Isolation of Four New Cyclic Depsipeptides, Antanapeptins A−D, and Dolastatin 16 from a Madagascan Collection of *Lyngbya majuscula*

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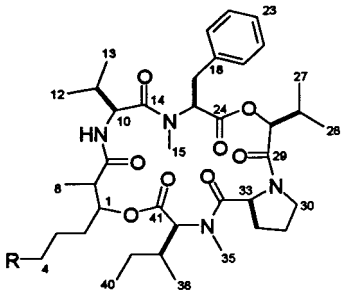
Examination of a *Lyngbya majuscula* collection from Antany Mora, Madagascar, led to the isolation of dolastatin 16 (**5**), a promising antineoplastic metabolite first reported from the marine mollusc *Dolabella auricularia*. In addition, a new series of depsipeptides, antanapeptins A−D (**1**−**4**), were discovered. Their structures were deduced by 2D NMR and mass spectrometry and are analogous to the molluscan kulomo'opunalides and the recently reported cyanobacterial metabolites, georgamide and the yanucamides. The antanapeptins were evaluated in several biological assays; however, this series did not exhibit activity.

Lyngbya majuscula is an established source of unique and bioactive secondary metabolites, especially peptides.[1] Compounds such as the lyngbyastatins, lyngbyabellins, majusculamides, and microcolins exhibit a variety of biological activities including cytotoxicity, immunosuppression, antiproliferation, and microbial toxicity.[2−5] As part of our ongoing search for structurally and pharmacologically interesting substances from this cyanobacterium, a detailed exploration of a Madagascan *L. majuscula* collection was undertaken. During this study, several depsipeptides were isolated, including dolastatin 16 (**5**), a potential antineoplastic metabolite first reported in 1997 from the marine mollusc *Dolabella auricularia*.[6] In addition, four new compounds, antanapeptins A−D (**1**−**4**), were discovered in the same organic extract. This report describes the chromatographic isolation and structure elucidation of this new series of *L. majuscula* peptides.



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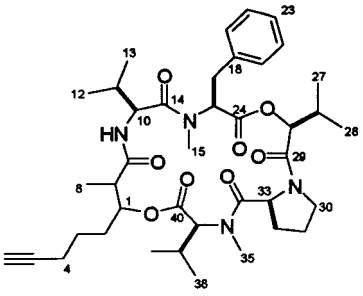

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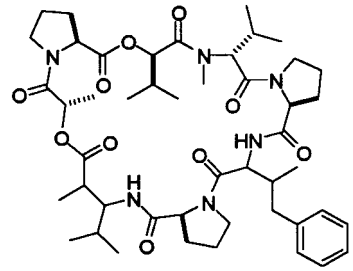

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Results and Discussion

The collection of *L. majuscula* acquired from Antany Mora, Madagascar, in April 2000, was extracted with CH_2Cl_2/MeOH (2:1) and fractionated by silica vacuum liquid chromatography. TLC and ^1H NMR analyses of the crude polar fractions indicated the presence of several structurally intriguing metabolites of peptide origin. The fraction eluting from the VLC with 85% EtOAc in hexanes was subjected to C_{18} solid-phase extraction and reversed-phase HPLC to afford four new depsipeptides, antanapeptins A−D (**1**−**4**).

Antanapeptin A (**1**) was isolated as a pale yellow oil with a molecular composition of $C_{41}H_{60}N_4O_8$, as determined by HRFABMS (obsd $[M + H]^+$ at *m/z* 737.4473). Analysis of 1D and 2D NMR spectra in $CDCl_3$ allowed construction of six partial structures (Table 1). Four standard amino acid residues were deduced as *N*-methyl isoleucine (*N*-Me-Ile), *N*-methyl phenylalanine (*N*-Me-Phe), proline (Pro), and valine (Val), while a fifth, non-amino acid moiety was identified as 2-hydroxyisovaleric acid (Hiv). The sixth and final residue required the composition $C_9H_{12}O_2$ to complete the molecular formula of **1**. The ^{13}C NMR spectrum showed two distinctive carbon signals at δ 83.5 and δ 68.9, consistent with a terminal acetylenic functionality. As previously observed, the carbon at δ 68.9 exhibited no HSQC correlations but showed a $^1J_{CH}$ coupling of 249 Hz to a methine proton at δ 1.92 in the HMBC spectrum.[7,8] This proton also exhibited a $^2J_{CH}$ HMBC correlation to the quaternary carbon at δ 83.5, confirming the presence of an acetylene. The remaining signals were then assembled

