

## Ulongapeptin, a Cytotoxic Cyclic Depsipeptide from a Palauan Marine Cyanobacterium *Lyngbya* sp.

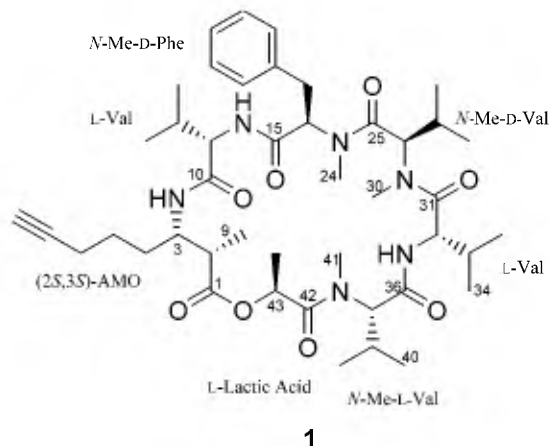
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Received January 31, 2003

Ulongapeptin (**1**), a cyclic depsipeptide, was isolated from a Palauan marine cyanobacterium *Lyngbya* sp. The gross structure was elucidated through one-dimensional TOCSY experiments and other spectroscopic techniques. The absolute and relative stereochemistry of the  $\beta$ -amino acid, 3-amino-2-methyl-7-octynoic acid (AMO), in **1** was determined by synthesis of the saturated  $\alpha$ -alkyl- $\beta$ -amino acid and Marfey's analysis of the acid hydrolysate of tetrahydro-**1**. Ulongapeptin (**1**) was cytotoxic against KB cells at an  $IC_{50}$  value of 0.63  $\mu$ M.

Cyanobacteria are photosynthetic microorganisms that inhabit diverse habitats ranging from marine to terrestrial. These ancient prokaryotes are believed to have played a key role in the evolution of the modern ecosystem since they were presumably the first organisms to produce molecular oxygen.<sup>1</sup> Filamentous cyanobacteria of the genus *Lyngbya* are the most frequently encountered cyanobacteria in tropical areas and have the ability to fix nitrogen through heterocysts. With few exceptions most secondary metabolites from *Lyngbya* arise by the incorporation of nitrogen into a combination of polyketide and peptide biosynthetic pathways.<sup>2</sup> Here we describe the isolation of a new nitrogen-containing compound, ulongapeptin (**1**), from a Palauan collection of *Lyngbya* sp.



### Results and Discussion.

Lyophilized VP755 was extracted with 1:1 methanol/ethyl acetate and the concentrated extract fractionated by normal and reversed-phase chromatography. After repeated RP-HPLC 1.1 mg of ulongapeptin (**1**), which had an  $IC_{50}$  of 0.63  $\mu$ M against KB cells,<sup>3</sup> was isolated in a yield of 0.10%.

Ulongapeptin (**1**) was found to have a molecular weight of 808 Da based on FABMS pseudo-molecular ion peaks

at  $m/z$  809, 831, and 847 for  $MH^+$ ,  $MNa^+$ , and  $MK^+$ , respectively. HR-MALDI, in conjunction with proton and carbon spectral data, established an elemental composition for the amorphous powder as  $C_{44}H_{68}N_6O_8$ . From the proton and carbon spectral data, 13  $sp^2$  carbons in the form of seven carbonyls and three carbon-carbon double bonds constituted 10 of the 14 degrees of unsaturation implied by the molecular formula of **1**. The six nitrogens could be accounted for by three secondary amide ( $\delta_H$  6.19, 8.18, and 8.24) and three tertiary *N*-methylamide groups ( $\delta_H$  2.75, 2.90, and 3.40) according to the  $^1H$  NMR data. In addition to a broad band at 1654  $cm^{-1}$  for amide carbonyls, the IR spectrum showed a strong vibration at 1727  $cm^{-1}$  characteristic of an ester moiety. The eight oxygens were therefore located in one ester and six amide carbonyl groups, suggesting that **1** was a depsipeptide consisting of one hydroxy and six amino acid units.

