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ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · APRIL 2010

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J Nat Prod. 2010 April 23; 73(4): 784–787. doi:10.1021/np100002q.

Nhatrangins A and B, Aplysiatoxin-Related Metabolites from the Marine Cyanobacterium *Lyngbya majuscula* from Vietnam

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Abstract

Two polyketide metabolites, nhatrangins A (**1**) and B (**2**), were isolated from a Vietnamese collection of *Lyngbya majuscula*. These compounds are related to the aplysiatoxin series of metabolites, which have also been isolated from this species of marine cyanobacterium. The use of 900 MHz cryoprobe NMR allowed the elucidation of the 2D structure of **1** from approximately 0.3 mg of compound. LC-MS analysis was utilized to direct the isolation of additional material as well as the isolation of **2**. Conformational analysis was completed using *J*-based coupling constant analysis and selective NOE experiments.

Cyanobacteria, in particular *Lyngbya majuscula*, have been shown to be a rich source of biologically active secondary metabolites.[1–3] Specifically, a series of toxins named the aplysiatoxins have been isolated from this species. Aplysiatoxins, which have been indicated as a causative agent for “swimmers itch”, were originally isolated from *Stylocheilus longicauda*, a sea hare.[4] It has been reported that *S. longicauda* preferentially feeds on *L. majuscula*, and chemical investigations of this cyanobacterium yielded aplysiatoxin and debromoaplysiatoxin, thus showing that the aplysiatoxins found in the sea hare were of dietary origin.[5] Since the first report of the isolation of aplysiatoxin from *L. majuscula*, aplysiatoxins and related analogues such as the oscillatoxins have been isolated from other cyanobacteria, such as *Schizothrix calcicola* and *Oscillatoria nigro-viridis*.[6] The aplysiatoxins and the related manauealide C have also been isolated from *Gracilaria coronopifolia*, a red alga.[7]

Many of the aplysiatoxins have tumor-promoting activity through the activation of protein kinase C.[8,9] This mechanism of action is the same as lyngbyatoxin, an another causative agent of contact dermatitis, i.e. “swimmer’s itch”.[10,11] Debromoaplysiatoxin has been reported to possess antiproliferative activity against a lymphocytic murine leukemia (P-388) cell line.[5] More recently, a synthetic analogue of aplysiatoxin was shown to have antiproliferative activity similar to bryostatin-1 in an eight-cell-line panel.[12]

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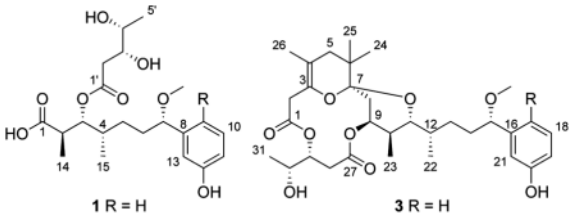
Supporting Information Available: ^{1}H, DEPTQ, gCOSY, TOCSY, gHSQC, and gHMBC spectra of **1** and **2**; selective ROE spectra of **1** in DMSO-d_6 with irradiation at 0.834, 0.946, 2.054, 2.265, 2.345, 3.154, 3.482, 3.750, and 4.744 ppm; semiselective HMBC spectrum of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

The chemistry and biological activity of these compounds have been well studied; however the biosynthetic pathway to these toxins has yet to be described. In this article, we describe the isolation and structure elucidation of nhatrangins A (**1**) and B (**2**), named after the collection site of Nha Trang Bay, Vietnam. The carbon skeleton of these molecules appears to be related to the carbon skeleton of the aplysiatoxins and may give an insight into the biosynthesis of these metabolites.

An initial organic extract (2.5 g) of *L. majuscula* displayed significant antiproliferative activity in a colon cancer cell line (CoL-2).[13] Bioassay-guided fractionation of the extract yielded anhydrodebromoaplysiatoxin (**3**) and anhydroaplysiatoxin (**4**). Chemical evaluation of the extract also yielded approximately 0.3 mg of nhatrangin A (**1**). Through the use of 900 MHz cryoprobe NMR spectroscopy and mass spectrometry, this amount was sufficient to elucidate the 2D structure.



