Lyngbyabellin B, a Toxic and Antifungal Secondary Metabolite from the Marine Cyanobacterium *Lyngbya majuscula*

Kenneth E. Milligan, Brian L. Marquez, R. Thomas Williamson, and William H. Gerwick*

College of Pharmacy, Oregon State University, Corvallis, Oregon 97331

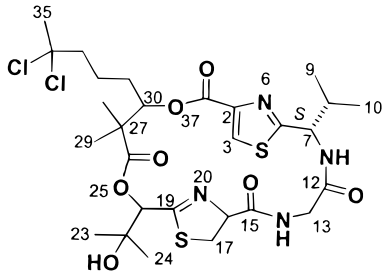
Received March 24, 2000

Lyngbyabellin B (**1**) was isolated from a marine cyanobacterium, *Lyngbya majuscula*, collected near the Dry Tortugas National Park, Florida. This new cyclic depsipeptide displayed potent toxicity toward brine shrimp and the fungus *Candida albicans*. The planar structure was deduced using 1D and 2D NMR spectroscopic methods, and the stereochemistry is proposed through a combination of NMR and chiral GC/MS analysis.

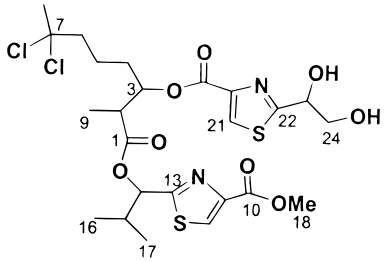
Cyanobacteria are phenomonal producers of structurally intriguing and biologically active secondary metabolites. Although reports abound in the literature of cyanobacterial metabolites active in medicinal and ecological assays,[1] they appear particularly robust in their production of toxic and antifungal natural products.[2] Some examples include hormothamnin A, the laxophycins, calophycin, fisherellin A, and majusculamide C.[3] The pantropical marine cyanobacterium *Lyngbya majuscula* Gomont (Oscillatoriaceae) is one of the more prolific producers of interesting cyanobacterial secondary metabolites, yielding no fewer than 100 reported compounds.[4] As part of an effort to discover biologically active natural products, we now report the isolation and structure elucidation of lyngbyabellin B (**1**),[5] a brine shrimp toxic and antifungal cyclic depsipeptide.

After extraction of the alcohol-preserved tissue [CH_2Cl_2/MeOH (2:1); 2.5 g extract], initial fractionation was accomplished by VLC (EtOAc/hexanes gradient) over Si gel. Successive LC fractionation steps culminated in the purification of **1** through ODS reversed-phase HPLC (70% MeOH/H_2O). Lyngbyabellin B (**1**) was isolated as a pale yellow oil (0.28% of the organic extract, 7.1 mg) and displayed a UV maximum at 246 nm (log ϵ 4.20) and an optical rotation of $[\alpha]^{25}_D$ +33° (MeOH, *c* 0.2). HRFABMS showed a pseudomolecular ion [$(M + H)^+$ at *m/z* 679.1794] consistent with the molecular formula $C_{28}H_{41}O_7N_4Cl_2S_2$ (Δ −2.3 mmu), indicating a structure with 10 degrees of unsaturation. The IR spectrum revealed absorptions indicative of NH/OH protons (3323 cm^{-1}), amide/ester carbonyls (1718, 1674 cm^{-1}), and *gem*-dimethyl moieties (1234 cm^{-1}). Analysis of the 1D ^1H and ^{13}C NMR data indicated a short aliphatic chain, two NH protons, three *gem*-dimethyl groups, a *gem*-dichloromethyl functionality, four methines bound to heteroatoms, and six downfield resonances in the amide/ester carbonyl or heterocyclic quaternary carbon chemical shift range (Table 1). Interpretation of 1D NMR data in concert with 2D NMR experiments, including $^1H-^1H$ DQF−COSY, $^1H-^{13}C$ HSQC, $^1H-^{13}C$ HSQC−TOCSY,[6] $^1H-^{13}C$ HMBC, and $^1H-^{15}N$ PEP−HSQC−TOCSY,[7] defined partial structures A−G (Figure 1).

