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Cyclic Depsipeptides, Grassypeptolides D and E and Ibu-epidemethoxylyngbyastatin 3, from a Red Sea *Leptolyngbya* Cyanobacterium

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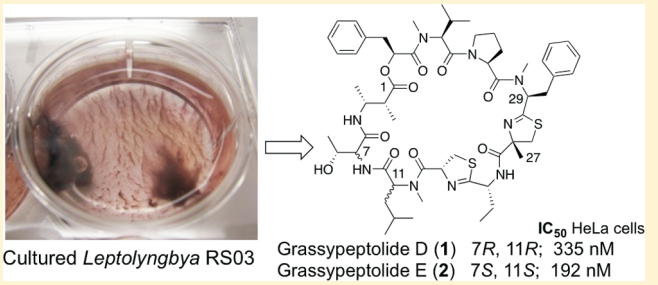
Supporting Information

ABSTRACT: Two new grassypeptolides and a lyngbyastatin analogue, together with the known dolastatin 12, have been isolated from field collections and laboratory cultures of the marine cyanobacterium *Leptolyngbya* sp. collected from the *SS Thistlegorm* shipwreck in the Red Sea. The overall stereostructures of grassypeptolides D (**1**) and E (**2**) and Ibu-epidemethoxylyngbyastatin 3 (**3**) were determined by a combination of 1D and 2D NMR experiments, MS analysis, Marfey's methodology, and HPLC-MS. Compounds **1** and **2** contain 2-methyl-3-aminobutyric acid and 2-aminobutyric acid, while biosynthetically distinct **3** contains 3-amino-2-methylhexanoic acid and the β-keto amino acid 4-amino-2,2-dimethyl-3-oxopentanoic acid (Ibu). Grassypeptolides D (**1**) and E (**2**) showed significant cytotoxicity to HeLa (IC_{50} = 335 and 192 nM, respectively) and mouse neuro-2a blastoma cells (IC_{50} = 599 and 407 nM, respectively), in contrast to Ibu-epidemethoxylyngbyastatin 3 (neuro-2a cells, IC_{50} > 10 μM) and dolastatin 12 (neuro-2a cells, IC_{50} > 1 μM).



Microbial metabolites appear to be characteristic of certain biotopes, on both an environmental and a species level, which has provided a diversity of chemical structures unparalleled by even the largest combinatorial libraries.[1] Our research has recently focused on the isolation and structure elucidation of biologically active natural products from microorganisms inhabiting unique environments. The Red Sea represents an unexplored repository of diverse cyanobacteria, although in low abundance. This may result from the low annual rainfall, minimal freshwater input, and high evaporation rate that make the Red Sea one of the most saline and pristine water bodies in the world.[2] Despite these conditions, we have collected specimens from a range of cyanobacterial genera including *Lyngbya*, *Phormidium*, *Symploca*, and the *Leptolyngbya* that is the subject of this report. Thus, we are interested to compare the biosynthetic capabilities of these Red Sea organisms with those collected pantropically. As is the case for natural products in general, cyanobacterial metabolites often occur as sets of related analogues that possess varying biological selectivity, putatively for optimal adaptation to a range of environments.[3] In addition, the capacity of any one cyanobacterium to produce several biosynthetically distinct metabolites,[4] as well as the production of the same or biosynthetically related natural products by different genera of cyanobacteria, is the subject of intense investigation.[5] One proposal is that common heterotrophic bacteria associated with cyanobacteria may be the biosynthetic origin of the isolated products. Alternatively, horizontal gene transfer between different cyanobacteria or between heterotrophic bacteria may account for the presence of multiple biosynthetic gene clusters in cyanobacterial genomes.[6] Remarkably, a Floridian *Lyngbya confervoides* has afforded lyngbyastatins 4−6,[7,8] pompanopeptins A and B,[9] largamides A−H,[10] tiglicamides A−C,[11] and grassypeptolides A−C.[12] Here we report the isolation of grassypeptolides D (**1**) and E (**2**) and Ibu-epidemethoxylyngbyastatin 3 (**3**), as well as the known dolastatin 12,[13] from a marine *Leptolyngbya* cyanobacterium collected from the Red Sea shipwreck *SS Thistlegorm* (46−98 ft). All four macrocyclic depsipeptides are also produced by the laboratory-cultured (monoclonal) Red Sea *Leptolyngbya*. While grassypeptolide D (**1**) is ∼1.5-fold less cytotoxic to HeLa cervical carcinoma and neuro-2a mouse blastoma cells than grassypeptolide E (**2**), these threonine/*N*-methylleucine diastereomers do not show the dramatic natural structure−activity relationship observed between the *N*-methylphenylalanine epimers grassypeptolides A and C.[12] Ibu-epidemethoxylyngbyastatin 3 (**3**, IC_{50} > 10 μM) was significantly

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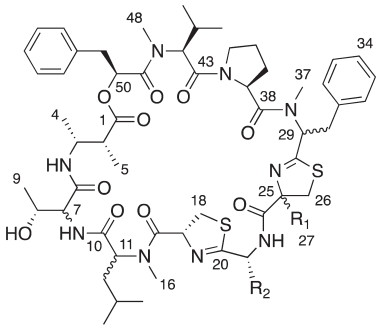
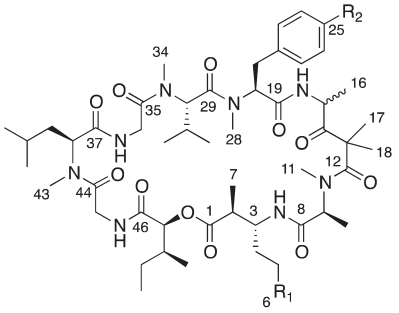
less cytotoxic to neuro-2a cells than the grassypeptolides and related dolastatin 12 (IC_{50} > 1 μM).



		R_1	R_2
Grassypeptolide D (**1**)	7*R*, 11*R*, 25*S*, 29*S*	Me	Et
Grassypeptolide E (**2**)	7*S*, 11*S*, 25*S*, 29*S*	Me	Et
Grassypeptolide A	7*R*, 11*R*, 25*R*, *29*R*	H	Et
Grassypeptolide B	7*R*, 11*R*, 25*R*, *29*R*	H	Me
Grassypeptolide C	7*R*, 11*R*, 25*R*, *29*S*	H	Et

* This is position 28 in the original report of grassypeptolides A-C



		R_1	R_2
Ibu-epidemethoxylyngbyastatin (**3**)	15*S*	Me	H
Dolastatin 12	15*S*	H	H
Lyngbyastatin 3	15*R*	Me	OMe

RESULTS AND DISCUSSION

A crude organic extract of the Red Sea *Leptolyngbya* was subjected to bioassay-guided fractionation via normal-phase VLC using a stepped gradient of hexanes to EtOAC to MeOH. The fraction eluting with 25% MeOH−EtOAC was highly cytotoxic to mouse neuro-2a neurobastoma cells (30 μg/mL reduced cell viability by 99.6%). This VLC fraction was separated by C_{18} reversed-phase (RP_{18}) solid-phase extraction (SPE) and exhaustive RP-HPLC to yield three minor cytotoxic metabolites (**1**, 1.5 mg; **2**, 0.5 mg; and **3**, 2.9 mg) and the known depsipeptide dolastatin 12 (5.0 mg) as the major component.

