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Structurally diverse new alkaloids from Palauan collections of the apratoxin-producing marine cyanobacterium *Lyngbya* sp.☆

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Abstract—Various collections of the apratoxin-producing marine cyanobacterium *Lyngbya* sp. from Palau have afforded several new nitrogenous metabolites. Lyngbyabellin C is a dichlorinated, thiazole hydroxy acid-containing, cytotoxic macrolide that is related to other lyngbyabellins, to dolabellin, and to hectochlorin. Its selective decomposition tendency to form homohydroxydolabellin suggests that the sea hare isolate dolabellin may be an isolation artifact. In addition to the known lyngbyapeptin A, two new related modified tetrapeptides, lyngbyapeptins B and C, have been isolated. Furthermore, a new cytotoxic *N*-acylpyrrolinone, termed palau'imide, was found. Structure elucidation of these metabolites involved extensive application of NMR spectroscopy.

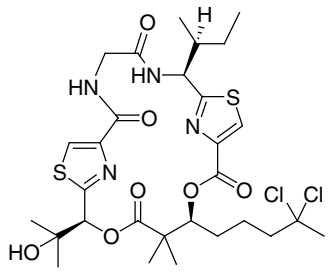
1. Introduction

Marine cyanobacteria, in particular those of the genus *Lyngbya*, have afforded numerous bioactive secondary metabolites over the past 25 years.[1] One particular *Lyngbya* sp. from Guam alone has provided us with many structurally intriguing compounds, some of which exhibited remarkable cytotoxicity.[2] Investigations of Palauan populations of this cyanobacterium for novel natural products with potential antitumor activity have been initiated and already yielded apratoxins A and C,[3] but also a structurally unrelated group of depsipeptides, the ulongamides.[4] Further examination of three Palauan cyanobacterial extracts afforded several new alkaloids along with compounds we had encountered in our Guamanian collections of this cyanobacterium. The isolation, structure elucidation, and cytotoxicity evaluation of those novel metabolites are the subject of the present report.

2. Results and discussion

2.1. Lyngbyabellin C

Cytotoxicity-guided fractionation of the lipophilic extract of *Lyngbya* sp. NIH309 from Short Dropoff, Palau, afforded apratoxins A and C as the major cytotoxins.[3] Total activity of the extract also included lyngbyabellin A (**1**)[5] and a new, weakly cytotoxic analogue in minute amounts, lyngbyabellin C (**2**), exhibiting IC_{50} values of 2.1 μM against KB and 5.3 μM against LoVo cells. Lyngbyabellin B that was isolated from the Guamanian producer of **1**, however, was not found.[6,7]



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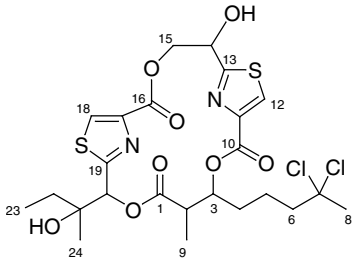

2

HRFABMS analysis combined with NMR data established the molecular formula for compound **2** as $C_{24}H_{30}Cl_2N_2O_{8-}$

☆ Taken in part from Luesch, H. PhD Thesis, University of Hawaii, 2002.

Keywords: cyanobacteria; cytotoxins; alkaloids; biosynthetic signatures; ^{15}N NMR spectroscopy.

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Table 1. NMR Spectral data for lyngbyabellin C (**2**) and homohydroxydolabellin (**3**) at 500 MHz (^{1}H) and 125 MHz (^{13}C) in $CDCl_3$

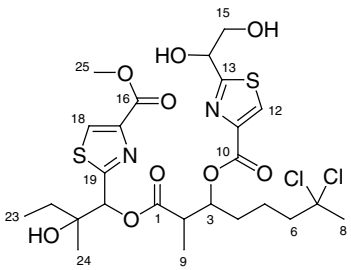
C/H no.	Lyngbyabellin C (**2**)			Homohydroxydolabellin (**3**)
	δ_H (J in Hz)	δ_C	HMBC[a,b]	δ_H (J in Hz)
1		173.8, s	H-2, H_3-9, H-20	
2	3.29, dq (10.2, 7.3)	42.6, d	H_3-9	2.96, qd (7.2, 4.3)
3	5.32, m	75.1, d	H-2, H_3-9	5.40, m
4a	1.75, m	30.2, t		1.76, m
4b	1.98, m			1.80, m
5	1.75, m, 2H	20.0, t	H_2-6	1.78, m, 2H
6a	2.15, m	49.3, t	H_3-8	2.17, m
6b	2.26, m			2.26, m
7		90.1, s	H_3-8	
8	2.12, s	37.3, q		2.12, s
9	1.29, d (7.3)	15.0, q		1.25, d (7.2)
10		159.8, s	H-3, H-12[c]	
11		145.4, s	H-12	
12	8.16, s	128.3, d		8.18, s
13		169.6, s	H-12, H-14, H-15a	
14	5.35, br s	69.8, d		5.07, dd (5.5, 4.2)
15a	4.49, dd (−11.5, 2.4)	69.4, t		4.00, dd (−11.5, 5.5)
15b	4.80, dd (−11.5, 0.8)			4.04, dd (−11.5, 4.2)
16		160.5, s	H_2-15, H-18[c]	
17		145.7, s	H-18	
18	8.18, s	129.4, d		8.25, s
19		166.4, s	H-18, H-20	
20	5.53, s	78.0, d	H_3-24	6.26, s
21		73.8, s	H-20, H_2-22, H_3-23, H_3-24	
22a	1.55, m	30.4, t	H_3-23, H_3-24	1.71, m
22b	1.73, m			1.78, m
23	0.96, t (7.5)	7.7, q		0.97, t (7.5)
24	1.25, s	23.1, q		1.09, s
25	–	–		3.95, s

[a] Protons showing long-range correlation with indicated carbon.
[b] If not indicated otherwise, correlations were observed after optimization for $^nJ_{CH}$=7 Hz.
[c] Correlation observed after optimization for $^nJ_{CH}$=4 Hz.

