Palmyramide A, a Cyclic Depsipeptide from a Palmyra Atoll Collection of the Marine Cyanobacterium *Lyngbya majuscula*†

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Bioassay-guided fractionation of the extract of a consortium of a marine cyanobacterium and a red alga (Rhodophyta) led to the discovery of a novel compound, palmyramide A, along with the known compounds curacin D and malyngamide C. The planar structure of palmyramide A was determined by one- and two-dimensional NMR studies and mass spectrometry. Palmyramide A is a cyclic depsipeptide that features an unusual arrangement of three amino acids and three hydroxy acids; one of the hydroxy acids is the rare 2,2-dimethyl-3-hydroxyhexanoic acid unit (Dmhha). The absolute configurations of the six residues were determined by Marfey's analysis, chiral HPLC analysis, and GC/MS analysis of the hydrolysate. Morphological and phylogenetic studies revealed the sample to be composed of a *Lyngbya majuscula*−*Centroceras* sp. association. MALDI-imaging analysis of the cultured *L. majuscula* indicated that it was the true producer of this new depsipeptide. Pure palmyramide A showed sodium channel blocking activity in neuro-2a cells and cytotoxic activity in H-460 human lung carcinoma cells.

Cyanobacteria are well known to produce a wide variety of secondary metabolites displaying significant structural diversity and biological activity.[1,2] Because the production of secondary metabolites depends on both the collection location and specific species, our program has focused on collections from a wide variety of tropical and subtropical locations including Panama, Papua New Guinea, Curaçao, Jamaica, and Puerto Rico, and this has generally been a productive approach.[2] In this vein, we recently had the opportunity to examine the unique marine flora and cyanobacteria of Palmyra Atoll in the Northern Pacific Ocean, a remote Pacific island with a unique biogeography.[3] Bioassay-guided fractionation of the extract of a cyanobacterial/red algal consortium led to the discovery of a novel cyclic depsipeptide, palmyramide A (**1**), along with the known compounds curacin D and malyngamide C.[4,5] This paper describes the isolation, structural determination including stereostructure, biological activity, and identification of the producing organism of **1**.

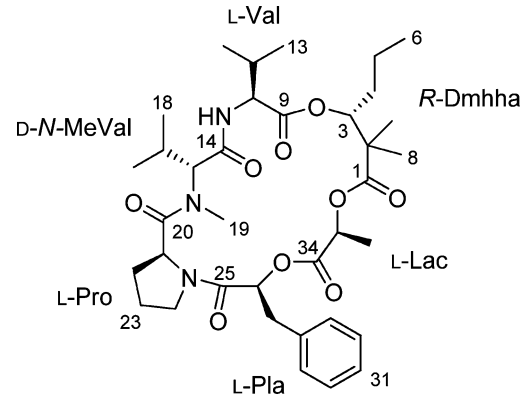

Figure 1. Structure of palmyramide A (**1**).

Results and Discussion

The crude extract of the consortium of cyanobacterium and red alga collected at Palmyra Atoll was subjected to silica gel vacuum liquid chromatography and assayed for sodium channel blocking activity and cytotoxic activity. The bioactive fraction eluted with 100% EtOAc and was further purified by C18 SPE cartridge and repetitive reversed-phase HPLC to yield palmyramide A (**1**) (Figure 1) as a colorless glassy solid.

High-resolution ESIMS of **1** gave $[M + H]^+$ and $[M + Na]^+$ peaks at *m*/*z* 672.3852 and 694.3669, respectively, indicating a molecular formula of $C_{36}H_{53}N_3O_9$ and requiring 12 degrees of unsaturation. The ^{1}H NMR spectrum possessed five aromatic protons at δ_H 7.23−7.28, an amide NH proton at δ_H 6.55, an *N*-methyl group at δ_H 2.85, and two singlet methyl groups at δ_H 1.16 and 1.21. Correspondingly, the ^{13}C NMR spectrum revealed the presence of six ester/amide carbonyls and a monosubstituted phenyl ring at δ_C 127.4, 128.6 (2C), 129.7 (2C), and 134.9, accounting for 10 of the 12 degrees of unsaturation.

Analysis of 1- and 2D-NMR spectra including COSY, TOCSY, HSQC, and HMBC led to the assignments of three amino acids [valine (Val), *N*-methylvaline (*N*-MeVal), and proline (Pro)] and three hydroxy acids (3-phenyllactic acid (Pla), lactic acid (Lac), and 2,2-dimethyl-3-hydroxyhexanoic acid (Dmhha)) (Table 1). The *N*-methyl group of *N*-MeVal was revealed by HMBC correlations (H-19/C-15, H-15/C-19) and the chemical shifts of the NMe (δ_H 2.85, δ_C 30.5). In the Pla residue, the proton chemical shifts at δ_H 7.23−7.28 (monosubstituted benzene ring), δ_H 3.13 and 2.95 (methylene), and δ_H 5.02 (methine) were very similar to those reported for phenylalanine. However, the carbon chemical shift of the methine (δ_C 70.9) was consistent with that of an oxymethine, indicating that this residue was not phenylalanine but rather 3-phenyllactic acid. The proton chemical shifts of the Lac residue at δ_H 1.47 (methyl) and 4.81 (methine) are also very similar to those reported for alanine, but once again the carbon chemical shift of the methine (δ_C 69.0) indicated that this residue was an α-hydroxy acid, namely, lactic acid. The identity of the Dmhha residue was deduced through analysis of TOCSY, COSY, and HMBC correlations as follows: the proton spin system from H-3 to H-6 was disclosed by TOCSY, and two singlet methyl proton resonances H-7 (δ_H 1.21) and H-8 (δ_H 1.15) gave HMBC cross-peaks to C-1, C-2, and C-3. Oxygenation at C-3 was once again

† Dedicated to the late Dr. John W. Daly of NIDDK, NIH, Bethesda, Maryland, and to the late Dr. Richard E. Moore of the University of Hawaii at Manoa for their pioneering work on bioactive natural products.