A molecular formula of $C_{57}H_{81}N_9O_{10}S_2$ for both grassypeptolides D (**1**) and E (**2**) was provided by HR-MS ($[M + Na]^+$ *m*/*z* 1138.5515 and 1138.5413, respectively) and supported by NMR spectroscopic (Table 1) data. The ^1H and ^{13}C NMR spectra for each compound were similar and indicated peptidic metabolites due to the presence of three NH doublets (δ_H 6.45−7.50), three *N*-methyl substituents (δ_H 3.1−3.5), nine α-H multiplets (δ_H 3.4−5.9), numerous overlapped methyl doublets (δ_H 0.74−0.99), and 10 putative ester/amide carbonyl ^{13}C signals (δ_C 168−175) in each case. These data suggested that **1** and **2** were structurally related to the Floridian *Lyngbya confervoides* metabolites grassypeptolides A−C and similar to grassypeptolide C in particular.[12] However, the 1D NMR spectra for both **1** and **2** contain a 3H singlet ($\delta_{H\text{-}27}$ 1.35 and 1.38, respectively) and a quaternary carbon ($\delta_{C\text{-}25}$ 84.0) not present in the spectra for grassypeptolide C. In addition, comparison of the α-CH chemical shifts for **1**, **2**, and grassypeptolide C revealed significant differences between compounds **1** and **2** (Table 1), whereas the α-CH chemical shifts for **1** more closely matched those for grassypeptolide C. These differences led us to investigate the structures of **1** and **2** more closely.

Analysis of the 2D NMR spectra in $CDCl_3$ for **1** and **2** (HSQC, HSQC-TOCSY, HMBC, COSY, ROESY) confirmed that grassypeptolides D (**1**) and E (**2**) had the same connectivity of planar structure as grassypeptolide C. An HSQC-TOCSY experiment for **1** identified spin systems for Thr, Leu, Pro, and Val amino acid side chains, and key HMBC (supported by COSY and ROESY) correlations established that Leu and Val were *N*-methylated as in grassypeptolide C. A pair of methyl doublets ($\delta_{H\text{-}4}$ 1.19 and $\delta_{H\text{-}5}$ 1.12) correlated to two vicinally coupled methine multiplets ($\delta_{H\text{-}2}$ 2.50 and $\delta_{H\text{-}3}$ 4.22) distinguished the β-amino acid residue 2-methyl-3-aminobutyric acid (Maba). COSY correlations from an upfield methyl doublet ($\delta_{H\text{-}23}$ 0.96) to a relatively shielded methylene ($\delta_{H\text{-}22}$ 2.11/1.86), in turn coupled to a methine ($\delta_{H\text{-}21}$ 4.55), supported a 2-aminobutyric acid (Aba) residue. An HMBC correlation to the putative carbonyl ^{13}C of Aba from a relatively deshielded methylene ($\delta_{H\text{-}19}$ 3.60/3.26), which also correlated to a second deshielded quaternary ^{13}C ($\delta_{C\text{-}17}$ 170.4), was consistent with an Aba-derived thiazoline carboxylic acid (Aba-thn-ca). Chemical shift comparison with grassypeptolide C and HMBC analysis (Table S1) also confirmed the presence of two aromatic residues, phenyllactic acid (Pla) and *N*-methylphenylalanine (*N*-Me-Phe). The latter residue is incorporated into a thiazoline carboxylic acid in grassypeptolide C. However, no $-CHCH_2-$ motif consistent with this remaining thiazoline ring was apparent in the spectra for grassypeptolide D (**1**). Instead, an AB spin system of a fifth isolated methylene ($\delta_{H\text{-}26}$ 3.74/3.17) showed HMBC correlations to a methyl ($\delta_{C\text{-}27}$ 24.3), a midfield quaternary ($\delta_{C\text{-}25}$ 84.0), and two deshielded quaternary ($\delta_{C\text{-}28}$ 173.9 and $\delta_{C\text{-}24}$ 173.7) carbons. This indicated the presence of a 2-methylthiazoline carboxylic acid derived from *N*-methylphenylalanine (*N*-Me-Phe-4-Me-thn-ca). Although none of the previously reported grassypeptolides A−C contain a methylated thiazoline carboxylic acid, this unit has been reported in largazole[14] and the hoiamides[15] from marine cyanobacteria, as well as several terrestrial bacteria.

Analysis of the 2D NMR data for **2** revealed the same sequence of units as found in **1**. However, the α-CH chemical shifts for Thr ($\delta_H/\delta_{C\text{-}7}$ 3.36/57.0) and *N*-Me-Leu ($\delta_H/\delta_{C\text{-}11}$ 5.15/54.6) in **2** were substantially different from those observed for **1** (Thr, $\delta_H/\delta_{C\text{-}7}$ 4.45/59.1; *N*-Me-Leu, $\delta_H/\delta_{C\text{-}11}$ 4.70/57.5) and grassypeptolide C (Thr, $\delta_H/\delta_{C\text{-}7}$ 4.44/59.2; *N*-Me-Leu, $\delta_H/\delta_{C\text{-}11}$ 4.70/57.6). Furthermore, the *N*-CH_3-16 singlet (δ_H 3.49) for **2** was shifted slightly upfield compared to those for **1** and grassypeptolide C (δ_H 3.20 and 3.17, respectively). Overall, these chemical shift differences suggested different configurations for both Thr and *N*-Me-Leu in **2** relative to **1** and grassypeptolide C.

The absolute configurations of grassypeptolides D (**1**) and E (**2**) were determined by a combination of acid hydrolysis and oxidative ozonolysis followed by chiral LC-MS or Marfey's analysis. Chiral LC-MS of the acid hydrolysates of **1** and **2** established the presence of L-Pla, while analysis by RP_{18} HPLC of the ozonolysis and acid hydrolysate products of **1** and **2** derivatized

Table 1. ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR Spectroscopic Data for Grassypeptolides D (1) and E (2) in $CDCl_3$