One-dimensional TOCSY experiments enabled us to identify the amino acids that constituted **1**. Excitation of the 2° amide proton signals at  $\delta_H$  8.18 and 8.24 produced spectra with signals for protons that were either directly or relay coupled to these NH groups and suggested the presence of two valine units. Likewise, excitation of the doublets at  $\delta_H$  3.95 (H-37) and 4.90 (H-26) generated spectra consistent with two more valine units whose nitrogens were part of tertiary amide groups. Analysis of the HMBC spectra showed  $^3J_{CH}$  cross-peaks from the methyl singlets at  $\delta_H$  3.40 (H-30) and 2.75 (H-41) to C-26 and C-37, respectively, verifying this conclusion and thereby expanded these fragments into two *N*-Me-Val units. Another unit was generated starting from the  $\alpha$ -proton signal at 4.99 ppm (H-16) that showed a strong  $^3J_{HH}$  coupling to a pair of geminal doublets of doublets at 3.90 and 2.53 ppm (H-17). This fragment was expanded into a *N*-methylphenylalanine unit via HMBC correlations to C-18 from H-17, to C-18 from H-20, and from the methylamide signal at  $\delta_H$  2.90 (H-24) to the  $\alpha$ -carbon (C-16).

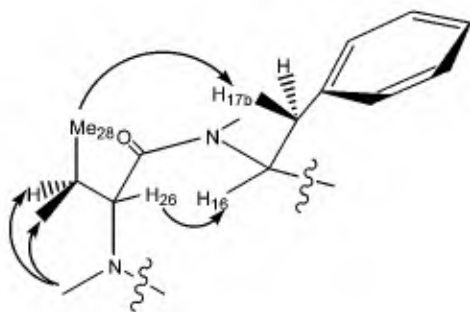
The two remaining isolated spin systems were assigned as follows. Irradiation of the methine quartet at  $\delta_H$  5.45 (H-43) in a 1D TOCSY experiment showed magnetization transfer to a methyl doublet at  $\delta_H$  1.50 (H-44) to form a two-carbon unit. A  $^1J_{CH}$  cross-peak obtained from the HSQC spectra connected this downfield methine to a carbon signal at  $\delta_C$  66.4 (C-43), a chemical shift suggestive of a carbinol and hence a lactic acid moiety. An initial one-dimensional TOCSY experiment on the final secondary

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**Figure 1.** Key ROESY correlations that suggest the D-configuration at C-26.

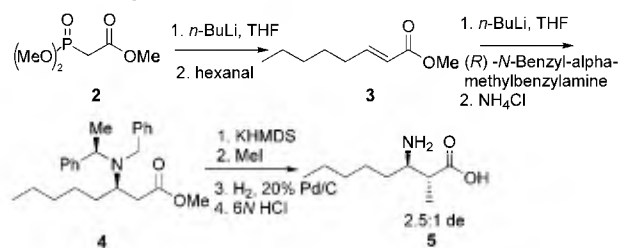
amide proton signal at  $\delta_H$  6.19 gave correlations to a nitrogen-bearing methine at  $\delta_H$  4.27 (H-3), a methyl doublet (H-9), a quartet of doublets at  $\delta_H$  2.82 (H-2), and the proton signals for H-4. These diagnostic resonances implied an  $\alpha$ -methyl- $\beta$ -amino acid unit common to cyanobacterial metabolites.<sup>2</sup> A second TOCSY experiment with a longer mixing time showed the secondary amide proton (3-NH) was relay-coupled to the terminal alkyne proton at  $\delta_H$  1.98 (H-8). Considering all the fragments previously identified and the constraints imposed by the molecular formula, this had to be a C<sub>8</sub> unit, viz., a 3-amino-2-methyloct-7-ynoic acid unit.

