Phytochemistry 69 (2008) 2067–2069

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Besarhanamides A and B from the marine cyanobacterium *Lyngbya majuscula*

Lik Tong Tan *, Ying Yan Chang, Tripathi Ashootosh

National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Singapore

ARTICLE INFO

Article history:
Received 6 December 2007
Received in revised form 28 April 2008
Available online 29 May 2008

Keywords:
Lyngbya majuscula
Oscillatoriaceae
Cyanobacteria
Brine shrimp toxicity
Fatty acid amides
Besarhanamides

ABSTRACT

Besarhanamides A (**1**) and B (**2**) are fatty acid amides purified from the marine cyanobacterium, *Lyngbya majuscula*, collected from Pulau Hantu, Singapore. The structure determination of these secondary metabolites was carried out using extensive 2D NMR spectral data as well as chemical manipulations including the Marfey's method. In addition, besarhanamide A exhibited moderate toxicity with LD_{50} at 13 μM in the brine shrimp toxicity bioassay.

1. Introduction

The prokaryotic marine cyanobacteria are a prolific source of structurally unique bioactive secondary metabolites. For instance, more than 300 nitrogen-containing compounds belonging to either the polypeptide or hybrid polyketide–polypeptide structural class have been reported in the literature (Gerwick et al., 2001; Ramaswamy et al., 2006; Tan, 2007). In addition, a number of these compounds, such as curacin A and dolastatins, have served as structural templates for the generation of synthetic analogues currently in either preclinical/clinical testing as anticancer agents (Simmons et al., 2005). In spite of the chemical richness of these microorganisms, no research was conducted on the chemistry of marine cyanobacteria from Singapore. As part of a research program on drug discovery from marine organisms, samples of the marine cyanobacterium *Lyngbya majuscula* Agardh ex Gomont, collected from Pulau Hantu Besar, Singapore, were investigated for its production of bioactive secondary metabolites. Bioassay-guided fractionation of its organic extract resulted in the isolation of two new fatty acid amides, besarhanamides A (**1**) and B (**2**). These compounds belong to the growing class of acyl amide-type natural products which are distinct marine cyanobacterial natural products consisting of unique fatty acids coupled through amide bond to a variety of amines. The discovery of these molecules in this paper adds to the growing number of this class of natural products unique to marine cyanobacterial secondary metabolism.

2. Results and discussion

Samples of the marine cyanobacterium, *L. majuscula*, collected from Pulau Hantu Besar, were extracted repeatedly in chloroform and methanol. The toxic organic extract obtained from solvent partitioning was subjected to vacuum flash chromatography using a combination of hexanes, EtOAc, methanol in increasing polarity. This is followed by semi-preparative RP-HPLC to yield compounds **1** and **2** (Fig. 1).

The isolation of besarhanamide A (**1**), an amorphous compound, was achieved using a combination of SEP-PAK RP-18 and semi-preparative HPLC on fraction 4 which was obtained from VFC of the organic extract of *L. majuscula*. Besarhanamide A showed an $[M+H]^+$ peak at *m/z* 382.3305 for a molecular formula of $C_{23}H_{44}NO_3$ by HR-ESI accounting for three degrees of unsaturation. The planar structure of besarhanamide A was determined mainly from detailed analysis of 1D (^1H and ^{13}C) and 2D NMR spectra (COSY, HSQC, and HMBC). The ^1H NMR spectrum was indicative of a fatty acid amide-type compound due to the tell-tale presence of proton signals attributable to a long aliphatic chain in the δ 1.20 to δ 1.40 window, a terminal methyl triplet at δ 0.90 as well as a broad amide doublet at δ 6.24 (Table 1). The three degrees of unsaturation were accounted for by an olefinic bond (δ 128.3 and δ 132.5), an amide carbonyl carbon resonance (δ 169.6), and an epoxide functional group. The presence of an epoxide ring was deduced from two midfield signals at δ 55.7 and δ 59.8 in the ^{13}C NMR spectrum and COSY data.