A second extract (10.58 g) from a larger re-collection of *L. majuscula*, from the same geographic area, was subjected to a similar separation scheme to the first extract. Selected fractions were subjected to HPLC-ESI-TOF-MS analysis to determine the presence of previously isolated metabolites, in particular nhatrangin A (**1**). Full scan data, *m*/*z* 200–1000, were acquired in negative ion mode. MS chromatograms for selected ions were then extracted during postacquisition processing. The mass spectrometric chromatogram (Figure 1, *m*/*z* 411) of one fraction (F9.2) revealed the presence of **1**. Upon the basis of the structures of anhydroaplysiatoxin and anhydrodebromoaplysiatoxin, we hypothesized that a brominated nhatrangin derivative would also be present. The MS chromatogram (Figure 1, *m*/*z* 489) for F9.2 revealed the presence of the brominated analogue, nhatrangin B (**2**). Both **1** and **2** were subsequently isolated using reversed-phase HPLC.

Nhatrangin A (**1**) was obtained as a pale orange oil. Both positive and negative mode HRESI-FTMS indicated a molecular formula of $C_{21}H_{32}O_8$ (435.1984 *m*/*z* $[M + Na]^+$ and 411.2034 *m*/*z* $[M - H]^-$) containing six degrees of unsaturation. The ^{1}H NMR spectrum (Table 1) was consistent with a 1,3-disubstituted aromatic ring (δ_H 6.63, 6.65, 6.67, and 7.10), four heteroatom-bearing methines (δ_H 3.48, 3.75, 3.93, and 4.74), a methoxy moiety (δ_H 3.07), and three tertiary methyl groups (δ_H 0.74, 0.83, and 0.95). The DEPTQ spectrum[14] confirmed the features described above and also indicated the presence of a carbonyl moiety (δ_C 171.1, C-1′). The HSQC spectrum showed that two of the protons in the ^{1}H spectrum (δ_H 2.35 and 2.05) shared the same carbon (C-2′, δ_C 38.3).

The ^{1}H and DEPTQ data accounted for $C_{20}H_{28}O_6$ and five of the six degrees of unsaturation. Due to the presence of water in the NMR sample, we were not able to assign the –OH protons; however there was still one carbon, two oxygen atoms, and one degree of unsaturation unassigned. Further evaluation of the HMBC spectra reveled a strong correlation from H-14 to a carbonyl carbon at δ_C 176.5. This additional carbonyl moiety (C-1) accounted for the missing one carbon and two oxygen atoms, as well as satisfied the missing degree of unsaturation. Interpretation of COSY, TOCSY, and HMBC experiments

allowed the elucidation of two separate parts of the molecule, C-1 through C-15 and C-1′ through C-5′ (Figure 2).

The main chain of the molecule consisted of C-1 through C-15. The seven-membered carbon alkyl chain (C-14, 2–7) with a methyl (C-15) attached at C-4 was deduced from the COSY spectrum (Figure 2). The downfield chemical shifts of C-3 and C-7, δ_C 78.0 and 83.3, respectively, and corresponding ^{1}H chemical shifts at δ_H 4.74 and 3.93, respectively, were consistent with carbons attached to an oxygen atom. Correlations from the HMBC spectrum revealed that the methoxy moiety was linked to the main chain via the oxygen attached at C-7. In addition, the HMBC spectrum showed correlations from H-7 to the carbons of the aromatic ring (C-9 and C-13). Thus, the aromatic ring was attached to the main chain by a bond between C-7 and C-8. The downfield shift of C-12 (δ_C 157.5) and the lack of additional HMBC correlations beyond the aromatic ring were consistent with a phenolic hydroxy moiety at C-12. The previously mentioned HMBC correlation from H-14 to C-1 revealed that the carbonyl was attached to C-2 (δ_C 40.4).

