Continuing Studies on the Cyanobacterium *Lyngbya* sp.: Isolation and Structure Determination of 15-Norlyngbyapeptin A and Lyngbyabellin D

Philip G. Williams,[†] Hendrik Luesch,[†] Wesley Y. Yoshida,[†] Richard E. Moore,*[,†] and Valerie J. Paul[‡]

Department of Chemistry, University of Hawaii at Manoa, Honolulu, Hawaii 96822, and University of Guam Marine Laboratory, UOG Station, Mangilao, Guam 96913

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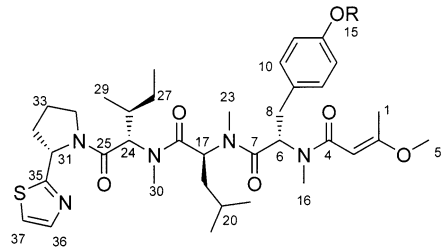
Re-collections of the cyanobacterium *Lyngbya* sp. have yielded two more members of the lyngbyapeptin and lyngbyabellin families. The gross structures of 15-norlyngbyapeptin A (**1**) and lyngbyabellin D (**3**) were deduced through standard 2D NMR techniques, with the absolute configuration of both elucidated through degradation and comparison with commercially available and synthetic standards. Degradation to the α-amino acid and NOE correlations determined the absolute and relative configuration of the 4-amino-3-hydroxy-5-methylhexanoic acid unit in **3**. Lyngbyabellin D (**3**) displayed an IC_{50} value of 0.1 μM against the KB cell line.

The innumerable environmental pressures faced by marine cyanobacteria have undoubtedly catalyzed a tremendous genetic diversity, which often manifests itself in the production of secondary metabolites. By one estimate, almost 10% of the cyanobacterial genome may be devoted to this cause.[1] We have spent the past few years involved in the chemical characterization of secondary metabolites from a particular strain of *Lyngbya* sp. (Oscillatoriaceae) found in Palau and Guam.[2] This field-collected cyanobacterium, easily identifiable by the presence of a small shrimp[3] within the algal mat, produces an extraordinary array of secondary metabolites. Collections of this cyanobacterium made over the previous 10 years have yielded eight distinct classes of metabolites, consisting of some 26 different compounds.[2,4]

Results and Discussion

Several large re-collections of the cyanobacterium were undertaken in Guam during the Spring of 2002 to facilitate further biological evaluation of two of these cytotoxins, the apratoxins[4] and lyngbyabellins.[2a] Reported here are the isolation and structure determination, from these extracts, of 15-norlyngbyapeptin A (**1**) and lyngbyabellin D (**3**), which were both isolated in 3×10^{-4}% of the cyanobacterium's dry weight. Lyngbyabellin D (**3**) displayed an IC_{50} value of 0.1 μM against the KB cell line.[5]



R=H 15-Norlyngbyapeptin A (**1**)
R=Me Lyngbyapeptin A (**2**)

The richly detailed proton NMR spectrum of **1** (Table 1) contained a pattern of resonances indicating **1** belonged to the family of metabolites known as the lyngbyapeptins.[2a,6]

