirical base frequencies and estimating the shape of the gamma distribution and proportion of invariant sites from the data. A resulting 50% majority-rule consensus tree (after discarding the burn-in of 25% of the generations) was determined to calculate the posterior probabilities for each node. The split-differential at ten million generations was below 0.01.

Fig. 2 depicts a phylogenetic tree reflecting the relationships of SSU rRNA gene sequences from select isolates. The tree topology was obtained from a bootstrapped neighbor-joining analysis, as described in the text. Nodes for which bootstrap values equaled or exceeded 70% are indicated by a numerical value. The bootstrap value derived from maximum likelihood analysis is also indicated (NJ/ML). Nodes supported by Bayesian analysis, with posterior probability values greater than 95%, are indicated with black circles. Nodes with posterior probability values greater than 70% are indicated with gray circles. Polytomies indicate branching points that were not consistently supported by bootstrap or Bayesian analyses. The scale bar indicates 0.02 substitutions per nucleotide position. Isolates are highlighted in gray.

Phylogenetic analysis of strain B025 – Strain B025 (GenBank Accesion number KM678242)[5] was isolated from a sediment sample collected from Lake Michigan. It shared 99% 16*S* rRNA gene sequence identity with the most closely related type strain, *Streptomyces koyangensis* (GenBank accession number NR025662).[35]

Fermentation and extraction of strain B025, and purification of 1 – Strain B025 was cultivated in two 2.8 L Fernbach flasks containing A1 freshwater media (500 mL filter-sterilized water from Lake Michigan, 500 mL deionized water, 10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 40 mg $Fe_2(SO_4)_3{\cdot}4H_2O$, 100 mg KBr) and shaken at 220 rpm for 7 days. Amberlite XAD-16 resin (15 $g{\cdot}L^{-1}$) was then added to each individual flask to adsorb the extracellular secondary metabolites in the culture medium. The 2 L cultures and resin were shaken at 220 rpm for 8 hours and filtered through cheesecloth to separate the resin from the culture medium. The resin and cheesecloth were shaken with acetone three times for three hours each, concentrated under vacuum, and partitioned between H_2O and EtOAc. The organic layer was dried in vacuo to yield 0.2 g of extract. The EtOAc layer from the liquid-liquid partition (0.2 g) was fractionated using silica gel solid-phase extraction (Phenomenex, *strata* SPE columns), eluting with a step gradient of hexanes/CH_2Cl_2 (50:50), hexanes/EtOAc (80:20), to EtOAc/CH_3OH (95:5) and (50:50), resulting in four fractions. Fraction 2 (56.8 mg) was separated using reversed-phase semi-preparative HPLC (Phenomenex Luna, C_{18}, 250 × 10 mm, 5 μm, 2.0 mL/min) under a gradient solvent system of 10% to 100% aqueous CH_3CN over 20 minutes, followed by a 10 minute isocratic flow of 100% CH_3CN, resulting in fourteen fractions. Subfraction F2-8 (3.0 mg, t_R = 18.6 min) was further fractionated using reversed-phase semi-preparative HPLC (Phenomenex Luna, C_{18}, 250 × 10 mm, 5 μm, 2.0 mL/min) using an isocratic flow of 50% aqueous CH_3OH over 30 minutes to yield **1**, which later degraded into **2** (1.1 mg, t_R = 19.8 min).

Deuteromethylactin B (2) – Colorless oil (1.1 mg). $[\alpha]_D^{20}$ −15.0 (*c* 0.001, 20 °C, $CHCl_3$). ^1H NMR (600 MHz, CD_3OD) and ^{13}C NMR (226.2 MHz, CD_3OD, $CDCl_3$), see Table 1. HRESI-ITTOF MS *m*/*z* 376.2804 $[M+H]^+$ (calcd. for $C_{20}O_6D_3H_{34}$: 376.2778), *m*/*z* 374.2592 $[M-H]^-$ (calcd. for $C_{20}O_6D_3H_{32}$: 374.2622), *m*/*z* 398.2620 $[M+Na]^+$ (calcd. for $C_{20}O_6D_3H_{33}Na$: 398.2598).