The second partial structure consisted of C-1′ to C-5′. The COSY spectrum displayed correlations consistent with an unbranched chain from C-2′ to C-5′. The DEPTQ and associated ^{1}H data for C-3′ (δ_C 70.6, δ_H 3.75) and C-4′ (δ_C 68.4, δ_H 3.48) indicated that these two carbons were substituted with oxygen. The HMBC correlations from H_2-2′ to C-1′ attached the carbonyl (C-1′) to C-2′. The downfield chemical shift of H-3 suggested an ester at the C-3 position. In order to support this ester linkage, a semiselective HMBC experiment focused around the two carbonyls (C-1 and C-1′) was performed. A correlation was observed between H-3 and C-1′. Thus, the two portions of the molecule are connected via an ester bond from C-1′ to C-3, and the planar structure was determined as shown above.

Nhatrangin B (**2**) was obtained as a clear oil. Negative mode HRESI-TOF-MS indicated a molecular formula of $C_{21}H_{31}BrO_8$ (489.1156 and 491.1139 *m*/*z* $[M - H]^-$), representing six degrees of unsaturation. The ^{1}H NMR and DEPTQ spectra showed the presence of similar structural features to those found in **1**, with the only difference being in the substitution pattern of the aromatic ring. The aromatic protons (H-10, H-11, and H-13) of **2** indicated the presence of a 1,2,5-trisubstituted aromatic ring (Table 1). The chemical shifts of C-8 (δ_C 142.1), C-9 (δ_C 118.3), and C-12 (δ_C 157.5) were consistent with alkyl, bromo, and hydroxy substitutions, respectively. The COSY, TOCSY, HSQC, and HMBC data of **2** revealed the identical carbon skeleton to that of **1**, and the planar structure is shown above.

Configurational Analysis

The two-dimensional structures of both nhatrangins A (**1**) and B (**2**) revealed the presence of six stereocenters (C-2, C-3, C-4, C-7, C-3′, and C-4′). The absolute configuration of C-7 was determined by circular dichroism and comparison with the CD spectrum of debromoaplysiatoxin.[6] Both **1** and **2** displayed positive molar ellipticities ($[\theta]_{274}$ +316 and $[\theta]_{275}$ +1054, respectively) in their CD spectra, which are similar to the values in the published CD spectrum of debromoaplysiatoxin, and thus **1** and **2** have identical absolute configurations at the benzylic carbon (C-7) as **3** and **4** (C-15). The relative configurations of the other five carbons (C-2, C-3, C-4, C-3′, and C-4′) were determined using 3-bond coupling constants, which included $^3J_{H,H}$ and $^3J_{C,H}$ values, and NOE correlations acquired from selective 1D ROE experiments (Figures 3 and 4).

Protons H-2 and H-3 displayed a large coupling constant ($^3J_{H\text{-}2,H\text{-}3}$ = 8.2 Hz), indicating them to be in an *anti* conformation. This allowed for only two of the six possible relative conformations for C-2 and C-3 (Figure 3a). A selective ROE experiment displayed a NOE correlation between H_3-14 and H-4, which indicated a relative configuration of 2*R** and 3*R**. The protons H-3 and H-4 displayed a small coupling constant ($^3J_{H\text{-}3,H\text{-}4}$ = 3.6 Hz),

which is consistent with a *gauche* conformation and produces four possible relative conformations (Figure 3b). Three of these conformations would satisfy a NOE correlation between H_3-15 and H-2; however only one, 3*R** and 4*S**, can also satisfy the previously described NOE correlation of H_3-14 (attached at C-2) and H-4. This relative configuration (2*R**, 3*R**, 4*S**) is identical to the relative configurations of C-10, C-11, and C-12 of anhydrodebromoaplysiatoxin (**3**).

The ester side chain of **1** contained two stereocenters (C-3′ and C-4′). The protons H-3′ and H-4′ displayed a small coupling constant ($^3J_{\text{H-3′,H-4′}}$= 4.2 Hz) and therefore are in a *gauche* conformation (Figure 4). The NOE correlation between H_3-5′ and H-2′a/b would be satisfied by three possible relative conformations, two 3′*R**, 4′*S** and one 3′*R**, 4′*R**. Since there were no other NOE correlations available to distinguish between these conformations, carbon–proton coupling constant analysis was used to determine the relative configuration.[15] The $^3J_{\text{C,H}}$ coupling constants were ascertained using a HSQMBC experiment.[16,17] The values for $^3J_{\text{C-2′,H-4′}}$ and $^3J_{\text{C-5′,H-3′}}$ were determined to be 4.6 and 5.0 Hz, respectively. Both of these values were considered to be “large” (5–7 Hz) and were consistent with a configuration of 3′*R** and 4′*R**.[15] The relative configurations of these carbons, C-3′ and C-4′, are identical to the relative configurations of C-29 and C-30 of **3**.

