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Merocyclophanes C and D from the Cultured Freshwater Cyanobacterium *Nostoc* sp. (UIC 10110)

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Abstract

Merocyclophanes C and D (**1** and **2**) were isolated from the cell extract of a cultured cyanobacterium, UIC 10110. The structures were determined by 1D NMR and HRESIMS and confirmed by 2D NMR techniques. The absolute configurations were determined using ECD spectroscopy. Merocyclophanes C and D represent the first known analogs of the merocyclophane core structure, a recently discovered scaffold of [7,7] paracyclophanes, characterized by an α -branched methyl at C-1 / C-14. **1** and **2** showed antiproliferative activity against the MDA-MB-435 cell line with IC₅₀ values of 1.6 μ M and 0.9 μ M, respectively. Partial 16S analysis determined UIC 10110 to be a *Nostoc* sp. and it was found to clade with UIC 10062 *Nostoc* sp., the only other strain known to produce merocyclophanes. The genome of UIC 10110 was sequenced and a biosynthetic gene cluster was identified that is proposed to encode type I and type III polyketide synthases that are potentially responsible for production of the merocyclophanes, yet further experiments will be required to verify the true function of the gene cluster. The gene cluster provides a genetic basis for the observed structural differences of the [7,7] paracyclophane core structures.

Graphical abstract

Cyanobacteria are a prolific source of bioactive secondary metabolites. Many of these secondary metabolites are cytotoxic and have potential as anticancer drug leads.^{1–3} For

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Microscopic photograph of UIC 10110, ¹H and DEPTQ spectra of **1** and **2** and COSY, HSQC, and HMBC of **1**, as well as ECD spectra of **1** and **2**.

example, dolastatin 10, a highly cytotoxic peptide was first isolated from the sea hare, *Dollabella auriculata*, and was later discovered to originate from cyanobacteria. In phase two clinical trials, dolastatin 10 lacked activity as an individual component drug.^{4,5} Synthetic analogues, while more active than dolastatin 10, demonstrated greater levels of toxicity. However, one of these analogues, monomethyl auristatin E, was conjugated to an antibody against CD30 and developed by Seattle Genetics as the monoclonal antibody-drug conjugate brentuximab vedotin, which obtained FDA approval for treatment of Hodgkins Lymphoma in 2011.^{6–8} Besides dolastatin 10, cyanobacteria produce a plethora of other cytotoxic compounds that could serve as novel anticancer drug leads.

Recently, a variety of [7,7] paracyclophane structures with cytotoxic and antibiotic activity have been discovered from freshwater and terrestrial cyanobacteria.^{9–12}

Cylindrocyclophanes, nostocyclophanes, and the recently discovered merocyclophanes represent the major structural classes of the [7,7] paracyclophanes identified to date, differing in the presence or absence of an alpha or beta methyl substituent (Figure 1).^{13–15} Initially, the biosynthetic origin of [7,7] paracyclophanes was proposed to be catalyzed by a polyketide synthase (PKS) by Moore *et al.* after performing ¹³C labeled acetate supplementation experiments and observing a PKS pattern of ¹³C incorporation.¹⁶ Nakamura *et al.* confirmed the PKS biosynthetic origin with the discovery of the cylindrocyclophane biosynthetic gene cluster from the cyanobacteria *Cylindrospermum licheniforme* ATCC 29412, and *Cylindrospermum* sp. ATCC 29204.¹⁷ The gene cluster indeed contained type I and type III PKS genes. However, unexpectedly, biosynthesis starts with the recruitment of a free fatty acid (decanoic acid) and the type I PKS contains only two modules responsible for two elongation reactions. Nakamura *et al.* demonstrated that the gene cluster encoded for the production of two halves of the core structure that complete the final macrocycle. The dimerization event is not yet completely understood, but involves the functionalization of an unactivated carbon.^{17,18} Recently, Preisitsch *et al.* has provided potential insight into the mechanism of the closing of the macrocycle with their discovery of the halogenated cylindrofridins.¹⁹ Preisitsch *et al.* also recently described the carbamidocyclophane biosynthetic gene cluster from the cyanobacterium CAVN2, a *Nostoc* sp. This gene cluster was quite similar to the cylindrocyclophane gene cluster, as both produce the cylindrocyclophane core structure, but contained a unique carbamoyl transferase which provided a genetic basis for the addition of carbamate groups to the cylindrocyclophane structure.²⁰ The single strain known to produce nostocyclophanes, UTEX B1932 *Nostoc linckia*, is no longer available through UTEX and no other producers have been reported since the initial discovery, limiting the data known about this group of [7,7] paracyclophanes. The unique chemical structures and prevalence of [7,7] paracyclophanes in members of the Nostocaceae family make these compounds of significant interest. Here we describe the isolation and structure elucidation of two new merocyclophanes, merocyclophane C and merocyclophane D, as well as the identification of a potential merocyclophane gene cluster from the cultured cyanobacterium UIC 10110, a *Nostoc* sp. The described gene cluster provides a genetic basis for the observed structural differences between the cylindrocyclophane and merocyclophane core structures. The newly discovered compounds, merocyclophane C and D, represent the only analogs of the merocyclophane structural core identified to date and display higher cytotoxic activity than

