Letter

Isolation of Polycavernoside D from a Marine Cyanobacterium

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Title

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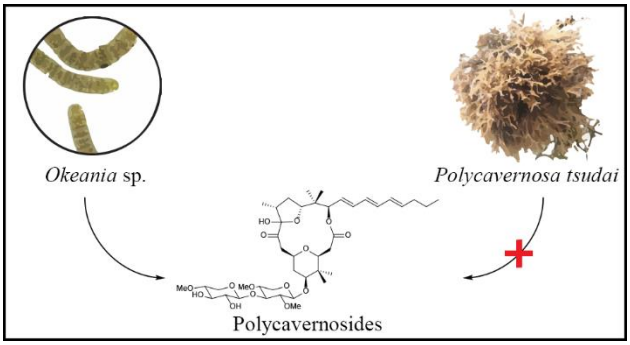

Abstract

The polycavernosides are a unique class of marine-derived macrolides that were implicated in the poisoning of 49 people in the South Western Pacific resulting in 11 deaths. The original source ascribed to these environmental toxins was from the edible red alga *Polycavernosa tsudai* (also known as *Gracilaria edulis*); however, the inability to re-isolate these metabolites from the alga, along with structural resemblance to several marine cyanobacterial natural products, suggested that these compounds derive from these latter photosynthetic prokaryotes. In this current study, we identified a new analog 'polycavernoside D' from an environmental sample of the marine cyanobacterium *Okeania* sp., thus providing the first experimental evidence that these lethal toxins are in fact cyanobacterial secondary metabolites. Moreover, the new metabolite was obtained from a Caribbean cyanobacterial collection, thus suggesting this toxin family to be of broader environmental occurrence than previously realized, and raising concerns about unrecognized human exposure in diverse tropical marine environments.

Introduction

The polycavernosides, originally isolated from red alga *Polycavernosa tsudai*,[1–3] are very potent toxins that were responsible for the deaths of eleven individuals and caused numerous illnesses.[4,5] The mode of toxin exposure was through ingestion of field collected samples of this normally edible red alga. Synthesis and structure-activity relationships (SAR) in mouse toxicity assays have shown that the polyene tail and the disaccharide appendage are both important for activity.[6] While the symptoms documented from the victims as well as the SAR work in mice indicated that the polycavernosides may function as neuromuscular junction toxins, subsequent mechanism of action studies were inconclusive. However, in these latter studies, which used neuro-2a human neuroblastoma cells, the toxic effects were only seen at 12 μM, suggesting that a cell line system poorly recapitulates the relevant neurotoxic

mechanism observed in mice.[7] As a result, many perplexing questions remain about the true source of the toxin, the extent of its distribution in the natural world, its mechanism of neurotoxic activity, and its potential biomedical utility.

Marine cyanobacteria have been an extraordinarily prolific source of structurally diverse secondary metabolites that elicit biological responses.[8] Recent examples include the cytotoxic veraguamides,[9] the anti-inflammatory honaucins and pitinoic acids,[10,11] and the potent anti-malarial lagunamides.[12] Analyses of marine cyanobacterial genomes have shown that these organisms remain underexplored for their unique secondary metabolites, and thus, a rich opportunity exists for new bioactive compound discovery from cyanobacteria.[13]

Based on their structural features, the inability to re-isolate the polycavernosides from the red alga in subsequent efforts, and their extremely low abundance in the algal source (0.00002% of the dry weight), these compounds have been hypothesized to derive from microbial metabolism, possibly that of marine cyanobacteria.[2] The macrolide skeleton with embedded tetrahydropyran ring, polyene tail and distinctive glycosylation are highly reminiscent of other marine cyanobacterial metabolites, such as cyanolide A and lyngbyaloside.[14,15] Additional marine cyanobacterial secondary metabolites have been mistakenly ascribed to other source organisms; for example, dolastatin 10 was thought to derive from the sea hare *Dolabella auricularia*, but later shown to be produced by its diet of marine cyanobacteria (e.g. *Symploca* sp.).[16] To date, however, experimental evidence that the polycavernosides derive from a marine cyanobacterium is lacking.