An alaninol moiety in besarhanamide A was deduced from COSY and HMBC data. For instance, H-1′ (δ 4.04) showed correlations with the amide proton at δ 6.24, methylene protons at

* Corresponding author. Tel.: +65 6790 3820; fax: +65 6896 9432.
E-mail address: LikTong.Tan@nie.edu.sg (L.T. Tan).

0031-9422/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2008.04.021

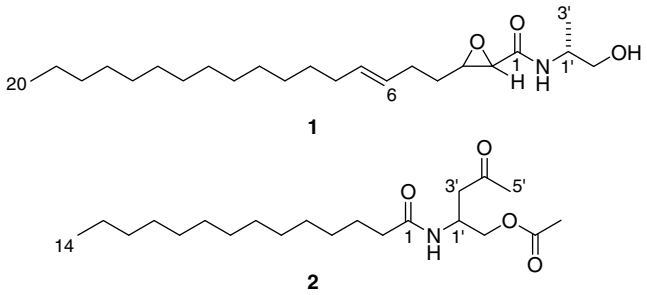

Fig. 1. Chemical structures of besarhanamides A (**1**) and B (**2**).

Table 1
^{1}H and ^{13}C NMR assignments for besarhanamide A (**1**) (400 MHz, $CDCl_3$)

Position	δ_H (*J* in Hz)	δ_C	HMBC
1		169.6	
2	3.27 *d* (2.1)	55.7	C-1
3	2.98 *dt* (6.6, 4.5, 2.1)	59.8	
4	1.78 *m*	32.3	C-2, C-3, C-5, C-6
	1.61 *m*		
5	2.18 *m*	29.0	C-3, C-4, C-6, C-7
6	5.43 *dt* (15.2, 6.4)	128.3	C-5
7	5.48 *dt* (15.2, 6.5)	132.5	C-8
8	2.00 *m*	32.9	C-6, C-7, C-9
9–17	1.28 *m*	30.1–29.6	
18	1.28 *m*	32.1	
19	1.28 *m*	23.1	
20	0.90 *t* (6.7)	14.5	C-18, C-19
1′	4.04 *m*	47.8	
2′	3.69 *dd* (11.0, 3.6)	67.3	
	3.58 *dd* (11.0, 6.0)		
3′	1.17 *d* (6.8)	17.3	C-1′, C-2′
*N*H	6.24 *d* (4.0)		

H_2-2′ (δ 3.69 and 3.58) as well as the methyl protons at H_3-3′ (δ 1.17) in the COSY spectral data. In addition, H_3-3′ gave correlations with C-1′ and C-2′ in the HMBC spectrum. The hydroxyl group was placed on C-2′ due to the carbon chemical signal of δ 67.3. The relative positions of the olefinic and epoxide groups in the fatty acid acyl chain moiety were determined from COSY and HMBC spectral data. The doublet proton signal at δ 3.27 (H-2) showed correlations with H-3 (δ 2.98) and H-3 in turn showed correlations with H_2-4 (δ 1.78 and δ 1.61) and H_2-5 (δ 2.18) protons in the COSY spectrum. Presence of a correlation between H-2 (δ 3.27) and C-1 (δ 169.6) in the HMBC spectrum placed the epoxide ring system adjacent to the carbonyl carbon. The placement of the olefinic group to the epoxide ring via two methylene carbons was indicated by 2D NMR spectral data. For instance, the methylene protons at δ 2.18 (H_2-5) gave correlation with C-3 (δ 59.8), C-4 (δ 32.3), C-6 (δ 128.3), and C-7 (δ 132.5) in the HMBC spectrum. The geometry of the olefin at C-6 was assigned as *trans* due to the coupling constant of 15.2 Hz measured for $^3J_{H\text{-}6/H\text{-}7}$. In spite of a number of 1D and 2D NOE spectral measurements (with various mixing times) made on besarhanamide A, no correlations were observed between H-2 and H-3 on the epoxide ring. This led to speculation of a *trans* configuration of H-2 and H-3, with the relative stereochemistry at C-2 and C-3 as either 2*S**, 3*R** or 2*R**, 3*S**. Although there are no HMBC correlations between the alaninol moiety and the fatty acid derived chain, the linkage through amide bond is the only option left to complete the structure of besarhanamide A. A D-configuration for the alaninol moiety was determined by Marfey's method using RP-18 HPLC in comparison with the retention time of the derivatized L/D-alaninol standards. Moderate toxicity was observed for this molecule when tested in the BSL assay with LD_{50} at 13 μM.