other [7,7] paracyclophanes. The identification of two new merocyclophanes and their biosynthetic gene cluster continues to provide evidence that cyanobacteria are prolific producers of bioactive secondary metabolites.

Results and Discussion

Merocyclophane C (**1**) and D (**2**) were identified as part of our ongoing screen of cultured freshwater cyanobacteria for compounds with cytotoxic activity. The cellular extract of *Nostoc* sp. UIC 10110 was active in an MTS cytotoxicity assay, with 50% cell viability at 25 $\mu\text{g/mL}$. Dereplication using high resolution LC-MS and ^1H NMR spectrum indicated the cytotoxic components to be two [7,7] paracyclophanes. One was identified as the previously reported merocyclophane A¹⁵, and one [7,7] paracyclophane was of unknown structure²¹.

Merocyclophane C (**1**) was obtained as pale yellow amorphous powder. HRESIMS data established the molecular formula as $\text{C}_{36}\text{H}_{56}\text{O}_5$. This indicated that **1** contained an additional oxygen atom when compared to the molecular formula of merocyclophane A (**3**), ($\text{C}_{36}\text{H}_{56}\text{O}_4$). The structure of **1** was elucidated using HRMS and NMR spectra. A triplet at δ_{H} 3.47 (H-30) was identified as unique to the ^1H spectra of **1** as compared to the published ^1H NMR spectrum of **3**.¹⁵ The triplet integrated to two protons and the chemical shift suggests the presence of an adjacent oxygen. Additionally, the aliphatic terminal methyl group (triplet at δ_{H} 0.83) only integrated to three protons in **1**; whereas, this triplet integrated to six protons in the symmetrical **3**. These data indicated that the additional oxygen atom in **1** was at one of the terminal methyl groups of the aliphatic chain of **3**. This placement would account for both the triplet coupling pattern as well as the deshielded shift. This conclusion was further substantiated by analysis of the DEPTQ spectrum, which contained a CH_2 signal at δ_{C} 63.5, again indicating a terminal carbon with an additional oxygen. The final structure of merocyclophane C was confirmed by analysis of the COSY, HSQC, and HMBC spectra (Figure 2) (Table 1). The absolute configuration of **1** was determined using circular dichroism (ECD) spectroscopy. A comparison of ECD spectrum of **1** to the ECD spectrum of **3** confirmed that it has the same stereoconfiguration at C-1, C-7, C-14, and C-20 (Figure S10).¹⁵

During the process of isolation of **1**, an additional minor [7,7] paracyclophane, was identified. Merocyclophane D (**2**) had a molecular weight of 584.4151 Da and a molecular formula of $\text{C}_{36}\text{H}_{56}\text{O}_6$. This indicated that **2** contained an additional oxygen atom as compared to **1**. Inspection of the proton NMR spectrum showed that the triplet at δ_{H} 3.47 now integrated to four protons. In addition, the terminal methyl group found in **1** was missing. These findings, in combination with the now symmetrical appearance of the proton NMR spectrum, indicated the placement of the additional oxygen in **2** at the terminal methyl group of **1** (Figure S7, S8). As above, the structure of merocyclophane D was confirmed by DEPTQ spectroscopy (Figure S9). The stereoconfiguration was determined by ECD spectroscopy (Figure S10).