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Table 1. NMR Spectral Data for Antanapeptin A (**1**) in $CDCl_3$

unit	position	^{13}C	^{1}H (*J* in Hz)	HSQC-TOCSY[a]	HMBC[b]
Hmoya	1	76.7	4.87, d (10.4)	2, 3, 4	2, 3, 7, 8, 9
	2	27.7	1.99, 1.60	1, 3, 4	1, 3, 4, 7
	3	25.2	1.63, 1.45	1, 2, 4	1, 2, 4, 5
	4	18.0	2.20, m	1, 3	2, 5, 6
	5	83.5			
	6	68.9	1.92, brt (2.6)	4	5
	7	42.5	3.36, dd (7.3, 1.5)	8	1, 2, 8, 9
	8	14.9	1.23, d (7.3)	7	1, 7, 9
	9	171.3			
Val	NH		6.01, d (8.9)		9, 10, 14
	10	52.4	4.40, dd (8.9, 7.3)	NH, 11, 12, 13	9, 11, 14
	11	31.2	1.46, m	10, 12, 13	10, 12, 13, 14
	12	17.7	0.62, d (6.4)	NH, 10, 11, 13	10, 11, 13
	13	18.8	0.28, d (6.4)	NH, 10, 11, 12	10, 11, 12
	14	173.4			
N-Me-Phe	15	30.5	2.95, s		10, 14, 16
	16	62.4	5.15, dd (10.5, 3.8)	17	14, 15, 17, 18, 24
	17	35.3	3.50, dd (14.3, 3.7)	16	16, 18, 19/20, 24
			3.00, dd (10.6, 3.7)		
	18	137.0			
	19/20	129.3	7.25		17, 21/22, 23
	21/22	128.9	7.30		18, 19/20
	23	127.2	7.24		19/20
	24	170.4			
Hiv	25	77.4	5.03, d (9.2)	26, 27, 28	24, 26, 29
	26	29.6	2.19, m	25, 27, 28	25, 27, 29
	27	17.9	0.96	25, 26, 28	25, 26, 28
	28	18.6	0.95	25, 26, 27	25, 26, 27
	29	165.6			
Pro	30	47.3	3.89, q (7.3)	31, 32, 33	29, 31, 32, 33
			3.65, q (7.3)		
	31	25.0	2.13, 1.97	30, 32, 33	30, 32, 33
	32	29.2	2.22, 1.83	30, 31, 33	30, 31, 33, 34
	33	56.9	4.99, dd (7.7, 3.9)	30, 31, 32	29, 30, 31, 32, 34, 36
	34	172.6			
N-Me-Ile	35	28.8	3.02, s		33, 34, 36
	36	64.2	4.17, d (10.4)	37, 39	34, 35, 38, 39, 37, 41
	37	34.5	2.08, m	36, 39	36, 38, 40
	38	15.5	0.97	36, 37	36, 40
	39	25.6	1.49, 0.96	36, 37	36, 37, 38, 40
	40	11.2	0.96	36, 37, 39	
	41	170.9			

[a] Proton showing TOCSY correlation to indicated proton. [b] Proton showing HMBC correlation to indicated carbon.

by 2D NMR, identifying this unit as 3-hydroxy-2-methyloctynoic acid (Hmoya), a substructure observed in the molluscan metabolites onchidin B and the kulomo'opunalides.[9,10] However, C_8 alkynoate moieties have also been reported recently from several cyanobacterial metabolites, including the yanucamides, apramides, dragonamide, and georgamide.[7,8,11,12]

HMBC connectivities were used to unambiguously sequence the residues of **1**, completing its planar structure (Table 1). The absolute stereochemistries of **1**, determined by Marfey's analysis, included L-*N*-Me-Ile, L-*N*-Me-Phe, L-Pro, and L-Val.[13] An L-configuration was also confirmed for the Hiv residue by a GC−MS comparison of the corresponding methyl ester derivative with methylated Hiv standards. However, the absolute configuration of the Hmoya residue was not established.