Table 2
^{1}H and ^{13}C NMR assignments for besarhanamide B (**2**) (400 MHz, $CDCl_3$)

Position	δ_H (*J* in Hz)	δ_C	HMBC
1		173.5	
2	2.14 *brt* (7.0)	37.2	C-1, C-3, C-4
3	1.58 *m*	26.0	C-1
4-11	1.20–1.30	29.6–30.9	
12	1.26 *m*	32.3	
13	1.25 *m*	23.1	
14	0.88 *t* (6.8)	14.5	C-12, C-13
1′	4.33 *ddq* (7.7, 7.4, 5.2)	49.1	C-2′
2′	4.02 *dd* (11.5, 5.2)	65.0	C-3′
	4.20 *dd* (11.5, 5.2)		C-1′
3′	3.04 *dd* (14.2, 5.2)	30.1	C-1′, C-2′, C-4′
	3.13 *dd* (14.2, 8.1)		C-1′, C-2′, C-4′
4′		196.7	
5′	2.36 *s*	30.1	C-4′
*O*Ac		171.3	
	2.09 *s*	21.2	*C*(O)CH_3
*N*H	5.80 *d* (8.0)		C-1

Besarhanamide B (**2**) gave an $[M+H]^+$ peak at 370.2962 *m*/z for a molecular formula of $C_{21}H_{39}NO_4$ by HR-ESI accounting for three degrees of unsaturation. The ^{1}H NMR spectrum of **2** was similar to **1** by having a fatty acid moiety due to long chain CH_2 proton signals from δ 1.20 to δ 1.30, a terminal methyl triplet at δ 0.88 as well as a broad amide doublet at δ 5.80 (Table 2). The three degrees of unsaturation were accounted by the presence of two carbonyl carbon at 171.3 ppm and 173.5 ppm and a ketone functional group at 196.7 ppm.

A 2-amino-4-oxo-pentanol moiety in compound **2** was deduced from 2D NMR spectra, including COSY, HSQC, and HMBC. From the HMBC data, the proton signal at δ 4.33 m (H-1′) showed correlation with C-2′ (δ 65.0) while the methylene protons at H_2-2′ (δ 4.02 dd and δ 4.20 dd) showed correlation with C-1′ (δ 49.1) and C-3′ (δ 30.1). In addition, the methylene ^{1}H signals at δ 3.04 dd (H-3′) and δ 3.13 dd (H-3′) both showed correlations with C-1′ (δ 49.1), C-2′ (δ 65.0), and C-4′ (δ 196.7) while the methyl protons at δ 2.36 s (H_3-5′) correlated with the carbonyl carbon at C-4′ (δ 196.7). The placement of the acetate group at C-2′ was the only position possible in compound **2**. The above moiety is linked through amide bond to a C-14 carboxylic acid derivative as shown by the correlation of a NH proton at δ 5.80 d and H_2-2 protons at δ 2.14 m to carbonyl carbon at δ 173.5 (C-1) establishing the complete structure of besarhanamide B (**2**). Several attempts were carried out to determine the absolute stereochemistry at C-1′ of besarhanamide B using acid hydrolysis and subsequent analysis of hydrolysate based on the advanced Marfey's method. However, these chemical manipulations were deemed unsuccessful and this could possibly be due to the labile nature of the freed moiety upon acid hydrolysis. Besarhanamide B was inactive in the brine shrimp toxicity assay when tested at 100 ppm. Both besarhanamides A (**1**) and B (**2**) are structurally related to the recently reported series of fatty acid amides, the semiplenamides, isolated from a Papua New Guinea strain of *L. majuscula* (Han et al., 2003). The alaninol moiety in compound **1** is also present in semiplenamides C and F. However, the 2-amino-4-oxo-pentanol moiety in compound **2** has never been reported from marine cyanobacterial acyl-amide-type compounds. It could be speculated that the biosynthetic origin of C-4′ and C-5′ in **2** could be derived from an acetate unit attaching to an alaninol-derived unit.