Merocyclophanes C and D represent the first analogs of the merocyclophane alpha-methyl branched core structure and provide further evidence that [7,7] paracyclophanes are structurally diverse compounds. Merocyclophanes C and D, as well as our library of [7,7]

paracyclophanes, were tested in a panel of three cell lines: MDA-MB-435 (melanoma), MDA-MB-231 (breast adenocarcinoma), and OVCAR3 (ovarian adenocarcinoma). IC₅₀ values were determined for each of the compounds, and merocyclophane D was found to be the most potent [7,7] paracyclophane examined in the assay (Table 2).

To assess the *in vivo* antitumor efficacy of the merocyclophanes, **1** was evaluated in a hollow fiber tumor cell assay. The more active **2** was not chosen due to its low abundance in the extract. The assay was performed as detailed in Ren *et al.*²² Merocyclophane C was dissolved in 30% DMSO, 30% Cremaphor EL, and 40% H₂O. Ncr *nu/nu* mice with intraperitoneally inserted hollow fibers, containing cultured cancer cells, were injected with the merocyclophane C solution for four days before being sacrificed. The hollow fibers were retrieved and the remaining tumor cells were analyzed for growth inhibition. The p-value for inhibition of MDA-MB-231 by **1** at 15 mg/kg when compared to the negative control was 0.051, which did not reach statistical significance. The experiment was repeated with a new batch of **1** with concentrations of 17 mg/kg and 15 mg/kg, but **1** proved too toxic at these concentrations to continue the study.

Members of the Nostocaceae family are well-established producers of biologically active secondary metabolites, and the isolation of two novel [7,7] paracyclophanes further expands the number of bioactive compounds from this family. Phylogenetic analysis of a portion of the 16S rRNA was performed in order to determine the taxonomy of the producing strain UIC 10110. A portion of the 16S rRNA was amplified using cyanobacterial specific primers 109F and 1509R and was sequenced via Sanger sequencing. UIC 10110 clades with other members of *Nostoc* group I, including UIC 10062 *Nostoc* sp., the producer of merocyclophane A. Analysis of the phylogenetic tree provided potential insight into the evolutionary relationships of different [7,7] paracyclophane producing strains (Figure 3). The two known merocyclophane producing strains cluster together, suggesting that they are distinct from the cylindrocyclophane and carbamidocyclophane producing strains. Interestingly, the carbamidocyclophane producing strains; CAVN2, CAVN10, UIC 10274; and the cylindrocyclophane producing strain UIC 10022a cluster separately from the other two cylindrocyclophane producing strains, PCC 7417 and UTEX 2014. This provides three distinct clades that are known to produce [7,7] paracyclophanes: *Nostoc* group III, *Nostoc* group I, and *Cylindrospermum*. As there are only a few strains from each clade currently identified, the relationships between these clades and the compounds they produce will become more conclusive as more strains that produce [7,7] paracyclophanes are described.

In order to identify the biosynthetic origin of the α -methyl branching of the merocyclophanes, genomic DNA isolated from *Nostoc* sp. UIC 10110 was sequenced by NGS on an Illumina MiSeq. Reads were trimmed and assembled using the SPAdes Assembler through the Illumina BaseSpace platform. The resulting assembly was analyzed by AntiSMASH 3.0²³ in order to identify the gene cluster responsible for production of merocyclophanes. A gene cluster designated as a type I PKS-type III PKS hybrid by AntiSMASH, bore close homology to the previously published cylindrocyclophane and carbamidocyclophane gene clusters and was further investigated. The gene cluster was analyzed by BLASTp and eight genes showed homology of at least 50 percent to described genes from the [7,7] paracyclophane gene clusters when transcribed. One gene seemed to be