Comparison of Nhatragin and Anhydrodebromoaplysiatoxin

A comparison of the structures of **1** and anhydrodebromoaplysiatoxin (**3**) displayed similarities of the main chain of **1** (C-2 to C-15) and the C-10 to C-23 portion of **3**. The major difference between the two substructures was that C-1 was a carboxylic acid in **1**, while the corresponding carbon, C-9, was an oxygen-bearing methine in **3**. In addition, the side chain of **1** (C-1′ to C-5′) was identical to the substructure of C-27 to C-31 of **3**. The relative configurations of C-2, C-3, C-4, C-3′, and C-4′ of **1** were shown to be identical to the relative configurations of C-10, C-11, C-12, C-29, and C-30 of **3**, respectively. Given that C-7 of **1** and C-15 of **3** have the same absolute configuration, we submit that C-2, C-3, C-4, C-3′, and C-4′ of **1** also share the same absolute configuration with their representative carbons in **3**. In addition, because **1** and **2** have the same absolute configuration of C-7 and likely share a common biosynthetic origin, we submit that **1** and **2** have identical overall absolute configuration.

From these structural similarities, we suggest that **1**–**4** have a common biosynthetic origin. It is possible that they share the same biosynthetic genes, with the nhatrangins (**1** and **2**) representing either an error in biosynthesis or a competing metabolic pathway. Alternatively, the nhatrangins might result from mutant copies of the aplysiatoxin biosynthetic genes. Unfortunately, we do not have direct access to the biological material used in this study and were unable to perform any genetic and/or biosynthetic studies. Nonetheless, we propose that the nhatrangins (C-1 to C-15) could represent a starting unit for aplysiatoxin biosynthesis and may provide clues to aid in the determination of this biosynthetic pathway.

Experimental Section

General Experimental Procedures

Optical rotations were determined on a Perkin-Elmer 421 polarimeter. UV spectra were obtained on a Varian Cary 50 Bio spectrophotometer. CD spectra were obtained on a Jasco J-710 spectropolarimeter. IR spectra were obtained on a Jasco FTIR-40 Fourier transform infrared spectrometer. The NMR spectra of **1** and **2** were acquired at 900 MHz for ^{1}H and 226 MHz for ^{13}C on a Bruker Avance spectrometer. The NMR spectra of **3** and **4** were acquired at 600 MHz for ^{1}H on a Bruker Avance spectrometer. The FT-MS was acquired on a Thermo Finnigan LTQ FT spectrometer, and the TOF-MS was acquired on a Shimadzu IT-TOF spectrometer. HPLC separations were performed on an Agilent 1100 Series liquid

chromatograph. HPLC-MS analyses were performed on a Shimadzu HPLC system consisting of dual pumps, SIL autosampler, PDA, and the IT-TOF spectrometer listed above.

Biological Material

Samples of *Lyngbya majuscula* Harvey *ex* Gomont (Oscillatoriaceae), growing on rocks, dead corals, and gravel in the lower intertidal to subtidal zone of shores and exposed to calm to moderate wave action, were collected in Vietnam at Hon Do locality (N 12°16.05′, E 109°12.23′) in Nhatrang of Khanh Hoa Province. The first (primary) sample was collected on May 15, 2006, while the second (re-collection) was obtained on May 20, 2007. The voucher specimens of both samples (Pham Huu Tri 038/PHT 038) have been deposited at the Marine Museum of the Institute of Oceanography, Nhatrang. The thallus of PHT 038 expanded up to 3 cm long, dull blue-green to brown or yellowish-brown in color, with very long and curved filaments, seldom only slightly coiled, the sheath colorless, lamellated, the trichomes blue-green, not constricted at the cross-wall, not attenuated at both ends, the calyptra absent.