Table 1. ^1H and ^{13}C NMR Data for 15-Norlyngbyapeptin A (**1**) in $CDCl_3$

C/H no.[a]	δ_H (*J* in Hz)	δ_C
1	2.18, s	19.1, q
2		170.4, s
3	5.17, s	90.4, d
4		167.9, s
5	3.64, s	55.0, q
6	5.81, dd (9.7, 5.2)	54.3, d
7		169.5, s
8	3.24, dd (−12.9, 9.7)	34.7, t
	2.68, dd (−12.9, 5.2)	
9		129.0, s
10/14	7.14, d (8.5)	130.7, d
11/13	6.67, d (8.5)	115.2, d
12		154.5, s
15	5.60, br s	
16	3.00, s	29.9, q
17	5.44, dd (9.4, 5.5)	51.7, d
18		171.2, s
19	1.63, ddd (−14.4, 9.4, 5.0)	37.9, t
	1.51, ddd (−14.4, 9.1, 5.5)	
20	1.30, m	23.4, d
21	0.94, d (6.4)	22.3, q
22	0.92, d (6.7)	24.5, q
23	2.80, s	30.2, q
24	4.97, d (11.2)	58.0, d
25		169.9, s
26	1.92, m	33.4, d
27	0.98, m	23.4, t
	0.75, m	
28	0.74, t (6.5)	10.2, q
29	0.83, d (6.7)	15.0, q
30	2.59, s	30.2, q
31	5.43, dd (9.4, 5.5)	58.4, d
32	2.33, m	31.5, t
	2.17, m	
33	2.12, m	24.3, t
	1.96, m	
34	3.94, m	47.4, t
	3.75, m	
35		171.7, s
36	7.67, d (3.3)	142.1, d
37	7.22, d (3.3)	118.0, d

[a] The numbering system for lyngbyapeptin A (**2**) has been adopted.[6a]

The NMR spectra of **1** and lyngbyapeptin A (**2**) were nearly superimposable, but **1** lacked the signals for the aromatic methoxy group on the tyrosine unit (C-15), which established **1** as 15-norlyngbyapeptin A. Fragments derived from

* To whom correspondence should be addressed. (R.E.M.) Tel: (808) 956-7232. Fax: (808) 956-5908. E-mail: moore@gold.chem.hawaii.edu.
[†] University of Hawaii at Manoa.
[‡] University of Guam Marine Laboratory.

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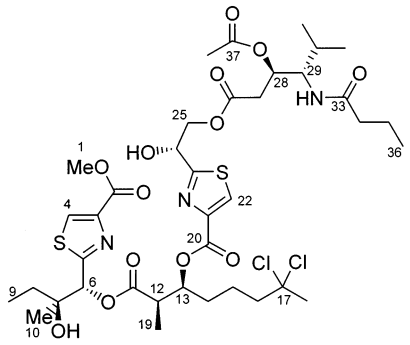
the sequential loss of Thz-pro, *N*-Me-Ile, and *N*-Me-Leu gave rise to major ion peaks at *m/z* 530, 403, and 276, respectively, which suggested the same amino acid sequence.



3

The absolute stereochemistry of the amino acid-derived units in **1** was determined by chiral HPLC after ozonolysis and acid hydrolysis. Comparison with authentic standards established the stereochemistry as 6*S*,17*S*,24*S*,26*S*,31*S*, analogous to lyngbyapeptin A (**2**).[6b] The configuration of the C-2/C-3 double bond was *E*, since irradiation of H-3 (δ_H 5.17) showed a NOE to the adjacent methoxy proton signal (H-5).

Perusal of subsequent HPLC peaks revealed another compound with familiar downfield resonances (vide infra) and yielded lyngbyabellin D (**3**). The molecular formula of **3** was established as $C_{38}H_{55}Cl_2N_3O_{13}S_2$ by high-resolution MALDI, indicating 12 degrees of unsaturation ascribed to six carbonyls, two carbon−carbon double bonds, two carbon−heteroatom double bonds, and two rings. The downfield singlets at δ_H 8.22 and 8.17 (H-22, H-4), in conjunction with the MS data, indicated the presence of two 2-alkylthiazole-4-carboxylate rings, which were consistent with the UV absorption at 223 nm. These resonances, plus the unusual quaternary carbon at δ_C 90.1 (C-17) and the downfield methyl singlet at δ_H 2.12 (H-18), implied that many of the abnormal structural features characteristic of this class of metabolites were still intact.

