Hermitamides A and B, Toxic Malyngamide-Type Natural Products from the Marine Cyanobacterium *Lyngbya majuscula*

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A Papua New Guinea collection of the marine cyanobacterium *Lyngbya majuscula* yielded two new and toxic natural products, hermitamides A (**1**) and B (**2**). The hermitamides were isolated using a brine shrimp (*Artemia salina*) toxicity assay. Planar chemical structures of **1** and **2** were established through 1D and 2D NMR, as well as FABMS data. Semisyntheses of hermitamides A (**1**) and B (**2**) were achieved by coupling the acid chloride derivative of 7(*S*)-methoxytetradec-4(*E*)-enoic acid (**4**), obtained from the same cyanobacterium collection, and the respective free amines, phenethylamine and tryptamine. Hermitamides A (**1**) and B (**2**) exhibited LD_{50} values of 5 μM and 18 μM in the brine shrimp bioassay, and an IC_{50} values of 2.2 μM and 5.5 μM to Neuro-2a neuroblastoma cells in tissue culture, respectively. Hermitamide A was mildly ichthyotoxic to goldfish, with an LD_{50} value of 19 μM, while hermitamide B was inactive at 25 μM.

Many tropical and subtropical shallow-water collections of the marine cyanobacterium *Lyngbya majuscula* produce a class of metabolites known as the malyngamides.[1−12] Most malyngamides are characterized as *N*-substituted amides of 7(*S*)-methoxytetradec-4(*E*)-enoic acid (**4**) and usually possess a terminal chloromethylene functionality. However, some deep-water varieties of *L. majuscula* contain amides of 7-methoxy-9-methylhexadec-4(*E*)-enoic acid (malyngamides D and E),[5] and a recent collection from the French Mediterranean yielded (−)-7(*S*)-methoxydodec-4(*E*)-enoic acid,[13] a fatty acid group previously seen only as a component of malyngamide G.[7] Additionally, two new serinol amide derivatives of 7(*S*)-methoxyeicos-4(*E*)-enoic acid were also recently isolated from an unidentified species of cyanobacterium collected from Northwestern Australia.[14] To date, more than 20 malyngamide-type metabolites have been reported in the literature and possess such biological properties as antifeedant activity (malyngamide A),[15] ichthyotoxicity (malyngamide H),[8] brine shrimp toxicity (malyngamides J and L),[10] and cytotoxicity to mouse neuroblastoma cells (malyngamides I acetate and N).[11]

Results and Discussion

In our continued search for pharmaceutically useful agents from marine cyanobacteria, we have isolated two new malyngamide-type secondary metabolites, hermitamides A (**1**) and B (**2**), from *L. majuscula* collected from coral reefs at Hermit Island Village, Papua New Guinea. Preliminary bioassay of the organic extract showed activity in the brine shrimp and the mouse neuroblastoma cell toxicity assays at 10 ppm. Guided by the brine shrimp toxicity assay, the natural products **1** and **2** were isolated as pale yellow oils using vacuum liquid chromatography and HPLC.

Hermitamide A (**1**) showed an $[M + H]^+$ peak at *m/z* 360.2910 for a molecular formula of $C_{23}H_{37}NO_2$ by HR-FABMS of **1** (6 degrees of unsaturation). The presence of a methoxy group in **1** was revealed by a diagnostic loss in the LRFABMS ($[M - OCH_3]^+$ peak at *m/z* 328). Structure elucidation of **1** was established mainly from analysis of 1D (^{1}H NMR, ^{13}C NMR, and DEPT) and 2D NMR (COSY, HSQC, and HMBC) spectra. The ^{1}H NMR spectrum of **1** was indicative of a malyngamide-type metabolite, especially in its presence of proton signals attributable to the "fatty acid" portion. These included an $-OCH_3$ singlet signal at δ 3.18 (H_3-15), olefinic signals at δ 5.47 (H-4) and 5.51 (H-5), an α-methoxy methine multiplet at δ 3.08 (H-7), protons of a long aliphatic chain in the δ 1.20−1.60 envelope, and a terminal $-CH_3$ triplet signal at δ 0.90 (Table 1).