		grassypeptolide D (1)		grassypeptolide E (2)	
unit	position	δ_H, mult. (J in Hz)	δ_C, mult.	δ_H, mult. (J in Hz)	δ_C, mult.
Maba	1		172.4, C		172.9, C
	2	2.50, dq (7.0, 6.8)	45.6, CH	2.51, dq (7.0, 4.5)	45.5, CH
	3	4.22, m	48.5, CH	4.23, m	47.0, CH
	4	1.19, d (6.7)	19.9, CH_3	1.10, d (7.0)	19.3, CH_3
	5	1.12, d (7.0)	14.4, CH_3	1.16, d (7.0)	14.4, CH_3
	NH	7.70, br		6.45, d (9.9)	
Thr	6		169.3, C		170.5, C
	7	4.45, (6.8, 6.1)	59.1, CH	3.36, dd (6.9, 3.3)	57.0, CH
	8	4.02, m	69.1, CH	3.30, m	67.9, CH
	9	1.22, d (6.4)	19.7, CH_3	0.87, d (6.5)	19.4, CH_3
	OH	4.00, br			
	NH	6.90, d (7.3)		6.90, d (6.9)	
N-Me-Leu	10		170.3, C		170.0, C
	11	4.70, br	57.5, CH	5.15, t (7.7)	54.6, CH
	12a	1.97, m	37.2, CH_2	1.78, m	37.1, CH_2
	12b	1.67, m			
	13	1.58, m	25.3, CH	1.52, m	25.3, CH
	14	0.98, d (6.4)	23.2, CH_3	0.98, d (6.6)	23.2, CH_3
	15	0.94, d (6.5)	22.8, CH_3	0.93, d (6.7)	22.4, CH_3
	16	3.20, s	33.2, CH_3	3.49, s	30.7, CH_3
Aba-thn-ca	17		170.4, C		169.3, C
	18	5.29, m	78.1, CH	5.07, m	75.9, CH
	19a	3.60, m	33.3, CH_2	4.15, br dd	35.5, CH_2
	19b	3.26, m		3.44, dd (−11.2, 8.5)	
	20		178.0, C		175.0, C
	21	4.55, m	54.2, CH	4.71, ddd (9.0, 4.0, 2.7)	53.2, CH
	22a	2.11, m	25.2, CH_2	1.95, dqd (−11.5, 7.4, 4.0)	28.5, CH_2
	22b	1.86, m		1.48, m	
	23	0.96, t (6.9)	11.6, CH_3	0.74, t (7.4)	10.0, CH_3
	NH	7.10, d (7.7)		7.50, d (9.0)	
N-Me-Phe-thn-ca	24		173.7, C		168.9, C
	25		84.0, C		84.0, C
	26a	3.74, d (−11.5)	43.1, CH_2	3.29, d (−11.6)	41.5, CH_2
	26b	3.17, d (−11.3)		3.18, d (−11.7)	
	27	1.35, s	24.3, CH_3	1.38, s	23.9, CH_3
	28		173.9, C		173.9, C
	29	5.39, dd (10.7, 6.4)	58.9, CH	5.87, dd (12.2, 3.0)	55.8, CH
	30a	3.24, m	36.0, CH_2	3.55, br	36.1, CH_2
	30b	3.16, m		3.28, m	
	31		135.5, C		137.7, C
	32/36	7.33, m	129.2, CH	7.18, m	129.6, CH
	33/35	7.22, m	128.5, CH	7.03, br	129.3, CH
	34	7.20, m	127.6, CH	7.08, m	127.3, CH
	37	3.21, s	30.7, CH_3	3.17, s	30.2, CH_3
Pro	38		172.7, C		171.2, C
	39	4.84, dd (8.8,5.1)	57.5, CH	5.10, br	58.8, CH
	40a	2.22, m	27.5, CH_2	2.30, m	31.2, CH_2
	40b	1.93, m		2.10, m	
	41a	1.99, m	24.9, CH_2	2.01, m	21.7, CH_2
	41b	1.63, m			
	42a	3.97, m	47.8, CH_2	3.80, m	46.9, CH_2
	42b	3.53, m		3.69, m	

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Table 1. Continued

		grassypeptolide D (**1**)		grassypeptolide E (**2**)	
unit	position	δ_H, mult. (*J* in Hz)	δ_C, mult.	δ_H, mult. (*J* in Hz)	δ_C, mult.
N-Me-Val	43		168.6, C		168.5, C
	44	4.98, d (11.0)	60.0, CH	5.01, d (10.8)	58.1, CH
	45	2.32, m	27.4, CH	2.35, m	28.1, CH
	46	0.98, d (6.4)	19.6, CH_3	0.95, d (6.7)	19.5, CH_3
	47	0.88, d (6.6)	18.0, CH_3	0.85, d (6.7)	18.6, CH_3
	48	3.12, s	30.0, CH_3	3.20, s	30.3, CH_3
Pla	49		170.2, C		171.4, C
	50	5.33, dd (9.9, 3.2)	72.4, CH	5.33, dd (9.7, 3.8)	72.4, CH
	51a	3.09, dd (−14.5, 9.9)	36.9, CH_2	3.16, m	36.9, CH_2
	51b	3.04, dd (−14.4, 3.2)		3.01, m	
	52		134.9, C		136.1, C
	53/57	7.22, m	129.1, CH	7.12, m	129.1, CH
	54/56	7.24, m	129.5, CH	7.31, m	129.5, CH
	55	7.27, m	127.6, CH	7.24, m	127.4, CH

with *N*-α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (Marfey's reagent) indicated the presence of *N*-Me-L-Val, L-Pro, *N*-Me-L-Phe, (2*S*)-MeCysA, D-Aba, L-Cya, and (2*R*,3*R*)-Maba. Consistent with the observed differences in the α-CH chemical shifts of Thr and *N*-Me-Leu described above, Marfey's analysis of the grassypeptolide D (**1**) ozonolysis and hydrolysis product matched the configurations observed for grassypeptolide C (D-*allo*-Thr and *N*-Me-D-Leu), while grassypeptolide E (**2**) hydrolysate retention times indicated the presence of L-Thr and *N*-Me-L-Leu.

Ibu-epidemethoxylyngbyastatin 3 (**3**) was isolated from the same first-tier RP-HPLC fraction as the grassypeptolides. The molecular composition of **3** was established as $C_{51}H_{82}N_8O_{11}$ from HR-FTMS data ($[M + Na]^+$ *m*/*z* 1005.5983). While the ^1H NMR spectrum for compound **3** also exhibited resonances typical of a peptide, it was significantly different from those for grassypeptolides **1** and **2**. In addition, inspection of the ^{13}C NMR spectrum for compound **3** revealed the absence of a midfield quaternary (δ_C 84.0) and the presence of a downfield quaternary carbon (δ_C 208.4), which, in combination with the reduced molecular mass, suggested a different planar structure for **3** relative to compounds **1** and **2**. Instead, the 1D spectra for **3** were similar to those for dolastatin 12, isolated as the major component from the preceding first-tier HPLC fraction. Examination of the 2D NMR data for **3** confirmed that an additional methyl triplet (δ_H 0.86) in the ^1H NMR spectrum for **3** and increase of 14 mass units relative to dolastatin 12 were attributable to the presence of a 3-amino-2-methylhexanoic acid moiety (Amha) in **3**, rather than the 3-amino-2-methylpentanoic acid (Ampa) in dolastatin 12. Thus, the planar structure of **3** could best be designated as demethoxylyngbyastatin 3, indicating replacement of the *N*,*O*-dimethylTyr in lyngbyastatin 3[16] with *N*-methylPhe in **3**. The configurational assignment of the Ibu unit in the lyngbyastatin/dolastatin series has proven arduous given the propensity of this unit to decarboxylate and epimerize during acid hydrolysis to yield (2*RS*)-2-amino-4-methylpentan-3-one (Amp).[4,17] The analysis and comparison of this series of depsipeptides containing Ibu is further confounded by whether or not the neighboring Ala unit is *N*-methylated. Williams and co-workers deduced that their cyanobacterial samples of dolastatin 12 and lyngbyastatins 1 and 3 each comprised Ibu epimeric mixtures following $NaBH_4$ reduction of the natural products, acid hydrolysis, and comparisons with the reduced Ibu standards, (3*RS*,4*S*)-4-amino-2,2-dimethyl-3-hydroxypentanoic acid (Adhpa).[16] It was shown that little enolization occurs during the $NaBH_4$ reduction itself, which is performed to avoid extensive enolization of the β-keto moiety in Ibu during subsequent acid hydrolysis. To investigate further the timing of epimerization at C-15 in **3** and dolastatin 12 during acid hydrolysis, we first undertook an alternate strategy. Compound **3** and dolastatin 12 were subjected to Marfey's analysis following acid hydrolysis under two different conditions: (A) hydrolysis with 6 N HCl for 50 s using a microwave and Ace high-pressure tube and (B) treatment with 6 N HCl at 110 °C for 18 h. Analysis of the reaction products of **3** by RP_{18} HPLC revealed the presence of both enantiomers of Amp in the *S*/*R* ratio of 4.1:1 and 1:2.2 for treatments A and B, respectively. A similar result was obtained in the analysis of dolastatin 12 under these conditions, suggesting that racemization of the Ibu unit likely occurs more slowly than peptide hydrolysis; a rapid acid hydrolysis reaction results in less enolization of the Ibu substrate. In tandem with the lack of extremely broad peaks (characteristic of the *R*-Ibu epimer lyngbyastatin 1[15]) in the ^1H NMR spectra for **3** and dolastatin 12, these data suggest that both compounds contain *S*-Ibu. Given the relatively sharp ^1H NMR signals for the natural products, it is unlikely that epimerization from an *R*-Ibu to *S*-Ibu occurred prior to amide hydrolysis, despite that the *S*-Ibu configuration is thermodynamically favored.[15] Noteworthy also is that the analysis of the *S*-Amp standard derivatized with Marfey's reagent under standard reaction conditions (40 °C, 1 h) showed the presence of both enantiomers in the *S*/*R* ratio of 6.7:1, whereas Marfey's derivatization for 24 h resulted in the ratio of 1.8:1. Thus, further enolization of the Amp unit in the natural product hydrolysate may occur during the derivatization process.

