Antillatoxin B, a Neurotoxic Lipopeptide from the Marine Cyanobacterium *Lyngbya majuscula*

Lisa M. Nogle, Tatsufumi Okino, and William H. Gerwick*

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

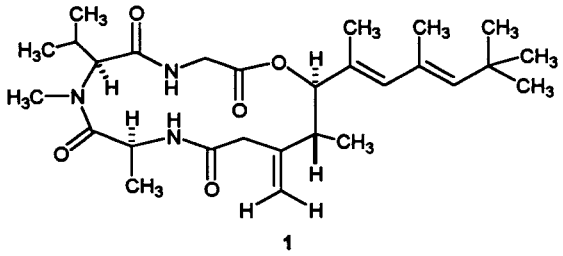
Received February 28, 2001

Bioassay-guided fractionation of organic extracts from two *Lyngbya majuscula* collections led to the isolation of a new secondary metabolite, antillatoxin B, an unusual *N*-methyl homophenylalanine analogue of the potent neurotoxin antillatoxin. Its structure was deduced from 2D NMR and data comparisons with antillatoxin. Antillatoxin B exhibited significant sodium channel-activating (EC_{50} = 1.77 μM) and ichthyotoxic (LC_{50} = 1 μM) properties.

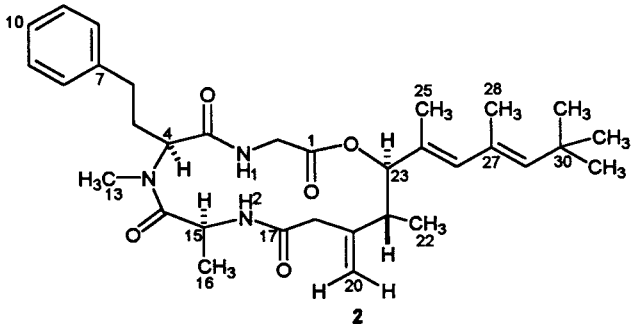
Marine microalgae are known for their production of noxious compounds responsible for human intoxications and extensive fish mortality (e.g., brevetoxins, ciguatoxins).[1] The marine cyanobacterium *Lyngbya majuscula* produces a variety of toxic secondary metabolites, including such structurally diverse substances as kalkitoxin, debromoaplysiatoxin, and the lyngbyatoxins.[1,2] In addition to their ecological and economic significance, such molecules possess considerable potential as biochemical and pharmacological tools.

An extremely potent ichthyotoxic *L. majuscula* metabolite, antillatoxin (**1**), was first reported from our laboratory in 1995[3] and subsequently shown to induce NMDA receptor-mediated neurotoxicity.[4] On the basis of this finding and the prospect of discovering new neurotoxins of novel structure and pharmacology, we evaluated our library of cyanobacterial extracts for sodium channel modulators using a mouse neuroblastoma cell line.[5] Approximately 150 cyanobacterial extracts were initially screened, identifying seven that displayed significant sodium channel activation. Assay-guided fractionation of two *L. majuscula* organic extracts from collections acquired in Puerto Rico and the Dry Tortugas led to the isolation of antillatoxin (**1**) and a novel structural homologue of **1** as the potently active constituents. Herein we report the structure and biological activity of this new metabolite, antillatoxin B (**2**).

Antillatoxin B (**2**) was isolated as a colorless oil using vacuum liquid chromatography and reversed-phase HPLC. The ^{1}H and ^{13}C NMR data of **2** indicated a close similarity of this metabolite to antillatoxin (**1**). By HR FABMS compound **2** analyzed for $C_{33}H_{48}N_3O_5$ and, hence, was larger than antillatoxin by a C_5H_2 unit, indicating that additional unsaturation was present in **2**. Moreover, several signals associated with the *N*-methyl valine moiety in **1** were absent and were replaced by two midfield methylenes and a monosubstituted aromatic ring. Two-dimensional NMR spectral data, especially HMBC, were used to delineate this new residue as an unusual *N*-methylated homophenylalanine residue (Table 1). While homophenylalanine has been detected previously in few freshwater cyanobacterial metabolites, such as [Dha^7]-microcystin-HphR[6] and nodulapeptins A and B,[7] it was previously unreported from marine cyanobacteria. Data comparisons and HMBC data indicated that the remainder of the planar structure of antillatoxin B (**2**) was identical to that of antillatoxin (**1**).