In addition, a phenyl moiety could be deduced from five low-field proton signals between δ 6.90 and 7.20 in the ^{1}H NMR spectrum. This grouping was confirmed by ^{13}C NMR in which carbon signals at δ 140.1 (C-3′), 129.5 (C-4′/-8′), 129.1 (C-5′/-7′), and 126.9 (C-6′) were observed. The remaining degrees of unsaturation were accounted for by an amide carbonyl (δ 171.4; IR absorption at 1644 cm^{-1}) and an olefin (δ 131.9 and 128.1), both present in the methoxy fatty acid portion of **1**. Placement of the methoxy group at C-7 was, in part, confirmed by an HMBC correlation between the $-OCH_3$ proton signal (δ 3.18) and the C-7 (δ 81.2) carbon signal. The geometry of the olefin group at C-4 and C-5 was determined as *E*, based on a $^3J_{H4-H5} = 15.3$ Hz when the ^{1}H NMR of **1** was measured in C_6D_6.

The phenethylamine moiety of **1** was established by analysis of COSY and HMBC spectral data. In the COSY spectrum, the methylene protons at δ 3.29 (H_2-1′) showed only two proton correlations, one to an amide proton at δ 4.58 and the second to a high-field methylene proton band at δ 2.56 (H_2-2′). The placement of the phenyl group at C-2′ (δ 36.5) was revealed by an HMBC correlation from H_2-2′ (δ 2.56) to C-3′ (δ 140.1). Finally, a correlation from H_2-1′ to the amide carbonyl signal at δ 171.4 (C-1) established the placement of the phenethylamine moiety as shown in **1**.

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Table 1. ^{1}H and ^{13}C NMR Data for Hermitamide A (**1**) in C_6D_6[a]

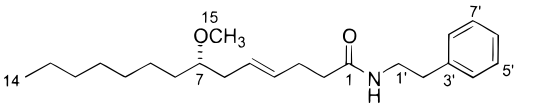
position	^{1}H mult	*J* (Hz)	^{13}C mult	HMBC[b]
1			171.4 s	
2	1.83 t	7.2	36.7 t	C-1, C-3, C-4
3	2.33 dt	7.4, 7.2	29.3 t	C-1, C-2, C-4, C-5
4	5.47 br dt	15.3, 6.4	131.9 d	C-3, C-6
5	5.51 br dt	15.3, 6.9	128.1 d	C-3, C-6
6	2.21 m		37.2 t	C-4, C-5, C-7
7	3.08 m		81.2 d	C-5, C-8, C-9, C-15
8	1.46 m		34.2 t	C-7, C-9, C-10
	1.54 m			C-6, C-7, C-9, C-10
9	1.36 m		26.1 t	C-10
	1.46 m			
10	1.28 m		30.6 t	
11	1.28 m		30.1 t	
12	1.28 m		32.6 t	
13	1.28 m		23.4 t	
14	0.90 t	7.2	14.7 q	C-12, C-13
15 ($-OCH_3$)	3.18 s		56.6 q	C-7
NH	4.58 m			
1′	3.29 dd	13.3, 7.0	41.2 t	C-1, C-2′, C-3′
2′	2.56 t	7.0	36.5 t	C-1′, C-3′, C-4′/-8′
3′			140.1 s	
4′/8′	6.99 m		129.5 d	C-2′, C-6′
5′/7′	7.14 m		129.1 d	C-3′
6′	7.06 m		126.9 d	C-4′/-8′

[a] Spectral data reported in ppm. [b] Optimized for 6 Hz.

HRFABMS of hermitamide B (**2**) gave an $[M + H]^+$ peak at 399.3013, yielding a molecular formula of $C_{25}H_{38}N_2O_2$ with eight degrees of unsaturation. In addition, an $[M - OCH_3]^+$ peak at *m/z* 367 was again present in the LR-FABMS. As it was for **1**, the planar chemical structure of **2** was elucidated using an assemblage of 1D and 2D NMR (COSY, TOCSY, HSQC, and HMBC) experiments. The ^{1}H NMR spectrum of compound **2** had several features strikingly similar to that of **1**, mainly in the proton signals attributable to the methoxy fatty acid portion of the molecule.

The main differences between compounds **1** and **2** were in the low-field aromatic proton signals between δ 7.00 and δ 8.20. The indolic moiety of **2** was confirmed through analysis of the low-field multiplicity patterns in the ^{1}H NMR [δ 7.60 br d (H-5′), δ 7.37 br d (H-8′), δ 7.02 br d (H-10′), δ 7.20 td (H-7′), and δ 7.12 td (H-6′)] as well as correlations in the COSY and TOCSY spectra. For example, the H-5′ to H-8′ spin system could be traced from ^{1}H$-^1$H correlations in the TOCSY spectrum of **2**. The COSY spectrum of **2** showed cross-peaks between H-5′ (δ 7.60)/H-6′ (δ 7.12) and H-7′ (δ 7.20)/H-8′ (δ 7.37). In addition, a cross-peak was observed between H-10′ (δ 7.02) and the indole NH at δ 8.22 in the TOCSY spectrum. The location of the tryptamine functionality in **2** was deduced mainly from HMBC data, which showed correlations from H_2-1′ (δ 3.60) and H_2-2′ (δ 2.96) to C-3′ (δ 113.2) of the indole. Finally, a cross-peak between H_2-1′ (δ 3.60) and the amide proton (δ 5.55) observed in both the COSY and TOCSY spectra, taken together with a correlation from H_2-1′ to the amide carbonyl signal at C-1 (δ 172.6) in the HMBC spectrum, confirmed the location of the tryptamine moiety in **2**.

