Isolation of Homodolastatin 16, a New Cyclic Depsipeptide from a Kenyan Collection of *Lyngbya majuscula*

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An examination of an organic extract of the cyanobacterium *Lyngbya majuscula*, collected from Wasini Island off the southern Kenyan coast, led to the isolation of the known cyclic depsipeptide antanapeptin A (**1**), recently isolated from a Madagascan collection of *L. majuscula*, and a new bioactive cyclic depsipeptide, homodolastatin 16 (**2**). The structures of these two compounds were determined from NMR and mass spectrometry data. Homodolastatin 16, a higher homologue of the potential anticancer agent dolastatin 16, exhibited moderate activity against oesophageal and cervical cancer cell lines.

The ubiquitous tropical cyanobacterium *Lyngbya majuscula* is a prolific producer of bioactive metabolites, and ca. 30% of all natural products reported from marine cyanobacteria have been isolated from this species.[1,2] The plethora of structurally diverse secondary metabolites isolated from *L. majuscula* exhibit a variety of bioactivities including antifeedant,[3] molluscicidal,[4] antiproliferative,[5] and immunosuppressive[6] activities. More than half of *L. majuscula*'s known secondary metabolites are either cyclic or linear lipopeptides,[1] of which cyclic depsipeptides comprise a significant proportion. Several of these cyclic depsipeptides, e.g., lyngbyalbellin A,[7] lyngbyastatin 1, and dolastatin 12,[8] possess moderate in vitro anticancer activity. As the profiles of secondary metabolites produced by populations of *L. majuscula* collected from various locations differ significantly, we have extended our search for new marine anticancer agents from western Indian Ocean marine organisms[9] to include an examination of an organic extract of *L. majuscula* collected near Wasini Island (southern Kenya) in December 2000. Investigations of the natural products chemistry of *L. majuscula* populations occurring along the tropical east coast of Africa have thus far been limited to Mozambique[10] and, more recently, Madagascar.[11−13] This study therefore represents the first study of the secondary metabolites produced by a population of *L. majuscula* occurring off the Kenyan coast.

Although the dichloromethane/methanol extract of the Wasini Island collection of *L. majuscula* was inactive in the standard brine shrimp assay,[14] the ^{1}H NMR spectrum of this extract revealed resonances suggestive of interesting lipopeptide-type metabolites. Consequently, an NMR-guided chromatographic protocol comprised of size exclusion and silica gel chromatography followed by reversed-phase HPLC of the *L. majuscula* extract afforded two cyclic depsipeptides: the known antanapeptin A (**1**)[13] and the new homodolastatin 16 (**2**), in similar low yields (ca. 2.5 × 10^{-5}%).