With the molecular formula satisfied, the gross structure was assembled via HMBC correlations. Cross-peaks from the amide proton signals to C-10, C-15, and C-36 established the sequences (AMO)-(Val)-(N-Me-Phe) [C-1 to C-24] and (Val)-(N-Me-Val) [C-31 to C-41]. These two fragments could be linked by  $^3J_{CH}$  correlations from the *N*-methylamide signals to the remaining units. Specifically, cross-peaks to C-25 from H-24, to C-31 from H-30, and to C-42 from H-41 connected all of these fragments into a linear chain, i.e., (AMO)-(Val)-(N-Me-Phe)-(N-Me-Val)-(Val)-(N-Me-Val)-(lactic acid). HMBC<sup>4</sup> and ROESY experiments failed to show any correlations that supported a connection between C-1 of the AMO unit and the lactic acid oxygen, but given the ester carbonyl vibration in the IR at 1727 cm<sup>-1</sup> and the degrees of unsaturation required by the molecular formula, alongapeptin had to be the cyclic depsipeptide **1**.

The absolute configuration of **1** was established by analysis of the degradation products. A small sample of **1** was hydrogenated to reduce the terminal alkyne and then hydrolyzed with 6 N HCl to liberate the amino acids. These were analyzed by chiral HPLC and the retention times compared with authentic standards. The proteogenic amino acids and the hydroxy acid were shown to have an L-configuration. Of the *N*-methylated amino acids, the phenylalanine-derived unit was clearly D, but both enantiomers of *N*-methylvaline were encountered in an equal amount. Hydrolysis of **1** at a lower temperature (90 °C) gave the same result and confirmed the presence of both enantiomers of *N*-Me-Val in **1**. Analysis of the ROESY data, specifically a cross-peak from H-16 to H-26 and a correlation between H-17b and H-28, suggested a D-configuration around C-26 (Figure 1). Molecular modeling of this dipeptide fragment with the stereochemistry of C-26 reversed [(N-Me-D-Phe)-(N-Me-L-Val)] did not yield a low-energy conformer consistent with the ROESY data. Correlations between the lactic acid moiety and the adjacent *N*-Me-Val unit were in line with this stereochemical conclusion and further suggested an L-configuration around C-37.<sup>5</sup>

The  $\alpha$ -methyl- $\beta$ -amino acid was synthesized as a 5:2 mixture of C-2 diastereomers (2*R*, 3*R* and 2*S*, 3*R*) as shown

### Scheme 1. Synthesis of (2*R*,3*R*)-AMO



in Scheme 1. The Horner–Wadsworth–Emmons elongation of hexanal produced a 1:1 mixture of *E*:*Z* methyl oct-2-enoate, which was easily separated by flash chromatography. Michael addition of *N*-benzyl- $\alpha$ -methylbenzylamine to *trans*-**3** afforded **4** after purification.<sup>6</sup> Subsequent methylation produced a mixture of diastereomers<sup>7</sup> that were separated after deprotection (**5**) as their 1-fluoro-2,4-dinitrophenyl-leucinamide derivatives (FDLA). Comparison with the derivatized hydrogenated hydrolysate established the absolute configuration of the 3-amino-2-methyloct-7-ynoic acid as 2*S*, 3*S* using standard Marfey's conditions.

The trivial name of **1** has been assigned after the collection site of the cyanobacterium. Ulong Channel has proven to be a rich source of interesting secondary metabolites including the lyngbyabellins<sup>8</sup> and the apramides,<sup>9</sup> both of which possess C<sub>8</sub> units similar to **1**. The structure of alongapeptin is an excellent example of the metabolic themes of cyanobacteria. Four of the five amino acid-derived units are valines, which is the most commonly encountered amino acid in cyanobacterial isolates. Two of the four valine units are *N*-methylated, a percentage that mirrors that found in the literature.<sup>2</sup> The presence of two amino acids that have been epimerized to a D-configuration is unusual though since over 90% of the amino acids incorporated into cyanobacterial metabolites have an L-configuration.<sup>2</sup> The AMO unit found in **1** has been previously identified in the mollusk metabolite onchidin.<sup>10</sup> Such  $\beta$ -amino acid units appear to be ubiquitous to cyanobacterial metabolites, and their appearance in compounds isolated from other marine organisms has often been suggested as indicative of either a dietary or symbiotic relationship between the two organisms.<sup>2</sup>