This study reports our isolation of a new polycavernoside analog, polycavernoside D (**1**), from a red-colored *Okeania* sp. in relatively high yields (0.004% of dry mass), thus identifying it as a metabolic source of these glycosylated macrolides (Figure 1). Bioassay guided fractionation of this extract led to the discovery of compound **1** which had moderate activity against the H-460 human lung carcinoma cell

line (EC_{50} = 2.5 μM). Importantly, the source cyanobacterial collection came from the Atlantic whereas all previous isolations of the polycavernosides came from the Western Pacific, indicating a broad distribution of this family of neurotoxic metabolites, and suggesting the possibility of additional human exposures. Using mass spectrometry and NMR spectroscopy, the planar structure of polycavernoside D was determined to have a slightly different carbon skeleton compared to previously identified polycavernosides. These structural changes may reflect a divergent evolution in their biosynthetic pathways as a result of their respective geographic isolation.

Materials and Methods

Cyanobacteria Collection and 16S Taxonomic Identification. The polycavernoside D producing cyanobacterium (VQR28MAR11-2) was collected by hand using snorkel gear at Punto de Vistas, Puerto Rico (approximately 1 m water depth). Approximately 10 g wet weight of cyanobacterium VQR28MAR11-2 was preserved in RNAlater solution (Qiagen). Morphological characterization of VQR28MAR11-2 used an Olympus IX51 epifluorescent microscope (100×, 20x, 4x) with an Olympus U-CMAD3 camera (SI Figure 1). Taxonomic identification was determined based on comparison of 16S rRNA sequence acquired from the RNA-later preserved environmental sample with previously identified 16S rRNA sequences from classified cyanobacterial species (see SI). Briefly, the 16S sequence was amplified using genomic DNA and cyanobacterial 16S rRNA primers, followed by TOPO cloning (Life Technologies pCR™-4-TOPO®) using the manufacturer's protocol. The plasmids containing the insert were Sanger sequenced using the vector primers M13F and M13R.

Disk Diffusion Assay. A 15 μL sample of the crude extract in DMSO was placed on a filter disk and applied to a matrix containing one of twelve mammalian cell lines (SI Table 1).[17] After 7 to 10 days of incubation, a zone of inhibition of colony formation was defined and quantified in mm by the radius of cell clearance. While the original VLC fraction 2126-H showed potent and selective cytotoxicity against

murine solid tumors, the limited amount of isolated material excluded our further investigation of **1** for its solid tumor selectivity.

H-460 Cytotoxicity Assay. H-460 cell viability was determined by mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, quantified at 570 and 630 nm. After culturing the H-460 cells in 250 µL of media with 2.5 µL of compound in dimethyl sulfoxide (DMSO) for 24 hours, cells were incubated with 1 mg/mL MTT at 37 °C for 25 min, the medium was aspirated, and cells resuspended in 100 µl DMSO for solubilization of the formazan dye. The percent survival was determined by comparison with the negative control group (cells treated with the DMSO vehicle). [18]

Bioassay Guided Fractionation of Polycavernoside D. The crude lipophilic extract from the Puerto Rican cyanobacterial collection VQR28MAR11-2 was fractionated using vacuum-liquid chromatography (VLC) (SI Figure 2) to yield 9 fractions; these were subsequently screened against a suite of mammalian cancer cell lines in the disk diffusion assay (SI Table 1).[17] The second most polar VLC fraction (2126-H) showed potent and selective activity against murine solid tumor cell line colon-38, but was only modestly cytotoxic against murine leukemia cell line L1210 as well as normal untransformed cells (SI Table 1). Fraction 2126-H was fractionated into five fractions with a C18 solid phase extraction (SPE) column using a 20% methanol stepped gradient, and tested for cytotoxicity using the H-460 human lung cancer cell line (SI Figure 3). [18] Fraction 2126-H-V (100% methanol) showed the highest cytotoxicity at both 30 and 3 µg/mL, and was thus selected for HPLC purification of the major constituent, compound **1** (0.3 mg, 8.5% of 2126-H-5). Purified compound **1** was analyzed using linear ion trap Fourier Transform Ion Cyclotron Resonance (LTQ-FT-ICR) mass spectrometry (MS), circular dichroism (CD), and 1D and 2D nuclear magnetic resonance (NMR) experiments to determine its planar and stereo-structure (see SI for more details).

