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Short Note

(5*S*)-5-[(4a*R*,8a*S*,9*E*,11*S*,13*R*,14*S*,16*R*,17*R*,19*S*)-11,19-Dihydroxy-8,10,13,16-tetramethyl-18-methylidene-3,4,5,6,8a,11,12,13,14,15,16,17,18,19,20,21-hexadecahydro-2*H*-14,17-epoxybenzo[2,3]cyclohexadeca[1,2-*b*]pyridine-7-yl]-3-methylfuran-2(5*H*)-one (12-Methylgymnodimine B)

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Academic Editor: Norbert Haider

Received: 25 February 2016; Accepted: 6 April 2016; Published: 14 April 2016

Abstract: A new member of the gymnodimine class of spiroimine toxins has been isolated from a laboratory culture strain of *Alexandrium ostenfeldii*. Extensive one-dimensional (1D) and two-dimensional (2D) NMR data analysis was used to elucidate its structure as 12-methylgymnodimine B.

Keywords: dinoflagellate; *Alexandrium ostenfeldii* (*peruvianum*); gymnodimine; spiroimine

1. Introduction

The threat from marine harmful algal blooms (HABs) to the world's oceans and to the seafood industry is increasing as the rate of toxic bloom detection is on the rise. In HAB toxin research, the dinoflagellate species *Alexandrium ostenfeldii* is an important member because it has repeatedly been shown to produce members of both the saxitoxin family and the spiroimine toxin groups, gymnodimines and spirolides [1–4]. HAB spiroimine toxins exert their toxicity primarily through the inhibition of nicotinic acetylcholine receptors [5]. Although still relatively uncommon, the occurrence of both spirolides and gymnodimines in the same organism is not surprising as they appear to share a closely linked biosynthetic pathway [1,6]. There have been 13 members of the spirolide class of toxins fully characterized and several more suggested based on mass spectral data [7–9]. In contrast, only five gymnodimines are reported, the most recent being gymnodimine D, recorded last year [1,10–13]. While analyzing the extracts of cultured *A. ostenfeldii* cells originating from a re-occurring bloom off the coast of North Carolina for the continued production of 12-methylgymnodimine (**2**) and 13-desmethylspirolide C, we detected the presence of a minor component at *m*/*z* 538 (**1**) which appeared to be a new gymnodimine congener. Detailed NMR, and high resolution mass spectral analyses allowed us to assign the structure of this new analog as 12-methylgymnodimine B (**1**) (Figure 1).

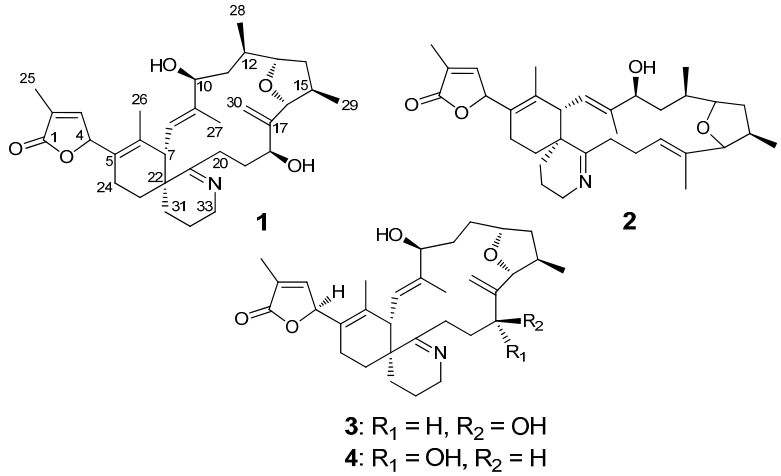

Figure 1. Structure of 12-methylgymnodimine B (**1**), 12-methylgymnodimine (**2**), gymnodimine B (**3**) and gymnodimine C (**4**).