### Experimental Section

**General Experimental Procedures.** The optical rotation was measured on a Jasco-DIP-700 polarimeter at the sodium D line (589 nm). The UV spectrum was determined on a Hewlett-Packard 8453 spectrophotometer, and the IR spectrum was recorded on a Perkin-Elmer 1600 FTIR instrument as a film on a NaCl disk. The NMR spectra of **1** were recorded in CDCl<sub>3</sub> on a Varian 500 operating at 500 and 125 MHz using the residual solvent signal as an internal reference. NMR analyses of the synthetic products were carried out at 300 and 75 MHz using a Varian spectrometer. FABMS and HR-MALDI were recorded in the positive mode on a VG ZAB2SE and a DE-STR spectrometer, respectively. HPLC separations were performed on a Beckman 110B apparatus coupled to an Applied Biosystems 759A absorbance detector. All synthetic reagents and amino acids were purchased from Aldrich, and all synthetic procedures were not optimized.

**Biological Material.** The dark reddish-black clumps of cyanobacterium, designated VP755, were collected at Ulong Channel in Palau. The sample was identified by V. J. Paul and a voucher is maintained at the Smithsonian Marine Station, Fort Pierce, FL.

**Extraction and Isolation of Ulongapeptin (1).** VP755 was extracted with 1:1 EtOAc/MeOH to yield 1.11 g of lipophilic extract that was partitioned between hexane and

**Table 1.** NMR Spectral Data for Ulongapeptin in CDCl<sub>3</sub>

C/H no.	$\delta_{\text{H}}$ ( $J$ in Hz)	$\delta_{\text{C}}$	$^1\text{H}-^1\text{H}$ COSY	HMBC	TOCSY	ROESY
AMO						
1		177.7, s		2, 9		
2	2.82, qd (7.0, 3.6)	41.8, d	3, 9	9		3, 9
3	4.27, m	49.4, d	2, 3-NH, 4	9		2, 9
3-NH	6.19, d (10.1)		3		2, 3, 4, 5, 6, 8, 9	4, 11-NH
4	1.59, m	33.0, t	3, 4b	2, 3		2, 3-NH, 5, 6, 19
	1.30, m		4a			
5	1.28, m	24.1, t		3		4a
6	2.18, m	17.7, t	5, 8			
	2.02, m		5, 8			
7		83.9, s				
8	1.98, t (2.6)	68.9, d	6			
9	1.13, d (7.0)	14.7, q	2			2, 3, 13
Val						
10		171.4, s		3-NH, 11		
11	4.28, t (10.2)	61.2, d	11-NH, 12			13, 14
11-NH	8.24, d (10.2)		11		11, 12, 13, 14	3-NH, 16
12	1.62, m	31.8, d	11, 13, 14	11, 13, 14		13, 14
13	0.97, d (6.7)	19.5, q	12	12, 14		9, 11, 12, 14
14	0.78, d (6.6)	20.3, q	12	11, 12, 13		11, 12, 13, 24
N-Me Phe						
15		167.5, s		11, 16, 17b, 11-NH		
16	4.99, dd (11.5, 1.9)	62.0, d	17	17a, 17b, 24	17a, 17b	11-NH, 17b, 26
17	3.90, dd (-13.2, 11.5)	35.8, t	17b			17b, 24
	2.53, dd (-13.2, 1.9)		17a			17a, 19, 28
18		137.6, s		17, 20		
19/23	7.26, d (7.4)	129.4, d	20	17, 19, 21		4, 17b, 28
20/22	7.30, dd (7.4, 6.0)	128.6, d	19, 21			
21	7.25, t (6.0)	126.7, d		19		
24	2.90, s	29.2, q				14, 17a
N-Me-Val						
25		170.9, s		16, 24, 26		
26	4.90, d (10.9)	58.4, d	27	28, 29, 30	27, 28, 29	16, 28, 29
27	2.39, m	28.5, d	26, 28, 29	28, 29		30
28	1.07, d (6.5)	20.1, q	27	27, 29		17b, 19, 27
29	0.94, d (6.4)	19.6, q	27	26, 27, 28		26, 30, 35
30	3.40, s	31.6, q		26		27, 29, 32, 35
Val						
31		175.7, s		26, 30, 32		
32	4.76, t (9.4)	54.6, d	32-NH, 33	34, 35		30, 34, 35
32-NH	8.18, d (9.4)		32		32, 33, 34, 35	37
33	2.06, m	31.2, d	32	32, 34, 35		34, 35
34	0.87, d (6.7)	18.6, q	33	32, 35		32, 33
35	0.84, d (6.8)	19.7, q	33	34		29, 35
N-Me-Val						
36		167.6, s		32, 32-NH, 37		
37	3.95, d (10.5)	66.5, d	38	39, 40, 41	38, 39, 40	32-NH, 39, 40, 43
38	2.46, m	26.1, d	37, 39, 40	37, 40		38, 40, 41
39	1.03, d (6.3)	20.7, q	38	37, 38, 40		37
40	0.87, d (6.7)	19.2, q	38	37, 38, 39		38, 44
41	2.75, s	29.3, q		37		34, 38, 40
Lac						
42		170.7, s		37, 41, 44		
43	5.45, q (6.6)	66.4, d	44	44	44	37, 44
44	1.50, d (6.6)	17.7, q	43	43		40, 43