Results and Discussion

Analysis of HRMS and NMR data for compound **1** resulted in the molecular formula of $C_{42}H_{66}O_{15}$, giving 10 degrees of unsaturation. Analysis of 2D NMR data (gCOSY, TOCSY, gHSQC, gHMBC) revealed six isolated spin systems (Figure 2). The first (**1a**) corresponded to an allylic conjugated decanol triene. All of the double bonds were determined to be *trans* based on coupling constant values (SI Table 2). The second spin system (**1b**) consisted of a methylene and hydroxy-methine, whereas the third spin system (**1c**) had a terminal oxygenated methine, followed by a methylene group next to an oxygenated methine, and then terminating with a methylene group. The fourth spin system (**1d**) was made up of a terminal oxygenated methine, next to a methylene group neighboring a methine, and lastly completing the spin system with a terminal methyl group. The fifth (**1e**) and sixth (**1f**) spin systems were two pentose pyranose rings. Analysis of the proton and carbon chemical shifts (SI Table 2) for the anomeric centers of the two saccharides revealed two β-pyranose anomers. In addition to these six spin systems, the ^{1}H NMR of compound **1** possessed signals for three *O*-methyl and two *gem*-dimethyl groups as well as a three *O*-H protons.

Key HMBC correlations from CH-15 and CH_2-2 to C-1 united spin systems **1a** and **1b** through an ester linkage (Figure 2). HMBC correlations from gem dimethyls CH_3-28 and CH_3-29 to C-3, C-4 and C-5 grouped spin systems **1b** and **1c** through quaternary carbon C-4. Likewise, HMBC correlations from hydroxy proton OH-30 to C-9, C-10, and C-11 joined spin systems **1c** and **1d** through an α-hemiketal ketone. This structural arrangement was corroborated by HMBC correlations from CH_2-8 to ketone C-9, and from CH_3-25 to hemiacetal C-10. HMBC correlations from gem dimethyls CH_3-26 and CH_3-37 to C-13, C-14 and C-15 joined spin systems **1a** and **1d** through quaternary carbon C-14, enabling closure of the 16-membered macrolide. This connectivity was also supported by HMBC correlations from CH-15 and CH_2-12 to quaternary carbon C-14.

Spin systems **1e** and **1f** were connected through a 1,3-disaccharide linkage as shown by HMBC correlation from CH-3_{xyl} to C-$1_{xyl'}$. The pattern of methoxy groups on these saccharides was identified by HMBC correlations from the methoxy methyl groups to their corresponding positions on the saccharide backbones (Figure 2). The disaccharide was connected to the macrolide via oxygenated carbon position C-5 as shown by reciprocal HMBC correlations between C-5 and the β-anomeric center C-1_{xyl}. The disaccharide connectivity was verified with MS/MS fragmentation which gave ions for both of the monosaccharides as well as the aglycone (SI Figure 4). Lastly, in order to satisfy the molecular formula and degrees of unsaturation, C-3 and C-7 were connected through an ether bond to form a tetrahydropyran ring whereas C-10 and C-13 were joined through a second ether bond to form a furan ring and a hemiketal functionality.

In order to determine the relative configuration for the macrolide portion of **1**, a combination of nOe correlations from ROESY data and J_{HH} values were analyzed. Key nOe correlations along the macrolide backbone for C-3 through C-16 suggested that the macrolide relative stereochemistry was similar to that of polycavernoside A (**2**) (Figure 2). In particular, the key nOe from CH_2-8 to oxygenated CH-13, which was also crucial in determining the relative stereochemistry of **2**, was also seen for compound **1**.[19] Indeed, comparison of the macrolide ^{1}H NMR shifts for **1** and **2** showed them to be nearly identical (SI Table 2), strongly suggesting that they share the same relative stereochemistry. This proposed relative stereochemistry is also supported by previous work on the total synthesis of epimers of **2** which revealed that they have very different chemical shifts.[20]