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Table 1. NMR Spectroscopic Data (600 MHz, $CDCl_3$) for Palmyramide A

residue	position	δ_C mult	δ_H (mult, *J* in Hz)	HMBC
Dmhha	1	174.9 qC		
	2		46.4 qC	
	3	77.2 CH	5.57 (dd, 9.6, 1.8)	C-1, C-2, C-4, C-5, C-7, C-8
	4a	31.6 CH_2	1.43 (m)	C-3, C-5, C-6
	4b		1.52 (m)	C-5, C-6
	5	19.6 CH_2	1.36 (m)	C-3, C-4, C-6
	6	13.9 CH_3	0.92 (t, 7.2)	C-4, C-5
	7	16.9 CH_3	1.21 (s)	C-1, C-2, C-3, C-8
	8	24.3 CH_3	1.16 (s)	C-1, C-2, C-3, C-7
Val	9	170.1 qC		
	10	58.3 CH	4.46 (dd, 5.4, 3.0)	C-9, C-12, C-13
	11	30.5 CH	2.38 (dqq, 3.0, 7.2, 7.2)	C-9, C-10, C-12, C-13
	12	17.8 CH_3	0.89 (d, 7.2)	C-10, C-11, C-13
	13	18.3 CH_3	0.98 (d, 7.2)	C-10, C-11, C-12
	NH		6.55 (d, 5.4)	C-10, C-14
N -MeVal	14	169.7 qC		
	15	61.7 CH	4.42 (d, 10.8)	C-14, C-16, C-17, C-18, C-19, C-20
	16	27.3 CH	2.13 (m)	C-14, C-15, C-17, C-18
	17	18.8 CH_3	0.70 (d, 6.6)	C-15, C-16, C-18
	18	19.3 CH_3	0.94 (d, 6.6)	C-15, C-16, C-17
	19 (*N*-CH_3)	30.5 CH_3	2.85 (s)	C-15, C-20
Pro	20	171.2 qC		
	21	56.7 CH	3.22 (dd, 7.8, 3.0)	C-22, C-23
	22	30.7 CH_2	1.50 (m)	C-20, C-23, C-24
	23a	22.0 CH_2	1.56 (m)	C-22, C-24
	23b		1.68 (m)	C-22, C-24
	24a	46.7 CH_2	3.42 (ddd, 12.0, 6.6, 6.6)	C-22, C-23, C-25
	24b		3.57 (ddd, 12.0, 8.4, 4.8)	C-22, C-23
Pla	25	167.2 qC		
	26	70.9 CH	5.02 (dd, 12.0, 4.8)	C-25, C-27, C-28, C-34
	27a	39.2 CH_2	2.95 (dd, 12.0, 4.8)	C-25, C-26, C-28, C-29/33
	27b		3.13 (dd, 12.0, 4.8)	C-25, C-26, C-28, C-29/33
	28	134.9 qC		
	29/33	129.7 CH	7.23 (m)	C-27, C-31
	30/32	128.6 CH	7.28 (m)	C-28, C-30/32
	31	127.4 CH	7.25 (m)	C-29/33
Lac	34	168.3 qC		
	35	69.0 CH	4.81 (q, 6.6)	C-34, C-36, C-1
	36	16.7 CH_3	1.47 (d, 6.6)	C-34, C-35

inferred from its ^{13}C NMR chemical shift at δ_C 77.2. The remaining required degree of unsaturation suggested the overall monocyclic nature of **1**.

The sequencing of these residues in **1** was accomplished by interpretation of HMBC correlations (Table 1). Correlations from the NH proton or *N*-methyl protons to the neighboring carbonyl carbons were observed between Val/*N*-MeVal and *N*-MeVal/Pro. Correlations from methine or methylene protons to carbonyl carbons of the neighboring amino or hydroxy acids were also observed between Pro/Pla, Pla/Lac, and Lac/Dmhha. Thus, the planar structure of **1** was determined as shown in Figure 1.

The absolute configurations of the three amino acids in palymramide A (**1**) were determined by HPLC analysis of the acid hydrolysate derivatized with Marfey's reagent [*N*α-(2,4-dinitro-5-fluorophenyl)-L-valinamide: DFVA].[6] LC/MS analysis of the derivatives eluting by a linear gradient MeCN/H_2O (0.1% HCOOH) revealed the presence of D-*N*-MeVal and L-Pro, whereas the configuration of the Val residue could not be determined because of the overlap between the peaks of L-Val and Marfey's reagent. After exploring a variety of HPLC conditions, LC/MS analysis using a linear gradient of MeOH/H_2O (0.1% HCOOH) and a Merck LiChrospher 100 RP-18 column enabled separation of the peaks of L-Val and the Marfey's reagent and, thus, allowed determination of the Val residue in **1** to be L. The absolute configurations of Pla and Lac were determined as L by chiral HPLC analysis. The absolute configuration of the β-hydroxy group of Dmhha was deduced by chromatographic comparisons with authentic *S*- and *R*-Dmhha, which were stereoselectively synthesized via a tertiary aldol reaction (Figure 2).[7] The absolute configurations of the intermediates **4a** and **4b** formed in these syntheses were confirmed

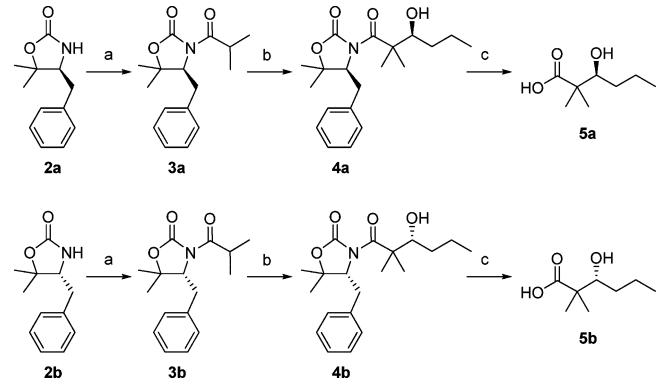

Figure 2. Stereoselective synthesis of *S*- and *R*-Dmhha. (a) 1. *n*-BuLi, THF, −78 °C. 2. Isobutyryl chloride. (b) 1. LDA, THF, −78 °C. 2. Ti(O-*i*-Pr)$_3$Cl. 3. Butanal. (c) 1. 30% aqueous H_2O_2. 2. LiOH/H_2O, THF/H_2O (4:1).

by the Mosher ester method[8] and optical rotations. Chiral GC/MS analysis of the Dmhha methyl ester liberated from **1** by acid hydrolysis revealed only the presence of *R*-Dmhha. Thus, the absolute configuration of palmyramide A (**1**) was determined as shown in Figure 1.