80% aqueous MeOH. After drying, the aqueous methanol residue was partitioned between water and *n*-butanol. The residue from the organic layer was subjected to normal-phase flash chromatography eluting with increasing amounts of methanol in dichloromethane. This resulted in the cytotoxicity concentrated primarily in the 5% methanol fraction. Subsequent separation on a C<sub>18</sub> column with increasing amounts of MeCN in H<sub>2</sub>O resulted in the activity being concentrated primarily in the 60% MeCN in H<sub>2</sub>O fraction. This sample was purified twice by RP-HPLC [Ultracarb 5 ODS 30, 10 × 250 mm, 3 mL/min, detection at 220 nm], first with 70% MeCN in H<sub>2</sub>O (*t*<sub>R</sub> 21.3 min) and then with 80% MeOH in H<sub>2</sub>O to yield 1.1 mg of **1** (*t*<sub>R</sub> 25.4 min).

**Ulongapeptin (1):** amorphous powder:  $[\alpha]_{\text{D}}^{25} -16^\circ$  (*c* 0.4, MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 204 (3.84) nm; IR (film)  $\nu_{\text{max}}$  3336, 1727, 1654 (br), 1508, 1458, 1259, 1078 cm<sup>-1</sup>; <sup>1</sup>H NMR, <sup>13</sup>C NMR, <sup>1</sup>H-<sup>1</sup>H COSY, HMBC, TOCSY, and ROESY data, see Table 1; FABMS *m/z* 809.4 [M + H]<sup>+</sup>; HR-MALDI *m/z* [M

+ H]<sup>+</sup> 809.5226 (calcd for C<sub>44</sub>H<sub>69</sub>N<sub>6</sub>O<sub>8</sub> 809.5171, 5.5 mDa error).

**Absolute Stereochemistry of the Amino Acid-Derived Units.** A 0.3 mg sample of **1** dissolved in 0.5 mL of methanol was placed in a 1 mL vial along with 1 mg of 30% Pd/C. The solution was stirred under an atmosphere of H<sub>2</sub> for 18 h at room temperature. The reaction mixture was filtered through a pad of Celite, which was then flushed with methanol. After removal of the solvent, the residue was taken up in 0.3 mL of 6 N HCl and heated to 90 °C for 18 h. The acid was removed under a stream of nitrogen and the hydrolysate analyzed by chiral HPLC, comparing the retention times of the components of the hydrolysate with those of authentic standards [Chirex Phase 3126 (D), 4.6 × 250 mm, Phenomenex; flow rate 1 mL/min, detection at 254 nm, solvent 2 mM CuSO<sub>4</sub> for the valines; 2 mM CuSO<sub>4</sub>/MeCN (95:5) for lactic acid and 85:15 for *N*-Me-Phe]. The retention times (*t*<sub>R</sub>, min) of the standards were L-Val (30.3), D-Val (54.5), *N*-Me-L-Val (23.4), *N*-Me-D-Val