3. Conclusions

Two new secondary metabolites, besarhanamides A and B, were isolated from the marine cyanobacterium, *L. majuscula*, collected from Pulau Hantu Besar, Singapore. Besarhanamide A (**1**) exhibited

moderate toxicity with LD_{50} of 13 μM in the brine shrimp toxicity assay. The present report represents the first of such studies on the isolation of natural products from local strain of marine cyanobacterium.

4. Experimental

4.1. General experimental procedures

Specific optical rotation of besarhanamides A and B were measured on a Perkin–Elmer Model 341 Polarimeter. IR and UV spectra were recorded on a Perkin–Elmer Spectrum One FT-IR and Shimadzu UV1601 spectrometers, respectively. NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer with the solvent $CDCl_3$ used as an internal standard (δ_H at 7.26). High and low mass spectra were recorded on an Agilent LCMS-TOF 1200. HPLC isolation of besarhanamides A and B were conducted on a Shimadzu LC-8A Preparative LC and Shimadzu SPD-M10A VP diode array detector while an Agilent 1100 HPLC system was used for the detection of the Marfey-derivatized L/D-alaninol standards and the alaninol moiety from besarhanamide A.

4.2. Marine cyanobacterial specimen

About 1.0 L of the marine cyanobacterium, *L. majuscula*, was collected from the west lagoon of Pulau Hantu Besar during low tides on June 29, 2005 and stored in 70% aqueous ethanol at −20 °C before extraction workup. The cyanobacterial filaments were examined under light microscope and found to be 54.3 to 74.1 μm wide with a hyaline, lamellated sheath. The trichomes were blue-green, cylindrical, non-constricted, and formed by homogeneous, disc-shaped cells. The cells were 3.7–4.9 μm long and 34.9–46.9 μm wide and had no granules at the septa when observed under the light microscope. The apical cells were rounded, non-capitate, and had no calyptra. A voucher specimen of this microalga is maintained at NIE under the code TLT/PHB/002.

4.3. Extraction and isolation

The extraction of *L. majuscula* from Pulau Hantu Besar were carried out repeatedly using $CHCl_3$/MeOH (1:1) to produce about 1.5 g of crude organic extract. The extract was then subjected through vacuum flash chromatography (VFC) on normal phase Si using a combination of hexanes, EtOAc, and MeOH of increasing polarity. Eight fractions were obtained and solvents were removed in vacuo using a rotary evaporator before storage in 4 dram vials in CH_2Cl_2. All fractions were assayed at 1 and 10 ppm in the brine shrimp lethality assay.

Fraction 4 obtained from VFC of the organic extract of *L. majuscula* was subjected to fractionation on a SEP-PAK RP-18 cartridge using a combination of MeOH and water into four sub-fractions. The brine shrimp active sub-fraction eluted with 10% H_2O in MeOH was subjected to further purification by preparative HPLC [Phenomenex Sphereclone 5 μm ODS, 250 × 10.00 mm, 9:1 MeOH/H_2O, detection at 215 nm) to give besarhanamides A (**1**, 1.5 mg) and B (**2**, 6.0 mg).

4.4. Absolute stereochemical determination of the alaninol moiety in besarhanamide A (*1*)

Hydrolysis of besarhanamide A (0.2 mg) was carried out in 6N HCl at 105 °C for 18 h. The hydrolysate was evaporated to dryness under a stream of N_2 and resuspended in 100 μL of H_2O. A 0.1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide solution in acetone (L-Marfey's reagent, 25 μL) and 1N $NaHCO_3$ (15 μL) were added to the resuspended hydrolysate and heated at 40°C for 40 min. The solution was cooled to room temperature, neutralized with 2 N HCl (15 μL) and evaporated to dryness and resuspended in 1.0 mL CH_3CN for analysis by reversed-phase HPLC on a Phenomenex Luna 5 μ C-18 (150 × 2.0 mm) column with a linear gradient of CH_3CN:H_2O (0.1% formic acid), 30:70 to 90:10 in 20 min at 0.2 mL/min (UV detection at λ 340 nm). The derivatized alaninol from besarhanamide A and the derivatized D-alaninol standard both eluted at 9.27 min while the derivatized L-alaninol standard eluted at 6.69 min.