As noted, palmyramide A (**1**) was isolated from an environmental sample composed of a consortium of a cyanobacterium and a red alga (Figure 3A and B) and obtained from two different populations collected from different sampling sites at Palmyra Atoll. Morphological and phylogenetic studies revealed that both samples were of similar composition and composed of *Lyngbya majuscula* Harvey

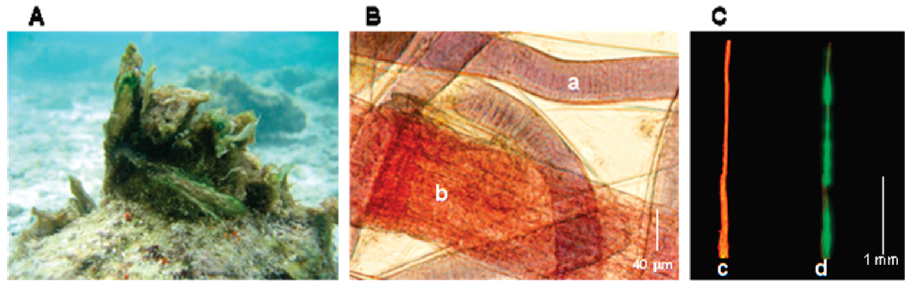

Figure 3. MALDI-TOF imaging of the palmyramide A (**1**)-producing cyanobacterium *Lyngbya majuscula*. (A) Photograph of the consortium of *Lyngbya majuscula* and *Centroceras* sp. at the time of collection. (B) Photomicrograph of the consortium of *Lyngbya majuscula* (a) and *Centroceras* sp. (b). (C) Epifluorescent image of the cultured *Lyngbya majuscula* at 590 nm (c, orange) and MALDI image of *m/z* 672 (= palmyramide A, **1**, d, green).

ex Gomont (Cyanophyta) and *Centroceras* sp. (Rhodophyta). The *L. majuscula* appeared as blackish tufts approximately 10 cm in height (Figure 3A). Microscopically, the *Lyngbya* filaments were long (>1 cm), straight or slightly waved, cylindrical, and 40.2 ± 3.4 μm (n = 3) wide. The trichomes were enclosed with distinct visible, colorless sheaths. The cells were disk-shaped (39.1 ± 2.8 μm wide and 3.5 ± 0.9 μm long; n = 30) with no or only very small constrictions at the cross-walls. The terminal cells were rounded and noncapitated, and lacked calyptras. Taxonomic identifications and evolutionary histories were inferred for both the *L. majuscula* and *Centroceras* sp. components of this consortium using the conserved 16S/18S (SSU) rRNA genes and the more variable RNA polymerase γ-subunit (rpoC1) gene. Furthermore, both genetic markers were obtained from the consortium obtained at both sampling sites and confirmed that these were the same two organisms in each case. An identical 16S rRNA gene (1373 base pairs or ~95% of the gene coverage; GenBank Acc. No. GQ231522) and rpoC1 gene (866 base pairs; GenBank Acc. No. GQ231521) were obtained from the *L. majuscula* of both populations. The evolutionary history was inferred from the 16S rRNA gene using the Minimum Evolution and MrBayes algorithms, which resulted in conserved tree topologies and similar bootstrap support. These collections of *L. majuscula* claded tightly (<1% sequence divergence) with other Pacific strains of *Lyngbya* within the "marine *Lyngbya* lineage" (Figure 4). The 18S rRNA genes (206 bp; GenBank Acc. No. GQ246180) obtained from the two collections of *Centroceras* sp. were also identical in sequence.

In order to determine which organism was responsible for production of palmyramide A (**1**), a natural product MALDI-TOF-imaging (npMALDI-I) approach was applied to the environmental samples stored in either EtOH/H_2O (1:1) at −20 °C or RNA stabilization solution (RNAlater) at −20 °C.[9,10] In principal, the npMALDI-I approach can visualize the spatial distribution of secondary metabolites in intact tissues of marine organisms. However, in this case the molecular weight of **1** was not detected in any of the *L. majuscula* or *Centroceras* sp. samples. We reasoned that this surprising result might be due to the instability of the palmyramide in the preserved biological samples. However, we were able to adapt into laboratory culture the cyanobacterial component of this consortium, and npMALDI-I of fresh *Lyngbya* filaments possessed a robust signal for a compound with the molecular weight of **1**, indicating that *L. majuscula* is the producer of this new depsipeptide (Figure 3C).

Pure palmyramide A (**1**) was assayed for sodium channel blocking and cancer cell cytotoxic activities (presumably due the presence of one *N*-methyl amide in **1**, it occurs as a 95:5 mixture of major and minor conformers by ^{1}H NMR; see Supporting Information).[11,12] In the sodium channel blocking assay, **1** inhibited the veratridine- and ouabain-induced sodium overload and resulting cytotoxicity in neuro2a cells, presumably by blocking the voltage-gated sodium channel with an IC_{50} value of 17.2 μM. In H-460 human lung carcinoma cells, palmyramide A (**1**) showed modest cytotoxic effects with an IC_{50} value of 39.7 μM.