HRFABMS data established the molecular compositions of **1** and **2** as $C_{41}H_{60}N_4O_8$ (obsd $[M + H]^+$ at *m/z* 737.4460) and $C_{48}H_{72}N_6O_{10}$ (obsd $[M + Cs]^+$ at *m/z* 1025.4364), respectively. The ^{1}H and ^{13}C NMR data of **1** were consistent with those of anatanapeptin A recently isolated from a Madagascan population of *L. majuscula*.[13] In the Kenyan *L. majuscula* extract we found no evidence of the other depsipeptides [antanapeptins B−D and dolastatin 16 (**3**)] reported by Nogle and Gerwick[13] from the Madagascan *L. majuscula* extracts. The molecular formula of **2**, together with the eight carbonyl (δ_C 169.0, 169.4, 169.6, 171.0, 171.1, 171.3, 172.2, 174.6) and four overlapped monosubstituted aromatic ring (δ_C 126.2, 128.3, 129.6, 140.6) resonances and the single *N*-methyl singlet (δ_H 3.08) observed in the ^{13}C and ^{1}H NMR spectra of **2**, initially suggested that this compound was either a higher homologue of **3** ($C_{47}H_{70}N_6O_{10}$),[15] differing by a single methylene group, or a hydrogenated analogue of kulokainalide-1 (**4**, $C_{48}H_{70}N_6O_{10}$),[16] differing by two hydrogen atoms. The latter alternative was eliminated by the absence of resonances corresponding to a terminal olefin in the ^{13}C NMR spectrum of **2**. Conversely, comparison of the ^{13}C chemical shift data of **2** with those of **3**[15] (Table 1) revealed substantial structural homology between these two compounds. The only significant difference between the two ^{13}C data sets was the incompatibility of the resonances assigned to the *N*-methylvaline (*N*-MeVal) residue in **3** with the analogous data for **2**, thus suggesting the presence of a different α-amino acid at this position in the structure of **2**. The contiguous coupling sequence, delineated in the COSY spectrum of **2**, from the deshielded methine (δ 5.27, d, J = 10.9 Hz, H-2) via methine (δ 2.17, H-3) and methylene (δ 2.14, H-5) protons to the methyl triplet (δ 0.91, J 7.3 Hz, H_3-6) provided the initial evidence for an *N*-methylisoleucine (*N*-Me-Ile) residue in **2**. Further support for the incorporation of an *N*-Me-Ile unit in the structure of **2** was provided from the HMBC data in which two- and three-bond HMBC correlations were observed from the deshielded H-2 methine proton to four surrounding carbon atoms (C-3, C-4, C-5, and C-7). An *N*-Me-Ile/*N*-Me-Val transposition is not an uncommon structural difference in cyclic depsipeptides from the same collection of *L. majuscula*, e.g., antanapeptins A and D,[13] and the yanucamides A and B.[17] The three partial structures

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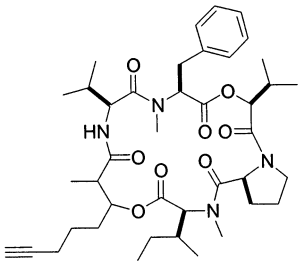

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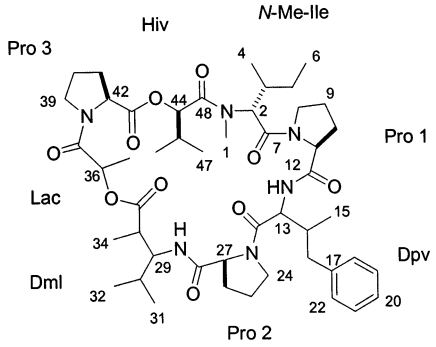

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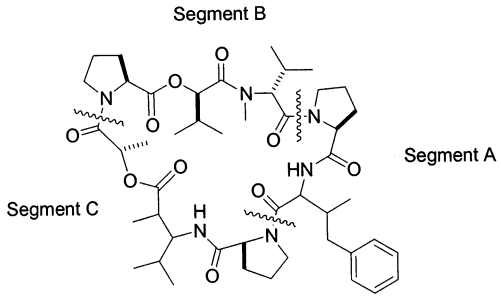

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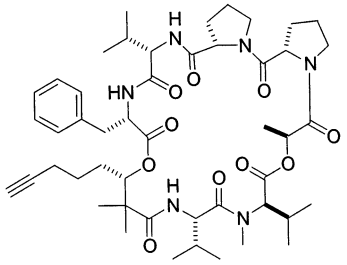

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(segments A−C) identified by Pettit et al.[15] from the HMBC, NOESY, and ROESY data for **3** were similarly identified from the HMBC and NOESY data of **2** (Table 1). Crucial NOESY correlations from H-2 to H_2-8, H-13 to H_2-24, and H-36 to H-42 provided unequivocal evidence for linking the three segments together in **2** in the same clockwise A−C−B order proposed for **3**.