To corroborate our assignment of *S*-Ibu following the method of Williams et al.,[16] compound **3** was reduced with $NaBH_4$ and subjected to microwave hydrolysis, and the Marfey's derivatives were compared to synthetically prepared Adhpa standards (see Supporting Information). Analysis of the reaction products of **3** by RP_{18} HPLC-MS revealed the presence of enantiomers of Adhpa in the *S*/*R* ratio 13.8:1. The absolute configurations of the remaining stereogenic centers in Ibu-epidemethoxylyngbyastatin

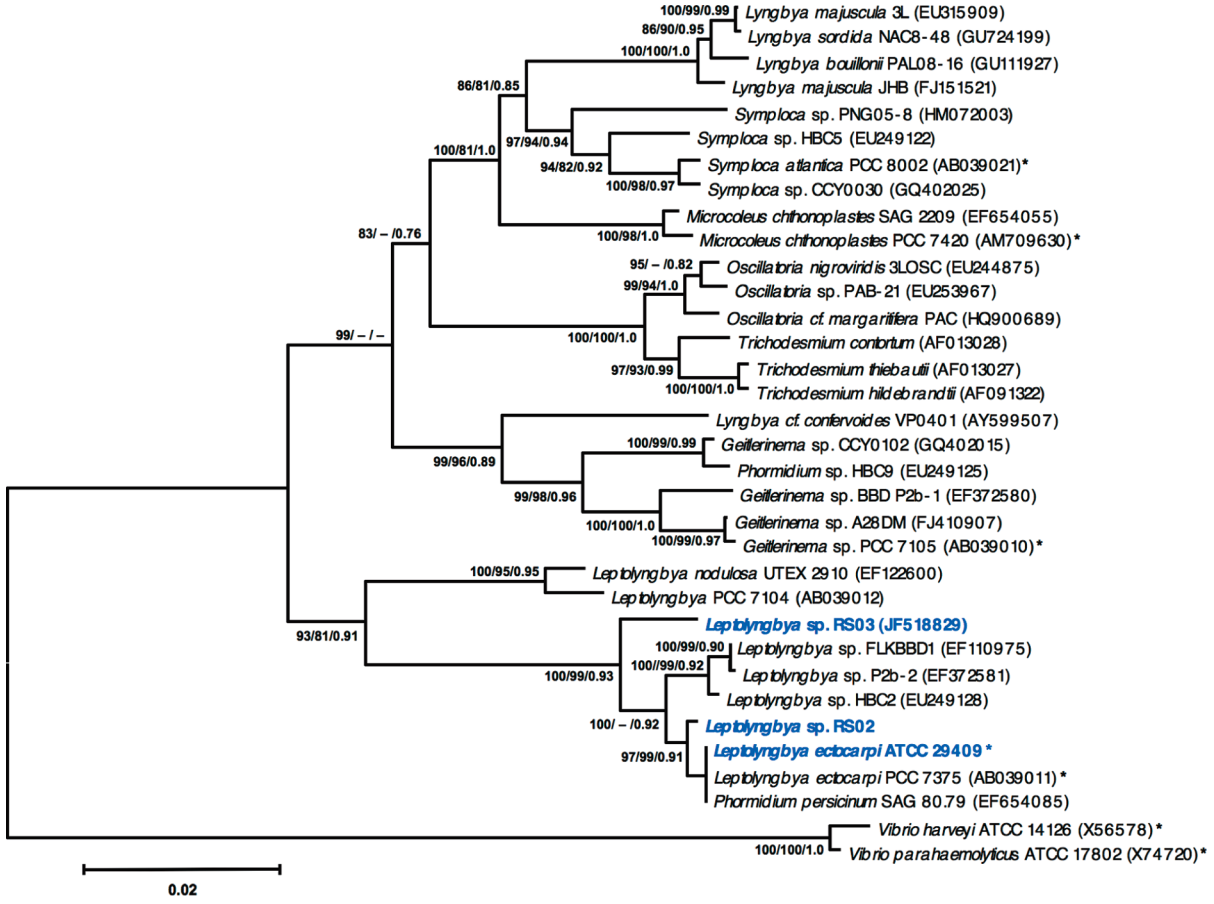

Figure 1. Phylogenetic relationship of the Red Sea *Leptolyngbya* sp. RS03 with marine filamentous cyanobacteria of the order Oscillatoriales (III) based on 16S rRNA nucleotide sequences. *Vibrio harveyi* ATCC 14126 and *V. parahemolyticus* ATCC 17802 (marine γ-proteobacteria) are included as outgroups. Labels on the terminal nodes indicate the species, strain, and GenBank accession numbers in parentheses; an asterisk designates reference strains. Strains with 16S rRNA gene sequences determined in this study are indicated in blue. Support values < 70 are not indicated. The scale bar indicates 0.02 nucleotide substitution per site.

3 were assigned by a combination of chiral LC-MS and Marfey's analysis using commercially available or synthetic (Amha) standards. Following acid hydrolysis of **3**, chiral LC-MS analysis revealed the presence of (2*S*,3*S*)-HMPA. Derivatization of the acid hydrolysate of **3** with Marfey's reagent, followed by RP_{18} HPLC or LC-MS, established the presence of *N*-Me-L-Ala, *N*-Me-L-Val, *N*-Me-L-Phe, *N*-Me-L-Leu, and (2*S*,3*R*)-Amha.