Antanapeptins B (**2**) and C (**3**) were isolated by RPHPLC from the crude fraction containing antanapeptin A (**1**). Their high structural homology to **1** was demonstrated by nearly identical ^{1}H and ^{13}C NMR chemical shifts (Table 2). However, both metabolites displayed obvious differences in the Hmoya unit. While lacking the characteristic acetylene carbon signals of **1**, antanapeptin B (**2**) contained new methine (δ 138.3, δ 5.75) and methylene signals (δ 115.1, δ 4.97, 4.94) indicative of a monosubstituted olefin. Similarly, the acetylenic carbons were absent from the ^{13}C spectrum of antanapeptin C (**3**) and were replaced by two additional high-field carbons at δ 22.7 and 14.1. These observations suggested that **2** and **3** were likely the reduced double bond and single bond equivalents of **1**, respectively. Subsequently, 2D NMR and HRFABMS confirmed these assignments (Table 2). Hydrolysis and stereoanalysis of metabolites **2** and **3** were not undertaken due to their limited quantities and the desire to preserve as much sample as possible for biological testing. However, due to the comparable spectroscopic properties of **2** and **3** to those of **1**, we propose that they are of the same enantiomeric series.

Antanapeptin D (**4**) also showed a close structural relationship to **1** (Table 2). However, HRFABMS data showed an $[M + H]^+$ ion at *m*/*z* 723.4336, indicating **4** differed from **1** by loss of a CH_2 unit. 2D NMR was again used to assemble the planar structure of **4**, confirming that antanapeptin D was identical to **1**, except for substitution of an *N*-methyl valine (*N*-Me-Val) residue for the *N*-Me-Ile in **1**. The absolute stereochemistries of **4** were determined in the same manner as **1** and found to be L-*N*-Me-Val, L-*N*-Me-Phe, L-Pro, L-Val, and L-Hiv.

The antanapeptins were screened for biological activity using brine shrimp toxicity,[7] sodium channel modulation,[14] and antimicrobial bioassays.[15] This structural series, however, proved inactive in all of these assays. Similarly, the related metabolite georgamide, the major secondary metabolite from an Australian cyanobacterial collection, was

Table 2. ^{1}H and ^{13}C NMR Data for Antanapeptins B (**2**), C (**3**), and D (**4**) in $CDCl_3$[a]

	antanapeptin B (**2**)		antanapeptin C (**3**)		antanapeptin D (**4**)	
position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	77.2	4.86	77.5	4.86	76.8	4.87
2	28.1	1.89, 1.35	29.0	1.89, 1.36	27.7	1.97, 1.60
3	25.0	1.65	26.3	1.39, 1.21	25.2	1.62, 1.44
4	33.3	2.35, 2.04	31.7	1.26	18.1	2.21
5	138.3	5.75	22.7	1.28	83.4	
6	115.1	4.97, 4.94[a]	14.1	0.87[a]	68.8	1.92
7	42.6	3.34	42.7	3.33	42.5	3.36
8	14.7	1.21	14.8	1.21	14.9	1.23
9	171.4		171.6		171.3	
NH		6.02		6.02		6.02
10	52.4	4.41	52.5	4.40	52.5	4.39
11	31.1	1.46	31.3	1.45	31.4	1.45
12	17.6	0.62	17.7	0.62	17.6	0.62
13	18.8	0.28	19.1	0.28	18.9	0.26
14	173.4		173.6		173.4	
15	30.5	2.94	30.6	2.95	30.6	2.95
16	62.4	5.15	62.6	5.15	62.6	5.14
17	35.3	3.51, 3.00	35.5	3.50, 3.00	35.4	3.50, 3.00
18	136.9		137.1		137.1	
19/20	129.3	7.25	129.4	7.25	129.4	7.25
21/22	128.9	7.30	129.2	7.30	129.1	7.30
23	127.3	7.24	127.3	7.24	127.3	7.24
24	170.4		170.6		170.4	
25	77.6	5.03	77.6	5.03	77.6	5.03
26	29.6	2.19	29.9	2.19	29.6	2.19
27	18.0	0.97	18.2	0.97	18.2	0.98
28	18.6	0.94	18.7	0.94	18.9	0.95
29	165.6		165.8		165.6	
30	47.3	3.89, 3.65	47.4	3.89, 3.65	47.4	3.90, 3.65
31	25.5	2.13, 1.98	25.3	2.13, 1.97	25.2	2.12, 1.98
32	29.2	2.23, 1.84	29.4	2.23, 1.83	29.4	2.25, 1.84
33	56.9	4.99	57.1	4.99	57.0	5.00
34	172.6		172.8		172.4	
35	28.9	3.01	29.1	3.01	28.9	3.02
36	64.2	4.15	64.4	4.16	65.3	4.07[a]
37	34.6	2.08	34.8	2.07	28.3	2.33
38	15.7	0.96	15.7	0.97	19.7	0.95
39	25.7	1.50, 0.96	25.9	1.50, 0.96	19.7	0.99
40	11.3	0.95	11.4	0.95	170.7	
41	171.0		171.0			