(41.8), L-lactic acid (18.5), D-lactic acid (31.5), *N*-Me-L-Phe (31.2), and *N*-Me-D-Phe (33.5). The retention times of the components in the hydrolysate were L-lactic acid (18.5), L-Val (30.3), *N*-Me-L-Val (23.4), *N*-Me-D-Val (41.8), and *N*-Me-D-Phe (33.5). The identities of the peaks were also confirmed by co-injection.

**Absolute Stereochemistry of the 3-Amino-2-methyloct-7-ynoic Acid.** The hydrogenated hydrolysate was derivatized with L-FDLA by the standard procedure and compared with the derivatized synthetic standards.<sup>11</sup> The analysis was carried out by RP-HPLC [YMC-Pack AQ-ODS, 10 × 250 mm, 50% MeCN in 0.01 N TFA, flow rate 2.5 mL/min, PDA detection]. The retention times (min) of the L-FDLA derivatized standards were (2*R*,3*S*)-**5** (25.7), (2*S*,3*S*)-**5** (26.7), (2*R*,3*R*)-**5** (45.6), and (2*S*,3*R*)-**5** (54.1)<sup>12</sup> with the retention times of L-FDLA-(2*R*,3*S*)-**5** and l-FDLA-(2*S*,3*S*)-**5** being inferred from the retention times of D-FDLA+(2*S*,3*R*)-**5** and d-FDLA+(2*S*,3*S*)-**5** respectively. The retention time of the β-amino acid in the hydrolysate was 26.7 min (2*S*, 3*S*), the identity of which was confirmed by co-injection of the (2*S*,3*S*)-**5**. The previously identified amino acids appeared at 12.0 (L-Val), 15.1 (*N*-Me-L-Val), and 20.5 min (*N*-Me-D-Val).

**Synthesis of 3-Amino-2-methyloctanoic Acid.** To 15 mL of THF in a 100 mL flask under N<sub>2</sub> were added trimethyl phosphonoacetate (**2**) (5.5 mmol) and *n*-BuLi (5.6 mmol). After stirring at -78 °C for 1 h, this mixture was cannulated into 20 mL of THF containing hexanal (5.5 mmol). The reaction was allowed to warm to room temperature overnight. The solvent was removed and the residue partitioned between diethyl ether and water. The residue from the organic layer was dissolved in a 20:1 mixture of petroleum ether/diethyl ether and chromatographed on silica to yield pure methyl oct-2(*E*)-enoate (**3**). This α,β-unsaturated ester was then treated according to Davies' procedure<sup>13</sup> except the diastereomers were not separated after methylation with KHMDS and iodomethane. The C-2 diastereomers were separated after deprotection as their FDLA derivatives in the manner previously described.

**D-FDLA + (2*R*,3*R*)-3-Amino-2-methyloctanoic Acid:** <sup>1</sup>H NMR (actone-*d*<sub>6</sub>, 500 MHz) δ (integration, multiplicity, *J* in Hz) 9.11 (1H, s), 8.93 (1H, d, 9.2), 8.55 (1H, d, 6.4), 7.55 (1H, br s), 6.63 (1H, br s), 6.11 (1H, s), 4.26 (1H, m), 3.99 (1H, m), 2.96 (1H, m), 1.86 (2H, m), 1.68 (1H, m), 1.40 (2H, m), 1.30 (6H, m), 1.23 (3H, d, 6.3), 1.01 (3H, d, 5.9), 0.93 (3H, d, 5.7), 0.85 (3H, t, 7.2).