Although proteinogenic L-amino acids overwhelmingly predominate in *L. majuscula* lipopeptides, the biosynthetic processes that construct these lipopeptides occasionally draw from the nonribosomal-derived pool of D- and *allo*-configured amino acids, e.g., dolastatin 16 (D-*N*-Me-Val)[15] and palau'imide (D-Val).[18] The absolute stereochemistry of the α-amino acids in **2** was unequivocally determined by HPLC analysis of three different diastereomeric derivatives of its 6 N hydrochloric acid hydrolysate. Marfey's reagent (*N*α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide, FDAA)[19] has traditionally been the precolumn chiral derivatizing agent of choice for the stereochemical analysis of cyanobacteria-derived amino acids. However, in systematic studies we found that the assignment of unnatural amino acids can be ambiguous when solely dependent on one chiral derivatizing agent.[20] Additional derivatization of portions of the acid hydrolysate with GITC (2,3,4,6-tetra-*O*-acetyl-*β*-glucopyranosyl isothiocyanate),[21] and S-NIFE [S-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester][22] can provide unequivocal confirmation of amino acid configurations. All three methods confirmed the presence of L-proline (L-Pro) and D-*N*-Me-Ile in **2**. A possibly significant difference in the optical rotation of the antanapeptin A ($[\alpha]_D$ −25°) isolated from the Kenyan *L. majuscula* from that reported recently from the Madagascan *L. majuscula* ($[\alpha]_D$ −50°) initiated a similar triplicate stereochemical analysis of the acid hydrolysate of this compound. The four amino acids in **1** (Pro, Val, *N*-Me-Ile, *N*-Me-Phe) were all found to have the expected L-configuration. The detection of small amounts of D-Pro in both hydrolysates of **1** and **2** suggested either slight racemization of proline had occurred during the hydrolysis of the cyclic depsipeptides and subsequent derivatization of the hydrolysate or the possibility that both D-Pro and L-Pro were present in the original cyclic depsipeptides. In situ partial racemization was confirmed from the similar rates of isomerization observed when D-Pro and L-Pro standards were subjected to the same hydrolysis and derivatization conditions.

Chiral GC comparison of the retention times of the methylated hydrolysate of **1** and **2** with similarly methylated D- and L-hydroxyisovaleric acid (Hiv) established an L- and D- configuration for this residue in **1** and **2**, respectively. Both assignments are in agreement with those reported for **1** and **3**. The L-configuration of the lactate residue in **3** was originally established from chiral HPLC data.[15] However, several attempts, using both chiral GC analysis and HPLC (with precolumn derivatization), failed to reproducibly establish the stereochemistry of the lactate residue in **2**. The stereochemistry of the 3-hydroxy-2-methyl-7-octynoic acid (Hmoya) fragment in **1** and the dolaphenvaline (Dpv) and dolamethylleuline (Dml) fragments in **2** remains unassigned.

Given the strong activity of dolastatin 16 against a wide variety of cancer cell lines, e.g., lung (NCI-H460: GI_{50} 0.00096 μg/mL), colon (KM20L2 GI_{50} 0.0012 μg/mL), brain (SF-295, GI_{50} 0.0052 μg/mL), and melanoma (SK-MEL5 GI_{50} 0.0033 μg/mL),[16] we were hopeful that the homodolastatin 16 isolated from the Kenyan *L. majuscula* would prove active against esophageal cancer. Black populations in certain regions of Southern Africa experience an abnormally high incidence of esophageal cancer.[23] The identification of novel agents with significant cytotoxic activity against esophageal cancer cells would substantially enhance our ability to treat this debilitating disease in Africa. However, homodolastatin 16 showed only moderate activity against two esophageal cancer cell lines (WHCO1 and WHCO6), exhibiting IC_{50} values of 4.3 and 10.1 μg/mL, respectively. Further screening of homodolastatin 16 against a cervical cancer cell line (ME180) yielded similar moderate activity (IC_{50} 8.3 μg/mL). Unfortunately, we did not have any dolastatin 16 in hand to carry out a comparative analysis in our esophageal and cervical cancer screens.