Extraction and Isolation

PHT 038 was extracted with 1:1 CH_2Cl_2/CH_3OH to yield 2.5 g of extract. Silica gel open column chromatography with increasing amounts of MeOH in CH_2Cl_2 yielded 14 fractions. Fraction 8 (333.9 mg) was further subjected to silica gel liquid column chromatography with a solvent gradient of hexane/EtOAc/EtOH/MeOH to yield 14 fractions. Subfractions 2–6 (F8.2–6) were recombined based upon TLC analysis (hexane/EtOAc, 1:1). Reversed-phase (C18) semipreparative HPLC of F8.2–6 yielded 2.5 mg of anhydrodebromoaplysiatoxin (**3**) and 9.8 mg of anhydroaplysiatoxin (**4**). Fraction 10 (24.4 mg) was subjected to reversed-phase HPLC (Alltech Altima C18, 10 × 250 mm, 9.5–95% aqueous CH_3CN with 10 mM NH_4OCOCH_3, 0.0–25.0 min) to yield **1** (6.12 min). The initial yield of **1** was 0.3 mg, determined gravimetrically.

A second collection of *L. majuscula* (PHT 038) yielded 10.58 g of extract (CH_2Cl_2/CH_3OH, 1:1). This extract was subjected to silica gel liquid column chromatography with a solvent gradient of CH_2Cl_2/CH_3OH to yield 17 fractions. Fraction 9 (5.37 g) was subjected to flash chromatography utilizing HP20SS Diaion resin and a H_2O/IPA gradient to yield eight fractions. These fractions were subjected to HPLC-ESI-TOF-MS analysis (Varian Microsorb C18, 2.0 × 250 mm, 5–95% aqueous CH_3CN with 0.1% acetic acid, 0–30 min) to determine the presence of previously isolated metabolites. Data were acquired in negative scan mode, 200–1000 *m*/*z*, with an event loop duration of 0.12 s. The mass spectrometric chromatogram (Figure 1) of subfraction 2 (F9.2) revealed the presence of **1** as well as **2**, which was not isolated from the first extract. The chromatogram of subfraction 4 (F9.4) revealed the presence of **3** and **4**. Semipreparative reversed-phase HPLC (Phenomenex Onyx C18, 4.6 × 100 mm, 10–50% aqueous acetonitrile, 0.0–6.0 min) of F9.2 yielded 2.0 mg of **1** (4.43 min) and 0.8 mg of **2** (4.70 min).

Nhatrangin A (1): pale orange oil; $[\alpha]_D$ none detected (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 223 (3.70), 274 (3.30) nm; CD (EtOH) λ_{max} ($\Delta\varepsilon$) 274 (0.096) nm; IR (neat) λ_{max} 3273, 2928, 1717, 1576, 1457, 1408, 1281, 1057 cm^{-1}; ^1H and ^{13}C NMR see Table 1; HR-ESI-FT-MS (+) *m*/*z* 435.1984 $[M + Na]^+$ (calcd for $C_{21}H_{32}O_8Na$ 435.1995) and (−) *m*/*z* 411.2034 $[M - H]^-$ (calcd for $C_{21}H_{31}O_8$ 411.2025).

Nhatrangin B (2): clear oil; $[\alpha]_D$ − 8.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 208 (3.64), 284 (2.97) nm; CD (EtOH) λ_{max} ($\Delta\varepsilon$) 275 (0.320) nm; IR (neat) λ_{max} 3463, 2931,

1684, 1593, 1439, 1292, 1210, 1140 cm^{-1}; ^{1}H and ^{13}C NMR see Table 1; HR-ESI-TOF-MS (−) *m*/*z* 489.1156 and 491.1139 $[M - H]^-$ (calcd for $C_{21}H_{30}BrO_8$ 489.1130 and 491.1127).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank C. Pennington and C. A. Crot of the Mass Spectrometry Laboratory at UIC for acquiring low-resolution MS and FT-MS data. Samples of *Lyngbya majuscula* were collected as part of a research cooperation between Nhatrang Institute of Oceanography, Nhatrang, and Institute of Chemistry of the Vietnam Academy of Science and Technology (VAST), Hanoi. Extracts of these samples were kindly provided to the University of Illinois at Chicago under a cooperative agreement between VAST and UIC, as part of an International Cooperative Biodiversity Groups (ICBG) program, under NIH grant 2 UO1-TW-01015-06 administered by the Fogarty International Center through funds from NIH, NSF, and the USDA Foreign Agricultural Service. This research was also supported by NIH grant R01GM078556.