unique to the potential merocyclophane gene cluster; based on BLASTp homology score, it appears to encode a cytochrome P450. A direct comparison of the two type I PKS genes in each biosynthetic gene cluster, CylD and CylH for cylindrocyclophane biosynthesis and MerC and MerD for merocyclophane biosynthesis, was performed in order to explain the differences of the alpha and beta methyl branching in the merocyclophane and cylindrocyclophane core structures. Superficially, the type I PKS domains of the biosynthetic gene clusters appear quite different, yet they are expected to produce a very similar core alkyl-resorcinol structure. The CylD homolog, MerC, contains ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains that should lead to full reduction of the carbonyl, in addition to a C-methyl transferase expected to catalyze the incorporation of the α -methyl, as opposed to the CylE, F, and G enzymes that install the beta branched methyl in cylindrocyclophanes (Figure 4). Accordingly, the CylH homolog, MerD, does not contain the ER and Enoyl-CoA Hydratase/Isomerase (ECH) domains that are involved in beta methyl formation. In both gene clusters, each type I PKS gene encodes a module that adds and processes one malonyl-CoA to the pre-loaded decanoic acid (Figure 4). The type III PKS would add two additional malonyl-CoA units similar to the type III PKS function in cylindrocyclophane biosynthesis. Comparison of the gene clusters illustrates the genetic basis for the observed branching differences in the cylindrocyclophanes and the merocyclophanes.

Thus, we propose a biosynthetic mechanism for the merocyclophanes similar to that of the biosynthesis of the cylindrocyclophanes proposed by Nakamura *et al.*¹⁷ MerI (fatty acid CoA ligase) and MerA (ACP) activate and load decanoic acid onto the PKS chain. MerC (Type I PKS) installs a malonyl-CoA unit, performs a full reduction of the ketone to the methylene, and installs the alpha methyl group via a C-methyl transferase domain. MerD (Type I PKS) then installs a second malonyl-CoA unit. MerE is a type III PKS which adds an additional two malonyl-CoA units and catalyzes the formation of the resorcinol ring. MerG is a cytochrome P450 which could potentially install the terminal hydroxy groups found in merocyclophanes C and D. MerB, MerF, and MerH have unknown functions, but are expected to be involved in dimerization of the two halves of the compound into the final macrocycle (Figure 5).

Isolation and structure elucidation of the cytotoxic compounds of UIC 10110 have led to the discovery of two new merocyclophane analogs. These compounds, merocyclophane C and merocyclophane D, were found to be cytotoxic, with IC₅₀ values of 1.6 μ M and 0.9 μ M respectively in the MDA-MB-435 cell line. Hollow fiber assays showed no statistically significant antitumor efficacy in Ncr *nu/nu* mice, with the highest concentrations proving too toxic. This increased toxicity in the second trial could be due to the use of a new batch of **1** which could have had a different level of purity than the first batch which would have led to slight differences in concentration between batches. Morphological analysis, validated by phylogenetic analysis of a portion of the 16S rRNA gene, determined the producing strain, UIC 10110, to be a *Nostoc* sp. The discovery of these two analogs corroborates the discovery of merocyclophanes as a novel [7,7] paracyclophane skeleton as proposed by Kang *et al.*¹⁵ The biosynthetic gene cluster that likely produces merocyclophanes has been identified from UIC 10110. The domains in MerC, a type I PKS, provide a proposed

biosynthetic basis for the alpha branched methyl group found in the merocyclophane structure. We also propose a biosynthetic scheme for the production of merocyclophanes based on the published biosyntheses of cylindrocyclophanes and carbamidocyclophanes, although further experiments will be needed to verify the functions of each of the genes.

Experimental Section

General Experimental Procedures

UV spectra were measured using a Shimadzu UV spectrometer UV2301 and scanned from 190 to 360 nm. ECD spectra were recorded on a JASCO J-710 CD spectrometer. IR spectra were measured using a Jasco FTIR-410 Fourier transform infrared spectrometer. 1D and 2D NMR spectra were obtained on a Bruker Avance 900 MHz spectrometer, with all chemical shifts referenced to the residual solvent signal (MeOH-*d*₄ δ_{H} 3.31 and δ_{C} 49.15). HMBC spectra were recorded with average $^3J_{\text{CH}}$ of 8Hz and HSQC spectra were recorded with average $^1J_{\text{CH}}$ of 140 Hz. HRESI mass spectra were acquired using a Shimadzu LC-IT-TOF mass spectrometer.