The absolute stereochemistry at C-7 for hermitamides A (**1**) and B (**2**) was suggested through the isolation of the co-occurring free methoxy acid from the organic extract. The optical rotation value of the isolated 7(*S*)-methoxytetradec-4(*E*)-enoic acid (**4**) had a close match to the reported value in the literature ($[\alpha]^{26}_D$ −12.5° (*c* 1.91, $CHCl_3$); lit. $[\alpha]^{26}_D$ −11.1° (*c* 3.9, $CHCl_3$),[1] suggesting a 7(*S*)-stereochemistry in **1** and **2**. This was confirmed through semisynthesis of both hermitamides.



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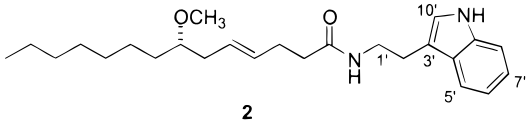

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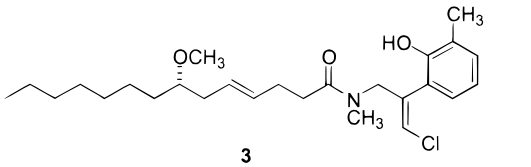

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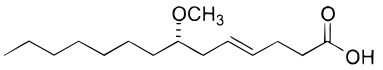

4

7(*S*)-Methoxy fatty acid (**4**) from *L. majuscula* (PNHV-11 Sep 98-04) was used in the semisynthesis of hermitamides A (**1**) and B (**2**). Two coupling reaction methods were employed. The first used dicyclohexylcarbodiimide (DCC) to couple 7(*S*)-methoxytetradec-4(*E*)-enoic acid and phenethylamine; however, the yield of product **1** was poor (22%) due to the formation of side products.[16] Improvement of the coupling yield was achieved through conversion of the free acid to the corresponding acid chloride and reacting this with either phenethylamine or tryptamine. 1D NMR spectra and FABMS of the semisynthetic compounds produced by either route were identical with the natural products **1** and **2**. The optical rotation values were −9.6° (*c* 0.49, $CHCl_3$) [natural −9.3° (*c* 0.45, $CHCl_3$)] for hermitamide A (**1**) and −4.9° (*c* 0.15, $CHCl_3$) [natural −4.5° (*c* 0.10, $CHCl_3$)] for hermitamide B (**2**).

Hermitamides A (**1**) and B (**2**) represent interesting examples of aromatized malyngamide-type compounds from the marine cyanobacterium *L. majuscula*. The first example of an aromatic-ring containing malyngamide, malyngamide M (**3**), was recently reported by Kan et al. from the Hawaiian red alga *Gracilaria coronopifolia*.[11] Because the malyngamides are well-known secondary metabolites of marine cyanobacteria, it has been suggested that the true producer of malyngamide M is a cyanobacterium growing epiphytically on *G. coronopifolia*.[11] This is supported by reports of cyanobacteria growing epiphytically on other species of *Gracilaria*.[17]

The hermitamides were evaluated for their biological activity in several systems. In the brine shrimp (*Artemia salina*) toxicity assay, hermitamides A (**1**) and B (**2**) showed LD_{50} values of 5 μM and 18 μM, respectively. Compound **1** exhibited modest ichthyotoxicity to goldfish, with an LD_{50} value of 19 μM, while compound **2** was inactive at 25 μM. Both **1** and **2** were inactive at 10 ppm in a molluscicidal (*Biomphalaria glabrata*) bioassay. The hermitamides were also found to be cytotoxic to Neuro-2a neuroblastoma cells in tissue culture, with IC_{50} values of 2.2 μM (A, **1**) and 5.5 μM (B, **2**). By comparison, malyngamide M was reported

to have only weak cytotoxicity to neuroblastoma cells (IC_{50} > 20 μM), while both malyngamides I acetate and N showed moderate cytotoxicity, with IC_{50} values at 12 μM.[11]

Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR spectra were measured on a Bruker DRX 600 MHz NMR spectrometer with the solvent, C_6D_6 or $CDCl_3$, used as an internal standard. HRMS were recorded on a Kratos MS50TC mass spectrometer. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. UV and IR spectra were recorded on Hewlett-Packard 8452A UV−vis and Nicolet 510 spectrophotometers, respectively. Isolation of compounds **1** and **2** was performed using a Waters Millipore model 590 pump and detected with a Waters Millipore Lambda-Max model 480 LC spectrophotometer.