1



2

The absolute stereochemistry of antillatoxin (**1**) has recently been firmly established by total synthesis.[8] The close comparability of NMR shifts and coupling constants at comparable centers (e.g., $^3J_{H21-H23}$ = 11.0 Hz as seen for **1**), along with its similar biological properties (see below), suggested that antillatoxin B (**2**) was of the same enantiomeric series. Additionally, the absolute stereochemistry of the alanine residue in metabolite **2** was determined by Marfey's analysis to be *S*, and the presence of a strong NOE between H-4 and H-15 supported an *S* stereochemistry for the *N*-methyl homophenylalanine residue as well.[9]

Both natural products **1** and **2** are potent activators of the voltage-sensitive sodium channel in mouse neuro-2a neuroblastoma cells with EC_{50} values of 0.18 and 1.77 μM, respectively. In addition, antillatoxin B (**2**) is strongly ichthyotoxic to goldfish, with an LC_{50} of 1.0 μM (antillatoxin LC_{50} = 0.1 μM). It is interesting to note that in both measures of antillatoxin B's biological activity, a 10-fold decrease in potency is observed compared to antillatoxin (**1**); it seems likely that substitution of a larger *N*-methyl homophenylalanine residue for an *N*-methyl valine residue accounts for this decrease.

Antillatoxin B (**2**) represents an interesting addition to the growing number of peptide-derived metabolites from *L. majuscula*. The presence of homophenylalanine is

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

10.1021/np010107f CCC: $20.00
Published on Web 06/29/2001

Table 1. NMR Spectral Data for Antillatoxin B (**2**) in $CDCl_3$

position	^{13}C	^{1}H (*J* in Hz)	TOCSY	HMBC[a]
1	167.9, s			
2	41.4, t	4.67, dd (18.4, 9.7)	NH-1	1/3
		3.57, dd (18.4, 1.5)	NH-1	1/3
NH-1		7.98, d (9.5)	2	1/3
3	167.8, s			
4	59.3, d	4.77, t (7.27)	5, 6	3, 5, 13, 14
5	29.9, t	2.39, 2.05	4, 6	3, 7
6	31.6, t	2.68, 2.54	4, 5	7
7	140.2, s			
8/12	128.0, d	7.19, d (7.27)	9/11	6, 10
9/11	128.5, d	7.31, t (7.27)	8/12, 10	7
10	126.1, d	7.22, d (7.27)	9/11	
13	28.5, q	2.88, s		4, 14
14	172.9, s			
15	42.9, d	5.06, m	NH-2, 16	16, 17
16	18.3, q	1.29, d (6.7)	NH-2, 15	14, 15
NH-2		6.46, d (9.1)	15, 16	17
17	170.9, s			
18	46.4, t	2.98, d (12.9)		17, 19, 20, 21
		2.80, d (12.9)		
19	144.6, s			
20	113.7, t	5.06, 5.02	18	18, 19, 21
21	38.9, d	2.17, m	22, 23	18, 19, 20, 22
22	18.8, q	0.87, d (7.0)	21, 23	19, 21, 23
23	83.3, d	5.17, d (11.0)	21, 22	1, 19, 21, 24, 25, 26
24	128.9, s			
25	12.2, q	1.56, obs[b]	26, 29	23, 24, 26
26	137.1, d	5.94, s	25, 28, 29	23, 25, 28, 29
27	130.2, s			
28	17.5, q	1.80, brs	26, 29	26, 27, 29
29	141.3, d	5.29, s	25, 26, 28	26, 28, 31−33
30	32.5, s			
31/32/33	30.8, q	1.13, s		29, 30