Biological Material

Nostoc sp. (UIC 10110) was collected from the Colorado River outside of Denver, Colorado in the summer of 2008 (40°17'32" N, 105°51'02" W). The unialgal strain (UIC 10110) was isolated using microisolation techniques and cultured in two 13 L flasks each containing 10 L of Z medium.^{24,25} The culture was grown with sterile aeration and a 18/6 h light/dark cycle with fluorescent lights at 2.6 klx. The culture room was maintained at 22 °C. After 8 weeks of growth, the cell material was harvested and lyophilized.

Strain Identification

UIC 10110 was identified as a *Nostoc* sp. based on morphological indicators including uniseriate trichomes with no branching, isopolar and flexuous with constrictions at the crosswalls of each cell. Cells are spherical and not differentiated at the end of the filament. Heterocytes are solitary and intercalary. Akinetes were undetectable in the filament, and the strain reproduces by disintegration of the colony. The taxonomic determination of UIC 10110 was made in accordance with modern taxonomic systems as designated by Komarek *et al.* 2014.²⁶

DNA Extraction and 16S Amplification

Approximately 250 mg wet cell mass, was centrifuged to pellet the cells. The media was decanted and the cell pellet was resuspended in lysis buffer (2.5 mL, 10 mM Tris, 0.1M EDTA, 0.5% w/v SDS, 20 µg/mL pancreatic RNase, pH 8.0) and lysozyme (1 mg/mL) and incubated in a water bath at 37 °C for 1 h. Proteinase K was added to the lysed cells to a final concentration of 100 µg/mL and incubated in a water bath at 50 °C for 1 h. The mixture was centrifuged to pellet the cells and the supernatant was decanted. DNA was then extracted from the pretreated cells using the Wizard Genomic DNA purification kit (Promega). Purity and concentration of the genomic DNA was determined by nanoDrop UV spectroscopy. A segment of the 16S rRNA gene was amplified by PCR using cyanobacterial specific primers, 109F and 1509R. The PCR reaction contained 2 µL of the genomic

template (25 ng/μL), 2 μL of each primer (10 μM), 1 μL dNTPs (10 μM), 0.5 μL Phusion high fidelity polymerase, 10 μL Phusion HF Buffer, and 32.5 μL nuclease free H₂O. PCR was performed in a Bio-Rad C1000 thermocycler with the following program: denaturation for 2 min at 95 °C, then 35 amplification cycles of 95 °C for 30 sec, 49 °C for 30 sec, 72 °C for 2 min, and a final extension of 72 °C for 5 min. Amplification was confirmed by gel electrophoresis, and the PCR product was purified using a MinElute kit (Qiagen). The purified PCR product was sequenced using the cyanobacterial specific primers stated above as well as an internal primer 359F. The GenBank accession code for the partial 16S rRNA of UIC 10110 is KY380004.

Phylogenetic Analysis

Phylogenetic confirmation of the taxonomy of UIC 10110 was conducted using MEGA 6.0. The partial 16S rRNA sequencing electropherogram was manually inspected and trimmed to a total of 1,115 nucleotides. The sequence was aligned with 42 cyanobacterial 16S rRNA sequences from GenBank including *Microcystis aeruginosa* PCC7941 as an outgroup. Some of the cyanobacterial 16S rRNA sequences selected represent reference strains from Bergey's Manual, and all sequences were at least 1 kb. A multiple sequence alignment was performed using ClustalW with default gap opening and extension penalties. Evolutionary distances were inferred using the neighbor-joining method, with 1000 bootstrap replicates to determine the accuracy of the branches in the tree (Figure 3).

Sequencing

DNA was extracted from UIC 10110 as described above and sequencing libraries were prepared using the Nextera DNA Library Preparation Kit. Sequencing was performed on an Illumina MiSeq. Reads were trimmed by quality score with a limit of 0.01 and all reads below 50 base pairs were discarded. Reads were assembled using the Spades assembler, available through the Illumina BaseSpace platform, using all default settings.²⁷ The assembled genome consisted of approximately 4,000 contigs which were then analyzed by AntiSMASH 3.0.²³ The GenBank accession code for the *mer* gene cluster is KY379971.