The relative configuration of xylose was determined by analysis of chemical shifts, J_{HH} values, and nOe correlations. The anomeric proton was determined to be in an axial position based on the upfield proton chemical shift and downfield carbon chemical shifts that are characteristic of this arrangement.[21–23] The proton coupling constant values of all of the oxygenated methines of this saccharide were larger than 7 Hz, corresponding to glycosyl methines with all protons in axial

configurations (SI Table 2). In agreement with this deduction, the ^{1}H NMR chemical shifts of all of the oxygenated methines were less than 3.65 ppm, characteristic of axially oriented methines.[21] Finally, the observed nOe correlations between CH-1_{xyl}/CH-3_{xyl} and CH-1_{xyl}/CH-5_{xyl} could only be rationalized with all of these methines in an axial configuration, and is consistent with the assignment of β-xylose. Lastly, the relative configuration between xylose and the macrolide was indicated by nOe correlations between CH-1_{xyl}/CH_3-4 and CH_3-6_{xyl}/CH_3-5.

Analogous to xylose, the relative configuration for xyl' was identified from the multiphasic analysis of chemical shifts, J_{HH} values, and nOe correlations. The anomeric proton was determined to be in an axial position based on its corresponding ^{1}H and ^{13}C NMR chemical shifts.[21–23] Despite the fact that the values for the coupling constants between protons were relatively small for an all axial configuration, the observed nOe correlations between CH-$1_{xyl'}$/CH-$3_{xyl'}$ and CH-$1_{xyl'}$/CH-$5_{xyl'}$ strongly supported this all axial configuration (Figure 2). Finally, the relative configuration between xyl and xyl' was determined by nOe correlations between CH-3_{xyl}/CH-$1_{xyl'}$ and CH_3-7_{xyl}/CH-$5'_{xyl'}$.

Previously, the absolute stereochemistry of polycavernoside A (**2**) was determined by comparing the circular dichroism (CD) spectrum of the natural substance with a synthetic standard.[19] We reasoned that the Cotton effect of the triene chromophore in polycavernoside D (**1**) would strongly depend on the configurational arrangement at C-15, and based on the relative configurational assignments outlined above, would be indicative of the overall stereoconfiguration. The CD spectra and Cotton effects at the triene absorption maxima for compounds **1** and **2** were very similar with similar signs and magnitudes (SI Figure 5), indicating the absolute configuration at C-15 was R. Thus, the absolute configuration of polycavernoside D is as shown in Figure 1.

While the polycavernosides were originally isolated from a commonly eaten red algal source, *Polycavernosa tsudai*, our isolation of this new polycavernoside analog was from a cyanobacterium with

a 98.8% 16S rRNA sequence identity to an *Okeania* sp,[24] thus providing the first evidence that strongly implicates the polycavernosides are of cyanobacterial origin (GenBank accessions: GU724195.1). Structurally, polycavernoside D (**1**) represents a unique carbon backbone with three significant changes compared to known polycavernosides. First, the polyene tail is not branched in compound **1** as it is in polycavernoside A (**2**), and moreover, appears to be extended by one additional two-carbon acetate unit. Second, on C-4 there appears to be an additional SAM mediated methylation in compound **1**. Lastly, the disaccharide in polycavernoside D is comprised of two xylose units versus the fucosyl-xylose formulation in **2**, suggesting a different selectivity in the terminal glycosyltransferase.

Polycavernoside D (**1**) was evaluated in a standard MTT-based cell toxicity assay using the H-460 human lung cancer cell line as previously described.[18] While compound **1** showed a dose dependent response with an EC_{50} value of 2.5 μM, the maximum cell growth inhibition achievable was only 50% (SI Figure 6). Interestingly, these results are in agreement with those obtained previously for other polycavernosides in that none of these metabolites are overtly potent cytotoxins.[1] In order to explain the potent animal toxicity, the polycavernosides likely modulate some aspect of intercellular interaction, such as at neurochemical junctions and receptors.