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

particularly unique in that only a few cyanobacterial metabolites have been shown to possess this structural feature. It is also worth noting that dolastatin 16, a cyclic peptide from the marine mollusc *Dolabella auricularia*, contains a similar aromatic residue (β-methyl homophenylalanine = dolaphenvaline).[10] It has been demonstrated that several of the dolastatins and related compounds are of cyanobacterial origin and are likely sequestered by these herbivorous animals from their cyanobacterial diet.[11]

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX 600 MHz NMR spectrometer, with the solvent $CDCl_3$ used as an internal standard. HRFABMS were recorded on a Kratos MS50TC mass spectrometer. Optical rotation was measured on a Perkin-Elmer 141 polarimeter. IR and UV spectra were recorded on Nicolet 510 and Beckman DU640B spectrophotometers, respectively. Isolation of **1** and **2** was performed using a Waters Millipore Lambda-Max model 480 spectrophotometer with a Waters Millipore model 590 pump.

Collection. Collections of the marine cyanobacterium *Lyngbya majuscula* (voucher specimens available from WHG as collection numbers PRLP-16 Sept 97-3 and DBK-16 Nov 95-2) were obtained from Collado Reef, Puerto Rico, and Bush Key, Dry Tortugas, on September 16, 1997, and November 16, 1995, respectively. The materials were stored in 2-propanol at −20 °C until extraction.

Extraction and Isolation. Approximately 170 g (dry wt) of the Puerto Rican cyanobacterium was extracted repeatedly with CH_2Cl_2/MeOH (2:1) to produce 6.8 g of crude organic extract. A portion of the extract (5.9 g) was subjected to Si VLC to produce 14 fractions. The fraction eluting with 45−50% EtOAc in hexanes was purified further using a C_{18} solid-phase extraction cartridge (7:3 MeOH/H_2O) and reversed-phase HPLC (4:1 MeOH/H_2O, Phenomenex Spherisorb 10 μ ODS) to yield 3.6 mg of antillatoxin (**1**). The fraction eluting with 55−65% EtOAc in hexanes was purified using a C_{18} solid-phase extraction cartridge (7:3 MeOH/H_2O) and reversed-phase HPLC (7:3 MeOH/H_2O, Phenomenex Spherisorb 10 μ ODS) to yield 0.4 mg of antillatoxin B (**2**). Similarly, the material collected from the Dry Tortugas (214 g dry wt) was extracted to produce 5.9 g of crude extract, a portion of which (4.0 g) was fractionated by Si VLC. The fraction eluting with 100% EtOAc was purified by ODS VLC (YMC gel ODS-A, 10% H_2O in MeOH) and reversed-phase HPLC (17:3 MeOH/H_2O, Phenomenex Sphereclone 5 μ ODS) to yield 2.7 and 3.2 mg of compounds **1** and **2**, respectively.

Biological Assays. The ichthyotoxicity of pure **1** and **2** to the common goldfish (*Carassius auratus*) was determined as previously described.[12] Activation of the voltage-sensitive sodium channel in mouse neuro-2a neuroblastoma cells was also determined as previously described.[5]

Antillatoxin B (2): clear oil; $[\alpha]^{23}_{D}$ −113.8° (*c* 0.21, MeOH); UV (MeOH) λ_{max} 209 nm (log ϵ 4.70), 240 nm (log ϵ 4.06); IR (neat) 3445, 3294, 3080, 2959, 1734, 1645, 1543, 1455, 1259 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; FABMS (3-NBA) obsd $[M + Na]^+$ at *m/z* 588 (18), $[M + H]^+$ 566 (34), 491 (41), 316 (15), 307 (21), 251 (53), 217 (15), 148 (100); HRFABMS *m/z* $[M + H]^+$ 566.3596 (calcd for $C_{33}H_{48}N_3O_5$, 566.3594).