Algal Collection. The marine cyanobacterium, *L. majuscula* (PNHV-11 Sep 98-04), was hand-collected at 8 m water depth using scuba from reefs near the Hermit Village Islands (*S* 1°28.621′, *E* 145°2.632′), Papua New Guinea on September 11, 1998. The specimens were preserved in isopropyl alcohol upon collection and stored at low temperature until workup.

Extraction and Isolation of Hermitamides A (1) and B (2) and Free Acid (4). The thawed algal material was homogenized in CH_2Cl_2/MeOH (2:1, v/v) and filtered, and the solvents were removed in vacuo to yield a residue that was partitioned between CH_2Cl_2 and H_2O. The marc was extracted repeatedly with CH_2Cl_2/MeOH (2:1, v/v), and the combined CH_2Cl_2 layers were reduced in vacuo to yield about 3.0 g of a dark green extract. The crude extract was fractionated using normal-phase Si gel vacuum liquid chromatography (VLC) through a stepwise gradient solvent system of increasing polarity starting from EtOAc in hexanes to EtOAc in MeOH. Fractions eluting with hexanes/EtOAc (3:7), 100% EtOAc, and EtOAc/MeOH (49:1) were combined and found to be the most active at 10 ppm in the brine shrimp toxicity assay. This recombined fraction was refractionated using Mega Bond RP_{18} SEP PAK. The most active material (85% toxicity at 1 ppm to brine shrimp) was eluted with 20% H_2O in MeOH and was further purified by HPLC [Phenomenex Sphereclone 5μ ODS (2), MeOH/H_2O (82:18); detection at 254 nm] giving both hermitamides A (**1**, 5.0 mg) and B (**2**, 1.1 mg). 7(*S*)-Methoxytetradec-4(*E*)-enoic acid (**4**), showing $[\alpha]^{26}_D$ −12.5° (*c* 1.91, $CHCl_3$), was isolated from fractions eluting from VLC with hexanes/EtOAc (4:1), Mega Bond RP_{18} SEP PAK (10% H_2O in MeOH), and HPLC [Phenomenex Sphereclone 5μ ODS (2), MeOH/H_2O (89:11); detection at 210 nm].

Hermitamide A (1): pale yellow oil; $[\alpha]^{26}_D$ −9.3° (*c* 0.45, $CHCl_3$), UV (EtOH) λ_{max} 216 nm (ϵ 5700); IR (neat) 3293, 2934, 2855, 1644, 1551, 1453, 1097, 970, 748, 699 cm^{-1}; ^1H NMR (600 MHz, C_6D_6) and ^{13}C NMR (150 MHz, C_6D_6), see Table 1; LRFABMS *m/z* 360 (100), 328 (92), 217 (15), 163 (70), 154 (16), 120 (11), 105 (39); HRFABMS (positive ion, 3-nitrobenzyl alcohol) *m/z* obsd $[M + H]^+$ 360.2910 ($C_{23}H_{38}NO_2$, 0.8 mmu dev.).

Hermitamide B (2): pale yellow oil; $[\alpha]^{26}_D$ −4.5° (*c* 0.10, $CHCl_3$); UV (EtOH) λ_{max} 224 nm (ϵ 13 600), 282 nm (ϵ 3600), 292 nm (ϵ 3000); IR (neat) 3301, 2930, 2854, 1646, 1549, 1455, 1095, 970, 740 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$), see Table 2; LRFABMS *m/z* 399 (77), 367 (39), 255 (3), 224 (5), 187 (2), 143 (100), 130 (31); HRFABMS (positive ion, 3-nitrobenzyl alcohol) *m/z* obs $[M + H]^+$ 399.3013 ($C_{25}H_{39}N_2O_2$, 0.2 mmu dev.).