[a] Resolved proton(s) with coupling constants differing from **1**: antanapeptin B (**2**) H-6 d (J = 10.5 Hz), antanapeptin C (**3**) H_3-6 t (J = 7.2 Hz), antanapeptin D (**4**) H-36 d (J = 10.5 Hz).

not reported to possess biological properties.[8] Interestingly, two other related metabolites, yanucamides A and B from a Fijian collection of *L. majuscula*, do display potent brine shrimp toxicity.[7] Thus, a specific biological function for this structural class of compounds remains uncertain.

Dolastatin 16 (**5**) was isolated as a white amorphous solid (11.0 mg, 0.09% extract) following reversed-phase chromatographic purification of the VLC fraction eluting with 100% EtOAc. The identity of this compound was established by comparison with the reported spectroscopic and physical data, including 1D and 2D NMR, HRFABMS, and optical rotation.[6] The recent isolations of dolastatins 3, 10, 12, and now 16, as well as several dolastatin analogues from various microalgal collections, demonstrate that this bioactive family of metabolites in fact originates in cyanobacteria.[2,16,17] Additionally, the high homology of the antanapeptins to the kulolides and kulomo'opunalides supports a cyanobacterial origin for these metabolites as well.[10]

Experimental Section

General Experimental Procedures. NMR spectra were recorded on Bruker DRX600 MHz and Bruker AM400 MHz spectrometers with the solvent $CDCl_3$ used as an internal standard. Mass spectra were recorded on a Kratos MS50TC mass spectrometer. IR and UV spectra were recorded on Nicolet 510 and Beckman DU640B spectrophotometers, respectively. Optical rotations were measured on a Perkin-Elmer 243 polarimeter. HPLC isolations were performed using a Waters Millipore model 590 pump and Waters Millipore Lambda-Max model 480 spectrophotometer.

Collection. The marine cyanobacterium *Lyngbya majuscula* (voucher specimen available from WHG as collection number MAM-25 Apr 00-1) was collected from shallow waters (1−3 m) in Antany Mora, Madagascar, on April 25, 2000. The material was stored in 2-propanol at −20 °C until extraction.

Extraction and Isolation. Approximately 602 g (dry wt) of the alga was extracted repeatedly with CH_2Cl_2/MeOH (2:1) to produce 13.5 g of crude organic extract. A portion of the extract (12.8 g) was then fractionated by silica gel vacuum liquid chromatography. The fraction eluting with 85% EtOAc in hexanes was further purified with a C_{18} solid-phase extraction (SPE) cartridge (7:3 MeOH/H_2O) and reversed-phase HPLC (9:1 CH_3CN/H_2O, Phenomenex Sphereclone 5 μ ODS) to yield 8.5 mg of antanapeptin A (**1**) and 1.0 mg of antanapeptin D (**4**). A second fraction eluting from the C_{18} cartridge (4:1 MeOH/H_2O) was also purified by reversed-phase HPLC (19:1 CH_3CN/H_2O, YMC-Pack 5 μ ODS-AQ) to yield 1.2 mg of antanapeptin B (**2**) and 1.0 mg of antanapeptin C (**3**). The fraction eluting with 100% EtOAc from the Si VLC was purified with a C_{18} cartridge (7:3 MeOH/H_2O) and reversed-phase HPLC (3:1 CH_3CN/H_2O, Shiseido C_{18} Capcell Pak 5 μ) to yield 11.0 mg of dolastatin 16 (**5**).

Antanapeptin A (1): pale yellow oil; $[\alpha]^{21}_{D}$ −50° (*c* 0.13, MeOH); UV (MeOH) λ_{max} 212 nm (log ϵ 4.5); IR (neat) 2966, 2936, 2876, 1734, 1650, 1447, 1243, 1182 cm^{-1}; ^{1}H and ^{13}C

NMR data, see Table 1; HRFABMS *m/z* $[M + H]^+$ 737.4473 (calcd for $C_{41}H_{61}N_4O_8$, 737.4489).

Antanapeptin B (2): pale yellow oil; $[\alpha]^{21}_D$ −42° (*c* 0.09, MeOH); UV (MeOH) λ_{max} 212 nm (log ϵ 4.6); IR (neat) 2964, 2931, 2874, 1734, 1651, 1447, 1241, 1183 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HRFABMS *m/z* $[M + H]^+$ 739.4636 (calculated for $C_{41}H_{63}N_4O_8$, 739.4646).