Absolute Stereochemistry of Alanine by Marfey's Derivatization.[9] Approximately 0.2 mg of antillatoxin B (**2**) was hydrolyzed with 6 N HCl (Ace high-pressure tube, microwave, 1.5 min), and the hydrolysate was evaporated to dryness and resuspended in H_2O (50 μL). A 0.1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide solution in acetone (Marfey's reagent, 20 μL) and 1 N $NaHCO_3$ (10 μL) were added, and the mixture was heated at 40 °C for 1 h. The solution was cooled to room temperature, neutralized with 2 N HCl (5 μL), and evaporated to dryness. The residue was resuspended in H_2O (50 μL) and analyzed by reversed-phase HPLC [Waters Nova-Pak C_{18}, 3.9 × 150 mm, UV detection at 340 nm] using a linear gradient (10% CH_3CN in H_2O containing 0.05% TFA to 50% CH_3CN). The retention time (t_R, min) of the derivatized alanine residue in the hydrolysate of antillatoxin B (**2**) matched that of L-Ala (25.8) but not that of D-Ala (30.2).

Acknowledgment. The authors would like to thank B. Marquez, T. Williamson, and the Biochemistry NMR Facility at OSU for NMR spectral data, B. Arbogast and the EIHS Mass Spectrometry Facility at OSU for mass spectral data, and the MFBS Center ESO3850 and the Japan Society for the Promotion of Science for financial support of this work. We also thank D. Ballantine and the University of Puerto Rico Marine Laboratory for assistance with the collection of marine specimens.

Supporting Information Available: Spectral data for antillatoxin B. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (a) Shimizu, Y. *Chem. Rev.* **1993**, *93*, 1685−1698. (b) Yasumoto, T.; Murata, M. *Chem. Rev.* **1993**, *93*, 1897−1909.
(2) Wu, M.; Okino, T.; Nogle, L. M.; Marquez, B. L.; Williamson, R. T.; Sitachitta, N.; Berman, F. W.; Murray, T. F.; McGough, K.; Jacobs, R.; Colsen, K.; Asano, T.; Yokokawa, F.; Shioiri, T.; Gerwick, W. H. *J. Am. Chem. Soc.* **2000**, *122*, 12041−12042.
(3) Orjala, J.; Nagle, D. G.; Hsu, V. L.; Gerwick, W. H. *J. Am. Chem. Soc.* **1995**, *117*, 8281−8282.
(4) Berman, F. W.; Gerwick, W. H.; Murray, T. F. *Toxicon* **1999**, *37*, 1645−1648.
(5) Manger, R. L.; Leja, L. S.; Lee, S. Y.; Hungerford, J. M.; Hokama, Y.; Dickey, R. W.; Granade, H. R.; Lewis, R.; Yasumoto, T.; Wekell, M. M. *J. AOAC Intern.* **1995**, *78*, 521−527.
(6) Namikoshi, M.; Sivonen, K.; Evans, W. R.; Carmichael, W. W.; Rouhiainen, L.; Luukkainen, R.; Rinehart, K. L. *Chem. Res. Toxicol.* **1992**, *5*, 661−666.
(7) Fujii, K.; Sivonen, K.; Adachi, K.; Noguchi, K.; Sano, H.; Hirayama, K.; Suzuki, M.; Harada, K. *Tetrahedron Lett.* **1997**, *38*, 5525−5528.

(8) (a) Yokokawa, F.; Fujiwara, H.; Shioiri, T. *Tetrahedron* **2000**, *56*, 1759−1775. (b) Yokokawa, F.; Fujiwara, H.; Shioiri, T. *Tetrahedron Lett.* **1999**, *40*, 1915−1916. (c) Yokokawa, F.; Shioiri, T. *J. Org. Chem.* **1998**, *63*, 8638−8639.
(9) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596; *Chem. Abstr.* **102**, 167132.
(10) Pettit, G. R.; Xu, J.; Hogan, F.; Williams, M. D.; Doubek, D. L.; Schmidt, J. M.; Cerny, R. L.; Boyd, M. R. *J. Nat. Prod.* **1997**, *60*, 752−754.
(11) Harrigan, G. G.; Leusch, H.; Moore, R. E.; Paul, V. J. *Spec. Publ. − R. Soc. Chem.* **2000**, *257*, 126−139.
(12) (a) Bakus, G. J.; Green, G. *Science* **1974**, *185*, 951−953. (b) Orjala, J.; Nagle, D.; Gerwick, W. H. *J. Nat. Prod.* **1995**, *58*, 764−768.

NP010107F