Finally, there are several implications of the finding that this cyanobacterial collection was obtained from the Atlantic Ocean whereas all previous polycavernosides were obtained from Pacific collections of "red algae" (but in all likelihood contaminated by cyanobacteria). First, because of the significant physical separation between these collections, it can be predicted that they have been geographically isolated for an extended period, and this may underlie the extent of the biosynthetic differences between their polycavernoside-type natural products. Second, because cyanobacteria of the genus *Okeania* are pantropical in their distribution,[24] the impact of isolating the polycavernosides from an *Okeania* sp. implies a potentially larger distribution and hence human exposure to polycavernoside-type environmental toxins.

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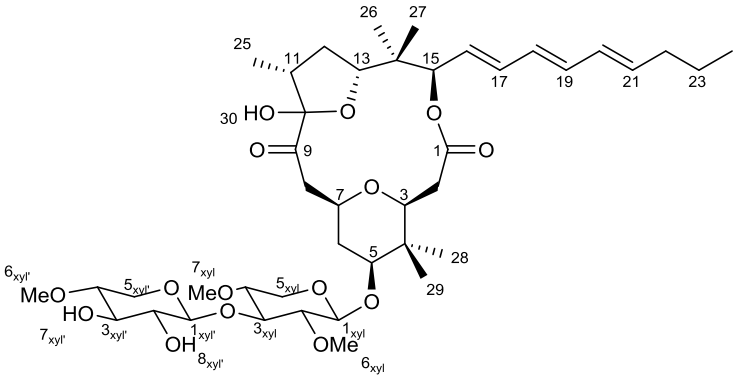
Supporting Information

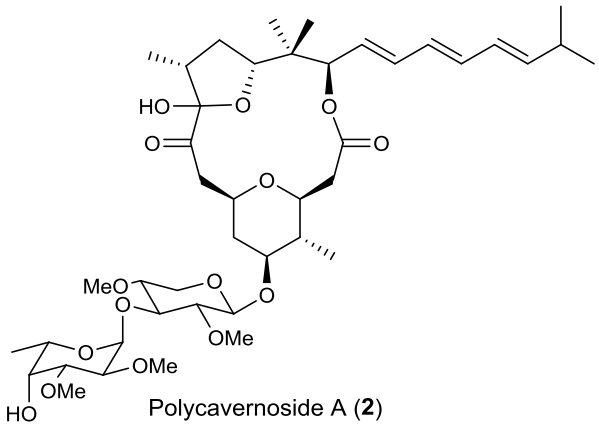
Supporting methods, VLC and C18-SPE extraction scheme, 2126 extract cytotoxicity, MS/MS analysis, CD spectrum, NMR data (SI Figure 7), and VQR28MAR11-2 microscope images are found in supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

212 **Figures**

213 **Figure 1**. Polycavernosides A (**2**) and D (**1**).



Polycavernoside D (**1**)



Polycavernoside A (**2**)