Semisynthesis of Hermitamide A (1). The acid chloride 7(*S*)-methoxytetradec-4(*E*)-enoic acid was prepared by adding 5 μL of pyridine to 11.7 mg of 7(*S*)-methoxytetradec-4(*E*)-enoic acid (**4**, 0.046 mmol) and then 200 μL of $SOCl_2$ and heating to 60 °C for 1 h under argon. Excess $SOCl_2$ was removed in vacuo. This was followed by addition of 3 molar equiv of phenethylamine (17 μL) and stirring for 1 h at room temperature. The crude mixture was successively washed with 5% HCl, 5% $NaHCO_3$, and H_2O and then extracted 3× with CH_2Cl_2. Semisynthetic hermitamide A (**1**, 10.0 mg, 60%) was purified using HPLC [Phenomenex Sphereclone 5μ ODS (2), CH_3CN/H_2O (91:9); detection at 254 nm] and showed $[\alpha]^{26}_D$ −9.6° (*c* = 0.49, $CHCl_3$).

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

position	^1H mult	*J* (Hz)	^{13}C mult	HMBC[b]
1			172.6 s	
2	2.17 m		36.7 t	C-1, C-3, C-4
3	2.29 m		28.8 t	C-1, C-2, C-4, C-5
4	5.42 m		131.0 d	C-3, C-6
5	5.42 m		127.7 d	C-3, C-6
6	2.14 m		36.5 t	C-4, C-5, C-7
7	3.12 ddd	5.8, 5.6, 5.3	80.9 d	C-5, C-8, C-9, C-15
8	1.40 m		33.5 t	C-7, C-9, C-10
9	1.26 m		25.4 t	
10	1.26 m		29.9 t	
11	1.26 m		29.5 t	
12	1.26 m		32.0 t	
13	1.26 m		22.8 t	
14	0.87 t	6.4	14.3 q	C-12, C-13
15 (−OCH_3)	3.30 s		56.6 q	C-7
NH	5.55 m			
1′	3.60 dd	12.8, 6.6	39.8 t	C-1, C-2′, C-3′
2′	2.96 t	6.6	25.5 t	C-1′, C-3′, C-4′, C-10′
3′			113.2 s	
4′			127.5 s	
5′	7.60 br d	7.9	118.9 d	C-3′, C-4′, C-6′, C-9′
6′	7.12 td	8.0, 1.3	119.4 d	C-4′, C-8′, C-9′
7′	7.20 td	8.0, 1.3	122.3 d	C-5′, C-9′
8′	7.37 br d	8.0	111.4 d	C-4′, C-7′
9′			136.6 s	
10′	7.02 br d	2.1	122.2 d	C-2′, C-3′, C-4′
NH	8.22 br s			

[a] Spectral data reported in ppm. [b] Optimized for 6 Hz.

Semisynthesis of Hermitamide B (2). The acid chloride of 7(*S*)-methoxytetradec-4(*E*)-enoic acid was prepared identically as described above for hermitamide A (**1**). However, in this case, 3 molar equiv of tryptamine (22 mg) in THF (300 μL) was added to the acid chloride and stirred for 1 h at room temperature. Workup was carried out as above for hermitamide A (**1**). Semisynthetic hermitamide B (**2**, 9.0 mg, 50%) was purified using HPLC [Phenomenex Sphereclone 5μ ODS (2), MeOH/H_2O (84:16); detection at 254 nm] and showed $[\alpha]^{26}_D$ −4.9° (*c* 0.15, $CHCl_3$).

Brine Shrimp Toxicity, Ichthyotoxicity, and Molluscicidal Bioassays. Evaluation of the crude extract, chromatography fractions, and pure compounds for brine shrimp (*A. salina*) toxicity was determined as detailed previously.[18] Icthyotoxicity of pure compounds using goldfish (*Carassius auratus*) was also measured as detailed previously.[19] Molluscicidal bioassays used the aquatic snail *B. glabrata* in protocols previously described.[20]

Cytotoxicity Assay.[21] Neuro-2a mouse neuroblastoma cells (ATCC CCL-131) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50 μg/mL streptomycin, and 50 units/mL penicillin in an atmosphere of 5% CO_2 at 37 °C. Growth medium (200 μL) containing the cell suspension (1×10^5 cells/mL) was placed in 96-well culture plates. After 24 h, 30 μL of the samples were added to the cells. The sample was dissolved in EtOH and serially diluted with medium to make the final concentration of EtOH less than 1%. Cultures were incubated for 24 h, and cytotoxicity determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay with colorimetric measurement at 570 nm.

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Supporting Information Available: FABMS, ^{1}H NMR, ^{13}C NMR, and 2D NMR spectra of hermitamides A (**1**) and B (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

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