S_2. $[M+Na]^+$ isotope peaks at m/z 631/633/635 in a ratio of approximately 5:4:1 clearly indicated the presence of two chlorine atoms. NMR analysis (^{1}H, ^{13}C, HSQC, ^{1}H–^{1}H COSY, and HMBC, see Table 1) ascertained the chlorine containing unit to be a 7,7-dichloro-3-acyloxy-2-methyl-octanoic acid residue (C-1 to C-9), thus lacking only one methylation at C-2 compared with lyngbyabellin A (**1**). Two singlets in the ^{1}H NMR spectrum at δ_H 8.16 (H-12) and 8.18 (H-18), correlating to carbons at δ_C 128.3 and 129.4 in the HSQC spectrum, suggested that two 2,4-disubstituted thiazole rings as in **1** were present, accounting for all sulfur and nitrogen atoms in the molecule. Long-range correlations in the HMBC spectrum (Table 1) within the heterocycles supported this conclusion. Furthermore, three-bond correlations of H-12 and H-18 to conjugated carbonyl carbons at δ_C 159.8 (C-10) and 160.5 (C-16), respectively, were indicative of carboxylic acid derived functionalities attached to the heterocycles as in compound **1**. NMR data (Table 1) revealed a slight structural variation between compounds **1** and **2**: the α,β-dihydroxyisovaleric acid residue in **1** is replaced by a homologous moiety in **2** (C-19 to C-24), viz. an α,β-dihydroxy-β-methylpentanoic acid unit, which is an intermediate in isoleucine biosynthesis.[8] Additional HMBCs from H-3 (δ_H 5.32) to C-10 and from H-20 (δ_H 5.53) to C-1 permitted the connection of the thiazole carboxylic acid units to the chlorinated residue via ester linkages. In contrast to **1**, compound **2** appeared to contain no amino acid since, based on the molecular formula, only $C_2H_4O_2$ were unaccounted for. Both yet unassigned carbons, a methine and a methylene carbon, were clearly oxygenated ($\delta_{C\text{-}14}$ 69.8 and $\delta_{C\text{-}15}$ 69.4). According to the ^{1}H–^{1}H COSY spectrum, the attached protons belonged to the same spin system. Consequently, another dihydroxylated moiety was present, and HMBCs from H-14 and H_2-15 to carbonyl carbons C-13 and C-16 of the thiazole carboxylic acid moieties (Table 1) permitted the placement of this unit, leading to the cyclic gross structure shown for **2**.

One ester linkage of lyngbyabellin C (**2**) appeared to be particularly prone to methanolysis. Treatment with MeOH (that was apparently slightly acidic) caused conversion to methyl ester **3** by regioselective ester cleavage at C-16, as was evident from the upfield-shift of H-15ab by 0.49 and 0.76 ppm and the appearance of one ester methyl group singlet (δ_H 3.95) in the ^{1}H NMR spectrum (Table 1). Compound **3** was named homohydroxydolabellin due to its structural analogy to the sea hare isolate dolabellin (**4**),[9] which is likely an artifact as well in light of the decomposition tendency of **2**. Indeed, MeOH had been used extensively during the isolation of dolabellin (**4**),[9] and the corresponding natural product could have been cyclic, as is lyngbyabellin C (**2**). Furthermore, IC_{50} values for in vitro cytotoxicity for homohydroxydolabellin (**3**), 2.2 μM against KB and 3.3 μM against LoVo cells, were almost identical to the ones for lyngbyabellin C (**2**) (see above). Methanolysis at any stage of the isolation procedure for dolabellin (**4**) would not have affected the total activity of the extract and thus would not have been noticed. Alternatively, ester bond cleavage could have already occurred in the acidic digestive

glands of the sea hare that had ingested the cyanobacterium certainly producing the metabolite. Chemical conversion of diet-derived metabolites in digestive glands of sea hares, e.g. of laurinterol into aplysin in *Aplysia californica*,[10] has previously been observed. This represents another reason to investigate the producer rather than the animals downstream of the food chain when looking for algal or cyanobacterial metabolites, in addition to ecological considerations and problems associated with low yields, especially in case of *generalist* herbivores such as *Dolabella auricularia*, from which dolabellin (**4**) was isolated.



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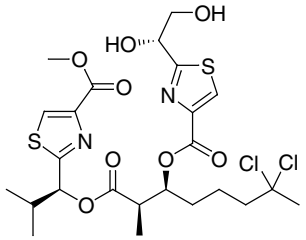