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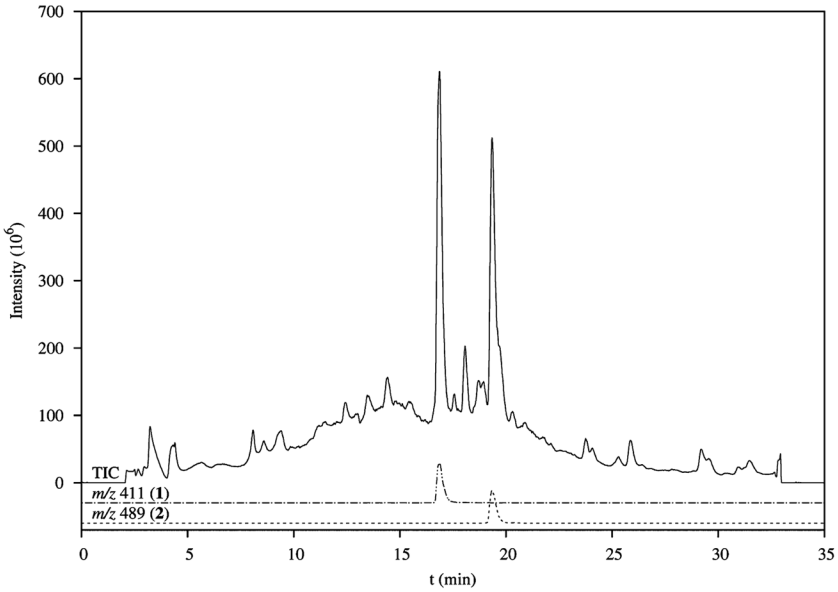

Figure 1.

HPLC-MS chromatogram of fraction 9.2. The total ion chromatogram (TIC) is depicted as a solid line with the extracted MS chromatograms for *m*/*z* 411 and 489, which are the $[M - H]^-$ ions for **1** and **2**, respectively. The baselines of the extracted chromatograms have been shifted downward for clarity.