Palmyramide A (**1**) is a novel cyclic depsipeptide that features an unusual arrangement of three amino acids and three hydroxy acids. Among them, Dmhha is the most unusual hydroxy acid and has previously been reported only in guineamides E and F, two metabolites isolated from Papua New Guinea collections of *Lyngbya majuscula*.[13] Since environmental samples of marine organisms are almost always collected as a consortium, as mixtures, or with symbionts, it is necessary to determine the true source of interesting secondary metabolites by other approaches. The npMALDI-I method is well suited to directly observing the physical location of metabolites in intact marine organisms or assemblages.[10] In this case, palmyramide A (**1**) was isolated from a consortium of two organisms, a cyanobacterium and a red alga; however, analysis by npMALDI-I showed that *L. majuscula* is the true producer. Indeed, the cyanobacterium *L. majuscula* is one of the most prolific producers of natural products, with more than 185 reported compounds to date.[2] In this study, our investigation of *L. majuscula* from a new geographical region was rewarded by the discovery of a new sodium channel blocking and cytotoxic cyclic lipopeptide, palmyramide A (**1**), and suggests that a continued investigation of this organism from new locations will be productive in the discovery of novel natural compounds with pharmaceutical potential.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO P-2000 polarimeter. IR and UV spectra were recorded with a Nicolet IR-100 FT-IR and Beckman-Coulter DU800 spectrophotometer, respectively. NMR spectra were recorded on Varian Inova 500 and Bruker Avance III DRX600 with the solvent $CDCl_3$ (δ_H at 7.26, δ_C at 77.0) used as an internal standard. High-resolution mass spectra were obtained on a Thermo Scientific LTQ-XL Orbitrap mass spectrometer. LC/MS analysis was carried out on a Finnigan LCQ advantage mass spectrometer with a Finnigan Surveyor HPLC system. HPLC was performed using a Waters 515 pump and a Waters 996 photodiode array spectrometer.

Collection. The consortium of a cyanobacterium and a red alga was collected by hand from shallow water (<1 m) at two different sites in the western lagoon south of Strawn Island, Palmyra Atoll, USA, in August 2008 (GPS coordinates: 05′53.04 N, 162′05.161 W and 05′52.172 N, 162′05.047 W). The environmental samples were stored in EtOH/H_2O (1:1) at −20 °C, while the genetic materials were preserved in RNA stabilization solution at −20 °C (RNAlater, Ambion Inc.). Voucher specimens are available from WHG as collection numbers PAL 8/17/08-2 and PAL 8/15/08-1.

Extraction and Isolation. Approximately 417.1 g (dry weight) of the consortium was extracted repeatedly with CH_2Cl_2/MeOH (2:1) to yield 7.1 g of crude extract. A portion of the extract (1.5 g) was subjected to silica gel vacuum liquid chromatography (VLC, hexanes/EtOAc/MeOH) to produce nine chemically distinct fractions. The fraction eluted with EtOAc was subjected to C18 SPE (MeOH/H_2O, stepwise) and repetitive reversed-phase HPLC (Phenomenex Jupiter C18, 10 × 250 mm, 70% MeCN, 3 mL/min) to obtain 6.1 mg of

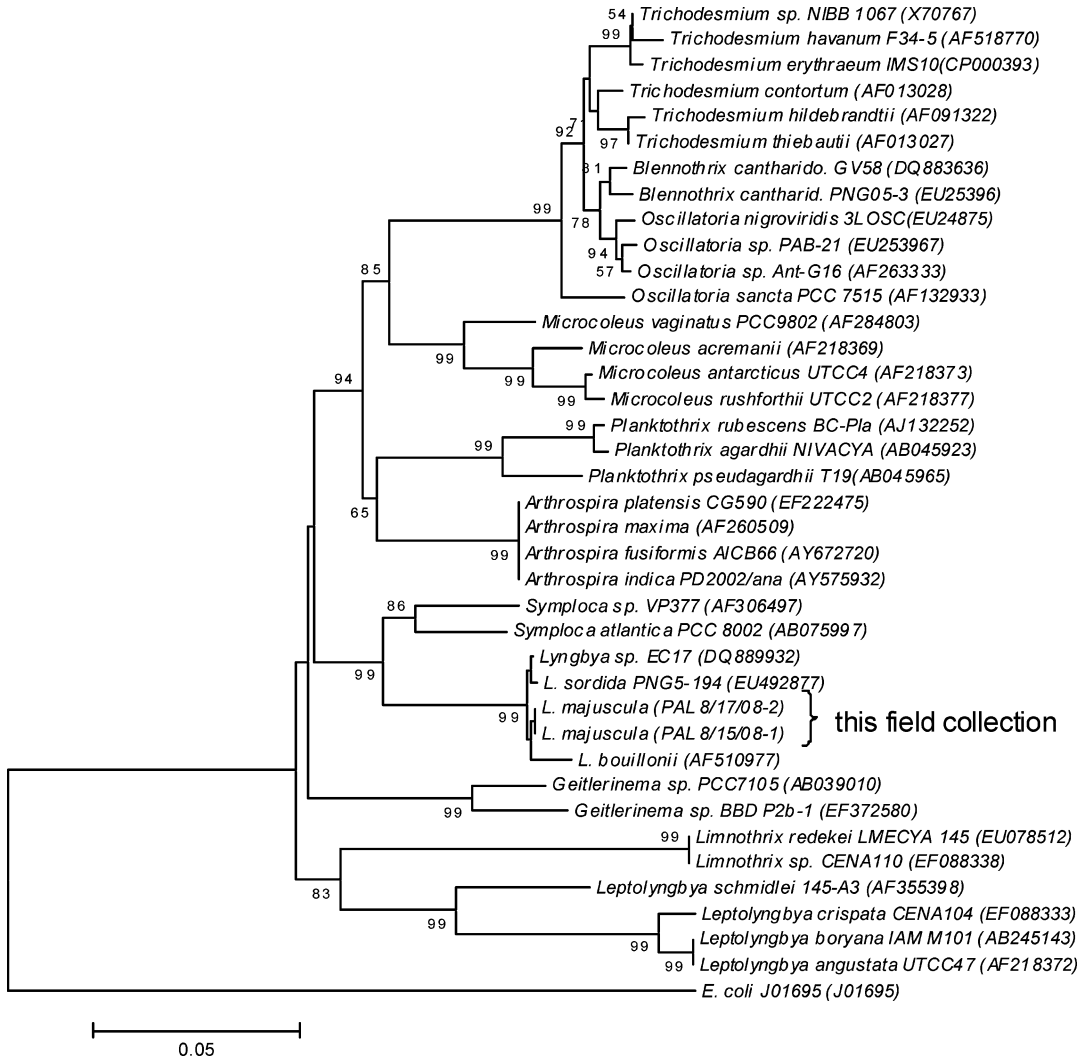

Figure 4. Phylogenetic tree of the cyanobacterial order Oscillatoriales based on the 16S rRNA gene and placement of this field collection of *Lyngbya majuscula*.

palmyramide A (**1**, 20.1 min) and 2.0 mg of malyngamide C (14.1 min). The fraction eluting with 40% EtOAc in hexanes was purified by silica gel column chromatography [hexane/EtOAc (6:1)] to yield 28.6 mg of curacin D.