Table 1. NMR Spectral Data for Dolastatin 16 (**3**) and Homodolastatin 16 (**2**) in $CDCl_3$[a]

unit	position	^{13}C (**3**)[15]	^{13}C (**2**)	^{1}H (**2**, *J* in Hz)	NOESY[b]	HMBC[c]
N-Me-Ile	1	29.3	29.6	3.08, s	3, 4	2, 48
	2	59.5	57.9	5.27, d (10.9)	4, 6, 8, 15	3, 4, 5, 7
	3	25.6	31.8	2.17, m	6	6
	4	19.7	13.8	0.79, d (6.8)	1, 5, 6, 14	2, 3, 5
	5		26.2	2.14, m	4, 6	6
	6	17.8	11.2	0.91, t (7.3)	4, 2	3, 5
	7	169.3	169.0			
Pro 1	8	47.6	47.6	3.92, m; 3.45, m	2, 9, 10	9, 10
	9	24.8	24.8	2.08, m; 1.99, m	8	10
	10	30.7	30.7	2.20, m	8, 11	12
	11	61.3	61.4	4.61, dd (8.8, 2.0)	10	8, 9, 10, 12
	12	172.2	172.2			
Dpv	NH	-	-	6.72, d (8.6)	1, 8, 9, 13, 15	12
	13	50.6	50.6	4.96, d (8.6)	14, 24	14, 15, 23
	14	40.9	40.9	1.74, m	13, 15, 16	
	15	15.1	14.9	0.80, d (6.6)	2, 10, 14, NH, 29	13, 14
	16	41.0	41.0	2.51, m; 2.39, m	13, 14, 24	13, 14, 15, 17,18/22
	17	140.6	140.6			
	18/22	129.6	129.6	7.35, d (7.1)	13, 14, 26	16, 19/22
	19/21	128.3	128.3	7.27, d (7.6)	18/22	17, 18/22
	20	126.2	126.2	7.17, dd (7.6, 7.1)	19/21	18/22
	23	171.3	171.3			
Pro 2	24	45.9	45.9	2.85, m; 2.52, m	13, 29, 31, 34	26
	25	25.0	25.0	1.84, m; 1.73, m	13, 24, 26	27
	26	25.5	25.5	2.40, m; 1.49, m	13, 24, 25	24, 28
	27	58.8	58.8	4.55, d (7.6)	1, 26	23, 24, 26
	28	171.0	171.1			
Dml	NH			7.70, d (10.1)	27, 29, 30, 31, 34	28, 29
	29	56.4	56.4	3.66, m	31, 32, 33, 34	30, 32, 33, 35
	30	32.3	32.3	1.54, m	31,32	29, 32
	31	19.7	19.7	1.06, d (7.1)	29, 30	29, 30
	32	20.3	20.3	0.87, d (7.1)	29, 30	29, 30, 31
	33	38.7	38.7	2.86, m	13, 16, 29, 34	34, 35
	34	14.9	15.1	1,01, d (7.1)	36	29, 33, 35
	35	174.6	174.6			
Lac	36	66.6	66.6	5.19, q (6.8)	37, 42	35, 37, 38
	37	17.2	17.2	1.44, d (6.6)	36	36, 38
	38	169.2	169.4			
Pro 3	39	46.4	46.4	3.67, m; 3.43, m	40	38, 40
	40	21.8	21.8	2.07, m; 1.95, m	39	42
	41	30.8	30.8	2.28, m; 2.17, m	42	40, 43
	42	57.8	57.8	4.45, dd (7.6, 1.1)	36, 41, 44	40, 43
	43	171.0	171.0			
Hiv	44	76.4	76.4	5.42, m	1, 45, 46, 47	43, 45, 46, 47
	45	28.3	28.3	2.18, m	46, 47	
	46	19.7	19.7	1.07, d (7.1)	2, 44, 47	44, 45, 47
	47	16.1	16.1	1.05, d (7.1)	2, 44, 46	44, 45, 46
	48	169.6	169.6			