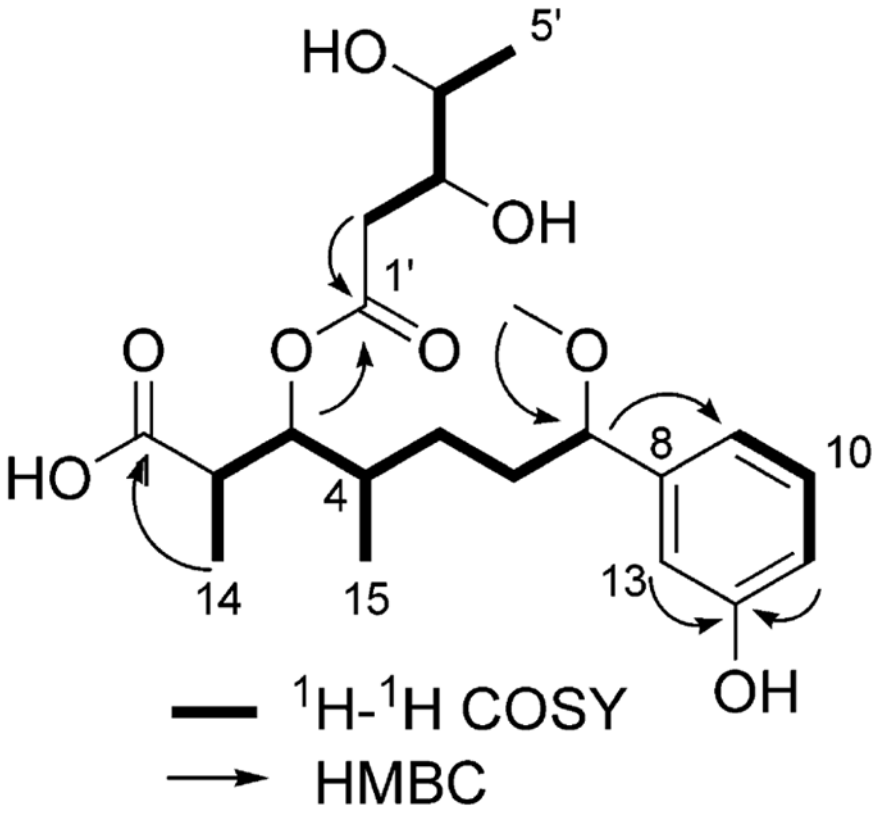

Figure 2.
Structural fragments determined by COSY data and key HMBC correlations of **1**.

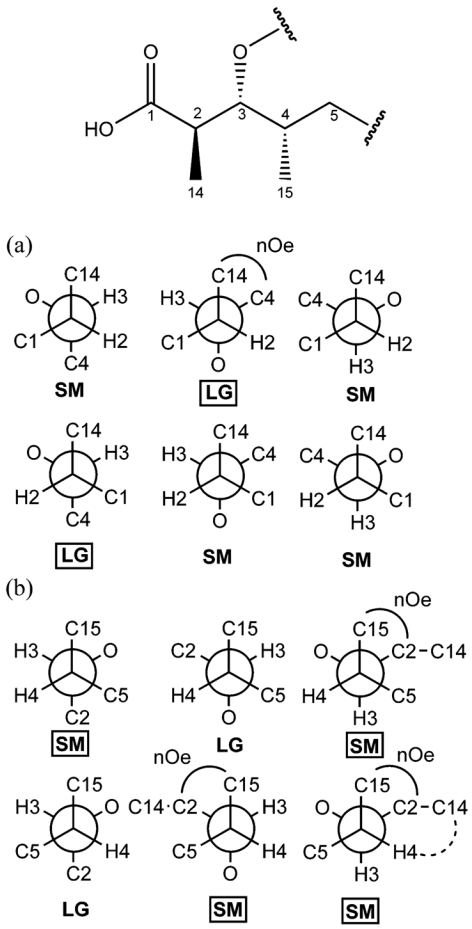

Figure 3.

Newman projections for (a) C-2/C-3 and (b) C-4/C-3. All possible relative conformations are shown: (a) (top) 2*R**,3*R** and (bottom) 2*S**, 3*R**; (b) (top) 3*S**, 4*S** and (bottom) 3*S**, 4*R**. Labels below projections denote predicted size of the $^{3}J_{H,H}$ coupling constant between the protons displayed. Predicted values highlighted by a box are consistent with observed values. Observed NOE correlations are presented as an (a) arched line, H-14/H-4, and (b) solid, H-2/H-15, and dashed line, H-14/H-4.