2. Results

During the purification of 12-methylgymnodimine (**2**) and 13-desmethyl spirolide C from *A. peruvianum* [2], an additional congener (0.9 mg from 100 g wet weight cell pellet *vs.* 2.6 mg of (**2**)) of gymnodimine was tentatively identified by UV-LC-MS, displaying an M + H parent ion at m/z 538. This corresponds to the molecular formula of a gymnodimine analog containing an additional oxygen compared with 12-methylgymnodimine (**2**) [1] or, alternatively, the addition of an extra methyl to either gymnodimine B or C (**3** or **4**) [11,12]. The latter proposal was confirmed by the HR-ESIMS data (M + H = 538.3531; calculated = 538.3532; $\Delta = -0.2$ ppm) which accounted for a molecular formula of $C_{33}H_{47}NO_5$. Subsequent one-dimensional (1D) and two-dimensional (2D) NMR experiments further confirmed this as a new member of the gymnodimine class of spiroimines. The planar structure of this new compound was assembled using ^{1}H, ^{13}C, HSQC, COSY, TOCSY, and HMBC data (Table 1). Comparison with the known spectral data for **2** confirmed that it is a very closely related congener, also containing methylation at the C-12 position (Table 2). Analysis of the NMR data allowed us to determine that the region differentiating this new congener from **2** corresponded to additional oxygenation at C-18 in conjunction with double bond isomerization, resulting in an exomethylene group (C-17 and C-30) (Table 2). Specifically, the resonances at position 18 in 12-methylgymnodimine (**2**) δ_H 5.40 δ_X 126.8) now appeared much further upfield δ_H 4.35, δ_C 70.9) in the new compound. Additionally, the resonance for C-17 was shifted downfield from 134.5 ppm in **2** to 156.3 ppm in **1** and the C-30 methyl of 12-methylgymnodimine (**2**) is now present as an exomethylene (δ_H 5.55, 5.11; δ_X 112.5). This oxygenation and isomerization is also observed in gymnodimines B and C (**3** and **4**) which differ from each other only in the configuration of the C-18 stereocenter.

Extensive ROESY analysis allowed us to determine the relative configuration of all stereocenters in 12-methylgymnodimine B with the exception of C-4 (Table 1, Figure 2). Based on biosynthetic considerations, it is likely that this center retains the same configuration as was determined for gymnodimine by X-ray analysis [14]. However, sample limitations precluded us from definitively assigning this center. Importantly, ROESY correlations between H-12 and H-10 and H-15 allowed us to confirm that the relative configuration of 12-methylgymnodimine B (**1**) corresponds with that established for 12-methylgymnodimine (**2**) at position 12. Additional ROESY correlations from H-18 to H-12, H-14a, and H-15 correspond to the configuration assigned to gymnodimine B (**3**) at position 18 (Figure 2) [1,11,12]. While the optical rotations of gymnodimines B and C were not reported, the optical rotation for (**1**) was determined to be $[\alpha]_D^{26} = -30$ (c. 0.2, MeOH) which is similar in value and sign to that reported for (**2**) [1].

Table 1. NMR data for 12-methylgymnodimine B in pyridine-d_5.