Diastereomeric grassypeptolides D (**1**) and E (**2**) both showed significant cytotoxicity to HeLa (IC_{50} 335 and 192 nM, respectively) and neuro-2a (IC_{50} 599 and 407 nM, respectively) cells. The small (~1.5-fold) difference in cytotoxicity between **1** and **2** indicates that the chirality of the *N*-Me-Leu and Thr α position is not critical for activity and that this region is not central to the pharmacophore of these structures. In contrast, Kwan et al.[12] conclude that the *N*-Me-Phe region of grassypeptolides A–C is critical for their cytotoxicity given that grassypeptolide C (*N*-Me-L-Phe) showed 16–23- and 65-fold greater potency, respectively, than grassypeptolides A and B (both *N*-Me-D-Phe) against colorectal adenocarcinoma HT29 and cervical carcinoma HeLa cells. In addition the ethyl side chain of the Aba-thn-ca in grassypeptolides A and C is associated with enhanced activity over the methyl of the Ala unit in grassypeptolide B.[12] Like grassypeptolide C, grassypeptolides D (**1**) and E (**2**) also contain Aba and *N*-Me-L-Phe thiazoline carboxylic acid units. The only structural difference between **1** (IC_{50} 335 nM, HeLa cells) and grassypeptolide C (IC_{50} 45 nM, HeLa cells) is a methylated thiazoline of opposite chirality (2*S*). The apparent ~7.5-fold difference in cytotoxicity between grassypeptolides D (**1**) and C supports the hypothesis that the *N*-Me-Phe-thn-ca-Aba-thn-ca motif is central to the pharmacophore of the grassypeptolides. While lyngbyastatin 3 was reported previously to be potently cytotoxic to KB (epithelial carcinoma, subline of HeLa) and LoVo (colon carcinoma) cell lines (IC_{50} = 32 and 400 nM, respectively),[16] we observed little effect of Ibu-epidemethoxylyngbyastatin 3 (**3**) on neuro-2a cells (IC_{50} > 10 μM). Similarly dolastatin 12 was only slightly more cytotoxic to these cells than **3** (IC_{50} > 1 μM).

A majority of the more than 300 known marine cyanobacterial metabolites have been reported from the genus *Lyngbya*.[3] However, metabolites closely related to these "*Lyngbya* compounds" have also been reported from non-*Lyngbya* species, as in the case of the four compounds reported here. This has led to an inability to define strong chemotaxonomic trends for cyanobacterial genera. The taxonomic description of cyanobacteria has transitioned from the traditional phycological system to the modern bacteriological system, supported by use of the 16S rRNA gene for phylogenetic determinations. Phylogenetic revision of cyanobacterial taxonomy is in progress for several genera, including *Lyngbya*.[5] In tandem with confirmation of the biogenetic source

of compounds isolated from mixed field collections, this may clarify chemotaxonomic relationships. Although it belongs to a less commonly reported genus and is collected from a unique habitat, the Red Sea *Leptolyngbya* sp. RS03 reported here shows biosynthetic capabilities comparable to cyanobacteria collected pantropically. Bis-thiazoline-containing grassypeptolides D (**1**) and E (**2**) are closely related to grassypeptolide C, one of three structural analogues reported from a Florida Keys collection of *Lyngbya confervoides*,[12] while Ibu-epidemethoxylyngbyastatin 3 (**3**) and dolastatin 12 are additional congeners of the large family of lyngbyastatins and micropeptins. A systematic classification of the cultured Red Sea *Leptolyngbya* sp. RS03 indicates that this organism is *Leptolyngbya ectocarpi* (Gomont) Anagnostidis et Komárek 1988, according to Komárek and Anagnostidis.[18] A phylogenetic analysis of the partial 16S rRNA sequences from *Leptolyngbya* sp. RS03 (GenBank acc. no. JF518829) and the marine *Leptolyngbya* reference strain *Leptolyngbya ectocarpi* ATCC 29409 revealed a distinct cluster of *Leptolyngbya* spp. from various marine habitats (Figure 1), with the exception of *Phormidium persicinium* SAG 80.79. However, the latter shares ~99.7% 16S rRNA gene sequence identity with the *Leptolyngbya* reference strains included in this analysis and may represent the same organism that has been maintained in different culture collections.[19] Noteworthy is that the Floridian *Lyngbya* cf. *confervoides* VP0401 clusters with members of the *Phormidium* subgenus *Geitlerinema* Anagnostidis et Komárek 1988, which seemingly supports the notion that horizontal gene transfer plays an important role in the evolution of cyanobacteria.[18] Also included in this analysis is a separate cultured Red Sea *Leptolyngbya* sp. (RS02) that produces the brominated macrolide phormidolide,[20] which was previously isolated from an Indonesian *Phormidium* sp. Further chemical and biological characterization of this second Red Sea *Leptolyngbya* is in progress. While it is well recognized that organisms isolated from their native environment may not produce the same secondary metabolites in laboratory culture,[21] LC-MS analysis of extracts of monoclonal cultures of the Red Sea *Leptolyngbya* sp. RS03 confirmed its production of the four metabolites reported here. The stereoisomerism and natural SAR within the grassypeptolide series present an intriguing example of the biosynthetic flexibility of cyanobacteria. Characterization and manipulation of the biosynthetic pathways for these natural products may provide exciting opportunities to produce new biologically active metabolites.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Jasco P-1010 polarimeter. UV spectra were measured on a SpectraMax190 (Molecular Devices). NMR data were acquired in $CDCl_3$ referenced to residual $CHCl_3$ chemical shifts (δ_C 77.2, δ_H 7.26) on a Bruker Avance III 700 MHz spectrometer equipped with a 5 mm ^{13}C cryogenic probe for compound **2**. NMR data for **1** and **3** were acquired in $CDCl_3$ on a Bruker DPX 400 MHz spectrometer equipped with a 5 mm BBI probe and also in methanol-d_4 (residual CH_3OH, δ_C 49.2, δ_H 3.31) on a Bruker DRX 600 MHz spectrometer equipped with a 5 mm TXI probe. HR-MS was performed in positive ion mode on Thermo Scientific LTQ FT Ultra Hybrid and AB SCIEX Triple TOF 5600 mass spectrometers. LC-ESIMS data were obtained on an AB SCIEX 3200 Q TRAP mass spectrometer. HPLC was performed using a Shimadzu dual LC-20AD solvent delivery system with a Shimadzu SPD-M20A UV/vis photodiode array detector.

Collection, Isolation, and Culture of the Red Sea *Leptolyngbya*. An apparent mixed assemblage of cyanobacteria was collected by hand using scuba from the *SS Thistlegorm* shipwreck (46−98 ft) in the Red Sea (N 27°48.849′ E 33°55.222′) on May 27, 2007. Morphological characterization was performed using a Zeiss phase contrast microscope (100× objective), and the specimen was identified systematically according to Komárek and Anagnostidis.[18] Live cyanobacteria were isolated microscopically (Olympus SZ40 stereomicroscope) and grown in triplicate in 24-well culture plates at 27 °C with a 12 h light−dark cycle (~5 μmol photons s^{-1} m^{-2} provided by 40 W cool-white fluorescent lights). Four different enrichment mediums were used to investigate optimal conditions for survival in laboratory culture. These included 0.2 μm-filtered seawater from the Red Sea (RSW); RSW amended with soil extract; BG-11 medium containing DN vitamin mix,[22] which had been modified to closely resemble conditions of the cyanobacterium's natural environment (RSM; pH 8.4 and salinity 41‰); and RSM + RSW (1:1). Monoclonal cultures of *Leptolyngbya* were isolated microscopically from serial dilutions of enrichment cultures in which exceptional growth was present (RSM:RSW) and maintained in RSM.