Diagnostic chemical shifts and HMBC correlations from the methine proton (H-3) to three quaternary carbons (C-1, -2, and -5) allowed assembly of the thiazole containing fragment **A**. Similarly, in fragment **D**, connectivity could be observed from the methylene protons (H-17) to a pseudo-α carbon (C-16) and a deshielded quaternary carbon (C-19), suggesting a thiazoline partial structure. A $^1H-^{15}N$ PEP−HSQC−TOCSY NMR experiment clearly demonstrated connectivity between N-11 and the corresponding α, *β*, and *γ* protons of fragment **B**. Likewise, both methylene protons of the glycinyl residue correlated to the nitrogen in fragment **C**. Partial structures **E** and **F** were assembled based on their chemical shifts and HMBC data. Particularly revealing for fragment **E** were HMBC correlations to the pseudo-α carbon (C-21) from both methyl groups of the *gem*-dimethyl pair, as well as connectivity observed from the proton of the tertiary alcohol to the midfield quaternary carbon (C-22) and one of the *gem*-dimethyl pair (C-23). Similarly, fragment **F** was defined by HMBC cross-peaks descriptive of a *gem*-dimethyl functionality adjacent to an amide/ester carbonyl. The aliphatic chain of partial structure **G** was primarily elucidated by following the $^1H-^1H$ spin system using $^1H-^{13}C$ HSQC−TOCSY. Definition of the *gem*-dichloro arrangement was accomplished by consideration of its ^{13}C NMR chemical shift (C-34, δ 90.01), which is comparable to the *gem*-dichloro functionality in dolabellin (**2**, C-7),[8] and HMBC correlations from the adjacent methyl (C-35) and methylene groups (C-33). These partial structures account for all atoms in the molecular formula of lyngbyabellin B (**1**).



1



2

* To whom correspondence should be addressed. Tel.: (541) 737-5801. Fax: (541) 737-3999. E-mail: Bill.Gerwick@orst.edu.

10.1021/np000133y CCC: $19.00
Published on Web 08/25/2000

Table 1. NMR Data for Lyngbyabellin B (**1**)[a]

residue[b]	atom no.	^{1}H (ppm, mult, *J* in Hz)	^{13}C (ppm)	HMBC[c]	^{1}H−^{15}N PEP−HSQC−TOCSY[d]
thiazole (**A**)	1		161.10		
	2		146.82		
	3	8.27 (s)	129.45	1, 2, 5	
	4				
	5		171.13		
	6				
valine (**B**)	7	5.10 (t, 9.6)	55.58	5, 8, 9, 10, 12	11
	8	2.36 (dq, 9.7, 6.7)	33.89	7, 10	11
	9	0.74 (d, 6.8)	19.73	7, 8, 10	11
	10	0.99 (d, 6.8)	19.30	7, 8, 9	11
	11	8.07 (bs)		12	11
glycine (**C**)	12		167.69		
	13a	3.61 (dd, 10.0, 2.3)	43.15	12, 15	14
	13b	4.64 (dd, 10.2, 10.0)	43.15	12, 15	14
	14	6.68 (dd, 9.8, 1.6)			14
thiazoline (**D**)	15		170.89		
	16	5.27 (d, 9.6)	78.67	15, 19	
	17a	3.27 (dd, 9.8, 1.8)	34.44	15, 16	
	17b	3.75 (dd, 10.6, 1.5)	34.44	15, 16, 19	
	18				
	19		177.63		
	20				
t-hydroxy valic acid (**E**)	21	5.72 (d, 1.6)	78.58	19, 26	
	22		74.13		
	23	1.81 (s)	26.19	21, 22, 24	
	24	1.44 (s)	29.29	21, 22, 23	
	OH	5.55 (br s)		22, 23	
	25				
DDHO[e] (**F** and **G**)	26		172.62		
	27		47.24		
	28	1.33 (s)	22.81	26, 27, 29, 30	
	29	1.41 (s)	24.45	26, 27, 28, 30, 31	
	30	5.32 (dd, 11.0, 3.3)	78.45	1, 26, 27[f], 33	
	31a	1.68 (m)	21.91	30, 32	
	31b	1.76 (m)	21.91	30, 32	
	32a	1.93 (m)	29.23	31	
	32b	1.70 (m)	29.23	31	
	33a	2.05 (m, obs)	48.80	31, 32, 34	
	33b	2.25 (ddd, 14.6, 11.3, 4.4)	48.80	31, 32, 34	
	34		90.01		
	35	2.05 (s)	37.43	33, 34	