Extraction and Isolation

The lyophilized cell material from two 10 L cultures yielded 9.22g of dried cell material. This was extracted via maceration with a solvent mixture of 1:1 CH₂Cl₂ and MeOH (500 mL) three times. The extract was dried in vacuo to yield 1.268 g, which was fractionated using a Diaion HP20 vacuum liquid chromatography column with an isopropanol:H₂O gradient to produce six fractions (60 mL each). Fractions eluted at the following mix of isopropanol:H₂O solvent, 0:100, 20:80, 40:60, 70:30, 90:10, 100:0. The fraction eluting at 70:30 was found to be most active against MDA-MB-435 cell lines. Dereplication of the active fraction revealed novel [7,7] paracyclopentane, merocyclopentane C, as well as the known merocyclopentane A. During isolation of merocyclopentane C, merocyclopentane D was also discovered. The two new merocyclopentanes were isolated by reversed phase semi-preparative HPLC (Varian Microsorb Dynamax C18 semi-preparative column 250 mm × 10 mm, 4 mL/min) with a gradient from 65% to 100% CH₃CN. Merocyclopentane C eluted at 19 minutes, merocyclopentane D eluted at 6 minutes, and merocyclopentane A eluted at 27

minutes. 21.7 mg of merocyclophane C, 2.1 mg of merocyclophane D, and 18.2 mg of merocyclophane A were isolated from the 1.268 g of extract.

Merocyclophane C (1)—Pale yellow amorphous powder; $[\alpha]_D^{25} -32$ (c0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 207 (4.55), 221 (4.02), 272 (3.24) nm; ECD (MeOH) λ_{max} (ϵ) 205.4 (−4.9), 215.8 (−6.0), 277.8 (0.02); IR (neat) ν_{max} 3371 (br), 2972, 2931, 2865, 1621, cm^{-1} ; ^1H and ^{13}C NMR spectroscopic data, Table 1; HRESIMS m/z 567.4173 $[\text{M}-\text{H}]^-$ (calcd for $\text{C}_{36}\text{H}_{55}\text{O}_5$, 567.4169)

Merocyclophane D (2)—White amorphous powder; $[\alpha]_D^{25} -26$ (c0.23, MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (4.6), 221 (4.02), 272 (3.24) nm; ECD (MeOH) λ_{max} (ϵ) 205.8 (−3.6), 216.2 (−4.5), 279.6 (0.1); IR (neat) ν_{max} 3378, 2970, 2932, 2859, 1656, cm^{-1} ; ^1H and ^{13}C NMR spectroscopic data, Table S7; HRESIMS m/z 583.4154 $[\text{M}-\text{H}]^-$ (calcd for $\text{C}_{36}\text{H}_{55}\text{O}_6$, 583.4159)

Cytotoxicity Assay

Following a previously described protocol,²² MDA-MB-435, MDA-MB-231, and OVCAR3 cells in log phase growth were harvested by trypsinization. Cells were seeded in 96-well plates and incubated at 37 °C in 5% CO_2 . Overnight cultures were then treated with either the samples, positive control (paclitaxel), or the vehicle (DMSO) for 72 h. The viability of the cells was evaluated by CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega.²¹ IC_{50} values were calculated from the vehicle control.

Animals

Protocols for the NCr *nu/nu* mice have been previously described.²² The procedure was approved by the University of Illinois at Chicago Animal Care and Use Committee (protocol number 13-057) and institutional guidelines for animal care were followed.

In Vivo Hollow Fiber Assay

Detailed protocols can be found in Ren *et al.*²² Merocyclophane C (1) was dissolved in 30% dimethyl sulfoxide, 30% cremaphor EL, and 40% water. Ncr *nu/nu* mice with intraperitoneally inserted hollow fibers, containing either MDA-MB-231 or OVCAR3, were injected with 1 at 15 mg/kg or 10 mg/kg for four days before being sacrificed. Mice were also injected for four days with paclitaxel at 5 mg/kg or the vehicle, as positive and negative controls, and sacrificed after four days. The hollow fibers were retrieved and the remaining tumor cells were analyzed for growth inhibition using the previously described cytotoxicity assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Core structures of [7,7] paracyclophanes discovered from freshwater cyanobacteria.

Figure 2.
Key 2D correlations used in structure verification of **1** and **2**

Figure 3.