DNA Extraction and Amplification of Cyanobacterial 16S rRNA. Prior to DNA extraction, heterotrophic bacteria within the cyanobacterial cultures were reduced by sequential treatment through sonication, washing, and addition of antibiotics as previously described.[23] Genomic DNA was then extracted from approximately 40 mg of freeze-dried cyanobacterial tissue using the Wizard Genomic DNA purification kit (Promega Inc., A1120) following the manufacturer's protocol. The isolated genomic DNA was subjected to further purification using an anion exchange column (Qiagen, Genomic-tip 20/G). DNA concentration and purity was measured on a Bio-Rad SmartSpec 3000 spectrophotometer. Approximately 650 bp of the upstream cyanobacterial 16S rRNA sequence was amplified from these genomic extracts using the cyanobacteria-specific primers CYA106F and CYA781Ra/b,[24] while the cyanobacteria-specific primer CYA359F and general bacterial primer 1509R were used to amplify 1150 bp downstream.[25] Sequences were amplified from approximately 50 ng of DNA using GoTaq Hot Start polymerase (0.5 uL, Promega) according to the manufacturer's specifications. Polymerase chain reactions (PCR) were performed in an Eppendorf Mastercycler gradient thermal cycler (Eppendorf, Hauppauge, NY, USA) as follows: initial denaturation at 95 °C for 3 min; 15 cycles of Touch Down PCR, 95 °C for 30 s, 65 °C for 45 s (decreased by 1 °C per cycle), and 72 °C for 1 min; 15 additional cycles of amplification, 95 °C for 20 s, 50 °C for 20 s, and 72 °C for 1.5 min; and final elongation at 72 °C for 3 min. PCR products were gel-purified, cleaned with the QIAquick gel extraction kit (cat. no. 28704, Promega), and directly sequenced on an ABI 3730 capillary sequencer by the Oregon State University Center for Genome Research and Biocomputing DNA Sequencing Core Facility. The amplification primers described above were used as sequencing primers with the addition of reverse complement primers CYA359R and CYA781Fa/b for additional sequence coverage at the end regions of the 16S rRNA gene amplification product. The 16S rRNA partial gene sequences were inspected visually and assembled using CAP3.[26] The resulting contig was analyzed for chimeric sequences using Pintail[27] and compared to sequences in the Ribosomal Database Project database (http://rdp.cme.msu.edu) and GenBank (http://www.ncbi.nlm.nih.gov). The consensus sequence was deposited in GenBank under accession number JF518829.

Phylogenetic Analysis. The 16S rRNA gene sequences of 29 marine cyanobacterial species within the Oscillatoriales (III) and two marine γ-proteobacteria strains, *Vibrio harveyi* ATCC 14126 and *Vibrio parahemolyticus* ATCC 17802 (included as outgroups), were collected from GenBank. The 16S rRNA gene library was screened for chimeric sequences using the computer program Mallard,[28] aligned using ClustalX in MEGA 5,[29] and the resulting alignment optimized with RASCAL[30] for a total of 1250 positions (89.7% identity) in the final data set covering the

V2 to V8 hypervariable regions within the 16S rRNA gene. Prior to phylogenetic predictions, a statistical selection of best-fit models of nucleotide substitutions for the 16S rRNA data set was made using Akaike and Bayesian information criteria (AIC and BIC) in jModelTest 0.1.1.[31] Phylogenetic trees were calculated using the minimum-evolution algorithm in MEGA 5 as well as the Bayesian (MrBayes)[32] and phylogenetic maximum likelihood (PhyML v3.0)[33] algorithms in Geneious 5.3.[34] The minimum evolutionary distances were determined using a maximum composite likelihood method performed with 1000 bootstrap replicates and the close-neighbor-interchange algorithm. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The PhyML analysis was performed with 500 bootstrap replicates using the GTR+I+G model (selected by AIC and BIC in jModelTest; proportion of invariable sites (pINV) = 0.465, shape parameter (α) = 0.387, number of rate categories = 4). Bayesian analysis was performed with the GTR+I+G substitution model (pINV = 0.452, α = 0.372, number of rate categories = 4). The Markov chain length (one cold and three heated) was set to 3 million with sampling performed every 100 generations (25% burn-in). The analysis was completed once convergence was achieved (~2.7 million generations), which was determined by an average standard deviation in split frequencies of <0.01.

Extraction and Isolation of Compounds 1−3 and Dolastatin 12. The field collection of *Leptolyngbya* (500 mL, collection code EHu5-27-07-1) for chemical extraction was stored in 2-propanol at −20 °C until extraction to yield 1.26 g of organic extract (CH_2Cl_2−MeOH, 2:1). The organic extract was subjected to bioassay-guided fractionation via NP VLC using a stepped gradient of hexanes to EtOAc to MeOH. The fraction eluting with 25% MeOH−EtOAc was further separated by RP-SPE using a stepped gradient of MeOH−H_2O from 50% MeOH−H_2O to 100% MeOH, followed by 100% CH_2Cl_2. Isocratic RP-HPLC (column: Synergi Fusion-RP, 10 × 250 mm, 70% MeCN−H_2O, 3 mL/min) of the SPE fraction C eluting in 70% MeOH−H_2O yielded two impure HPLC peaks targeted for further purification. RP-HPLC (Chirobiotic TAG, 4.6 × 250 mm, 98% EtOH−H_2O, 0.5 mL/min) of the less polar of these fractions yielded Ibu-epidemethoxylyngbyastatin 3 (**3**, 2.9 mg) and a mixture of grassypeptolides D (**1**, 1.5 mg) and E (**2**, 0.5 mg), which were separated on the Chirobiotic TAG column using 75% MeOH−H_2O (0.5 mL/min). Dolastatin 12 was also isolated from the 25% MeOH−EtOAc NP VLC fraction H. Repeated isocratic RP-HPLC (column: Synergi Fusion-RP, 10 × 250 mm, 70% MeCN−H_2O, 3 mL/min) of the SPE fraction (H2) eluting in the 70% MeOH−H_2O fraction yielded dolastatin 12 as the major component. Subsequent LC-MS profiling (Synergi Fusion-RP, 2 × 100 mm, 0.2 mL/min, linear gradient of 65% to 100% MeCN in 0.1% (v/v) aqueous TFA) of extracts from monoclonal *Leptolyngbya* cultures (2 × 1.5 L) yielded *m/z* 1138.6 (**1** and **2**, $[M + Na]^+$) and 1005 (**3**, $[M + Na]^+$) at the same retention times as **1**, **2**, and **3** purified from the original field collection.

Grassypeptolide D (1): colorless, amorphous solid; $[\alpha]^{21}_D$ +25.9 (*c* 0.15, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 212 (3.82), 260 (3.56); ^1H and ^{13}C NMR data, see Table 1, Tables S1 and S4; HR-FTMS *m/z* $[M + Na]^+$ 1138.5515 (calcd for $C_{57}H_{81}N_9O_{10}S_2Na$, 1138.5445), *m/z* $[M + H]^+$ 1116.5458 (calcd for $C_{57}H_{82}N_9O_{10}S_2$, 1116.5620).