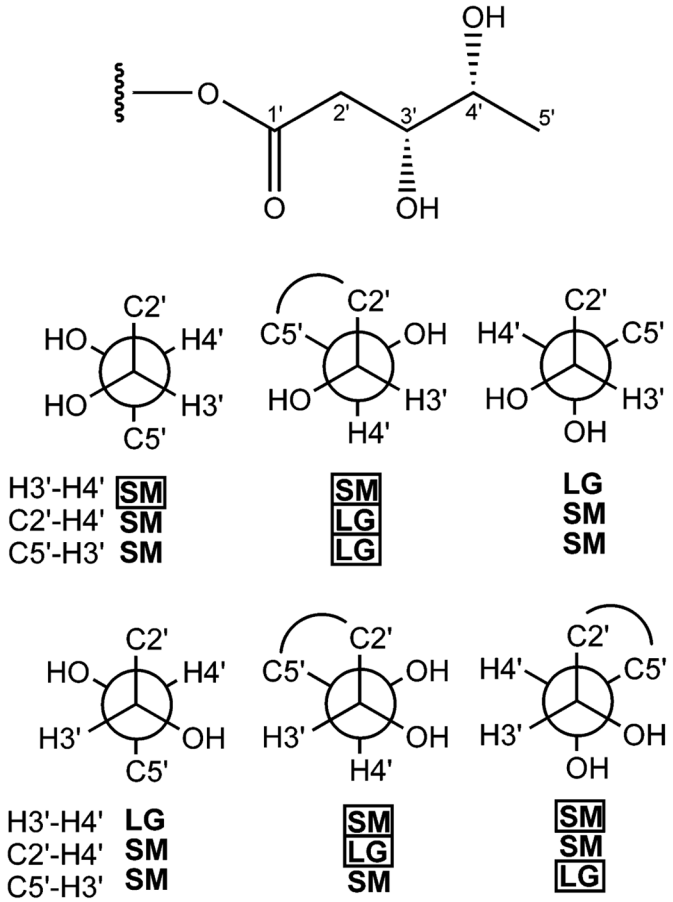

Figure 4.

Newman projections for C-4′/C-3′. All possible relative configurations are shown: (top) 3′*R**, 4′*R** and (bottom) 3′*S**, 4′*R**. Labels below projections denote predicted size of the $^{3}J_{H,H}$ and $^{3}J_{C,H}$ coupling constants for atoms displayed. Predicted values highlighted by a box are consistent with observed values. The observed NOE correlation is presented as a solid arch (H-5′/H-2′a).

J Nat Prod. Author manuscript; available in PMC 2011 April 23.

Table 1

NMR Data of Nhatrangins A and B (**1** and **2**) in DMSO-d_6

	1				2			
position	**δ_C, mult.[a]**	**δ_H[b]**	**m (*J* in Hz)**	**HMBC**	**δ_C, mult.[a]**	**δ_H[b]**	**m (*J* in Hz)**	**HMBC**
1	176.5,[c] C				176.8,[c] C			
2	40.4, CH	2.26	dq (8.3, 7.0)		48.7, CH	2.28	dq (7.7, 7.1)	1
3	78.0, CH	4.74	dd (8.3, 3.6)		78.7, CH	4.77	dd (7.7, 3.9)	
4	33.3, CH	1.67	qd (6.7, 3.6)		33.2, CH	1.77	m	
5	30.0, CH_2	1.23	m	4	30.1, CH_2	1.22	m	4
6	35.2, CH_2	1.60	m	5, 7, 8	34.1, CH_2	1.63	m	
						1.51	m	
7	83.3, CH	3.93	dd (7.4, 5.4)	5, 6, 9, 13, 7-OMe	81.7, CH	4.33	dd (8.0, 3.9)	8,7-OMe
7-OMe	56.0, CH_3	3.07	s	7	56.6, CH_3	3.12	s	7
8	144.0, C				142.1, C			
9	113.1, CH	6.65	m	7, 10	118.3, C			
10	129.2, CH	7.10	t (7.8)	8, 12	133.2, CH	7.31	d (8.6)	8, 12
11	116.9, CH	6.63	m		116.7, CH	6.63	dd (8.6, 2.9)	
12	157.5, C				157.5, C			
13	114.3, CH	6.67	m		114.3, CH	6.82	d (2.9)	9, 12
14	15.1, CH_3	0.83	d (7.0)	1, 2, 3	15.8, CH_3	0.87	d (7.1)	1,2,3
15	14.0, CH_3	0.74	d (6.7)	3, 4, 5	13.9, CH_3	0.77	d (6.7)	3,4
1′	171.1, C				171.1, C			
2′a		2.35	dd (14.3, 3.2)	1′, 3′		2.37	dd (14.6, 3.3)	1′,3′
2′b	38.3, CH_2	2.05	dd (14.3, 10.5)	1′, 3′	38.6, CH_2	2.09	dd (14.6, 10.5)	1′,3′
3′	70.6, CH	3.75	ddd (10.5, 4.2, 3.2)		70.7, CH	3.77	dd (10.5, 4.5, 3.3)	
4′	68.4, CH	3.48	qd (6.4, 4.2)	2′, 3′	68.5, CH	3.49	qd (6.3, 4.5)	3′
5′	18.0, CH_3	0.95	d (6.4)	3′	18.1, CH_3	0.96	d (6.3)	3′,4′

[a] DEPTQ experiment recorded at 226 MHz.

[b] Recorded at 900 MHz.

[c] Chemical shift determined from gHMBC experiment recorded at 900 MHz.