[a] ^{13}C NMR data for **5** acquired at 125 MHz; ^{1}H and ^{13}C NMR data for **2** acquired at 400 and 100 MHz, respectively. [b] Protons showing NOESY correlation with indicated proton(s). [c] Protons showing long-range correlation with indicated carbon.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 141 polarimeter at the sodium D line (589 nm). Infrared spectra were recorded on a Perkin-Elmer Spectrum 2000 FT-IR spectrometer with compounds as films (neat) on NaCl disks. NMR spectra were recorded on a Bruker 400 MHz Avance spectrometer in $CDCl_3$, and chemical shifts are reported in ppm and referenced to residual $CHCl_3$ resonances (δ_H 7.25, δ_C 77.2). FABMS data were acquired using a JEOL SX102 mass spectrometer. Derivatized amino acids were separated and analyzed on an HP1100 LC-MSD consisting of a binary pump, a degasser, an autosampler, a DAD detector, an 1100 MSD, and a ChemStation for data acquisition and processing. HPLC isolations were performed using a Spectra Physics P100 pump and a Waters 410 differential refractometer with either a Whatman's Magnum 9 Partisil 10 normal-phase or a Phenomenex Luna C-18 reversed-phase column.

Collection. The marine cyanobacterium *L. majuscula* Harvey ex Gomont (family Oscillatriaceae Harv. ex Kirchn.) was collected from the shallow waters (1−3 m) off Wasini Island, Kenya, during December 2000. A voucher specimen is available from MD-C as collection number TDWAI. The material was stored in 2-propanol at −20 °C until extraction.

Extraction and Isolation. Approximately 136 g (dry wt) of the cyanobacterium was exhaustively extracted with CH_2Cl_2 (2:1). The extract was concentrated under reduced pressure and partitioned between CH_2Cl_2 and water. The CH_2Cl_2 partition fraction (700 mg) was chromatographed twice through Sephadex LH-20 (5:2:1 CH_2Cl_2/hexane/MeOH and 1:1 EtOAc/MeOH) to eventually give three fractions. Fraction 2 (66.0 mg) was further purified by chromatography on Si gel ($CHCl_3$/EtOAc) to yield four fractions, of which the third fraction (15.4 mg) was subject to reversed-phase C_{18} (7:8 CH_3CN/H_2O) HPLC to give anatanapeptin A (**1**, 3.5 mg) and homodolastatin 16 (**2**, 3.7 mg).

Antanapeptin A (1): pale yellow oil; $[\alpha]^{22}_D$ −25° (*c* 0.11, MeOH) [lit.[13] −50° (*c* 0.13)]; IR, ^{1}H and ^{13}C NMR data consistent with published data;[13] HRFABMS *m/z* $[M + H]^+$ 737.4460 (calcd for $C_{41}H_{61}N_4O_8$, 737.4489).

Homodolastatin 16 (2): pale yellow oil; $[\alpha]^{22}_D$ −25° (*c* 0.19, MeOH); IR (neat) cm^{-1}; 3394, 3321, 2931, 2877, 2362, 1745, 1733, 1651, 1638, 1509, 1460, 1426, 1388, 1298, 1185, 1091, 753, 702; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* $[M + Cs]^+$ 1025.4364 (calcd for $C_{48}H_{73}N_6O_{10}Cs$, 1025.4364).