Analysis of the 2D NMR data (Table 2) rapidly led to fragments that included an acetate, a butyric acid, a 2-(1,2-dihydroxyethyl)thiazole-4-carboxylate unit, and a methyl 2-(1,2-dihydroxy-2-methylbutyl)thiazole-4-carboxylate unit. A 7,7-dichloro-3-hydroxy-2-methyloctanoic acid fragment was also easily sequenced, and these combined units suggested a similarity to the recently reported degradation product homohydroxydolabellin.[2a]

The remaining $C_7H_{12}NO_2$ was clearly attached to the β-hydroxyl group of the glyceric acid-derived moiety via an ester linkage, on the basis of an HMBC correlation to C-26. The exchangeable amide proton signal at δ_H 5.47 (29-NH) showed a strong COSY correlation to H-29, which showed three vicinal couplings ($^3J_{HH}$ = 11.8, 8.4, 3.2 Hz). Using HMBC and COSY correlations, this fragment was expanded into a modified valine unit (C-26 to C-32) that accounted for the proton−proton coupling (8.4 Hz) between H-29 (δ_H 4.25) and H-28 (δ_H 5.11). A $^1J_{CH}$ from H-28 to a downfield carbon at 70.3 ppm indicated this carbon was oxygenated, while a COSY correlation to the methylene protons (H-27) and two HMBC correlations from carbonyls at 170.6 and 169.5 (C-37, C-26) to H-28 established the acetylated structure depicted.

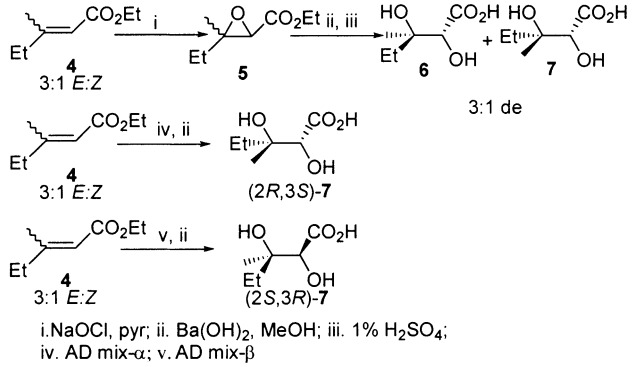
The configuration of **3** was determined by analysis of the degradation products. Ozonolysis and base hydrolysis liberated C-5 through C-10 as 2,3-dihydroxy-3-methylpentanoic acid (Dhmp) and C-23 through C-25 as glyceric acid. Standards of the former were prepared by diastereoselective and enantiospecific routes[7] (Scheme 1). Comparison with the hydrolyzate by chiral HPLC firmly established the stereochemistry of the Dhmp unit and the glyceric acid as shown in **3**, viz., 6*R*,7*S*,24*R*.

The stereochemistry of the polyketide-derived β-hydroxy acid is believed to be 12*R**,13*S** by comparison of coupling

Table 2. Spectral Data for Lyngbyabellin D (**3**) in $CDCl_3$

C/H no.	δ_H (*J* in Hz)	δ_C	COSY	HMBC
1	3.95, s	52.5, q		
2		161.7, s		1
3		145.9, s		4
4	8.17, s	128.8, d		
5		167.4, s		4, 6
6	6.22, s	76.9, d		8, 10
7		74.4, s		6, 8, 9, 10
8	1.78, q (7.4)	31.5, t	9	9, 10
9	0.934, t (7.4)	8.1, q		
10	1.06, s	21.58, q		
11		170.9, s		6, 12, 19
12	2.98, dq (7.1, 6.9)	43.8, d	12, 19	19
13	5.41, m	74.7, d	12, 14	19
14	1.78, m	31.7, t	13	15
	1.67, m			
15	1.85, m	21.56, t		16
16	2.24, m	49.0, t	15	18
	2.20, m		15	
17		90.1, s		18
18	2.12, s	37.4, q		
19	1.25, d (7.1)	13.6, q	12	12
20		160.9, s		
21		146.7, s		22
22	8.22, s	129.1, d		
23		172.8, s		22, 24-OH, 25
24	5.25, ddd (8.7, 5.5, 3.3)	69.6, d	24-OH, 25	24-OH, 25
24-OH	5.60, d (5.5)		24	
25	4.56, dd (−11.3, 3.3)	68.1, t	24, 25b	
	4.44, dd (−11.3, 8.7)		24, 25a	
26		169.5, s		25, 27, 28
27	2.71, dd (−15.5, 5.7)	36.5, t	28	
	2.63, dd (−15.5, 5.7)		28	
28	5.11, dt (8.4, 5.7)	70.3, d	27, 29	27
29	4.25, ddd (11.8, 8.4, 3.2)	54.4, d	28, 29-NH, 30	27, 31, 32
29-NH	5.47, d (11.8)		29	
30	1.93, m	27.6, d	29, 31, 32	31, 32
31	0.930, d (7.1)	20.2, q	30	32
32	0.84, d (6.9)	16.0, q	30	31
33		173.7, s		34, 35
34	2.20, t (7.4)	38.9, t	35	35, 36
35	1.67, m	19.2, t	34, 36	34, 36
36	0.95, t (7.4)	13.7, q	35	34, 35
37		170.6, s		28, 38
38	2.08, s	21.0, q		