[a] All ^{1}H and ^{13}C chemical shifts referenced to residual $CHCl_3$ (δ 7.27 and 77.00), respectively. [b] Residue names given by dominant moiety type. For partial structures see Figure 1. [c] HMBC data are reported as proton (atom no.) displaying connectivity to indicated carbon. [d] ^{1}H−^{15}N PEP−HSQC−TOCSY data are reported as proton (atom no.) displaying connectivity to indicated nitrogen. [e] 7,7-dichloro-2,2-dimethyl-3-hydroxy octanoic acid. [f] Weak correlation.

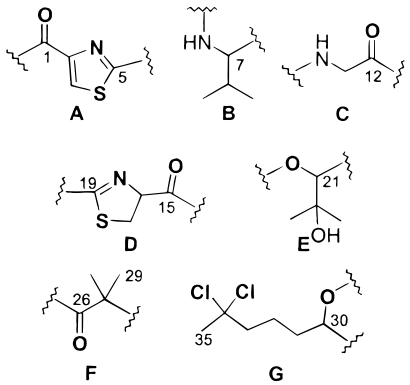


Figure 1. Partial structures of lyngbyabellin B (**1**).

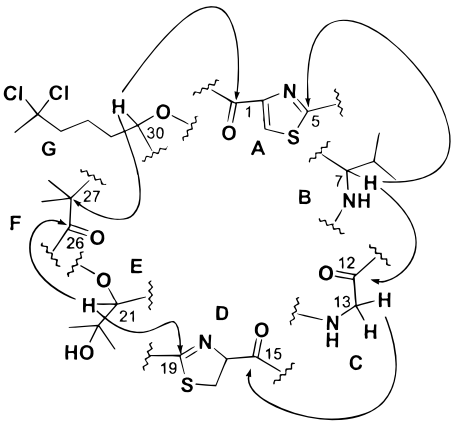


Figure 2. Key HMBC correlations used in the sequencing of partial structures **A**−**G** of lyngbyabellin B (**1**).

Assembly of the planar structure of **1** was accomplished primarily through HMBC correlations (Figure 2). The thiazole-containing partial structure **A** was placed adjacent to **B** by HMBC correlations observed between the pseudo-α hydrogen (H-7, δ 5.10) of the valine-like residue **B** to the sp^2 hybridized carbon of residue **A** (C-5, δ 171.13). H-7 also displayed heteronuclear connectivity to the carbonyl terminus, C-12 (δ 167.69), of the glycinyl residue (**C**). The methylene protons of this latter residue (H_2-13, δ 3.61 and 4.64) showed HMBC cross-peaks to C-15 (δ 170.89) of partial structure **D**. Correlations were also observed between C-19 (δ 177.63) of partial structure **D** and the pseudo-α hydrogen of **E** (H-21, δ 5.72). Substructures **E** and **F** were connected through an ester bond by observing long-range heteronuclear coupling between H-21 and the C-26 carbonyl of **F** (δ 172.62) and consideration of the ^{13}C

NMR chemical shift of C-21 (δ78.58). Correlations between H-30 (δ 5.32) and the quaternary carbon containing a *gem*-dimethyl group (C-27, δ 47.24) allowed connection of **G** and **F**. The planar structure of lyngbyabellin B (**1**) was completed by linkage of partial structures **G** and **A** by observation of an HMBC correlation between H-30 and the C-1 carbonyl (δ 161.10). The ester nature of this linkage was established by consideration of the chemical shift of C-30 (δ 78.45).