Grassypeptolide E (2): colorless, amorphous solid; $[\alpha]^{21}_D$ +13.2 (*c* 0.15, CH_2Cl_2); UV (MeOH) λ_{max} (log ε) 212 (3.73), 256 (3.48); ^1H and ^{13}C NMR data, see Table 1, Table S2; HR-TOFMS *m/z* $[M + Na]^+$ 1138.5413 (calcd for $C_{57}H_{81}N_9O_{10}S_2Na$, 1138.5445), *m/z* $[M + H]^+$ 1116.5603 (calcd for $C_{57}H_{82}N_9O_{10}S_2$, 1116.5620).

Ibu-epidemethoxylyngbyastatin 3 (3): white, amorphous solid; $[\alpha]^{21}_D$ −48.6 (*c* 0.5, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 212 (3.98), 256 (3.81); ^1H and ^{13}C NMR data, see Table S3; HR-FTMS *m/z* $[M + Na]^+$ 1005.5983 (calcd for $C_{51}H_{82}N_8O_{11}Na$, 1005.6001), *m/z* $[M + H]^+$ 983.6162 (calcd for $C_{51}H_{83}N_8O_{11}$, 983.6175).

Dolastatin 12: white, amorphous solid; $[\alpha]^{21}_D$ −79.8 (*c* 0.5, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 212 (3.71), 256 (3.57); ^1H and ^{13}C NMR data, see Supporting Information;[13] HR-TOFMS *m/z* $[M + H]^+$ 969.6035 (calcd for $C_{50}H_{81}N_8O_{11}$, 969.6019).

Absolute Configuration of Grassypeptolides D (1) and E (2). The amino acid standards relevant to compounds **1** and **2** were obtained commercially or as gifts and prepared as 50 mM solutions in H_2O. Standards for (2*R*)- and (2*S*)-methylcysteine were kindly provided by Dr. W. H. Gerwick, Scripps Institution of Oceanography, University of California, San Diego. A portion of each standard (5.0 mg) was dissolved in 720 μL of HCO_2H at 0 °C. Next, 80 μL of H_2O_2 (30%) was added dropwise with continuous stirring, and the reaction was carried out at 0 °C for 2 h to yield either (2*R*)- or (2*S*)-methylcysteic acid (MeCysA). The product mixture was dried under a steady stream of N_2 gas and resuspended in H_2O (50 mM). The *N*-benzoyl *O*-methyl ester of (2*R*,3*S*)-2-methyl-3-aminobutyric acid was gratefully received from Dr. Hendrik Luesch, Department of Medicinal Chemistry, University of Florida. Approximately 0.4 mg was deprotected with 500 μL of 6 N HCl at 110 °C for 24 h, evaporated to dryness, and resuspended in H_2O (50 mM). Each standard was derivatized for Marfey's analysis by adding 10 μL of 1 M $NaHCO_3$ and 50 μL of *N*-α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA or D-FDLA, 1% w/v in acetone) to 25 μL of each standard solution. The mixture was heated at 40 °C for 1 h with continuous stirring, cooled to room temperature, acidified with 5 μL of 2 N HCl, evaporated to dryness, and resuspended in 250 μL of MeCN−H_2O (1:1).

Approximately 0.1 mg of **1** and 0.2 mg of **2** were dissolved separately in 3 mL of CH_2Cl_2 (−78 °C). Ozone was then bubbled through each solution for 15 min. The solution was dried under a stream of N_2 gas, followed by an oxidative workup of the residue (0.6 mL of H_2O_2−HCOOH, 1:2, at 70 °C for 20 min). The oxidation product was concentrated under vacuum and hydrolyzed with 1 mL of 6 N HCl at 110 °C for 18 h. The hydrolyzed products were resuspended in 25 μL of H_2O and derivatized for Marfey's analysis in a similar manner to the derivatized chromatographic standards. The Marfey's products of **1** and **2** were resuspended in 50 μL of MeCN−H_2O (1:1) and analyzed by RP-HPLC (Gemini C_{18} 110 A, 4.6 × 150 mm, 5 μm, 1.0 mL/min, UV detection at 340 nm) using a linear gradient of 30% to 70% MeCN in 0.1% (v/v) aqueous TFA over 50 min. The retention time (t_R, min) of the residues in the hydrolysate of **1** matched standards for D-Aba (21.9; L-Aba, 17.1), L-Cya (7.1; D-Cya, 6.5), (2*S*)-MeCysA [6.2; (2*R*)-MeCysA, 7.8], *N*-Me-D-Leu (27.8; *N*-Me-L-Leu, 24.5), (2*R*,3*R*)-Maba L-FDLA [18.8; (2*R*,3*R*)-Maba D-FDLA, 25.6; (2*R*,3*S*)-Maba L-FDLA, 18.6; (2*R*,3*S*)-Maba D-FDLA, 20.8], L-Pro (14.1; D-Pro, 17.0), *N*-Me-L-Phe (22.7; *N*-Me-D-Phe, 24.6), *N*-Me-L-Val (21.2; *N*-Me-D-Val, 25.6), D-*allo*-Thr (12.6; L-Thr, 10.2; L-*allo*-Thr, 11.1; D-Thr, 14.4). The retention times for the residues in the hydrolysate of **2** were consistent with the results for **1**, with the exception of *N*-Me-L-Leu (24.5) and L-Thr (10.2) standards matching the corresponding residues in the hydrolysate of **2**. The configuration of the Pla residue in the hydrolysates of both **1** and **2** was determined by chiral LC-MS. The retention time [Chirobiotic TAG, 4.6 × 250 mm; MeOH−10 mM NH_4OAc (3:2, pH 5.50); flow rate, 0.4 mL/min; detection by ESIMS in negative ion mode] of the natural product hydrolysate matched that for L-Pla (7.4 min; D-Pla, 8.5).

Absolute Configuration of Ibu-epidemethoxylyngbyastatin (3). Approximately 0.4 mg of **3** in 0.2 mL of anhydrous MeOH was added to a solution of $NaBH_4$ (2 mg) in anhydrous MeOH at 0 °C. After stirring for 30 min, the solution was acidified with 1 N HCl until pH 6. The solution was then partitioned between EtOAc and H_2O, and the organic layer concentrated to dryness for analysis by RP-HPLC (Synergi Fusion-RP, 10 × 250 mm, 62% MeCN−H_2O, 3.5 mL/min). A single product consistent with the dihydro form of **3** (t_R 18.9 min) was present, while unreduced compound **3** (t_R 21.3 min) was not detected. This reduction product and an additional ~0.4 mg of **3** were separately