Results and Discussion

Cultivation-independent analysis of Actinobacteria in Lake Michigan sediment – The search for novel actinomycete taxa in freshwater environments is significant, and our program is applying a similar paradigm that afforded marine researchers a wealth of chemical diversity and successes in drug-lead discovery.[18] Since freshwater systems harbor distinct environmental selection pressures and growth conditions, it was suspected that globally endemic freshwater microbial populations existed. This postulate was argued until the advent of molecular-based screening techniques. Indeed, several studies have shown that some freshwater-derived actinomycetes were taxonomically distinct in comparison to their terrestrial and marine counterparts (*e.g.*, the acI – acIV clades), which are well summarized by Newton et al.[19] Cultivation-independent studies of freshwater bacterial communities around the world have indicated the presence of actinomycete clades that are exclusive to freshwater environments, the most abundant of which appear to fall within the acI lineage.[20-24] Few cultured representatives of these clades exist.[24,25] However, the majority of these studies focused on bacterioplankton communities (samples collected from the water column) rather than sediment actinomycetes.

In order to assess the population of actinomycetes in Lake Michigan sediment, we collected four sediment samples off of the coast of Milwaukee, Wisconsin, from depths ranging between 56 and 145 m. After extracting genomic DNA from sediment samples, we PCR amplified the DNA with primers targeting the V4 region of the small subunit 16S rRNA gene of bacteria. After next-generation sequencing and subsequent bioinformatic analysis of sequences from all four locations, we assessed the distribution of bacterial communities in sediment. Sequences derived from members of the phylum Proteobacteria accounted for the majority (54%) of the reads, while sequences from Actinobacteria were approximately 11% of the total sequence library. Interestingly, according to cultivation- independent analysis *within* the phylum Actinobacteria, two of the most common families that account for the majority of actinomycete secondary metabolites were scarcely represented (Streptomycetaceae and Micromonosporaceae, 0.04 and 0.11% of total Actinobacteria reads, respectively; data not shown), while 31% of reads were attributed to families that remain uncharacterized.

Phylogenetic analysis of cultivatable Lake Michigan actinomycetes – The majority of isolates obtained through

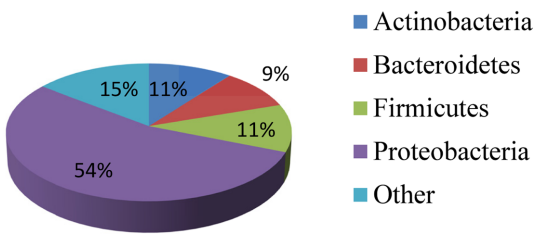


Fig. 1. Composition of bacterial community in collected Lake Michigan sediment.

cultivation of strains from the four Lake Michigan sediment samples were members of the families Streptomycetaceae and Micromonosporaceae (Fig. 2). Phylogenetic analysis of the Actinobacteria isolates from Lake Michigan revealed that the organisms belonged to three different genera: *Micromonospora, Streptosporangium* and *Streptomyces*. Most of the isolates (19 of 29) belonged to the genus *Micromonospora*. No organisms with rRNA genes similar to so-called "lake Actinobacteria" (*i.e.* clades acI-acIV) were detected.[22] Actinobacterial lineages that so far are known as exclusive to freshwater environments have primarily been detected through cultivation-independent molecular assays, and further efforts will be needed to isolate representatives of these more divergent lineages. However, it is important to note that traditionally, the process of isolating actinomycetes from petri dishes for drug-lead discovery has been heavily biased toward larger, spore-forming colonies with specific morphological features, and in our case this collection of strains does not represent the true population that exists on the plates. Our lab is currently developing an assay to rapidly assess the total bacterial community on agar plates, so that it is possible to assess the recovery of actinomycetes from sediment and potentially prioritize sediment locations based off of potential of a sediment sample to yield novel *cultivatable* actinomycete taxa.