Palmyramide A (1): colorless glass; $[\alpha]_D$ +19.6 (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 203 (4.27); IR (neat) ν_{max} 3409, 2965, 2876, 1741, 1660, 1504, 1454, 1387, 1261, 1197, 1146, 1100 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m*/*z* 672.3852 $[M + H]^+$ (calcd for $C_{36}H_{54}N_3O_9$, 672.3855).

Marfey's Analysis. Palmyramide A (0.5 mg) in 0.5 mL of 6 N HCl was heated in a sealed tube at 110 °C for 17 h, and the reaction mixture extracted with EtOAc. The aqueous layer was dried under N_2, and the residue dissolved in 400 μL of H_2O. Subsequently, 200 μL of this solution was treated with 40 μL of 1% DFVA solution in acetone and 8.5 μL of 1 M $NaHCO_3$ and heated at 90 °C for 5 min. After cooling to room temperature, 20 μL of 1 N HCl was added and the solution was analyzed by LC/MS on a Merck LiChrospher 100 RP-18 (4 × 125 mm) at a flow rate of 0.8 mL/min using a linear gradient from MeCN/H_2O (30:70, 0.1% HCOOH) to MeCN/H_2O (70:30, 0.1% HCOOH) over 60 min. The DFVA derivatives from the acid hydrolysate were identified by comparing the retention times with those of authentic standards, as follows: D-*N*-MeVal 20.9 min (L-*N*-MeVal 15.8 min) and L-Pro 8.0 min (D-Pro 10.6 min). The configuration of Val could not be determined in the above solvent system because L-Val and the Marfey's reagent coeluted. However, employing a linear gradient from MeOH/H_2O (30:70, 0.1% HCOOH) to MeOH/H_2O (70:30, 0.1% HCOOH) over 60 min cleanly resolved these peaks and identified the configuration of Val in **1** to be L (L 34.1 min, D 51.5 min).

Chiral HPLC Analysis. Palmyramide A (0.5 mg) in 0.5 mL of 6 N HCl was heated in a sealed tube at 110 °C for 17 h, and the reaction mixture extracted with EtOAc. The organic layer was dried under N_2, and the residue dissolved in 200 μL of MeOH. The hydrolysate was analyzed by chiral HPLC on a Phenomenex Chirex 3126 (4.6 × 250 mm) at a flow rate of 0.7 mL/min with UV detection at 254 nm. The isocratic solvent system of 2.0 mM $CuSO_4$ in H_2O was used to determine the configuration of L-Lac (L 16.4 min, D 20.3 min). The isocratic solvent system of 2.0 mM $CuSO_4$ in H_2O/2-propanol (85:15) was used to determine the configuration of L-Pla (L 53.2 min, D 74.1 min).

(*S*)-4-Benzyl-3-isobutyryl-5,5-dimethyloxazolidin-2-one (3a). Oxazolidinone **2a** (102 mg, 0.497 mmol) was dissolved in dry THF (1.3 mL) and treated with *n*-BuLi (1.42 M in hexanes, 350 μL, 0.497 mmol, 1 equiv) dropwise at −78 °C. After 1 h, isobutyryl chloride (130 μL, 1.242 mmol, 2.5 equiv) in THF (0.8 mL) was added at −78 °C. The reaction went to completion within 1 h, as monitored by TLC, and was quenched with H_2O (2.1 mL). The mixture was extracted with Et_2O (4 × 4 mL), dried over $MgSO_4$, and concentrated *in vacuo*. The product was purified by flash chromatography on silica gel 60 (Et_2O/hexanes, 1:19), yielding the title compound as a nearly colorless oil (132.5 mg, 97% yield): $[\alpha]_D$ −34.0 (*c* 0.87, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.15 (3H, d), 1.17 (3H, d), 1.36 (3H, s), 1.38 (3H, s), 2.89 (1H, dd), 3.09 (1H, dd), 3.76 (1H, m), 4.51 (1H, dd), 7.21−7.32 (5H, m); HRMS *m*/*z* 275.1516 $[M]^+$ (calcd for $C_{16}H_{21}NO_3$, 275.1516).

(*R*)-4-Benzyl-3-isobutyryl-5,5-dimethyloxazolidin-2-one (3b). Oxazolidinone **3b** was prepared in the same way as oxazolidinone **3a** from the D-phenylalanine-derived oxazolidinone **2b** (81% yield, due to a partial, accidental loss of product): $[\alpha]_D$ +33.6 (*c* 1.08, $CHCl_3$); ^1H

NMR ($CDCl_3$) δ 1.16 (3H, d), 1.17 (3H, d), 1.36 (3H, s), 1.38 (3H, s), 2.89 (1H, dd), 3.09 (1H, dd), 3.76 (1H, m), 4.51 (1H, dd), 7.21−7.32 (5H, m); HRMS *m/z* 275.1517 $[M]^+$ (calcd for $C_{16}H_{21}NO_3$, 275.1516).