Antanapeptin C (3): pale yellow oil; $[\alpha]^{21}_D$ −42° (*c* 0.09, MeOH); UV (MeOH) λ_{max} 212 nm (log ϵ 4.6); IR (neat) 2963, 2932, 2873, 1734, 1651, 1450, 1242, 1183 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HRFABMS *m/z* $[M + H]^+$ 741.4815 (calcd for $C_{41}H_{65}N_4O_8$, 741.4802).

Antanapeptin D (4): pale yellow oil; $[\alpha]^{21}_D$ −30° (*c* 0.05, MeOH); UV (MeOH) λ_{max} 212 nm (log ϵ 4.4); IR (neat) 2965, 2936, 2874, 1734, 1650, 1448, 1242, 1182 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HRFABMS *m/z* $[M + H]^+$ 723.4336 (calcd for $C_{40}H_{59}N_4O_8$, 723.4333).

Biological Assays. The brine shrimp (*Artemia salina*) toxicity assay was performed as previously described with all samples at 10 μg/mL.[7] Modulation of the voltage-sensitive sodium channel in mouse neuro-2a neuroblastoma cells was also examined as previously described.[14] Antimicrobial activity was evaluated using standard paper disk/agar plate methodology against *Candida albicans* (ATCC 14053), *Pseudomonas aeruginosa* (ATCC 10145), and *Escherichia coli* (ATCC 11775).[15] The antanapeptins displayed no antimicrobial activity to any of the test organisms at concentrations of 100 μg/disk.

Marfey Analysis of Antanapeptins A and D.[13] Approximately 0.8 mg of antanapeptin A (**1**) and 0.3 mg of antanapeptin D (**4**) were separately hydrolyzed with 6 N HCl (Ace high-pressure tube, microwave, 1.0 min). The hydrolysates were evaporated to dryness and resuspended in H_2O (100 μL). A 0.1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide solution in acetone (L-Marfey's reagent, 20 μL) and 1 N $NaHCO_3$ (10 μL) were added to a portion of each hydrolysate, and the mixtures were heated at 40 °C for 1 h. The solutions were cooled to room temperature, neutralized with 2 N HCl (5 μL), and evaporated to dryness. The residues were resuspended in H_2O (50 μL) and analyzed by reversed-phase HPLC [LiChrospher 100 C_{18}, 5 μ, 4 × 125 mm, UV detection at 340 nm] using a linear gradient of 9:1 50 mM triethylammonium phosphate (TEAP) buffer (pH 3.1)/CH_3CN to 1:1 TEAP/CH_3CN over 60 min.

The retention times (t_R, min) of the derivatized residues in the hydrolysate of **1** matched L-Val (28.5; D-Val, 35.7), L-Pro (22.3; D-Pro, 25.8), and *N*-Me-L-Phe (38.4; *N*-Me-D-Phe, 39.3). Given that only *N*-Me-L-Ile and *N*-Me-L-*allo*-Ile were commercially available, the D-Marfey's reagent was synthesized and used to make the *N*-Me-D-Ile and *N*-Me-D-*allo*-Ile derivatives. The retention time of the hydrolysate matched that of *N*-Me-L-Ile (41.0; *N*-Me-L-*allo*-Ile, 41.5; *N*-Me-D-Ile, 44.9; *N*-Me-D-*allo*-Ile, 45.5). Similarly, the retention times of the derivatized residues in the hydrolysate of **4** matched L-Val, L-Pro, *N*-Me-L-Phe, and *N*-Me-L-Val (36.4; *N*-Me-D-Val, 40.2).

Chiral GC−MS Analyses of the Hiv Moieties in 1 and 4. Portions of each hydrolysate of **1** and **4** were separately diluted in 50 μL of MeOH and treated with diazomethane for 10 min. Excess CH_2N_2 and solvent were removed with a stream of N_2, and the residues were resuspended in CH_2Cl_2. Capillary GC−MS analyses were conducted using a Chirasil-Val column (Alltech, 25 m × 0.25 mm) using the following conditions: column temperature held at 40 °C for 10 min after injection of the sample, then increased from 40 °C to 100 °C at a rate of 3 °C/min, then from 100 °C to 150 °C at a rate of 15 °C/min. The retention time of the methylated Hiv residue in both **1** and **4** matched that of the methylated L-Hiv standard (7.7 min) but not the methylated D-Hiv standard (8.5 min).

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