Although many strains had identical or nearly identical SSU rRNA gene sequences, these strains did not necessarily have similar profiles of secondary metabolite production. For example, Strain B006 is a producer of the diazaquinomycin antibiotic class.[4] Five strains clustered together with strain B006 (B026, B009, B008, B027, and B028), but via LCMS analysis of fermentation extracts only B006 and B026 produced that antibiotic class. This observation highlights the complexity and limitations of using taxonomic uniqueness to guide the discovery of novel chemistry.

Although the isolates recovered in this study were highly similar by rRNA gene sequence to previously isolated organisms, we were still able to identify strains

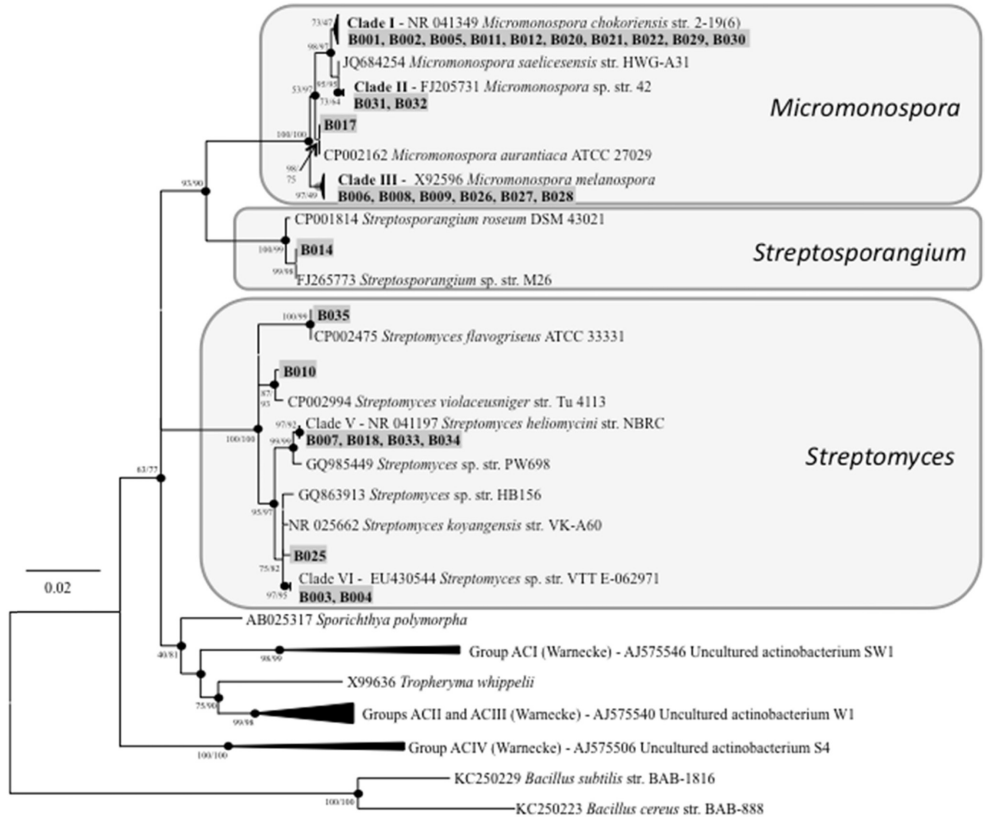
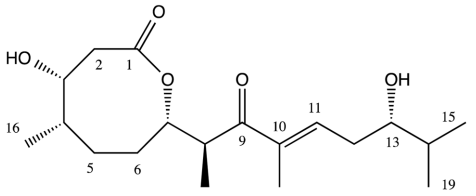
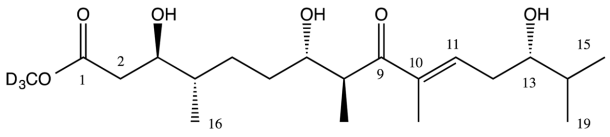


Fig. 2. Phylogenetic analysis of cultivatable actinomycete strains from Lake Michigan.