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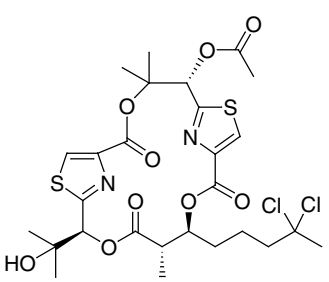
Due to the minute amounts of compounds **2** and **3** (~100 and ~50 μg, respectively) which were used rather to obtain cytotoxicity data, the absolute stereochemistry was not established by chemical methods. On biogenetic grounds it is probable that C-20 has *S* configuration like lyngbyabellin A (**1**) where the stereochemistry was proven by total synthesis.[11] The coupling constant between H-2 and H-3 in homohydroxydolabellin (**3**) is small compared to the one reported for compound **4** (4.3 vs. 7.3 Hz),[9] indicating that the relative stereochemistry in the chlorinated unit is different. Most likely C-3 will have 3*S* configuration as dolabellin (**4**) and lyngbyabellin A (**1**). C-2 would then possess 2*S* configuration in homohydroxydolabellin (**3**) (and therefore also in the parent compound, **2**), in contrast to dolabellin (**4**) which exhibits 2*R* configuration. This proposal is supported by NMR data for the recently described and synthesized, closely related *Lyngbya* metabolite hectochlorin (**5**) that possesses all-*S* configuration and has a similar optical rotation.[12]



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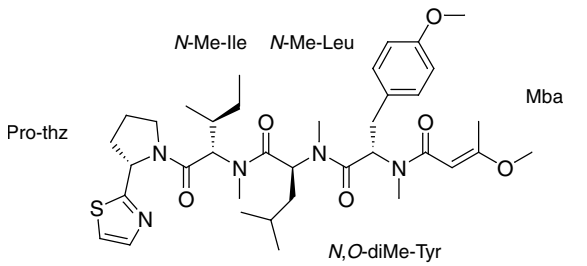
2.2. Lyngbyapeptins B and C

In our Guamanian collections, apratoxin A and lyngbyabellin A (**1**) have usually been encountered together with the modified tetrapeptide lyngbyapeptin A (**6**).[6,13] Several apratoxin-affording cyanobacterial collections around Palau (see Section 4) have indeed also yielded compound **6**, but additionally analogues of **6**, which we consequently named lyngbyapeptins B (**7**) and C (**8**). Like compound **6**, compounds **7** and **8** were noncytotoxic at <5 μM against KB and LoVo cells.



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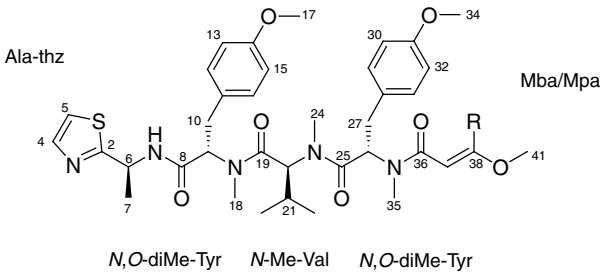

7 R = Me
8 R = Et

Compound **7** possesses a molecular formula of $C_{38}H_{51}N_5O_7S$ according to HRMS (MALDI) analysis and NMR data. Its close relationship to lyngbyapeptin A (**6**) became evident by inspecting the ^1H and ^{13}C NMR spectra of compound **7** (Table 2). Signals were identified that indicated the presence of a terminal thiazole ring (δ_C/δ_H 142.3/7.66 for C/H-4, 119.0/7.24 for C/H-5) and a methyl enol ether functionality (δ_C/δ_H 90.5/5.10 for C/H-37, δ_C/δ_H 55.0/3.60 for C/H_3-41), the most remarkable features of lyngbyapeptin A (**6**). Detailed 2D NMR (^1H–^1H COSY, HSQC, HMBC) analysis established the presence of two *N*,*O*-dimethyltyrosine residues, an *N*-methylvaline unit, a thiazole-containing modified alanine (Ala-thz) unit, and a 3-methoxy-2-butenoic acid (Mba) moiety (Table 2). The *E* geometry of the double bond in the Mba moiety was concluded from cross-peaks in the ROESY spectrum between signals at δ_H 5.10 (H-37) and 3.60 (H_3-41). Sequencing of the partial structures was achieved by HMBC (Table 2) and gave the gross structure represented by structure **7**. The absolute stereochemistry was found to be all *S* by chiral HPLC analysis of the methylated amino acids

Table 2. NMR spectral data for lyngbyapeptins B (**7**) and C (**8**) at 500 MHz (^{1}H) and 125 MHz (^{13}C) in $CDCl_3$ (major conformers)