The absolute stereochemistry at C-7 was defined using chiral GC/MS analysis (Alltech capillary column, CHIRASIL-VAL Phase 25 m × 0.25 mm).[9] Compound **1** was ozonized (0.2 mg, CH_2Cl_2, 1 min, ambient temperature), hydrolyzed (6 N HCl, 18 h, 118 °C), and derivatized (Alltech PFP−IPA Amino Acid Kit). The resulting derivatized valine residue of **1**, and identically derivatized valine standards (1 mg each of L- and D,L-valine) were subjected to chiral GC/MS analysis (see Experimental Section). The valine derivative obtained from **1** eluted at t_R 14.34 min, as did the standard L-valine derivative, thus defining C-7 as having *S* stereochemistry (the D-valine derivative eluted with t_R 13.59 min).

Correlations were observed between H-7 and H-16, H-16 and H-21, and H-21 and H-30 in the ROESY (300 ms) of lyngbyabellin B (**1**), suggesting that all of these protons are on the same face of the macrocycle. From these ROESY data and the definition of C-7 as *S* by chemical methods, it appears that all of the remaining stereocenters are also of *S* configuration. These tentative assignments are consistent with the stereochemical assignments at two comparable chiral centers in the related sea hare metabolite, dolabellin (**2**) [lyngbyabellin B C-21 and C-30; dolabellin (**2**) C-14 and C-3].[8] However, further experimentation is necessary to confirm these assignments unequivocally. Unfortunately, the limited supply of lyngbyabellin B (**1**) precludes this; other approaches are underway.

Interestingly, of these seven partial structures (**A**−**G**), only one is for an underivatized amino acid residue (glycinyl partial structure **C**). Partial structures **A** and **D** may be formed by condensation of cysteine residues to produce thiazole and thiazoline rings, respectively.[7] Partial structures **B** and **E** are modified valine residues. Partial structures **F** and **G** are similar to the *gem*-dichloro aliphatic chain of dolabellin (**2**), a metabolite isolated from the sea hare *Dolabella auricularia*.[8]

Lyngbyabellin B (**1**) was toxic to brine shrimp (*Artemia salina*),[5,10] with an LD_{50} value of 3.0 ppm, and active against *Candida albicans* (ATCC 14053) in a disk diffusion assay,[5c,11] giving a 10.5-mm zone of inhibition at 100 μg/disk and a slight halo at 10 μg/disk. The striking structural parallel between lyngbyabellin B (**1**) and dolabellin (**2**) strongly supports a cyanobacterial origin for the latter metabolite.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker DRX 600 MHz spectrometer. Mass spectra were recorded on a Kratos MS50TC mass spectrometer. UV spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer, while FT-IR spectra were recorded on a Nicolet 510 spectrophotometer. Chiral GC/MS analysis was accomplished on a Hewlett-Packard 5890 Series II gas chromatograph with a Hewlett-Packard 5971 mass selective detector using an Alltech capillary column (CHIRASIL-VAL Phase 25 m × 0.25 mm). HPLC separation was accomplished with a Waters M-6000A pump, a Rheodyne 7010 injector, and a Waters Lambda-Max 480 spectrophotometer. Optical rotation measurements were recorded on a Perkin-Elmer model 141 polarimeter.

Biological Material. The marine cyanobacterium, *L. majuscula*, was collected by hand from shallow water (2 m) on 15 November 1995, at Bush Key, Dry Tortugas, FL, and stored at −20 °C in (IPA) until workup. A voucher sample is available from WHG as collection number DBK-15 NOV 95-4.