Position	δ_H (mult, J in Hz)	δ_C (mult)	COSY	HMBC	ROESY
1		175.3 C(q)			
2		130.2 C(q)			
3	7.15 (br s)	148.8 CH	4,25	1,2,4,25	25
4	5.95 (br s)	81.3 CH	3,25	2, 3,5, 6, 24	26
5		125.0 C(q)			
6		134.1 C(q)			
7	3.66 (m)	46.1 CH	8	6,8,9,22	20a,26,27
8	5.34 (d, 11.0)	125.3CH	7	7,10, 27,22	10, 26, 27, 31a,32a
9		142.9 C(q)			
10	4.52 (d, 10.5)	77.2 CH	11a,b	8,11, 27	8, 11b, 12, 28
11a	2.81 (t, 12.0)	39.9 CH_2	10, 11b, 12	9, 10,12, 13, 26	27
11b	1.47 (m)		10, 11a, 12	9, 10,12, 26	10, 13
12	1.40 (m)	35.7 CH	11a,b, 28	11,13, 28	10, 15, 18
13	3.71 (m)	83.6 CH	12, 14a,b	11,15,16, 28	11b,14b, 16, 28
14a	2.15 (m)		13,15	15,16, 29	15,18
14b	1.58 (m)	38.4 CH_2	13,15	15,16, 29	13, 16, 28, 29
15	2.94 (m)	36.8 CH	14 a,b, 16, 29	14,16,17, 29	12, 14a,18,19a
16	4.08 (d, 8.5)	92.1 CH	15	15,17, 18, 29, 30	13, 14b, 29
17		156.3 C(q)			
18	4.35 (d, 10.0)	70.9 CH	19a,b	16, 17, 19,20,30	12,14a,15,19a
19a	2.58 (m)		18, 19b, 20a,b	18, 20,21	15, 18
19b	2.04 (m)	34.6 CH_2	18, 19a, 20a,b	18, 20,21	
20a	2.98 (m)	31.1 CH_2	19a,b, 20b	18, 19,21	7
20b	2.37 (m)		19a,b, 20a	18, 19,21	
21		174.3 C(q)			
22		41.9 C(q)			
23a	1.71 (m)	32.9 CH_2	23b, 24 a,b	5,7,24,31	
23b	1.27 (m)		23a	5,7,21,22,24,31	
24a	2.04 (m)	19.8 CH_2	23a, 24b		
24b	1.59 (m)		23a, 24a	5,6,22	
25	2.00 (s)	11.0 CH_3	3,4	1, 2, 3, 4	3
26	1.66 (s)	17.0 CH_3		6, 7, 8	4,7
27	2.14 (s)	12.0 CH_3		7, 8, 9, 10	7,11a
28	0.96 (d; 6.5)	17.3 CH_3	12	11,12,13	10,13,14b
29	1.06 (d; 6.5)	17.6 CH_3	15	14,15,16	14b, 16
30a	5.55 (m)	112.5 CH_2	30b	16,17,18	
30b	5.11 (m)		30a	16,17,18	
31a	1.83 (m)	26.4 CH_2	31b, 32a,b	7, 21, 22,23	8
31b	1.35 (m)		31a, 32a,b	7, 21, 22,23	
32a	1.58 (m)	21.3 CH_2	31a, 32b, 33a,b	22,33	
32b	1.44 (m)		31a, 32a, 33a,b	22,33	
33a	3.64 (m)	49.5 CH_2	32a,b, 33b	21, 31	
33b	3.45 (m)		32a,b, 33a	21, 31	

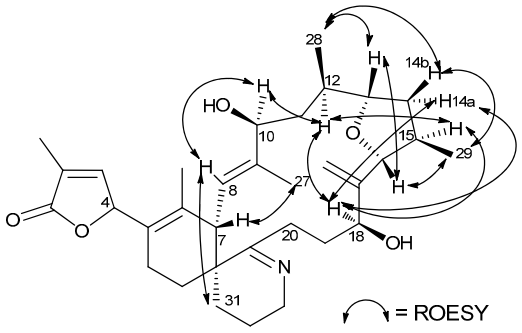

Figure 2. Relative configuration **1** shown with key ROESY correlations.

Table 2. Proton and carbon assignments in pyridine-d_5 for 12-methylgymnodimine B (**1**) and 12-methylgymnodimine (**2**).

Position	12-Methyl Gym B δ_H	12-Methyl Gym B δ_C	12-Methyl Gym δ_H	12-Methyl Gym δ_C
1		175.3		175.4
2		130.2		130.3
3	7.15	148.8	7.13	148.7
4	5.95	81.3	5.95	81.4
5		125.0		125.3
6		134.1		134.1
7	3.66	46.1	3.63	46.6
8	5.34	125.3	5.34	126.2
9		142.9		141.7
10	4.52	77.2	4.58	77.3
11a	2.81	39.9	2.92	40.3
11b	1.47		1.61	
12	1.40	35.7	1.52	37.1
13	3.71	83.6	3.84	83.7
14a	2.15	38.4	1.78	36.0
14b	1.58		1.52	
15	2.94	36.8	2.14	38.6
16	4.08	92.1	4.12	89.8
17		156.3		134.5
18	4.35	70.9	5.40	126.8
19a	2.58	34.6	2.77	22.8
19b	2.04		2.11	
20a	2.98	31.1	2.65	31.4
20b	2.37		2.48	
21		174.3		171.8
22		41.9		41.8
23a	1.71	32.9	1.71	34.4
23b	1.27		1.32	
24a	2.04	19.8	2.06	20.0
24b	1.59		1.59	
25	2.00	11.0	2.00	11.1
26	1.66	17.0	1.69	17.0
27	2.14	12.0	2.10	11.7
28	0.96	17.3	0.92	17.0
29	1.06	17.6	1.06	20.6
30a	5.55	112.5	1.54	14.9
30b	5.11			
31a	1.83	26.4	1.89	27.4
31b	1.35		1.32	
32a	1.58	21.3	1.54	21.4
32b	1.44		1.47	
33a	3.64	49.5	3.75	50.6
33b	3.45		3.40	