C/H no.	Lyngbyapeptin B (**7**)			Lyngbyapeptin C (**8**)	
	δ_H (J in Hz)	δ_C	HMBC[a]	δ_H (J in Hz)	δ_C
2		171.7, s	H-4, H-6, H_3-7		171.6, s
4	7.66, d (3.3)	142.3. d		7.67, d (3.2)	142.3, d
5	7.24, d (3.3)	119.0, d		7.24, d (3.2)	119.0, d
6	5.33, quint (7.0)	47.1, d	H_3-7	5.33, quint (7.0)	47.1, d
7	1.54, d (7.0)	21.7, q	H-6	1.54, d (7.0)	21.7, q
NH	6.85,[b] d (7.0)			6.85,[b] d (7.0)	
8		169.2, s	NH, H-9, H-10a		169.2, s
9	5.35, dd (10.9, 5.4)	57.5, d	H_2-10, H_3-18	5.35, dd (10.8, 5.4)	57.5, d
10a	2.70, dd (−14.7, 10.9)	32.3, t	H-9, H-12/16	2.71, dd (−14.6, 10.8)	32.3, t
10b	3.12, dd (−14.7, 5.4)			3.13, dd (−14.6, 5.4)	
11		128.5, s	H-9, H_2-10, H-13/15		128.5, s
12/16	6.86, d (8.7)	129.8, d	H-16/12	6.89, d (8.6)	129.8, d
13/15	6.76, d (8.7)	113.8, d	H-15/13	6.77, d (8.6)	113.8, d
14		158.4, s	H-12/16, H-13/15, H_3-17		158.4, s
17	3.81, s	55.2, q		3.80, s	55.2, q
18	2.47, s	30.5, q	H-9	2.50, s	30.4, q
19		171.0, s	H-9, H_3-18, H-20		171.0, s
20	4.95, d (10.4)	58.9, d	H_3-22, H_3-23, H_3-24	4.97, d (10.6)	58.9, d
21	2.21, m	26.7, d	H-20, H_3-22, H_3-23	2.23, m	26.7, d
22	0.71, d (6.8)	17.8, q	H_3-23	0.72, d (6.9)	17.8, q
23	0.90, d (6.2)	20.1, q	H_3-22	0.90, d (6.4)	20.1, q
24	2.19, s	29.0, q	H-20	2.25, s	29.1, q
25		169.8, s	H-20, H_3-24, H-26, H_2-27		169.9, s
26	5.56, dd (9.4, 5.5)	54.2, d	H_2-27, H_3-35	5.60, dd (9.2, 6.0)	54.1, d
27a	2.65, dd (−13.2, 5.5)	34.6, t	H-26	2.66, dd (−13.3, 6.0)	34.6, t
27b	3.15, dd (−13.2, 9.4)			3.15, dd (−13.3, 9.2)	
28		129.5, s	H-26, H_2-27, H-29/33		129.5, s
29/33	7.25, d (8.7)	130.8, d	H-33/29	7.25, d (8.6)	130.8, d
30/32	6.83, d (8.7)	113.6, d	H-32/30	6.83, d (8.6)	113.6, d
31		158.3, s	H-29/33, H-30/32, H_3-34		158.3, s
34	3.77, s	55.7, q		3.77, s	55.3, q
35	2.94, s	31.3, q	H-26	2.94, s	31.4, q
36		167.7, s	H_3-35, H-37, H_3-39		167.6, s
37	5.10, s	90.5, d	H_3-39	5.02, s	89.8, d
38		169.9, s	H-37, H_3-39, H_3-41		174.0, s
39	2.16, s	18.8, q		2.56, q (7.5), 2H	25.4, t
40	–	–		1.06, t (7.5)	12.0, q
41	3.60, s	55.0, q		3.60, s	55.0, q

[a] Protons showing long-range correlation with indicated carbon.
[b] Signal obscured by aromatic protons. Chemical shift determined from the ^{1}H–^{1}H COSY spectrum.

liberated after acid hydrolysis and of alanine liberated after ozonolysis-acid hydrolysis.

Lyngbyapeptin C (**8**) appeared to contain an additional methylene unit since HRMS (MALDI) analysis suggested a molecular formula of $C_{39}H_{53}N_5O_7S$. NMR data (Table 2) were nearly identical to the ones for compound **7**. The ^{1}H NMR spectrum of compound **8** lacked the methyl singlet at δ_H 2.16 for H_3-39 present in **7** and displayed a methylene quartet at δ_H 2.56 (H_2-39) and a methyl triplet at δ_H 1.06 (H_3-40) instead. HMBC analysis clarified that the N-terminal unit was the homologue of Mba, a 3-methoxy-2-pentenoic acid (Mpa) residue. Long-range correlations of H_3-39 and H_3-40 to C-38 permitted this assignment. The sequence of all units as in **7** was confirmed by HMBC, the *E* geometry of the double bond by ROESY, and the *S* configuration of all amino acid derived units by chiral HPLC analysis of degradation products.

In comparison to lyngbyapeptin A (**6**), the peptides **7** and **8** possess the same (for **7**) or similar (for **8**) characteristic C and N-terminal modification and differ by containing other amino acid units in between.

2.3. Palau'imide

Another cytotoxic constituent of this *Lyngbya* sp., which has been assigned the trivial name palau'imide (**9**), was structurally unrelated to any other cytotoxin or compound isolated from the organism from Guam and was only encountered in one Palauan extract. Compound **9** exhibited IC_{50} values for in vitro cytotoxicity of 1.4 and 0.36 μM against KB and LoVo cells, respectively.

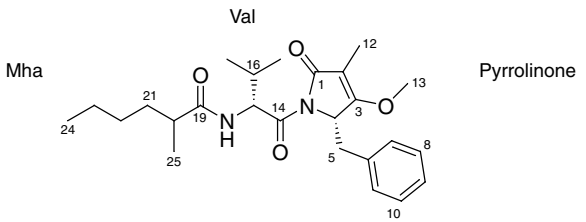

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Table 3. NMR spectral data for palau'imide (**9**) at 500 MHz (^{1}H) and 125 MHz (^{13}C)

C/H no.	δ_H (J in Hz)[a]	δ_C[a]	δ_N[b]	HMBC[c]	ROESY[d]
1		170.9, s		H_3-12	
2		103.9, s		H_3-12	
3		170.0, s		H-4, H_2-5, H_3-12, H_3-13	
4	4.70, br	58.1, d		H_2-5	H_2-5, H-7/11
5a	3.14, dd (−13.9, 2.9)	34.9, t		H-7/11	H-4
5b	3.38, dd (−13.9, 5.1)				H-4
6		134.3, s		H_2-5, H-8/10	
7/11	6.95, m	129.5, d		H_2-5, H-11/7, H-8/10	H-4, H-16, H_3-17
8/10	7.19, m	128.0, d		H-10/8	
9	7.18, m	127.1, s		H-7/11	
12	1.75, d (0.7)	7.7, q			H_3-13
13	4.08, s	58.7, q			H_3-12
N			−215.4	H-5a[b]	
14		171.7, s		H-16	
15	5.64, br	56.2, d			H-16, H_3-18
16	2.13, m	30.5, d		H_3-17, H_3-18	H-7/11, H-15
17	0.93, d (7.0)	16.0, q		H-16, H_3-18	H-7/11, NH
18	1.03, d (6.7)	20.5, q		H-16, H_3-17	H-15
NH	6.13, br		−265.0		H_3-17, H-20
19		176.7, s		H-20, H-21b, H_3-25	
20	2.28, hex (7.0)	41.8, d		H_3-25	NH
21a	1.38, m	33.9, t		H-20	
21b	1.64, m				
22	1.28, m	29.6, t		H-20, H_2-21, H_3-24	
23	1.29, m	22.7, t		H-21b, H_3-24	
24	0.88, t (7.0)	14.0, q			
25	1.17, d (7.0)	18.2, q		H-20	