(*S*)-4-Benzyl-3-(3-hydroxy-2,2-dimethylhexanoyl)-5,5-dimethyloxazolidin-2-one (4a). Oxazolidinone **3a** (47.6 mg, 0.173 mmol) was dissolved in dry THF (2 mL) and added dropwise to a solution of LDA (1.5 equiv) in dry THF (1.76 mL) at −78 °C [prepared by addition of 1.35 M *n*-BuLi (192 μL, 0.259 mmol, 1.5 equiv) to a solution of diisopropylamine (39 μL, 0.277 mmol, 1.6 equiv) in dry THF at −78 °C, the solution was warmed to 0 °C for 15 min, then cooled to −78 °C]. After 30 min, a solution of chlorotriisopropoxy titanium IV (1 M in THF, 692 μL, 0.691 mmol, 4 equiv) was added dropwise, and the solution was warmed to −40 °C. After 1 h, the solution was cooled to −78 °C, butyraldehyde (46.5 μL, 0.519 mmol, 3 equiv) in THF (2 mL) was added dropwise, and the solution was warmed to −40 °C. After 3 h, the reaction was quenched with saturated NH_4Cl (4 mL) and stirred with Celite until warmed to room temperature. The filtrate was extracted with EtOAc (4 × 4 mL), washed with brine (4 mL), dried over Na_2SO_4, and concentrated *in vacuo*. The product was purified by flash chromatography on silica gel 60 (DCM/hexanes/MeCN, 49.5:49.5:1), yielding the title compound as a nearly colorless oil (32.3 mg, 54% yield): $[\alpha]_D$ −33.0 (*c* 0.48, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, t), 1.28 (2H, m), 1.33 (3H, s), 1.34 (3H, s), 1.35 (3H, s), 1.37 (3H, s), 1.60 (2H, m), 2.39 (1H, brd), 2.90 (1H, dd), 3.10 (1H, dd), 4.11 (1H, m), 4.56 (1H, dd), 7.20 − 7.35 (5H, m); HRMS *m/z* 370.1992 $[M + Na]^+$ (calcd for $C_{20}H_{29}NO_4Na$, 370.1989).

(*R*)-4-Benzyl-3-(3-hydroxy-2,2-dimethylhexanoyl)-5,5-dimethyloxazolidin-2-one (4b). This was prepared in the same way as **4a** from **3b** (35% yield): $[\alpha]_D$ +32.4 (*c* 0.69, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, t), 1.28 (2H, m), 1.33 (3H, s), 1.34 (3H, s), 1.35 (3H, s), 1.37 (3H, s), 1.60 (2H, m), 2.39 (1H, brd), 2.90 (1H, dd), 3.10 (1H, dd), 4.11 (1H, m), 4.56 (1H, dd), 7.20−7.35 (5H, m); HRMS *m/z* 370.1991 $[M + Na]^+$ (calcd for $C_{20}H_{29}NO_4Na$, 370.1989).

(*S*)-3-Hydroxy-2,2-dimethylhexanoic Acid (5a). A hydrogen peroxide solution (30%, 13 μL, 0.115 mmol, 3.6 equiv) and lithium hydroxide monohydrate (2.1 mg, 0.051 mmol, 1.6 equiv), dissolved in H_2O (61 μL), were added successively to a solution of **4a** (11 mg, 0.031 mmol) in 4:1 THF/H_2O (275 μL) at 0 °C. After stirring 1 h, 20 min at 0 °C, sodium sulfite (16.6 mg, 0.132 mmol, 4.2 equiv) was added. THF was removed *in vacuo*, and the residual aqueous solution was partitioned between CH_2Cl_2 (3 × 1 mL) and H_2O. The combined aqueous layers were acidified to pH 1 with 1 N HCl. The aqueous layer was extracted with Et_2O (3 × 1 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo*, yielding the title compound as a colorless oil (3.8 mg, 75% yield): $[\alpha]_D$ −35.5 (*c* 0.24, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.95 (3H, t), 1.20 (3H, s), 1.25 (3H, s), 1.25−1.70 (4H, m), 3.66 (1H, dd); HRMS *m/z* 183.0995 $[M + Na]^+$ (calcd for $C_8H_{16}O_3Na$, 183.0992).

(*R*)-3-Hydroxy-2,2-dimethylhexanoic acid (5b). This was prepared in the same way as **5a** from **4b** (69% yield): $[\alpha]_D$ +26.5 (*c* 0.28, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.95 (3H, t), 1.20 (3H, s), 1.25 (3H, s), 1.25−1.70 (4H, m), 3.66 (1H, dd); HRMS *m/z* 183.0994 $[M + Na]^+$ (calcd for $C_8H_{16}O_3Na$, 183.0992).

Chiral GC/MS Analysis. Palmyramide A (0.5 mg) in 0.5 mL of 6 N HCl was heated in a sealed tube at 110 °C for 17 h, and the reaction mixture extracted with EtOAc. The organic layer was dried under N_2, and half of the residue was dissolved in 600 μL of 1:1 Et_2O/MeOH and treated with diazomethane for 25 min. Solvent and excess diazomethane were removed with N_2 gas, and the residue was resuspended in CH_2Cl_2. Standards of the *R*-Dhhma and *S*-Dhhma methyl esters were prepared similarly. Capillary GC/MS analysis was conducted using a Cyclosil B column (Agilent Technologies J&W Scientific, 30 m × 0.25 mm) under the following conditions: the initial oven temperature was 40 °C, followed by an immediate ramp from 40 to 90 °C at a rate of 5 °C/min, and held at 90 °C for 55 min. The Dhhma methyl ester derived from **1** eluted at 42.00 min, while the *R*-Dhhma methyl ester eluted at 41.90 min. The *S*-Dhhma methyl ester eluted at 39.82 min. Co-injection of the Dhhma methyl ester derived from compound **1** with the *R*-Dhhma methyl ester yielded a single peak at 41.95 min, while co-injection with the *S*-Dhhma methyl ester yielded a peak at 39.87 min and a peak at 42.00 min, confirming that the β-ester linkage of the Dhhma residue of **1** is *R*.

Mosher's Method. To a solution of **4a** (5.4 mg, 0.016 mmol) in CH_2Cl_2 (1 mL) were added Et_3N (0.010 mL, 0.078 mmol), *R*-MTPA chloride (0.015 mL, 0.078 mmol), and DMAP (2.0 mg, 0.017 mmol). The mixture was stirred at room temperature overnight, followed by purification on a silica gel column to yield the *S*-MTPA ester **6a** (5.7 mg, yield 62.5%). The same procedure for the synthesis of **6a** was used for the synthesis of the *R*-MTPA ester **6b** (2.2 mg, yield 29.6%). The absolute configuration of oxymethine carbon of **4a** was determined to be *S* by $\Delta\delta$ values for the protons adjacent to the MTPA esters moieties.