Extraction and Isolation of Lyngbyabellin B (1). The IPA-preserved alga (476 g dry wt) was extracted with CH_2Cl_2/MeOH (2:1) two times to give a crude extract of 2.6 g. A portion of this (2.5 g) was fractionated using vacuum liquid chromatography (VLC) on Si gel with a stepwise gradient of hexanes/EtOAc/MeOH to give eight fractions. Because fractions 6 and 7 (eluted 50−75% EtOAc/hexanes) contained **1**, they were recombined and subjected to VLC (hexanes/EtOAC/MeOH) and collected as seven fractions. Fractions 5 and 6 (60−100% EtOAc/hexanes) both possessed **1** and were recombined. This fraction was further purified over a Waters Sep-Pak C_{18} solid-phase extraction cartridge (75% MeOH/H_2O). ODS reversed-phase HPLC (Phenomonex ODS, 250 × 10 mm, 5 μm) in 90% MeOH/H_2O afforded a single peak, which was further purified using ODS reversed-phase HPLC (Phenomonex ODS, 250 × 4.60 mm, 5 μm) in 70% MeOH/H_2O to yield **1** (peak centered at 14 min, 7.1 mg, 0.3% of extract).

Lyngbyabellin B (1): pure; $[\alpha]^{25}_D$ +33° (CH_2Cl_2, *c* 0.2); UV λ_{max} (MeOH) 246 nm (log ϵ 4.20); IR ν_{max} (film) 3323, 2959, 1718, 1674, 1513, 1234, 1145; FABMS (3-NBA) obsd *m/z* (rel int) 679 (100), 665 (15), 201 (30), 166 (70); HR FABMS (3-NBA) obsd $[M + H]^+$ *m/z* 679.1794 for $C_{28}H_{41}O_7N_4Cl_2S_2$ (Δ −2.3 mmu); ^1H and ^{13}C data, see Table 1.

Stereoanalysis of 1. Lyngbyabellin B was ozonized (0.2 mg of **1** in 1 mL CH_2Cl_2, 1 min, ambient temperature), immediately dried in vacuo, and hydrolyzed (1 mL 6 N HCl, 110°, 18 h). The hydrolysate was dried under nitrogen and derivatized using an Alltech PFP−IPA Amino Acid Kit (#18093). The dried hydrolysate was treated with 0.2 N HCl (5 min at 110 °C), and then again dried under nitrogen. To this, 150 μL of acetyl chloride and 500 μL of IPA were added and heated at 110 °C for 45 min. After drying with nitrogen, the derivatizing agent, pentafluoropropyl isopropionic acid (1 mL dissolved in 2 mL of CH_2Cl_2), was added and the solution heated at 115 °C for 15 min, blown dry with nitrogen, and then solubilized in hexanes. For standards, 1 mg each of the L and D/L valine were subjected to the identical derivatization sequence as the ozonized hydrolysate of **1**. All samples were analyzed by gas chromatography under identical conditions, with a sustained initial oven temperature of 50 °C (4 min), a 3 °C/min ramp from 50 °C to 150 °C, and a concluding 20 °C min ramp from 150 °C to 180 °C. The derivatized lyngbyabellin B fragment as well as the derivatized L-valine standard coeluted at t_R = 14.34 min. The derivatized d-valine eluted at t_R = 13.59 min.

Brine Shrimp Toxicity Bioassay. Evaluation for brine shrimp toxicity was performed as previously described using *Artemia salina* as the test organism.[5,10] After a 24-h hatching period, aliquots of a 10-mg/mL stock solution of **1** were added to test wells containing 5 mL of artificial seawater and brine shrimp to achieve a range of final concentrations from 1 to 50 ppm. After 24 h, the live and dead shrimp were tallied.

Antimicrobial Assay. Antimicrobial activity of lyngbyabellin B was evaluated using standard paper sensitivity disk-agar plate methodology (disk diameter, 6 mm).[5c,11] Lyngbyabellin B showed a 10.5-mm zone of inhibition at 100 μg and a slight halo at 10 μg against *C. albicans* (ATCC 14053). No activity was observed against *Pseudomonas aeruginosa* (ATCC 10145), *Escherichia coli* (ATCC 11775), *Salmonella choleraesuis* subsp. *choleraesuis* (ATCC 14028), *Bacillus subtilis* (ATCC 6051), or *Staphylococcus aureus* (ATCC 12600).