**L-FDLA + (2*R*,3*R*)-3-Amino-2-methyloctanoic Acid:** <sup>1</sup>H NMR (actone-*d*<sub>6</sub>, 500 MHz) δ (integration, multiplicity, *J* in Hz) 9.11 (1H, s), 8.91 (1H, d, 8.9), 8.61 (1H, d, 6.3), 7.54 (1H, br s), 6.73 (1H, br s), 6.11 (1H, s), 4.26 (1H, m), 3.98 (1H, m), 2.93 (1H, m), 1.89 (2H, m), 1.68 (1H, m), 1.44 (2H, m), 1.31 (6H, m), 1.20 (3H, d, 7.3), 1.02 (3H, d, 6.2), 0.94 (3H, d, 6.1), 0.85 (3H, t, 7.9).

**D-FDLA + (2*S*,3*R*)-3-Amino-2-methyloctanoic Acid:** <sup>1</sup>H NMR (actone-*d*<sub>6</sub>, 500 MHz) δ (integration, multiplicity, *J* in Hz) 9.10 (1H, s), 8.54 (1H, d, 8.9), 8.52 (1H, d, 5.5), 7.71 (1H, br s), 6.64 (1H, br s), 6.20 (1H, s), 4.35 (1H, m), 4.25 (1H, m), 2.92 (1H, m), 1.84 (2H, m), 1.68 (1H, m), 1.40 (2H, m), 1.30 (6H, m), 1.23 (3H, d, 7.0), 1.02 (3H, d, 6.0), 0.94 (3H, d, 6.2), 0.83 (3H, t, 7.0).

**L-FDLA + (2*S*,3*R*)-3-Amino-2-methyloctanoic Acid:** <sup>1</sup>H NMR (actone-*d*<sub>6</sub>, 500 MHz) δ (integration, multiplicity, *J* in

Hz) 9.11 (1H, s), 8.93 (1H, d, 9.2), 8.55 (1H, d, 6.4), 7.55 (1H, br s), 6.63 (1H, br s), 6.11 (1H, s), 4.30 (1H, m), 4.11 (1H, m), 2.85 (1H, m), 1.86 (2H, m), 1.70 (1H, m), 1.47 (2H, m), 1.27 (6H, m), 1.22 (3H, d, 7.1), 1.04 (3H, d, 5.7), 0.95 (3H, d, 5.9), 0.85 (3H, t, 8.0).

**Acknowledgment.** We would like to thank the Republic of Palau for the marine research permit and the National Cancer Institute for providing support through R01 grant CA12623 and NCPDDG grant CA53001. The upgrades to the NMR spectrometers used in this research were funded by grants from the CRIF Program of the National Science Foundation (CHE9974921), the Air Force Office of Scientific Research (F49620-01-1-0524), and the Elise Pardee Foundation. The cyanobacterium was collected by J. Starmer and identified with the assistance of E. Cruz-Rivera. G. Tien at the University of Hawaii, Department of Chemistry carried out the bioassays. The UCR Mass Spectrometry Facility, Department of Chemistry, University of California, performed the mass spectral analyses at Riverside.

**Supporting Information Available:** The <sup>1</sup>H, <sup>13</sup>C, and 2D NMR data of **1** and the full model for Figure 1 are available free of charge via the Internet at <http://pubs.acs.org>.

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- Two HMBC experiments with <sup>3</sup>J<sub>CH</sub> optimized for 7 and 4 Hz, respectively, failed to show any correlation between C-1 and H-43.
- ROESY cross-peaks between H-37 and H-43 indicate a *cis* amide bond, while correlations between H-44 and H-40 of the L-lactic acid and adjacent *N*-Me-L-Val units indicate the two side chains are on the same face of the molecule. Analysis of models suggested that H-40 was responsible for the latter correlation rather than the overlapping H-34. Unfortunately too little material remains to conclusively establish which methyl group shows a ROESY correlation to H-44.
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NP030050S