Stereoanalysis of Amino Acid Residues in Anatanapetin A and Homodolastatin 16. Approximately 100 μg each of anatanapeptin A (**1**) and homodolastatin 16 (**2**) were separately hydrolyzed with 6 N HCl (108 °C, 16 h). To remove excess HCl, the hydrolysates were twice evaporated to dryness and resuspended in H_2O (100 μL). To an aliquot (1 μL) of an aqueous solution (1 μg/μL) of the amino acid standard or hydrolysate mixture were added 6% triethylamine (10 μL) and a 1% acetone solution of chiral derivatizing agent (10 μL) and allowed to react at room temperature for 10 min (GITC), for 20 min (S-NIFE), or at 50 °C for 1 h (FDAA). The reaction mixture was diluted with 5% acetic acid (10 μL), and an aliquot (20 μL) was analyzed by reversed-phase HPLC-MS [Zorbax SB column (C_{18} 150 × 2.1 mm), column temperature (50 °C), flow rate (250 μL min^{-1})] using a linear gradient of aqueous acetic acid (5%, pH 2.6) and CH_3CN/MeOH (10:1) to 1:1 aqueous acetic acid CH_3CN/MeOH over 50 min. The DAD detector was set to 340 nm (FDAA derivatives) and 254 nm (GITC and S-NIFE derivatives), respectively. The effluent was directed into the mass spectrometer after a 4−5 min delay. The molecular mass of the samples was determined using the Agilent HP MSD mass spectrometer in positive ion mode. Scanning range was from m/z = 300 to m/z = 1000. Internal standards of the derivatizing agents were used as follows: m/z = 348 for FDAA (t_R = ca. 38 min), m/z = 463 for GITC (t_R = ca. 38 min), and for S-NIFE (t_R = ca. 45 min.).

The relative retention times (t_R, min) of the FDAA derivatized residues in the hydrolysates of **1** matched L-Val (0.860; D-Val, 1.011), L-Pro (0.693; D-Pro, 0.742), L-*N*-Me-Ile (1.064; D-*N*-Me-Ile, 1.146), and L-*N*-Me-Phe (1.021; D-*N*-Me-Phe, 1.034); the GITC derivatized residues matched L-Val (0.868; D-Val, 0.925), L-Pro (0.686; D-Pro, 0.734), L-*N*-Me-Ile (1.055; D-*N*-Me-Ile, 1.127), and l-*N*-Me-Phe (1.071; D-*N*-Me-Phe, 1.118), and the S-NIFE derivatized residues matched L-Val (0.701; D-Val, 0.799), L-Pro (0.631; D-Pro, 0.659), L-*N*-Me-Ile (0.899; D-*N*-Me-Ile, 0.942), and l-*N*-Me-Phe (0.930; D-*N*-Me-Phe, 0.957). Similarly the relative retention times of the derivatized residues in the hydrolysate of **2** matched L-Pro and D-*N*-Me-Ile.

Chiral GC Analyses of the Hiv Moieties in 1 and 2. Portions of each hydrolysate of **1** and **2** were separately diluted in 50 μL of MeOH and treated with diazomethane (10 min). Excess CH_2N_2 and solvent were removed with a stream of dry N_2, and the residues resuspended in $CHCl_3$. Capillary GC analyses were conducted using a Chirasil-Val column (Alltech, 25 m × 0.25 mm) using the following conditions: column temperature increased from 30 to 40 °C at 1 °C min^{-1}, held at 40 °C for 3 min, then increased to 200 °C at 30 °C min^{-1}. The retention times of the methylated Hiv residue in **1** and **4** matched those of the L-methylated Hiv standard (13.08 min) and the D-methylated Hiv standard (13.67 min), respectively.

Cell Culture. Cells were routinely maintained at 37 °C and 5% CO_2. WHCO1 and WHCO6 cells were maintained in DMEM and supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin, and 100 μg/mL streptomycin. ME180 (ATCC #HTB-33) cells were maintained in McCoy's 5A supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin, and 100 μg/mL streptomycin.

MTT Assay. IC_{50} determinations were carried out using the MTT kit from Roche (Cat #1465007), according to manufacturer's instructions. Briefly, 1500 cells were seeded per well in 96-well plates. Cells were incubated (24 h), after which aqueous DMSO solutions of **2** (10 μL, with a constant final concentration of DMSO = 0.1%) were plated at various concentrations. After 48 h incubation, observations were made, and MTT (10 μL) solution was added to each well. After a further 4 h incubation, solubilization solution (100 μL) was added to each well, and plates were incubated again overnight. Plates were finally read at 595 nm on an Anthos microplate reader.

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