Acknowledgment. We acknowledge J. V. Rossi for initial collections of this organism; the Dry Tortugas National Park for permission to make these collections; and M. A. Roberts, Oregon State University (OSU), for species identifications. We thank V. Hsu, OSU Department of Biochemistry and Biophys-

ics, for use of the 600 MHz NMR and B. Arbogast, Environmental Health Sciences Center, OSU, for obtaining HRFABMS data. This project was supported by NIH (NCI 52955), and the Oregon Sea Grant (R/BT-18).

Supporting Information Available: ^{1}H NMR, ^{13}C NMR, HSQC, HSQC−TOCSY, HMBC, ^{1}H−^{15}N PEP−HSQC−TOCSY, and FABMS of **1**. This material is available free of charge via the Internet.

References and Notes

(1) (a) Ling-Hua, A.; Langley, R. E.; Koehn, F. E. *Life Sci.* **1997**, *60*, 751−762. (b) Nagle, D. G.; Paul, V. J.; Roberts, M. A. *Tetrahedron Lett.* **1996**, *37*, 6263−6266. (c) Orjala, J.; Gerwick, W. H. *J. Nat. Prod.* **1996**, *59*, 427−430.

(2) Kulik, M. M. *Eur. J. Plant Pathol.* **1995**, *101*, 585−599.

(3) (a) Gerwick, W. H.; Jiang, Z. D.; Agarwal, S. K.; and Farmer, B. T. *Tetrahedron* **1992**, *48*, 2313−2324. (b) Frankmolle, W. P.; Larsen, L. K.; Caplan, F. R.; Patterson, G. K.; Levine, I. A.; Moore, R. E. *J. Antibiotics* **1992**, *45*, 1458−1466. (c) Moon, S. S.; Chen, J. L.; Moore, R. E.; Patterson, G. L. *J. Org. Chem.* **1992**, *57*, 1097−1103. (d) Carter, D. C.; Moore, R. E.; Mynderse, J. S.; Niemczura, W. P.; Todd, J. S. *J. Org. Chem.* **1984**, *49*, 236−241.

(4) (a) Gerwick, W. H.; Proteau, P. J.; Nagle, D. G.; Hamel, E.; Blokhin, A.; Slate, D. L. *J. Org. Chem.* **1994**, *59*, 1243−1245. (b) Wu, M.; Milligan, K. E.; Gerwick, W. H. *Tetradhedron* **1997**, *53*, 15983−15990. (c) Singh, I. P.; Milligan, K. E.; Gerwick, W. H. *J. Nat. Prod.* **1999**, *62*, 1333−1335. (d) Tan, L. T.; Williamson, R. T.; Gerwick, W. H.; Watts, K. S.; McGough, K.; Jacobs, R. *J. Org. Chem.* **2000**, *65*, 419−425.

(5) In our laboratory, compound **1** was originally named "tortugamide" to reflect its origin from *L. majuscula* collected in the Dry Tortugas, FL. However, upon recognition that compound **1** was discovered simultaneously by both our group and that of Professor R. E. Moore (University of Hawaii) and in light of the earlier publication of lyngbyabellin A,[12] we have adopted the name lyngyabellin B for compound **1**.

(6) Lerner, L.; Bax, A. *J. Magn. Reson.* **1986**, *69*, 375−380.

(7) Williamson, R. T.; Marquez, B. L.; Gerwick, W. H. *Tetrahedron* **1999**, *55*, 2881−2888.

(8) Sone, H.; Kondo, T.; Kiryu, M.; Ishiwata, H.; Ojika, M.; Yamada, K. *J. Org. Chem.* **1995**, *60*, 4774−4781.

(9) McDonald, L. A.; Ireland, C. M. *J. Nat. Prod.* **1992**, *55*, 376−379.

(10) Meyer, B. N.; Ferrigni, J. E.; Putnam, J. E.; Jacobson, L. B.; Nichols, D. E.; McLaughlin, J. L. *Planta Med.* **1982**, *45*, 31−34.

(11) Gerwick, W. H.; Mrozek, C.; Moghaddam, M. F.; Agarwal, S. K. *Experientia* **1989**, *45*, 115−121.

(12) Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J.; Mooberry, S. L. *J. Nat. Prod.* **2000**, *63*, 611−615.

NP000133Y