hydrolyzed with 6 N HCl (method A: Ace high-pressure tube, 1200 W microwave for 50 s and immediately cooled to 0 °C; or method B: 110 °C for 18 h), evaporated to dryness, and resuspended in H_2O (50 mM). Standards for the 3-amino-2-methylhexanoic acid unit were kindly provided by Dr. David Horgen, College of Natural Sciences, Hawaii Pacific University. The other amino acid standards relevant to compound **3** were available commercially or from synthesis (Amp and Adhpa, see Supporting Information) and also prepared as 50 mM solutions in H_2O. Marfey's derivatization was performed by adding 10 μL of 1 M $NaHCO_3$ and 50 μL of *N*-α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA, 1% w/v in acetone) to 25 μL of each 50 mM solution. The mixture was heated at 40 °C for 1 h with stirring, cooled to room temperature, acidified with 5 μL of 2 N HCl, and evaporated to dryness. The derivatized product was resuspended in 250 μL of MeCN−H_2O (1:1) for each standard or 100 μL for the hydrolysate of **3** and analyzed by RP-HPLC (Gemini C_{18} 110A, 4.6 × 150 mm, 5 μm, 1.0 mL/min, UV detection at 340 nm) using a linear gradient of 30% to 70% MeCN in 0.1% (v/v) aqueous TFA over 50 min. The retention times (t_R min) of the derivatized residues in the hydrolysate of **3** matched *N*-Me-L-Ala (16.2; *N*-Me-D-Ala, 16.8), (2*S*,3*R*)-Amha [28.6; (2*S*,3*S*)-Amha, 23.2; (2*R*,3*R*)-Amha; (2*R*,3*S*)-Amha, 22.5], and *N*-Me-L-Val (21.2; *N*-Me-D-Val, 25.5). The retention times of *N*-Me-L-Phe (23.2) and *N*-Me-D-Phe (24.5) standards overlapped with (2*S*,3*S*)-Amha (23.2) and *N*-Me-L-Leu (24.5) standards. Thus, the derivatized hydrolysate was subjected to LC-MS analysis (Gemini C_{18}, 2.0 × 150 mm, 3 μm, 0.2 mL/min, UV and ESIMS detection, 340 nm and negative ion mode, respectively) using a linear gradient of 30% to 70% of 0.1% (v/v) HCO_2H in MeCN and 0.1% (v/v) HCO_2H in H_2O over 50 min. The retention times (t_R, min, base peak m/z) of the derivatized residues in the hydrolysate matched *N*-Me-L-Phe (23.1, 472.1) and *N*-Me-L-Leu (24.9, 439.1). For the assignment of the Ibu unit, both the decarboxylated product, (2*S*)-amino-4-methylpentan-3-one (Amp), and the reduced Ibu unit, (3*RS*,4*S*)-amino-2,2-dimethyl-3-hydroxypentanoic acid (Adhpa), were prepared (see Supporting Information). Portions were separately derivatized with L-FDLA and D-FDLA reagents and analyzed by RP-HPLC (Kinetex XB-C_{18} 110A, 4.6 × 100 mm, 2.6 μm, 1.8 mL/min, UV detection at 340 nm) using a linear gradient of 30% to 70% MeCN in 0.1% (v/v) aqueous TFA over 15 min or LC-MS (Gemini C_{18}, 2.0 × 150 mm, 3 μm, 0.2 mL/min, UV and ESIMS detection, 340 nm and negative ion mode, respectively) using a linear gradient of 30% to 70% of 0.1% (v/v) HCO_2H in MeCN and 0.1% (v/v) HCO_2H in H_2O over 50 min. For the hydrolysate of **3** for method A, both *S*- and *R*-Amp were detected by RP-HPLC at t_R = 9.8 and 10.0 min, respectively, in the ratio of 4.1:1. For the hydrolysate of **3** for method B, both *S*- and *R*-Amp were detected by RP-HPLC at t_R = 9.8 and 10.0 min, respectively, in the ratio of 1:2.2. The retention times (t_R, min; *S*/*R* ratio) of the Amp-derivatized standards were as follows: L-FDLA-*S*-Amp (9.8, 6.7:1) and D-FDLA-*S*-Amp (10.0, 1:5.9). For the hydrolysate of the reduction product of **3** (method A), the assignment of the reduced Ibu unit was determined by LC-MS analysis due to overlap in the HPLC trace. The retention time (t_R, min, 4*S*/4*R* ratio, base peak m/z) of the reduced natural product hydrolysate matched L-FDLA-(3*RS*,4*S*)-Adhpa (19.7, 13.8:1, 454.1), while D-FDLA-(3*RS*,4*S*)-Adhpa eluted at 20.6 min.

Synthesis of (2*S*)-2-Amino-4-methylpentan-3-one (Amp) and (3*RS*,4*S*)-4-amino-2,2-dimethyl-3-hydroxypentanoic acid (Adhpa). See Supporting Information.

Preparation and Chiral LC-MS of 2-Hydroxy-3-methylpentanoic acid (HMPA) for 3. Diazotization of L-Ile (100 mg, 0.75 mmol) dissolved in 50 mL of 0.2 N $HClO_4$ (0 °C) was carried out by the dropwise addition of a cold (0 °C) 20 mL solution of $NaNO_2$ (1.4 g, 20 mmol) with rapid stirring. The solution was stirred at room temperature until the evolution of N_2 subsided (~60 min). The solution was then brought to boiling for 3 min, cooled to room temperature, saturated with NaCl, and extracted with 20 mL of EtOAc. The extract was dried with anhydrous Na_2SO_4 and concentrated under vacuum to afford (2*S*,3*S*)-HMPA as an oil. The three other stereoisomers (2*S*,3*R*)-HMPA, (2*R*,3*R*)-HMPA, and (2*R*,2*S*)-HMPA were synthesized in a similar manner from L-*allo*-Ile, D-Ile, and D-*allo*-Ile, respectively. A portion of each standard was analyzed using chiral LC-MS. The retention time of the natural product hydrolysate matched that for (2*S*,3*S*)-HMPA (7.5 min; Chirobiotic TAG, 4.6 × 250 mm; MeOH−10 mM NH_4OAc, 3:2, at pH 5.50; flow rate, 0.4 mL/min; detection by ESIMS in negative ion mode). The retention times of the remaining HMPA standards were as follows: (2*S*,3*R*)-HMPA (6.5 min), (2*R*,3*R*)-HMPA (9.2 min), (2*R*,2*S*)-HMPA (8.1 min).

Cell Viability Assays. Mouse neuroblastoma neuro-2a or HeLa cells (ATCC, Manassas, VA) were cultured in RPMI-1640 media with 2 mM L-glutamine, pH 7.4 (Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1 mM sodium pyruvate (Mediatech), and 1% penicillin/streptomycin (Mediatech) at 37 °C in a humidified chamber containing 5% CO_2. Cells were seeded into 96-well plates (neuro-2a, 20 000 cells per well; HeLa, 3000 cells per well) in 90 μL of medium 4 h before treatment. Purified compounds were added to cells at final concentrations ranging from 10 nM to 10 μM (neuro-2a cells) or 2 μM (HeLa cells), each added in a 10 μL aliquot generated by serial dilution in serum-free medium on the day of the experiment, from stock solutions of 200 μM (**1** and **2**, both cell lines) or 2 mM (**3** and dolastatin 12, neuro-2a cells only) compound in 100% DMSO (neuro-2a cells) or 100% EtOH (HeLa cells). Each 96-well plate also contained untreated and vehicle-treated control cells. Neuro-2a cells were also treated with 30 μg/mL of the parent 25% MeOH−EtOAC fraction as a positive control. Cell viability was determined after 48 h treatment using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, MTT reagent (0.5 mg/mL in PBS; Sigma, St. Louis, MO) was added to each well and incubated for 2 h at 37 °C. The medium was then aspirated from all wells, and the purple formazan product solubilized with DMSO. The optical density of each well was determined at 550 nm using a BioTek Synergy HT microplate reader with Gen5 software (Bio-Tek, Winooski, VT). The cytotoxicity of each purified compound was assessed in at least three independent cultures, with the viability of vehicle-treated control cells defined as 100% in all experiments.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details for the syntheses of Amp and Adhpa. Tables of 1D and 2D NMR data for compounds **1**, **2**, and **3**. Selected NMR spectra in $CDCl_3$ and CD_3OD for compounds **1**, **2**, and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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