3. Experimental Section

A clonal culture of *Alexandrium ostenfeldii* AP0411 (previously called *A. peruvianum*) was grown and harvested according to our previously reported protocol [2,6].

The *A. peruvianum* cells were filtered onto glass microfiber (VWR, 691-, Radnor, PA, USA) paper and the media was extracted with XAD resin to collect the remaining organics. The organics were then rinsed from the resins with 100% MeOH and 100% acetone washes. The cells were extracted with 2× 400 mL 80% MeOH and 1× 400 mL 100% MeOH. All organic extracts were combined and dried to HP20 resins for desalting and primary fractionation. The extract-bound resins were washed with 100% water and the remaining de-salted extract was eluted with increasing concentrations of acetone. The dried desalted extract was then fractionated on a 20 g C_{18} SPE cartridge with a stepwise elution of MeCN and H_2O (0.1% TFA). LC-MS monitoring indicated that the spiroimines eluted in the 40% and 60% MeCN fractions. The spiroimine-containing fractions were then purified by reversed phase HPLC

(semi-prep Gemini 5 µm C_{18} 110A 250 × 10 mm column) under isocratic conditions (37% MeCN:63% H_2O (0.05% formic acid)) 2 mL/min.

Optical rotations were measured with a Rudolph Research Analytical Autopol® III automatic polarimeter (Hackettstown, NJ, USA) in methanol. NMR analyses were performed using a Bruker®Avance 1 500 MHz system (Billerica, MA, USA) run by TopSpin version 2.0. HPLC isolation was performed using a Waters Breeze HPLC system (Waters, Manchester, UK) with a Waters dual wavelength detector. LC-MS monitoring was performed on a Waters Micromass ZQ mass spectrometer tandem to an Agilent 1600 high performance liquid chromatography (HPLC) instrument (Santa Clara, CA, USA). All solvents were of HPLC grade and were used without further purification. The HRMS spectrum was obtained on Waters UPLC i-Class system couples to a Waters Xevo-G2XS QToF-MS mass spectrometer. The system was operated in electrospray positive mode (ESI^+) with the capillary voltage set at 2.5 kV, source and desolvation temperatures at 100 °C and 550 °C, respectively, and desolvation gas flow at 800 L/h. The MS-MS data was obtained with the same instrument under the same conditions with a set mass of 538.4 and a collision energy of 35 eV. All solvents were of mass spectrometry grade and were used without further purification.

4. Discussion

This new congener of the gymnodimine class of spiroimine toxins further expands the structural variety of this group and hence needs to be incorporated into any shellfish monitoring program. In addition to the fast acting toxicity profile for spiroimine toxins, recent literature indicates potential therapeutic value for these compounds in combating neurodegenerative diseases such as Alzheimer's disease, including the closely related gymnodimine C [15,16] as well as the spirolides [17,18]. Increasing the library of structurally diverse spiroimines available for this research is important for enhancing drug discovery efforts.

Supplementary Materials: The molfile, and 1D and 2D NMR spectra, and high resolution MS/MS fragmentation data can be found at: http://www.mdpi.com/1422-8599/2016/2/M896.

Acknowledgments: The authors would like to thank Ryan M. Van Wagoner for isolation of the initial gymnodimine material and would also like to acknowledge funding from the NC MARBIONC program and from the UNCW Carl B. Brown Trust to J.L.C.W.

Author Contributions: W. Strangman performed the structure elucidation. M. Anttila assisted with structure determination. C. Tomas cultured the *A. ostenfeldii* biomass. J. Wright contributed to the project conceptualization and experimental design. All authors contributed to the writing of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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