New Analogue of Gymnodimine from a *Gymnodinium* Species

Christopher O. Miles,*,† Alistair L. Wilkins,‡ David J. Stirling,§ and A. Lincoln MacKenzie‖

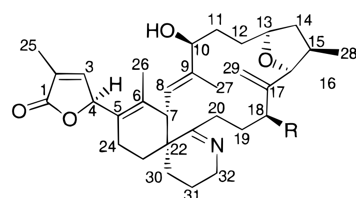
New Zealand Pastoral Agriculture Research Institute Ltd., Ruakura Agricultural Research Centre, Private Bag 3123, Hamilton, New Zealand; Chemistry Department, The University of Waikato, Private Bag 3105, Hamilton, New Zealand; Institute of Environmental Science and Research Ltd., Kenepuru Science Centre, P.O. Box 50-348, Porirua, New Zealand; and Cawthron Institute, 98 Halifax Street, Private Bag 2, Nelson, New Zealand

A spiroimine, gymnodimine B (**1**), was isolated from cells recovered by filtration from cultures of a marine dinoflagellate, *Gymnodinium* sp. Its structure was identified by one- and two-dimensional NMR spectroscopy and mass spectrometry. Gymnodimine B is similar in structure to gymnodimine (**2**) but contains an exocyclic methylene at C-17 and an allylic hydroxyl group at C-18.

Keywords: *Gymnodimine; Gymnodinium; shellfish toxin; spiroimine*

INTRODUCTION

Samples of New Zealand shellfish occasionally give positive responses in the standard mouse bioassay for neurotoxic shellfish poisoning (NSP) but do not contain NSP toxins. These false positives have been attributed to the presence of gymnodimine (**2**) (Figure 1), a novel toxic spiroimine isolated from toxic shellfish (Seki et al., 1995; Stewart et al., 1997; Stewart, 1997) associated with blooms of a marine dinoflagellate *Gymnodinium* sp. (MacKenzie et al., 1996). [Note added in proof: The name *Gmnodinium selliforme* has recently been proposed for this organism (Haywood et al., 2000).] Although **2** is structurally related to the spiroimine shellfish toxins of the spirolide and pinnatoxin groups (Seki et al., 1995; Stewart et al., 1997), no close analogues of **2** are known. Here we report the isolation of gymnodimine B (**1**) from the cells of cultures of the *Gymnodinium* sp. and determination of its structure by NMR spectroscopy and mass spectrometry. A preliminary account of this work has appeared elsewhere (Miles et al., 1999).



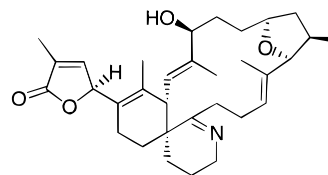


Figure 1. Structures of gymnodimine B (**1**) showing the probable configuration at C-18 and of gymnodimine (**2**). Also shown is 18-deoxygymnodimine B (**3**), a hypothetical isomer of **2** from which **1** may have originated.

EXPERIMENTAL PROCEDURES

General. Preparative and analytical HPLC was performed on a C-18 RadPak cartridge (Waters, 4 μm, 8 × 100 mm), with acetonitrile/water (55:45, 2.5 mL min^{-1}) containing methanol (0.8% v/v) and triethylamine (0.005% v/v) as eluent. Eluting compounds were detected by UV absorbance at 215 nm with a diode array detector (Hewlett-Packard 1040M Series II) and a UV absorbance detector (Perkin-Elmer LC 75).

One- and two-dimensional NMR spectra were determined at 400.13 MHz from $CDCl_3$ solutions at 30 °C, using a Bruker DRX 400 spectrometer fitted with an inverse 5-mm dual probehead. HSQC and HMBC NMR spectra were acquired with gradient selection. Molecular modeling was performed with Chem3D Pro 4.0 (CambridgeSoft Corp., Cambridge, MA) using the supplied MM2* parameters and minimization routines.

Mass spectra were obtained with a VG70 S double-focusing magnetic sector mass spectrometer (VG-Micromass, Manchester, U.K.). Electron impact spectra were obtained from the standard EI source at 1000 and 5000 resolving powers (RP) at 8 kV source potential, with 2 mA current and 70 eV electron energy. The sample was applied in methanol to a quartz glass solids probe desorption tube and heated to 400 °C. A scanning rate of 2 s/decade was used at 5000 RP, and perfluorokerosene calibrant was run concurrently to measure the accurate mass to four decimal places.