[a] Recorded in $CDCl_3$.
[b] Values deduced from ^{1}H–^{15}N 2D NMR experiments in MeOH-d_3, referenced externally to $MeNO_2$ at δ_N 0.0.
[c] Protons showing long-range correlation with indicated carbon or nitrogen.
[d] Selected correlations listed.

HRMS analysis (DCI) provided the molecular composition of compound **9** to be $C_{25}H_{36}N_2O_4$ by showing a $[M+H]^+$ peak at m/z 429.2741. Inspection of ^{1}H NMR, ^{13}C NMR, and HSQC data in $CDCl_3$ (Table 3) suggested a modified lipopeptide structure. ^{1}H–^{1}H COSY and HMBC analysis (Table 3) permitted the assignment of a valine moiety (C-14 to C-18), a 2-methylhexanoic acid (Mha) residue (C-19 to C-25), and a phenylalanine-derived unit containing a conjugated methyl enol ether (C-1 to C-13). The only exchangeable proton (δ_{NH} 6.13) belonged to the Val residue since it correlated to the α-proton of the Val unit (H-15). Since C-4 was clearly nitrogenated (δ_C 58.1) and hence all atoms were accounted for, a pyrrolinone structure was proposed at this point. ROESY cross-peaks between methyl group signals for H_3-12 (δ_H 1.75) and H_3-13 (δ_H 4.08) were in agreement with the geometry of the double bond in such a ring system. The proposal was consistent with an observed homoallylic coupling of H-4 with H_3-12, suggesting a rigid (ring) structure. The small $^3J_{H,H}$ couplings of H-4 to both benzylic protons H-5ab (J=2.9, 5.1 Hz) were even more suggestive of a ring structure, since one such vicinal coupling is commonly large (~10 Hz) in linear compounds. Long-range correlations across the three residues were not observed in the HMBC spectrum. This fact was probably due to the broadness of H-15 and NH signals in the case of couplings involving those signals. The lack of an HMBC correlation between C-14 and H-4 (as well as C-1 and H-4) was likely a result of the small magnitude of $^3J_{C\text{-}14,H\text{-}4}$ (as well as $^3J_{C\text{-}1,H\text{-}4}$), as one would predict based on a Karplus-type relationship. However, the assembly of the partial structures could be done by default since the Mha unit and the pyrrolinone unit had to be terminal by nature, thus flanking the Val unit as depicted for structure **9**. ROESY cross-peaks (Table 3) supported the connectivities.

The fragmentation pattern in the DCI-MS spectrum (Fig. 1) is also in agreement with structure **9**, displaying a base peak at m/z 218 for the C-terminal unit and a less intense peak at m/z 130 for the N-terminal unit.

The proposed mixed amide–imide structure was substantiated by ^{1}H–^{15}N 2D NMR carried out in MeOH-d_3 (Table 3). While amide nitrogens typically resonate at approximately δ_N −270, imide nitrogens are observed at ca. δ_N −200 in the ^{15}N NMR spectrum (^{15}N chemical shifts referenced to $MeNO_2$ in MeOH-d_3 at δ_N 0.0).[14] As expected, a ^{1}H–^{15}N HSQC experiment (optimized for $^1J_{NH}$=90 Hz) yielded only one correlation, i.e. of the Val-NH (δ_H 7.76 in MeOH-d_3) to δ_N −265.0, proving the amide linkage. A ^{1}H–^{15}N HMBC experiment (optimized for $^nJ_{NH}$=4 Hz) provided a long-range correlation between H-5a (δ_H 3.16 in MeOH-d_3) and δ_N −215.4, consistent with

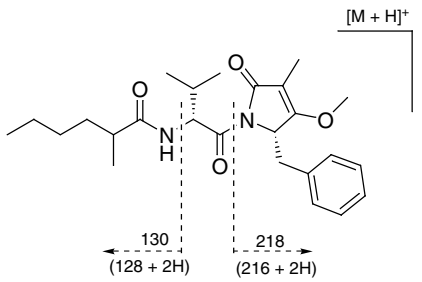

Figure 1. Mass spectral fragmentation of compound **9** by DCI.

an imide functionality involving the phenylalanine-derived moiety.

The configuration of C-4 and C-15 was deduced by analysis of degradation products. Ozonolysis followed by acid hydrolysis liberated L-phenylalanine and D-valine. The absolute stereochemistry of the amino acids was ascertained by chiral HPLC. The determination of the stereocenter at C-20 in the Mha residue was not pursued due to lack of material.