Scheme 1. Synthesis of α,β-Dihydroxy-β-methylpentanoic Acid Standards



constants and chemical shifts with other members of this family. Specifically H-12 exhibits two large couplings of approximately 7 Hz, almost identical to the $^3J_{H,H}$ for dolabellin, whose configuration was determined by synthesis.[8,9] The stereochemistry of C-13 is probably *S* as with all other members of this family.[8,10,11]

There are few reports of γ-amino-β-hydroxy acids, and no general method for determining the configuration of the chiral centers. However, reports in the literature indicated that 4-amino-3-hydroxy-5-methylheptanoic acid, found in the didemnins, underwent an epimerization at C-3 via an acid-catalyzed dehydration/hydration sequence, and that significant quantities of the intermediate α,β-unsaturated acid existed after prolonged hydrolysis.[12] This suggested the absolute configuration of C-29 could be determined by acid hydrolysis and subsequent ozonolysis, with oxidative workup, to D- or L-valine. This was indeed the case and chiral HPLC showed unambiguously that C-29 was derived from L-valine. Fortuitously, H-28 and H-29 exhibited an 8.4 Hz coupling, enabling the relative configuration to be determined by a NOE experiment,[13] specifically between 29-NH and H-27, thus completing the stereochemical assignment of lyngbyabellin D (**3**) as 6*R*,7*S*,12*R*,13*S*,24*R*,-28*S*,29*S*.

In summary, further investigation into the chemical composition of a particular *Lyngbya* sp. has expanded the lyngbyapeptin and lyngbyabellin structural classes. Lyngbyabellin D (**3**), the largest member of this family, contains a rare γ-amino-β-hydroxy acid. The presence of a methyl ester in **3** and the derivatization of C-25 with this γ-amino-β-hydroxy acid further clouds the issue as to whether dolabellin is a degradation product, as suggested by the lability of a related cyclic analogue to preferential methanolysis of a C-1 to C-25 ester linkage.[2a]

Evidently, this organism is able to produce concurrently both cyclic and acyclic analogues of these compounds with a variety of stereochemical configurations,[2a,8,10,11] a tactic that, if these metabolites are antifeedants, may provide an evolutionary advantage by delaying the development of detoxification mechanisms by predators.[14]

Experimental Section

General Experimental Procedures. The optical rotations were measured on a Jasco-DIP-700 polarimeter at the sodium D line (589 nm). The UV spectra were determined on a Hewlett-Packard 8453 spectrophotometer, and the IR spectra were recorded on a Perkin-Elmer 1600 FTIR instrument as a film on a NaCl disk. HRFAB-MS data were recorded in the positive mode on a VG ZAB2SE spectrometer, and high-resolution MALDI-MS data were recorded on a DE-STR mass spectrometer. The NMR spectra were recorded in $CDCl_3$ on a Varian Unity INOVA 500 operating at 500 and 125 MHz using the residual solvent signal as the internal reference. NMR analyses of the synthetic products were carried out at 300 and 75 MHz using a Varian spectrometer. 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.