6a: ^{1}H NMR ($CDCl_3$) δ 0.83 (3H, t), 1.14 (2H, m), 1.30 (3H, s), 1.32 (3H, s), 1.34 (3H, s), 1.42 (3H, s), 1.50 (1H, m), 1.61 (1H, m), 2.85 (1H, dd), 3.12 (1H, dd), 3.54 (3H, s), 4.42 (1H, dd), 6.15 (1H, dd), 7.20−7.56 (10H, m); HRMS *m/z* 586.2389 $[M + Na]^+$ (calcd for $C_{30}H_{36}F_3NO_6Na$, 586.2387)

6b: ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t), 1.26 (2H, m), 1.28 (3H, s), 1.30 (3H, s), 1.33 (3H, s), 1.38 (3H, s), 1.48 (1H, m), 1.56 (1H, m), 2.82 (1H, dd), 3.04 (1H, dd), 3.55 (3H, s), 4.44 (1H, dd), 6.36 (1H, dd), 7.20−7.56 (10H, m); HRMS *m/z* 586.2385 $[M + Na]^+$ (calcd for $C_{30}H_{36}F_3NO_6Na$, 586.2387).

Morphological Characterization. Taxonomic identification of the cyanobacterium was performed in accordance with bacteriological systems[14] and current phycological systems.[15] Morphological characterizations were performed using an Olympus IX51 epifluorescent microscope (100× objective) equipped with an Olympus U-CMAD3 camera. Size measurements were calculated as an average with standard deviation of 10 neighboring cells from three different filaments of a population.

DNA Extraction, PCR-Amplification, and Cloning. DNA was extracted from approximately 40 mg of cleaned cyanobacterial filaments using the Wizard Genomic DNA purification kit (Promega Inc.) following the manufacturer's specifications. The isolated DNA was further purified using a Genomic-tip 20/G kit (Qiagen Inc.). DNA concentration and purity were measured on a DU 800 spectrophotometer (Beckman Coulter Inc.). The 16S rRNA genes were PCR-amplified using the cyanobacterial-specific primers 106F and 1509R,[16] the rpoC1 genes using the degenerate primers LrpoC1-F (5′-CYTGYTTNCCYTCDATDATRTC-3′) and LrpoC1-R (5′-YTNAARCCNGARATGGAYGG-3′), and the 18S rRNA gene using the universal primers U1F and U1R, as previously described.[17] The PCR reaction volumes were 25 μL containing 0.5 μL (50 ng) of DNA, 2.5 μL of 10× PfuUltra IV reaction buffer, 0.5 μL of dNTP mix (25 mM each of dATP, dTTP, dGTP, and dCTP), 0.5 μL of each primer (10 μM), 0.5 μL of PfuUltra IV fusion HS DNA polymerase, and 20.5 μL of dH_2O. The PCR reactions were performed in an Eppendorf Mastercycler gradient as follows: initial denaturation for 2 min at 95 °C, 25 cycles of amplification followed by 20 s at 95 °C, 20 s at 50 °C, and 1.5 min at 72 °C, and final elongation for 3 min at 72 °C. PCR products were analyzed on a (1%) agarose gel in SB buffer and visualized by EtBr staining. PCR products were subcloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen) into the pCR-Blunt IV TOPO vector and then transformed into TOPO cells and cultured on LB-kanamycin plates. Plasmid DNA was isolated using the QIAprep Spin miniprep kit (Qiagen) and sequenced with pCR-Blunt IV TOPO vector specific primers M13F and M13R. Sequencing of the 16S rRNA genes' middle regions was improved using the internal primers 359F and 781R.[16] Gene sequences were analyzed for anomalies using the Pintail software with the cutoff size set at >600 bp before submission to GenBank/EMBL/DDBJ.[18] The gene sequences are available in the GenBank/EMBL/DDBJ databases under accession numbers *L. majuscula* 16S rRNA gene (GQ231522), *L. majuscula* rpoC1 (GQ231521), and the *Centroceras* sp. 18S rRNA gene (GQ246180).

Phylogenetic Analysis. The gene sequences were aligned in ClustalW XXL in MEGA 4.0 with standard gap opening and extension penalties without gaps.[19] The evolutionary histories of the cyanobacterial 16S rRNA genes were inferred using the Minimum Evolution (ME) algorithm in MEGA 4.0 as well as the Bayesian (MrBayes algorithm) method using TOPALi v2.5.[20] All algorithms were performed with 1000 bootstrap replicates. The evolutionary distances (pairwise sequence divergence) were computed using the Maximum Composite Likelihood method. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1.[21] All positions containing gaps and missing data were eliminated from the data set (complete deletion option) for a total of 855 bp.

Culture. Specimens for culturing were isolated under microscopy and cleaned by running individual filaments through 1% agar SWBG-11 plates. The isolated specimens were cultured in SWBG-11 medium

at 28 °C with 35 g/L Instant Ocean (Aquarium Systems Inc.). The cultures were kept in a 16 h light/8 h dark cycle with a light intensity of ~7 μmol photon/s/m^2 provided by 40 W cool white fluorescent lights.

Filament Sample Preparation. Filaments were removed from the parent culture using small, sterile, blunt-tip tweezers and transferred to a Petri dish containing a small amount of distilled H_2O in order to remove excess salt and media; this process was repeated. Using the same tweezers, the filament was carefully removed and laid horizontally on a Bruker MSP 96 stainless steel microflex target plate. Excess liquid on the surface was carefully blotted using the corner of a Kimwipe. The plate and filament were allowed to dry at room temperature until visibly dry.

Image Acquisition. After the filament had dried on the target plate, a photograph (Nikon Coolpix, 3 megapixel) of the plate was taken to use as spatial teach reference for the Bruker Microflex MALDI instrument. Next, epifluorescent images of the length of the filament were acquired at 4× magnification using an Olympus IX511 microscope with an excitation filter of 590 nm. The resulting images were tiled together using Photoshop CS to create a single, epifluorescent image of the filament.