N-Acylpyrrolinone units are commonly found in cyanobacterial metabolites. Microcolins A and B from the marine cyanobacterium *L. majuscula* are immunosuppressive lipopeptides with such functionality.[15] Examples of cytotoxic imides of this type include majusculamide D and deoxymajusculamide D from a marine cyanobacterium,[16] and mirabimides A–E from a terrestrial cyanobacterium.[17] Dysidin is a naturally occurring *N*-acylpyrrolinone from the sponge *Dysidea herbacea*, albeit most likely produced by a symbiotic cyanobacterium.[18] Almost the same phenylalanine-derived pyrrolinone unit as in compound **9**, only lacking the methylation at C-2 and named dolapyrrolidone, has been found in dolastatin 15, isolated from the sea hare *D. auricularia*.[19] The lipid chain in palau'imide (**9**) has precedence in cyanobacterial metabolites as well, as it was recently encountered in malevamide A.[20]

3. Conclusion

Various apratoxin-producing cyanobacterial populations from Palau have yielded additional new alkaloids with amide-, imide-, and thiazole functionalities. Biosynthetic signature units of cyanobacterial metabolites have been recognized. The possibility was presented that the sea hare isolate dolabellin (**4**) is not a natural product but an artifact, which should further stimulate the isolation of microbial metabolites directly from the producers rather than from grazing animals in whose digestive glands a chemical conversion can occur. The high number of novel cytotoxins and other compounds from the investigated cyanobacterium as well as their structural diversity demonstrate the enormous biosynthetic potential of cyanobacteria.

4. Experimental

4.1. General

All NMR experiments were run on a Varian Unity Inova 500 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, using residual solvent signals as internal references. The HSQC experiments were optimized for $^{1}J_{CH}$=140 Hz, and the HMBC experiments for $^{n}J_{CH}$=7 or 4 Hz, the latter for $^{n}J_{CH}$=7 Hz if not indicated otherwise. Experimental conditions for the $^{1}H-^{15}N$ 2D NMR experiments are given in the text. HRMS data were obtained in the positive ion mode either by FAB on a VG-ZAB mass spectrometer, by MALDI using a DE-STR spectrometer, or by DCI using a VG-7070 spectrometer. IR spectra were recorded on a Perkin–Elmer 1600 Series FTIR spectrometer. UV spectra were obtained on a Hewlett–Packard 8453 spectrophotometer. Optical rotations were measured on a JASCO DIP-370 polarimeter.

4.2. Biological material

Lyngbya sp. NIH309 was collected on April 7, 1999, at Short Dropoff, Palau. The isolation of apratoxins A and C and ulongamides C–E from this collection has already been reported.[3,4] Minute cyanobacterial collections of other populations of this cyanobacterium were also carried out on April 5 at Lighthouse Channel, on April 7 at Ngerkuul Pass, and on April 8, 1999, at Ngederrak Lagoon and Big Dropoff, Palau. Those were combined before extraction and are hereafter referred to as 'mixed collection'. We have already reported the isolation of ulongamides A and E from this collection.[4] On April 18, 2000, a population of this organism was collected at Ulong Channel, Palau, producing ulongamides B–F.[4] All collections were acquired by scuba diving at 10–15 m depth. The cyanobacterium closely matches *L. bouillonii* Hoffmann et Demoulin in its general habitat as well as in its morphology.[21]

4.3. Extraction and isolation

Extraction and fractionation of NIH 309 from Short Dropoff has previously been described.[3,4] While lyngbyabellin A (**1**) eluted from silica gel with 8% *i*-PrOH in CH_2Cl_2, lyngbyabellin C (**2**) eluted in the earlier fraction with 6% *i*-PrOH in CH_2Cl_2 along with apratoxins A and C and ulongamides C–E. Separation of these compounds was achieved by semipreparative reversed-phase HPLC (Ultracarb, 5 ODS 30, 250×10 mm, 3.0 mL/min; UV detection at 220 nm) using an isocratic system of 65% aqueous MeCN, affording pure **2** (0.3 mg, t_R 11.8 min).

Extraction and fractionation of the mixed collection has also been reported previously.[4] Following solvent partition of the lipophilic extract, the concentrated organic phase was subjected to reversed-phase flash chromatography (YMC-ODS-A). The mixture eluting with 70% aqueous MeCN contained lyngbyapeptins A (**6**) and C (**8**) and ulongamides A and E. Partial purification of **8** was achieved by reversed-phase HPLC using the Ultracarb column and the conditions above (65% aqueous MeCN, 3.0 mL/min). Impure **8** (1.0 mg, t_R 29.5 min) was rechromatographed on the same column, but using 80% aqueous MeCN as the solvent (3.0 mL/min) to obtain pure compound **8** (0.5 mg, t_R 10.0 min). The fraction eluting with 80% aqueous MeCN from the flash column was most cytotoxic. It was also subjected to reversed-phase HPLC using the Ultracarb column from above (65% aqueous MeCN, 3.0 mL/min), yielding additional amounts of **6** (1.2 mg, t_R 32.0 min), palau'imide (**9**) (1.2 mg, t_R 46.5 min), and apratoxin A (0.8 mg, t_R 69.5 min). A fraction eluting at t_R 29.5 min (0.7 mg) contained compound **8** which was purified by HPLC as before by using 80% aqueous MeCN (0.3 mg, t_R 10.0 min).

The collection from Ulong Channel was extracted and fractionated as detailed previously.[4] The fraction eluting with 5% *i*-PrOH in CH_2Cl_2 during the silica gel chromatography step was concentrated and subjected to reversed-phase HPLC as above (Ultracarb, 65% aqueous MeCN,

3.0 mL/min) to separate the lyngbyapeptins from ulongamide F and other impurities. Lyngbyapeptin B (**7**) eluted at t_R 20.0 min (1.2 mg) and lyngbyapeptin C (**8**) at t_R 29.5 min (0.3 mg). The fraction eluting subsequently from silica gel (with 6% *i*-PrOH in CH_2Cl_2) was most cytotoxic. Purification by reversed-phase HPLC as above yielded, in addition to ulongamides B–E, compound **7** (0.5 mg, t_R 20.0 min), apratoxin C (0.7 mg, t_R 55.0 min), and apratoxin A (0.7 mg, t_R 69.5 min). The fraction eluting with 8% *i*-PrOH in CH_2Cl_2 was treated likewise, yielding lyngbyabellin A (**1**) (0.5 mg, t_R 16.0 min).