Biological Material. Several collections of *Lyngbya* sp. VP417, from Finger's Reef, Apra Harbor, Guam, were carried out from February to April 2002 and combined for a total weight of 300 g. A voucher is maintained in formalin at the UOG.

Extraction and Isolation of VP417. The cyanobacterium was initially extracted and separated as previously described except the *n*-BuOH/H_2O partition was omitted.[4] The 5% *i*-PrOH fraction contained lyngbyapeptin A (**2**), the 6% apratoxin A, and the 8% a mixture of apratoxin A, lyngbyastatin 2, lyngbyabellin A, **1**, and **3**. This 8% fraction was separated by RP-HPLC on an Ultracarb column (250 × 10 mm, 80% aqueous CH_3CN, 3 mL/min, detection at 220 nm) and provided a mixture of **1** and **3** (t_R 7.5 min). Further purification using 65% aqueous CH_3CN afforded pure 15-norlyngbyapeptin A (**1**) (0.8 mg, t_R 14.2 min) and lyngbyabellin D (**3**) (0.8 mg, t_R 18.9 min).

15-Norlyngbyapeptin A (1): white powder: $[\alpha]^{22}_D$ −31° (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 201 (4.20), 226 (4.02) nm; IR (film) ν_{max} 3367, 2849, 1635, 1456, 1338 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRFABMS *m/z* $[M + Na]^+$ 706.3649 (calcd for $C_{36}H_{53}N_5O_6SNa$, 706.3614 Δ 3.5 mDa).

Lyngbyabellin D (3): white powder: $[\alpha]^{25}_D$ +20° (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 202 (7.31), 223 (6.81) nm; IR (film) ν_{max} 3365, 1731, 1650, 1538, 1455, 1232, 1097 cm^{-1}; ^1H NMR, ^{13}C NMR, $^1H-^1H$ COSY, and HMBC data, see Table 2; HRMALDI-MS *m/z* $[M + Na]^+$ 918.2386 (calcd for $C_{38}H_{55}{}^{35}Cl_2N_3O_{13}S_2Na$, 918.2445 Δ 5.9 mDa).

Absolute Stereochemistry of 15-Norlyngbyapeptin A (1). A 0.1 mg sample of **1** was degraded as described[6b] for **2** and analyzed by chiral HPLC [column Chirex phase 3126 (D) (4.6 × 250 mm), Phenomenex, 0.8 mL/min, detection at 254 nm]. The retention times (min, % CH_3CN/2 mM $CuSO_4$) of the standards were L-Pro (13.0, 5%), D-Pro (21.7, 5%), *N*-Me-L-allo-Ile (30.4, 5%), *N*-Me-L-Ile (32.3, 5%), *N*-Me-D-Ile (41.0, 5%), *N*-Me-D-allo-Ile (41.1, 5%), *N*-Me-L-Tyr (14.1, 15%), *N*-Me-D-Tyr (16.2, 15%). The hydrolyzate contained peaks for L-Pro (13.0), *N*-Me-L-Ile (32.3), and *N*-Me-L-Tyr (14.1).

Synthesis of α,β-Dihydroxy-β-methylpentanoic Acid (Dhmp 6,7). A 3:1 mixture of the unsaturated ethyl ester[15] (100 mg) was heated to 60 °C for 12 h in 7 mL of pyridine and 11 mL of NaOCl.[16] The mixture was thrice partioned between EtOAc and H_2O, the organic layers were combined and dried over $MgSO_4$, and the solvent was removed in vacuo. This mixture was then stirred with 300 mg of $Ba(OH)_2$ in 0.5 mL of methanol at room temperature overnight before the addition of 1% H_2SO_4 until the solution was at pH 3. The cloudy solution was allowed to stir overnight, before pelleting the $BaSO_4$ by centrifugation. The supernatant was evaporated under N_2 to yield a mixture of all four diastereomers, in a ratio of approximately 3:1:1:3 as determined by chiral HPLC (vide infra).