Octalactin B (**1**)



Deuteromethylatctin B (**2**)

Fig. 3. Structure of octalactin B (**1**), and deuteromethylactin B (**2**).

with novel secondary metabolites. Of the strains classified under Streptomycetaceae, we identified B025 as being distinct from its *Streptomyces* counterparts. Upon fermentation it was found to produce a rare class of eight-membered lactone secondary metabolites, a member of which has been investigated for its antitumor properties.

Stereochemical determination of 1 – The identity of **1** as octalactin B was partially confirmed through comparison of ^{1}H NMR data to those reported for the natural product.[26] However, in its original publication the relative configuration of stereocenters in **1** was determined through X-ray crystallographic analysis, and most subsequent reports assembled this scaffold through stereoselective synthesis.[27-31] Thus, if **1** is isolated from a natural source, it is difficult to confirm the absolute configuration using data found in peer-reviewed literature (only OR data was published for **1**, and to our knowledge no group published a CD spectrum). Herein, we contribute spectroscopic and chemical derivitization data of **1** that will facilitate future efforts to rapidly confirm the identity of the natural product octalactin B and other 8-membered lactones with similar stereochemical orientations. The relative configuration of C-1–C-8 was confirmed through comparison of ^{1}H NMR chemical shifts and analysis of NOESY data. We observed the chemical shift of H-3 (δ_H 4.04, **1**) to match exactly as that reported by Tapiolas et. al[26], which suggests that these two are in the same chemical environment and would have the same configuration. An NOE correlation was observed between H-3 and H-4, confirming a *cis*-orientation. Also supporting a *cis* orientation was a clear ddd (J = 9.4, 5.2, 3.4 Hz) for H-3 in the hydrolysis product

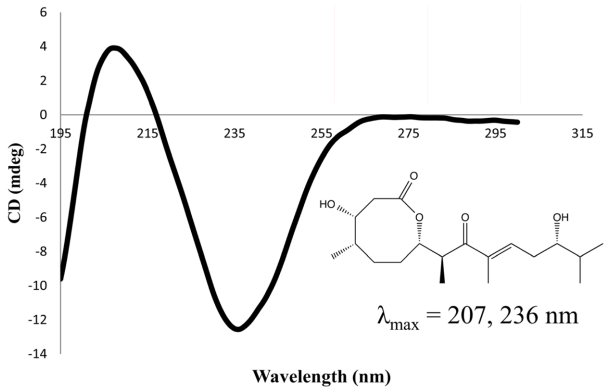


Fig. 4. CD Spectrum of **1**.

2 of octalactin, which represents two gauche- and one anti- interactions; this is described in further detail in the following section. Comparison of optical rotation data with synthetic octalactin B allowed us to assume the absolute configuration of stereocenters in C-1–C-8 to be 3*R*, 4*S*, 7*S,* 8*S*.[27,32] To confirm the absolute configuration at C-13, we performed modified Mosher's Ester analysis.[33,34] Upon analysis of the spectral data from resulting *R* and *S*-Mosher derivatives of **1**, we determined the absolute configurations at C-13 to be *S*. We then acquired a CD spectrum of **1** (Fig. 4), which will facilitate rapid identification of similar eight-membered lactones from natural product extracts in the future.

Structure elucidation of 2 – The structure of **2** was assigned by a combination of high-resolution MS and one- and two-dimensional NMR (Fig. 5). Pseudomolecular ions at *m/z* 376.2804 $[M+H]^+$, 374.2592 $[M-H]^-$, and 398.2620 $[M+Na]^+$ were observed in the mass spectrum, suggesting a molecular formula of $C_{20}O_6D_3H_{33}$. This molecular formula afforded three degrees of unsaturation. Analysis of the ^{13}C spectrum indicated the presence of twelve aliphatic sp^3 carbons, three secondary alcohols, an ester carbonyl (δ_C 173.9, C-1), a ketone carbonyl (δ_C 207.7, C-9), an olefinic methine carbon (δ_C 142.5, C-11), and a quaternary olefinic carbon (δ_C 139.4, C-10), which collectively account for the index of hydrogen deficiency suggested by the molecular formula.