Neighbor-joining tree with 1000 bootstrap replicates showing the evolutionary relationships of known [7,7] paracyclophane producing strains. Monophyletic branches have been collapsed, designated by wedged lines, for a more clear tree. Strains capable of producing [7,7] paracyclophanes with terminal-alkyl chain halogenation are colored in red.

Figure 4. Comparison of merocyclophane, carbamidocyclophane, and cylindrocyclophane biosynthetic gene clusters showing similarities in the functions of the genes and structure of the gene clusters.

CYP merG leads to hydroxy-merocyclophane C and D (substrate unknown: dimer, monomer or starter unit?)

Figure 5. Proposed biosynthesis of merocyclophanes based on identified gene clusters of cylindrocyclophane and carbamidocyclophane.^{17,28}

Table 1Spectroscopic Data (^1H 900 MHz, ^{13}C 225 MHz, MeOH d_4) of Merocyclophane C (1).

merocyclophane C (1)		
Position	δ_{C} , type	δ_{H} (J in Hz)
1	42.1, CH	2.30, m
2	40.9, CH ₂	1.45/1.32, m
3	31.1, CH ₂	0.91/0.67, m
4	32.8, CH ₂	1.27/0.88, m
5	30.9, CH ₂	1.3/0.99, m
6	35.52, CH ₂	1.31, m
7	37.0, CH	3.14, dddd (10.1, 12.1, 5.9, 4.1)
8	116.4, C	
9	158.78, C	
10	104.7, CH	6.04, s
11	147.0, C	
12	109.4, CH	6.0, s
13	157.14, C	
14	42.1, CH	2.30, m
15	40.9, CH ₂	1.45/1.32, m
16	31.1, CH ₂	0.91/0.67, m
17	32.8, CH ₂	1.27/0.88, m
18	30.8, CH ₂	1.3, 0.99, m
19	35.5, CH ₂	1.31, m
20	37.0, CH	3.14, ddd (10.1, 12.1, 5.9, 4.1)
21	116.1, C	
22	158.8, C	
23	104.7, CH	6.04, s
24	146.9, C	
25	109.4, CH	6.0, s
26	157.08, C	
27	35.1, CH ₂	1.96, m
28	31.9, CH ₂	1.19, 1.11 m
29	34.1, CH ₂	1.53/1.45, m
30	63.5, CH ₂	3.47, t (6.8)
31	35.0, CH ₂	1.92, m
32	25.8, CH ₂	1.24/1.21, m
33	24.2, CH ₂	1.30/1.22, m
34	14.7, CH ₃	0.83, t (6.8)
35	23.8, CH ₃	1.14, d (7.2)
36	23.8, CH ₃	1.15, d (7.2)

Table 2

IC₅₀ Values Expressed in μ M of [7,7] Paracyclophanes Against Three Cell Lines MDA-MB-435 (Melanoma), MDA-MB-231 (Breast Cancer), and OVCAR3 (Ovarian Epithelial Cancer)

Compound	MDA-MB-435	MDA-MB-231	OVCAR3
Merocyclophane A (3)	9.8	6.2	5.1
Merocyclophane C (1)	1.6	1.4	1.4
Merocyclophane D (2)	0.9	1.0	2.0
Carbamidocyclophane A	2.4	1.0	4.3
Carbamidocyclophane F	3.2	1.2	4.2
Cylindrocyclophane D	12	13	10
Paclitaxel	0.0031	0.0026	0.0075

Table 3

Genes in the Merocyclophane Biosynthetic Gene Cluster Including Length, BLAST Proposed Identities, and Percent Similarity to the Cylindrocyclophane Biosynthetic Gene Cluster

Gene	Length	Percent Identity to Cyl Gene Cluster	BLAST Proposed Identity Based on Homology Scores
MerA	104 aa	56%	Acyl Carrier Protein
MerB	468 aa	72%	Unkown Protein
MerC	2569 aa	71%	Type I PKS
MerD	1396 aa	59%	Type I PKS
MerE	374 aa	77%	Type III PKS
MerF	199 aa	75%	Methyl Transferase
MerG	460 aa	0%	Cytochrome P450
MerH	921 aa	66%	Hemolysin/Beta Propeller Containing
MerI	612 aa	61%	Acyl-CoA Synthase