Algal Culture. Batch cultures of *Gymnodinium* sp. (CAWD79) (Ponikla, 1999; MacKenzie et al., 1996) were grown in polycarbonate carboys (20 L) containing 50% GP+Se amended seawater medium (Loeblich and Smith, 1968) under a 14/10 h light/dark cycle at ~100 μeinsteins m^{-2} s^{-1} and 18−20 °C. Cells from 20-day-old cultures (297 L, 7.53 × 10^3 cells mL^{-1}) were harvested by filtration under vacuum (−15 kPa) through glass fiber filters (130 mm diameter, Sartorius 13430-130-G). The filtrate was then passed through a column (50 × 800 mm) containing HP-20 resin (Supelco, Bellefonte, PA) at a flow rate of ~300 mL min^{-1}.

Cell Extract. The filter disks containing the algal cells were extracted with $MeOH/H_2O/HOAc$ (80:19:1, 3 × 300 mL) and partitioned according to the method of Stewart et al. (1997)

* Author to whom correspondence should be addressed (e-mail milesc@agresearch.cri.nz; telephone +64-7-8385041; fax +64-7-8385189).

† New Zealand Pastoral Agriculture Research Institute Ltd.

‡ The University of Waikato.

§ Institute of Environmental Science and Research Ltd.

‖ Cawthron Institute.

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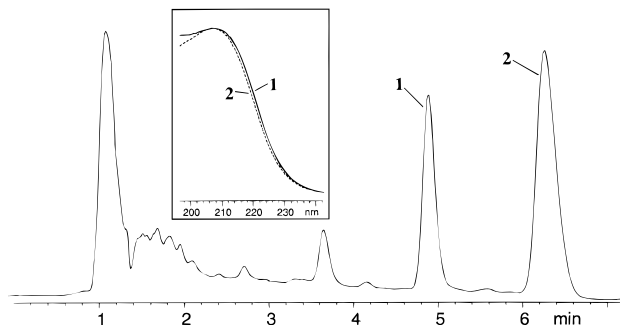

Figure 2. Semipreparative HPLC chromatogram of the fractionated extract from cells of the *Gymnodinium* culture. The inset shows normalized UV absorbance spectra for gymnodimine (**2**) and gymnodimine B (**1**) obtained from the chromatogram by means of a diode array detector.

with minor modifications. Briefly, after evaporation of the MeOH in vacuo, the extract was adjusted to pH 8.5 with NH_4OH and partitioned against CH_2Cl_2. The organic layer was evaporated to dryness and partitioned between diethyl ether and H_2O/HOAc (199:1). The aqueous layer was adjusted to pH 8.5 with NH_4OH and extracted with CH_2Cl_2, and the extract was evaporated to dryness to give crude gymnodimine (1.7 mg), which appeared to contain polar gymnodimine analogues (see Figure 2). HPLC analysis of this fraction indicated, with the assumption that the analogues possess identical extinction coefficients at 215 nm, that gymnodimine (**2**) and the major analogue (**1**) were present in a ratio of 2.6:1.

This material was fractionated by preparative HPLC. Fractions containing compounds with gymnodimine-like UV absorbance spectra (Figure 2) were evaporated to dryness in vacuo (<35 °C), dried under high vacuum for 4−6 h, then dissolved in a few drops of methanol, and stored in the dark at −20 °C under nitrogen until required. Immediately prior to NMR analysis the solvent was evaporated with a stream of dry nitrogen and the sample subjected to high vacuum for 20 min. The residue was transferred to the NMR tube with $CDCl_3$, and the tube was flushed with nitrogen and capped. Two compounds were isolated and identified from this material: gymnodimine (**2**) (0.3 mg) (see below for analytical data) and a novel analogue, which we name gymnodimine B (**1**) (<0.2 mg) (Figure 1).

Gymnodimine B (**1**): EI-MS *m/z* 523.3303 (34%, M^+; $C_{32}H_{45}NO_5$ requires *m/z* 523.3298), 505 (43), 503 (64), 487 (48), 485 (100). For UV absorbance and HPLC retention data, see Figure 2. For ^1H and ^{13}C NMR assignments, see Tables 1 and 2.