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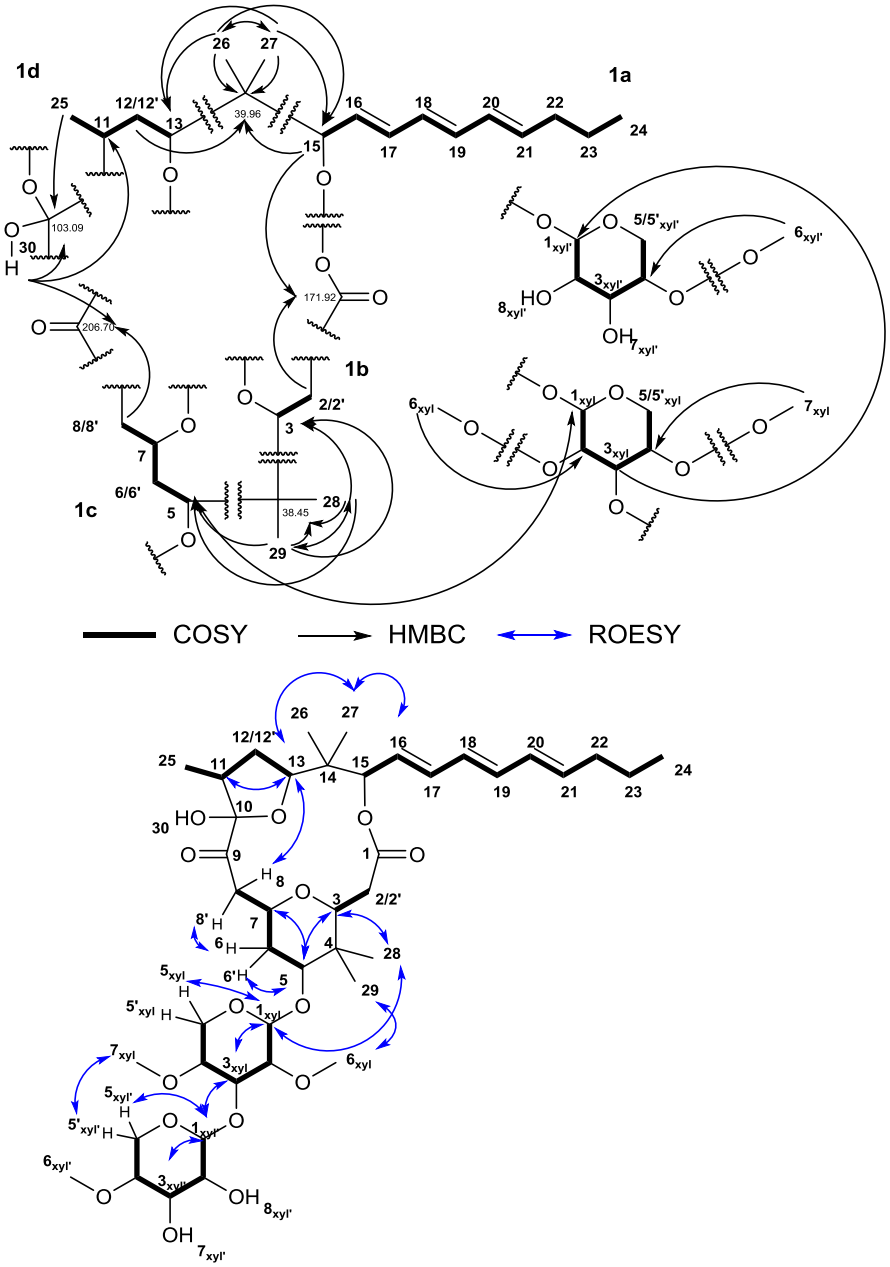
Figure 2. Structure determination of polycavernoside D (**1**).



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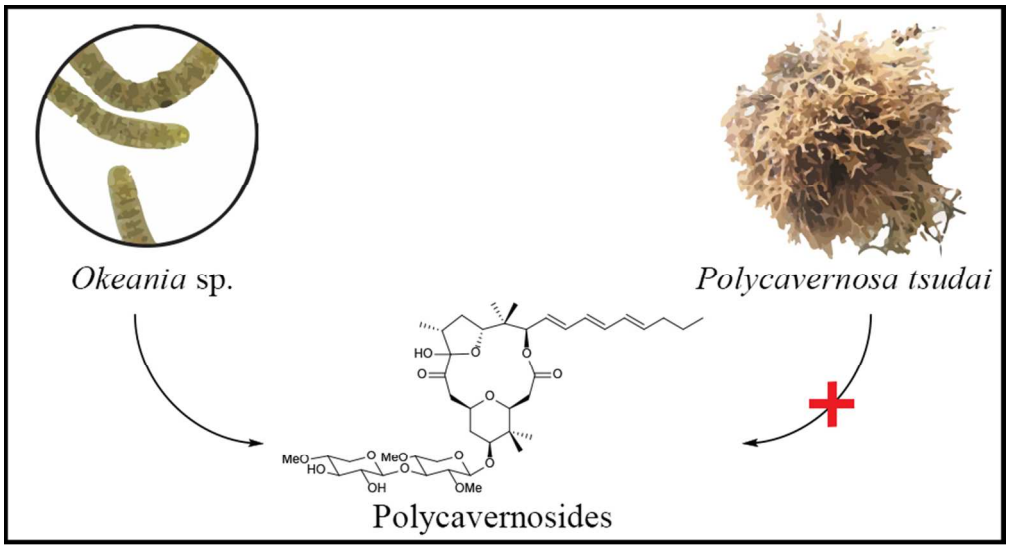

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