4.5. Besarhanamide A (*1*)

White amorphous solid; $[\alpha]_D^{26} + 12.5°$ (*c* 0.16, $CHCl_3$); UV (MeOH) λ_{max} 205 nm (ε 5000); IR (neat) 3295, 2919, 2849, 1657, 1559, 1465, 1047 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HRFABMS *m/z* $[M+H]^+$ 382.3305 (Calcd. for $C_{23}H_{44}NO_3$, 382.3315).

4.6. Besarhanamide B (*2*)

White amorphous solid; $[\alpha]_D^{26} + 3°$ (*c* 0.68, $CHCl_3$); UV (MeOH) λ_{max} 205 nm (ε 5500); IR (neat) 3290, 2915, 2849, 1696, 1650, 1551, 1223, 1047 cm^{-1}; ^1H and ^{13}C NMR data see Table 2; HRFABMS *m/z* $[M+H]^+$ 370.2962 (Calcd. for $C_{21}H_{40}NO_4$, 370.2951).

4.7. Bioassay

The brine shrimp (*Artemia salina*) toxicity assay is a slight modification of the original bioassay for measuring toxicity described by Meyer et al. (1982). About 16 newly hatched brine shrimp in about 0.5 mL seawater were added to each well containing various concentrations of test samples in 4 dram glass vials and samples and controls were performed in duplicate. After incubation for 24 h at 29 °C, the brine shrimp larvae were counted using a dissecting microscope and the percentage of live vs total brine shrimp was calculated to determine LD_{50} levels.

Acknowledgements

The authors would like to acknowledged the SIBiol RTF and the NIE AcRF (RI 8/05 TLT) for financial support as well as Dr. Chia Lian Sai (NSSE, NIE) for the use of the Shimadzu Preparative HPLC system. In addition, the authors would like to thank Say Guek Yee-Lee (NSSE, NIE), Chin Chye Teo (NSSE, NIE), Doris Tan (Technical Support Department at ICES), Yogeswari Selvaraja, Christopher Talbot, Soon Chai Lee, and Lalitha Kasi Pandiyan for technical assistance.

References

Gerwick, W.H., Tan, L.T., Sitachitta, N., 2001. Nitrogen-containing metabolites from marine cyanobacteria. In: Cordell, G.A. (Ed.), The Alkaloids: Chemistry and Biology, vol. 57. Academic Press, San Diego, pp. 75–184.

Han, B., McPhail, K.L., Ligresti, A., Di Marzo, V., Gerwick, W.H., 2003. Semiplenamides A–G, fatty acid amides from a Papua New Guinea collection of the marine cyanobacterium *Lyngbya semiplena*. J. Nat. Prod. 66, 1364–1368.

Meyer, B.N., Ferrigni, J.E., Putnam, J.E., Jacobson, L.B., Nicholas, D.E., McLaughlin, J.L., 1982. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med 45, 31–34.

Ramaswamy, A.V., Flatt, P.M., Edwards, D.J., Simmons, T.L., Han, B., Gerwick, W.H., 2006. The secondary metabolites and biosynthetic gene clusters of marine cyanobacteria. Applications in biotechnology. In: Proksch, P., Muller, W.E.G. (Eds.), Frontiers in Marine Biotechnology. Horizon Bioscience, pp. 175–224.

Simmons, T.L., Andrianasolo, E., McPhail, K., Gerwick, W.H., 2005. Maring natural products as anticancer drugs. Mol. Cancer Ther. 4, 333–342.

Tan, L.T., 2007. Bioactive natural products from marine cyanobacteria for drug discovery. Phytochemistry 68, 954–979.