4.3.1. Lyngbyabellin C (2). Colorless, amorphous solid; $[\alpha]_D^{25}=-10°$ (*c* 0.10, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 202 (4.47), 235 (4.04) nm; ^1H, ^{13}C NMR and HMBC data, see Table 1; FABMS *m/z* 631/633/635 (100:75:20, $[M+Na]^+$ ion cluster), HRFABMS *m/z* $[M+Na]^+$ 631.0732 (calcd for $C_{24}H_{30}^{35}Cl_2N_2O_8S_2+Na$, 631.0718).

4.3.2. Homohydroxydolabellin (3). Colorless, amorphous solid; $[\alpha]_D^{25}=-10°$ (*c* 0.05, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 203 (4.47), 238 (4.06) nm; ^1H NMR data, see Table 1; FABMS *m/z* 631/633/635 (100:75:20, $[M+Na]^+$ ion cluster), HRFABMS *m/z* $[M+Na]^+$ 663.0982 (calcd for $C_{25}H_{34}^{35}Cl_2N_2O_9S_2+Na$, 663.0980).

4.3.3. Lyngbyapeptin B (7). Colorless, amorphous solid; $[\alpha]_D^{25}=-50°$ (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε) 200 (4.53), 227 (4.24), 245 (sh) (4.00) nm; IR (film) ν_{max} 2966, 1637 (br), 1507, 1443, 1243, 1173, 1108, 1032 cm^{-1}; ^1H, ^{13}C NMR, HMBC data, see Table 2; HRMS (MALDI) *m/z* $[M+Na]^+$ 744.3416 (calcd for $C_{38}H_{51}N_5O_7S+Na$, 744.3401). In $CDCl_3$, lyngbyapeptin B (**7**) exists as a 3:1 mixture of rotamers, and NMR data are given for the major rotamer only.

4.3.4. Lyngbyapeptin C (8). Colorless, amorphous solid; $[\alpha]_D^{25}=-52°$ (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 200 (4.53), 227 (4.26), 245 (sh) (4.00) nm; IR (film) ν_{max} 2960, 1638, 1510, 1442, 1245, 1170, 1110, 1030 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; HRMS (MALDI) *m/z* $[M+Na]^+$ 758.3533 (calcd for $C_{39}H_{53}N_5O_7S+Na$, 758.3558). In $CDCl_3$, lyngbyapeptin C (**8**) exists as a 4:1 mixture of rotamers, and NMR data are given for the major rotamer only.

4.3.5. Palau'imide (9). Colorless, amorphous solid; $[\alpha]_D^{25}=+50°$ (*c* 0.33, MeOH); UV (MeOH) λ_{max} (log ε) 201 (3.78), 252 (3.44) nm; IR (film) ν_{max} 3378, 2954, 2919, 2860, 1720, 1660, 1455, 1384, 1320, 1243, 1196, 1108, 979 cm^{-1}; ^1H, ^{13}C, ^{15}N NMR, HMBC, and ROESY data, see Table 3; MS (DCI/NH_3) *m/z* 429 $[M+H]^+$ (57), 218 (100), 170 (27), 130 (22), 91 (19); HRMS (DCI/NH_3) *m/z* $[M+H]^+$ 429.2741 (calcd for $C_{25}H_{37}N_2O_4$, 429.2753).

4.4. Decomposition of lyngbyabellin C (2)

After sitting in MeOH, compound **2** (~0.2 mg) partially converted to homohydroxydolabellin (**3**), presumably catalyzed by trace amounts of acid. Both compounds were separated by reversed-phase HPLC on the Ultracarb column using 65% aqueous MeCN as the solvent system (3.0 mL/min). Compound **3** eluted at t_R 8.0 min (~0.05 mg), and leftover **2** at t_R 11.8 min (~0.1 mg). Compound **3** was the only product formed according to the crude ^1H NMR spectrum and the HPLC trace.

4.5. Absolute stereochemistry of lyngbyapeptins B (7) and C (8)

Samples of compounds **7** and **8** (0.1 mg each) were subjected to acid hydrolysis (6N HCl, 110°C, 12 h). The hydrolyzates, after concentration to dryness, were analyzed by chiral HPLC [column, Chirex phase 3126 (D) (4.6×250 mm), Phenomenex; flow rate, 1.0 mL/min; detection at 254 nm). *N*-Me-Val eluted at t_R 12.2 min using 2 mM $CuSO_4$–MeCN (95:5), matching with the retention time for the L-isomer (t_R of *N*-Me-D-Val standard: 17.0 min). *N*,*O*-diMe-Tyr eluted only at t_R 79.5 min using 2 mM $CuSO_4$–MeCN (90:10), corresponding to the retention time of the L-isomer (t_R of *N*,*O*-diMe-D-Tyr standard: 85.0 min) and indicating that both *N*,*O*-diMe-Tyr units in **7** and **8** had L-configuration. Expectedly, a peak for *N*-Me-L-Tyr was detected as well due to partial O-demethylation (t_R 22.0 min). Both hydrolyzates showed the same HPLC profile. When samples of **7** and **8** were ozonized (−78°C, CH_2Cl_2) before being subjected to acid hydrolysis, L-Ala was additionally liberated from both compounds, eluting at t_R 6.7 min using 2 mM $CuSO_4$–MeCN (95:5) as the solvent system (t_R of D-Ala standard: 8.0 min).