Correlations observed in the COSY spectrum were crucial in determining major spin systems in **2**. COSY correlations established the occurrence of two major fragments: a nine carbon spin system between carbonyls at C-1 and C-9, and a six carbon spin system directly adjacent to C-10. HMBC correlations from H-11 to the ketone carbon C-9, and from H_2-12 to quaternary alkene carbon C-10, confirmed the presence of an α,β-unsaturated ketone moiety consistent with the observed UV maximum

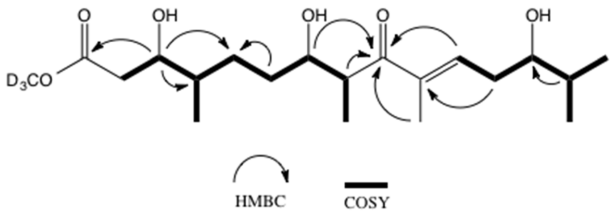


Fig. 5. Key 2D NMR correlations of **2**.

of 235 nm. Additional HMBC correlations from H-7 and H-8 to the ketone carbon C-9 linked the two spin systems in **2**. Finally, an HMBC correlation from the secondary alcohol H-3 to C-1 placed a terminal ester moiety adjacent to C-2. The structural assignment of the -OCD_3 group in the ester moiety was determined by the extra 34 mass units required by the molecular formula as well as the chemical shift value of C-1 at δ_C 173.9. In addition to acquiring ^{13}C data in CD_3OD, the experiment was also performed in $CDCl_3$ in order to confirm the presence of the carbon atom in the -OCD_3 group, which is overlapped with the solvent resonance in CD_3OD. The carbon atom was assigned at 52.0 ppm and the two-dimensional structure was thus established as **2**.

We suspected **2** to be an unnatural degradation product of **1**. After approximately 24 hours in an NMR tube, the ^1H spectrum appeared to be different from that of **1**. The molecular formula of **1** was determined to be $C_{19}H_{32}O_5$ on the basis of the pseudomolecular ion at *m/z* 341.2211 (calcd. for $C_{19}H_{33}O_5$; $[M+H]^+$) in the HRESI mass spectrum, affording four degrees of unsaturation and a molecular weight 35 mass units lower than that of **2**. The extra degree of unsaturation afforded by the molecular formula of **1** is consistent with the presence of a lactone moiety, whereas the three degrees of unsaturation afforded by the molecular formula of **2** suggest the open-chain conformation. Further analysis of the ^1H NMR spectra of **1** and **2** revealed a marked difference in the chemical shift of H-7 before (δ_H 4.83, **1**) and after (δ_H 3.74, **2**) the degradation, characteristic of sp^3 oxymethine resonances of lactone and open chain protons, respectively. Thus, the combined NMR and HRMS evidence suggested that a hydrolysis reaction occurred, resulting in the open-chain form of **1**. It is likely that residual acid in the NMR tube (from an unrelated experiment) catalyzed a methanolysis reaction between **1** and the CD_3OD. Based on the likely origin of **2** as a degradation product of **1**, the absolute configuration of stereocenters in **2** are conserved as 3*R*, 4*S*, 7*S*, 8*S*, 13*S*.

Octalactins A and B were originally isolated and characterized by Tapiolas et al. in 1991, who reported the strong cytotoxicity of octalactin A against HCT-116

human colon tumor and B-16-F10 murine melanoma cell lines.[26] Octalactin B was found to be not significantly active in these assays, presumably due to the loss of epoxide function between the two compounds. Many research groups have since undertaken efforts to develop facile total syntheses of these molecules, as their structural uniqueness and therapeutic potential have made them an interesting topic of study. The occurrence of saturated eight-membered lactones in natural products is rare, and future efforts to explore the structural and biological properties of such molecules will enable us to better assess their therapeutic potential.

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