Asymmetric Synthesis of α,β-Dihydroxy-β-methylpentanoic Acids (ent-7). A round-bottom flask with 700 mg of AD mix-α was stirred at room temperature with 10 mL of a 1:1 mixture of *t*-BuOH and water until the organic layer was yellow.[17] To this was added 50 mg of methanesulfonamide and the mixture cooled to 0 °C before the addition of 50 mg of **4**. After 18 h at 0 °C, 700 mg of sodium sulfite was added and the solution allowed to warm to room temperature over 30 min. This was then partioned between EtOAc and water, the organic layer dried over $MgSO_4$, and the solvent removed to yield the pure dihydroxy ester. Subsequent saponification with $Ba(OH)_2$ and acid workup followed by centrifugation provided the enantiomerically pure (2*R*,3*S*)-**7** from the supernatant. Dihydroxylation with AD mix-β produced the corresponding enantiomer by the same procedure.

(2*R*,3*S*)-2,3-Dihydroxy-3-methylpentanoic acid [(2*R*,3*S*)-7]: $[\alpha]_D{}^{24}$ −8° (*c* 1.23, H_2O, lit. $[\alpha]_D{}^{20}$ −16, *c* 1.23 in H_2O);[18] ^1H NMR (D_2O) δ (multiplicity, integration; *J* in Hz) 0.70 (t, 3H; 7.5), 0.99 (s, 3H), 1.35 (dq, 1H; −13.8, 7.5), 1.45 (dq, 1H; −13.8, 7.5), 3.87 (s, 1H).[18]

(2*S*,3*R*)-2,3-Dihydroxy-3-methylpentanoic acid [(2*S*,3*R*)-7]: $[\alpha]_D{}^{24}$ +7° (*c* 1.23, H_2O); ^1H NMR (D_2O) δ (multiplicity, integration; *J* in Hz) 0.70 (t, 3H; 7.5), 0.99 (s, 3H), 1.35 (dq, 1H; −13.8, 7.5), 1.45 (dq, 1H; −13.8, 7.5), 3.87 (s, 1H).

Absolute Stereochemistry of Lyngbyabellin D (3). A solution of 100 μg of **3** was ozonized and saponified as previously described.[10] The resulting mixture was analyzed by chiral HPLC [column CHIRALPAK MA(+), (4.6 × 50 mm), 0.8 mL/min, detection at 254 nm]. The retention times (min) with 5% CH_3CN/2 mM $CuSO_4$ of the α,β-dihydroxy-β-methylpentanoic acid standards prepared by the diastereoselective route were (2*R*,3*R*)-Dhmp (29.2), (2*R*,3*S*)-Dhmp (32.1), (2*S*,3*R*)-

Dhmp (42.3), and (2*S*,3*S*)-Dhmp (53.5) in a ratio of approximately 3:1:1:3. The retention times of the standards prepared by asymmetric dihydroxylation with the AD-α and -β mixes were (2*R*,3*S*)-Dhmp (32.1) and (2*S*,3*R*)-Dhmp (42.3), respectively. The base hydrolyzate was found to contain (2*R*,3*S*)-Dhmp (32.1), which was confirmed by co-injection of the appropriate standard. Glyceric acid was determined under the same system as **1**, except with a solvent system of 0.5 mM $CuSO_4$. Commercially available D-glyceric and L-glyceric acid eluted in 12.0 and 8.6 min, while the hydrolyzate showed a peak for the former at 12.0 min.

A solution of 100 μg of **3** in CH_2Cl_2 was ozonized, the solvent removed, and the residue hydrolyzed at 118 °C for 24 h in 6 N HCl. The acid was removed under a stream of N_2 and the residue ozonized for 30 min in 2 mL of methanol at −78 °C. After removal of the solvent, the residue was dissolved in 2:1 98% formic acid/30% H_2O_2 and the mixture was stirred overnight before refluxing for 1 h at 100 °C.[19] The sample was analyzed like the hydrolyzate of **1**. Comparison with authentic standards (L-Val [20.3 min, 5%] and D-Val [34.5 min]) established the presence of L-Val (20.3 min) in the hydrolyzate.

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