4.6. Absolute stereochemistry of palau'imide (9)

Compound **9** (0.3 mg) was dissolved in 3 mL of CH_2Cl_2 and subjected to ozonolysis at −78°C. The solvent was evaporated, and the residue incubated with 6N HCl for 12 h at 110°C. The hydrolyzate was concentrated to dryness and analyzed by chiral HPLC. The Chirex phase 3126 column used above (solvent, 2 mM $CuSO_4$–MeCN (95:5); flow rate, 0.8 mL/min) served for the detection of valine. The retention times (t_R, min) of authentic standards were: L-Val (22.0) and D-Val (29.5). The HPLC profile of the hydrolyzate exhibited only a peak at t_R 29.5 min, indicating the presence of D-Val. Phenylalanine was detected on a different chiral column (column, CHIRALPAK MA(+) (4.6×50 mm), Daicel Chemical Industries, Ltd.; solvent, 2 mM $CuSO_4$–MeCN (90:10); flow rate, 0.8 mL/min; detection at 254 nm). The retention times (t_R, min) of authentic phenylalanine samples were: D-Phe (8.9) and L-Phe (12.0). The HPLC profile of the hydrolyzate showed a peak at t_R 12.0 but not 8.9 min, thus indicating that L-Phe was present in the mixture.

4.7. Cytotoxicity assays

Fractionation of extracts was guided by monitoring cytotoxicity toward KB and LoVo cell lines. The IC_{50} values for cytotoxicity in vitro were determined using the sulforhodamine B assay.[22]

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References

1. Burja, A. M.; Banaigs, B.; Abou-Mansour, E.; Burgess, J. G.; Wright, P. C. *Tetrahedron* **2001**, *57*, 9347–9377.
2. Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J.; Corbett, T. H. *J. Am. Chem. Soc.* **2001**, *123*, 5418–5423.
3. Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *Bioorg. Med. Chem.* **2002**, *10*, 1973–1978.
4. Luesch, H.; Williams, P. G.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **2002**, *65*, 996–1000.
5. Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J.; Mooberry, S. L. *J. Nat. Prod.* **2000**, *63*, 611–615.
6. Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **2000**, *63*, 1437–1439.
7. Milligan, K. E.; Marquez, B. L.; Williamson, R. T.; Gerwick, W. H. *J. Nat. Prod.* **2000**, *63*, 1440–1443.
8. Hill, R. K.; Yan, S.-J. *Bioorg. Chem.* **1971**, *1*, 446–456.
9. Sone, H.; Kondo, T.; Kiryu, M.; Ishiwata, H.; Ojika, M.; Yamada, K. *J. Org. Chem.* **1995**, *60*, 4774–4781.
10. Faulkner, D. J. In *Ecological Roles of Marine Secondary Metabolites*. Paul, V. J., Ed.; Cornell University: Ithaca, NY, 1992; pp 119–163.
11. Yokokawa, F.; Sameshima, H.; Shioiri, T. *Tetrahedron Lett.* **2001**, *42*, 4171–4174.
12. Marquez, B. L.; Watts, K. S.; Yokochi, A.; Roberts, M. A.; Verdier-Pinard, P.; Jimenez, J. I.; Hamel, E.; Scheuer, P. J.; Gerwick, W. H. *J. Nat. Prod.* **2002**, *65*, 866–871. Cetusic, J. R. P.; Green, III., F. R.; Graupner, P. R.; Oliver, M. P. *Org. Lett.* **2002**, *4*, 1307–1310.
13. Klein, D.; Braekman, J.-C.; Daloze, D.; Hoffmann, L.; Castillo, G.; Demoulin, V. *Tetrahedron Lett.* **1999**, *40*, 695–696.
14. Moore, R. E.; Bornemann, V.; Niemczura, W. P.; Gregson, J. M.; Chen, J.-L.; Norton, T. R.; Patterson, G. M. L.; Helms, G. L. *J. Am. Chem. Soc.* **1989**, *111*, 6128–6132. Mason, J. In *Multinuclear NMR*. Mason, J., Ed.; Plenum: New York, 1987; pp 335–367.
15. Koehn, F. E.; Longley, R. E.; Reed, J. K. *J. Nat. Prod.* **1992**, *55*, 613–619.
16. Moore, R. E.; Entzeroth, M. *Phytochemistry* **1988**, *27*, 3101–3103.
17. Carmeli, S.; Moore, R. E.; Patterson, G. M. L. *Tetrahedron* **1991**, *47*, 2087–2096. Paik, S.; Carmeli, S.; Cullingham, J.; Moore, R. E.; Patterson, G. M. L.; Tius, M. A. *J. Am. Chem. Soc.* **1994**, *116*, 8116–8125.
18. Hofheinz, W.; Oberhänsli, W. E. *Helv. Chim. Acta* **1977**, *60*, 660–669.
19. Pettit, G. R.; Kamano, Y.; Dufresne, C.; Cerny, R. L.; Herald, C. L.; Schmidt, J. M. *J. Org. Chem.* **1989**, *54*, 6005–6006.
20. Horgen, F. D.; Yoshida, W. Y.; Scheuer, P. J. *J. Nat. Prod.* **2000**, *63*, 461–467.
21. Hoffmann, L.; Demoulin, V. *Belg. J. Bot.* **1991**, *124*, 82–88.
22. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl Cancer Inst.* **1990**, *82*, 1107–1112.