MALDI Matrix Deposition. After image acquistion, a MALDI matrix composed of 35 mg/mL α-cyanohydroxycinnamic acid, 35 mg/mL DHB, 75% MeCN, and 0.2% TFA was coated onto the MALDI MSP 96 plate using a Paasche airbrush (www.paascheairbrush.com) and repeated side-to-side strokes until an even, thin crystalline layer occluded the background of the plate. The target plate containing the sample and matrix was placed in an empty Petri dish until analysis.

MALDI MS and Imaging. The target plate containing the sample was inserted into a Microflex Bruker Daltonics mass spectrometer outfitted with the Compass 1.2 software suite (consists of FlexImaging 2.0, FlexControl 3.0, and FlexAnalysis 3.0). The sample was run in positive mode, with 80 μm raster intervals in X and Y and 20−25% absolute laser power. Briefly, a photomicrograph of the sample to be imaged by mass spectrometry was loaded onto the Fleximaging command window. Three teach points were selected in order to align the background image with the sample target plate. After the target plate calibration was complete, the AutoXecute command was used to run the sample. The settings under the FlexControl panel were used as previously described.[10]

Image Visualization and Analysis. Using the Bruker FlexImaging 2.0 software, a window containing the *m/z* values corresponding to the palmyramide A (**1**) isotope cluster was selected and assigned a bright green color. It was clear that the spatial distribution of the specified mass window colocalized precisely with the filament contained in the teach image. These visual results were exported and overlaid on the epifluorescent images of the filament acquired before matrix application using Photoshop CS.

Biological Activity. Sodium channel blocking activity was measured as previously described.[11] Neuro2a mouse neuroblastoma cells were seeded in 96-well plates at 3.0 × 10^5 cells/mL in 200 μL of RPMI medium with FBS. After 16 h, the test compounds were dissolved in DMSO and diluted into medium without FBS and then added to the wells. A solution of 10 mM ouabain and 1 mM veratridine was applied to wells to cause sodium overload cytotoxicity. After 24 h, cell viability was determined by the MTT assay and sodium channel blocking activity was calculated as percentage of recovery from the toxicity of ouabain and veratridine.

Cytotoxicity was measured in NCI H-460 human lung tumor cells using the MTT assay.[12] Cells were seeded in 96-well plates at 3.3 × 10^4 cells/mL in 180 μL of RPMI medium with FBS. After 16 h, the test compounds were dissolved in DMSO and diluted into medium without FBS and then added to the wells. After 48 h, cell viability was determined by the MTT assay.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HSQC, and HMBC spectra in $CDCl_3$ for palmyramide A (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Tan, L. T. *Phytochemistry* **2007**, *68*, 954–979.
(2) Tidgewell K.; Clark B. R.; Gerwick W. H. In *Comprehensive Natural Products Chemistry*; Pergamon Press, 2009; Vol. 8, in press.
(3) Dinsdale, E. A.; Pantos, O.; Smriga, S.; Edwards, R. A.; Angly, F.; Wegley, L.; Hatay, M.; Hall, D.; Brown, E.; Haynes, M.; Krause, L.; Sala, E.; Sandin, S. A.; Thurber, R. V.; Willis, B. L.; Azam, F.; Knowlton, N.; Rohwer, F. *PLoS ONE* **2008**, *3*, e1584.
(4) Marquez, B.; Verdier-Pinard, P.; Hamel, E.; Gerwick, W. H. *Phytochemistry* **1998**, *49*, 2387–2389.
(5) Ainslie, R. D.; Barchi, J. J.; Kuniyoshi, M.; Moore, R. E.; Mynderse, J. S. *J. Org. Chem.* **1985**, *50*, 2859–2862.
(6) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591–596.
(7) Nunnery, J. K.; Suyama T. L.; Gerwick W. H. Manuscript in preparation.
(8) Hoye, T. R.; Jeffrey, C. S.; Shao, F. *Nat. Protoc.* **2007**, *2*, 2451–2458.
(9) Simmons, T. L.; Coates, R. C.; Clark, B. R.; Engene, N.; Gonzalez, D.; Esquenazi, E.; Dorrestein, P. C.; Gerwick, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 4587–4594.
(10) Esquenazi, E.; Coates, C.; Simmons, L.; Gonzalez, D.; Gerwick, W. H.; Dorrestein, P. C. *Mol. Biosyst.* **2008**, *4*, 562–570.
(11) Manger, R. L.; Leja, L. S.; Lee, S. Y.; Hungerford, J. M.; Wekell, M. M. *Anal. Biochem.* **1993**, *214*, 190–194.
(12) Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. L.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. *Cancer Res.* **1988**, *38*, 589–601.
(13) Tan, L. T.; Sitachitta, N.; Gerwick, W. H. *J. Nat. Prod.* **2003**, *66*, 764–771.
(14) Castenholz, R. W. In *Bergey's Manual of Systematic Bacteriology*; Springer: New York, 2001; Vol. 1, pp 473−553.
(15) Komárek, J. K. Anagnostidis. In *Süsswasserflora von Mitteleuropa*; Elsevier/Spektrum: Heidelberg, 2005; pp 1−759.
(16) Nübel, U.; Garcia-Pichel, F.; Muyzer, G. *Appl. Environ. Microbiol.* **1997**, *63*, 3327–3332.
(17) Rivas, R.; Velázquez, E.; Zurdo-Piñeiro, J. L.; Mateos, P. F.; Molina, E. M. *J. Microbiol. Methods* **2003**, *56*, 413–426.
(18) Ashelford, K. E.; Chuzhanova, N. A.; Fry, J. C.; Jones, A. J.; Weightman, A. *Appl. Environ. Microbiol.* **2005**, *71*, 7724–7736.
(19) Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. *Mol. Biol. Evol.* **2007**, *24*, 1596–1599.
(20) Milne, I.; Lindner, D.; Bayer, M.; Husmeier, D.; McGuire, G.; Marshall, D. F.; Wright, F. *Bioinformatics* **2009**, *25*, 126–127.
(21) Nei, M.; Kumar, S. In *Molecular Evolution and Phylogenetics*; Oxford University Press: New York, 2000; 99−108.

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