Culture Filtrate. A slurry of the HP-20 (through which the culture filtrate had been passed, above) in H_2O was placed in a 15 cm diameter column. The column was washed with acetone/H_2O (3:7, 1 L, then 2:3, 1 L) and eluted with acetone/H_2O/HOAc (40:59:1, 1 L). The eluate was adjusted to pH 8.5 with NH_4OH and concentrated in vacuo, and the residue was taken up in MeOH (2 mL). This material was passed through an Extract-Clean amino SPE column (500 mg, Alltech, Auckland, NZ), and the column was washed with MeOH (2 × 5 mL). The eluted fractions were combined (gymnodimine is not retained on such a column), and the solvent was removed in vacuo. The residue was dissolved in H_2O/NH_4OH (3 mL, pH 8.5) and extracted with CH_2Cl_2 (5 × 2 mL), and the CH_2Cl_2 fraction was evaporated to dryness to give crude gymnodimine (8.2 mg). Gymnodimine (**2**) (1.8 mg) was obtained as a colorless solid by HPLC purification of this material in the manner described above. No gymnodimine B was detected during analytical or preparative HPLC of this fraction.

Gymnodimine (2). For UV absorbance and HPLC retention data, see Figure 2. The ^1H NMR spectrum was essentially identical to that reported for gymnodimine (Stewart, 1997) (for assignments, see Table 1). The following ^{13}C signal assignments (± 0.4 ppm) were indirectly determined for **2** in HMBC and HSQC NMR experiments: 10.7 (C-25), 11.1 (C-27), 14.6 (C-29), 17.0 (C-26), 19.3 (C-24), 20.2 (C-28), 20.7 (C-31), 22.0 (C-19), 26.4 (C-30), 31.0 (C-20), 31.7 (C-11), 32.5 (C-12), 33.6 (C-23), 37.5 (C-14), 37.8 (C-15), 46.1 (C-7), 49.6 (C-32), 77.8 (C-13), 79.5 (C-10), 80.3 (C-4), 89.7 (C-16), 124.5 (C-18), 124.8 (C-5), 127.0 (C-8), 130.3 (C-2), 132.9 (C-6), 134.4 (C-17), 139.7 (C-9), 147.1 (C-3), 174.8 (C-1). These assignments correspond closely to those reported by Stewart (1997) and are similar to those determined for **2** in C_5D_5N (Seki et al., 1995).

Table 1. ^1H NMR Chemical Shifts (δ) for Gymnodimine B (1) and Gymnodimine (2) in $CDCl_3$

	1	2[a,b]
H-3	6.91 (br m)	6.90 (br m)
H-4	5.84 (br s)	5.82 (br s)
H-7	3.63 (br d)	3.63 (br d)
H-8	5.28 (br d, J = 10.7 Hz)	5.28 (d, J = 11.0 Hz)
H-10	3.94 (dd, J = 11.6, 2.8 Hz)	4.00 (br d)
H-11	2.08, 1.48	2.04, 1.67
H-12	1.40,[c] 1.15	1.42, 1.16
H-13	4.09 (br t)	4.08 (br m)
H-14	1.77−1.82	1.60, 1.49
H-15	2.71 (m)	2.18 (m)
H-16	3.85 (d, J = 10.5 Hz)	3.97 (br s)
H-18	4.11 (d, J = 10.1 Hz)	5.06 (br s)
H-19	1.49, 2.40[e]	2.03, 2.47
H-20	2.23,[e] 2.64	2.48, 2.48
H-23	1.54,[c] 1.77	1.49, 1.77
H-24	1.54,[c] 2.06	1.55, 2.07
H-25	1.96 (br t, J = 1.5 Hz)	1.96 (br t, J = 1.7 Hz)
H-26	1.71 (br s)	1.69 (br s)
H-27	1.91 (br s)	1.81 (br s)
H-28	0.96 (d, J = 6.3 Hz)	1.09 (d, J = 7.1 Hz)
H-29	5.32 (br d, J = 1.4 Hz, H-29_E),[d] 5.19 (br s, H-29_Z)	1.52 (br s)
H-30	1.54,[c] 1.95	1.51, 1.94
H-31	1.54,[c] 1.54[c]	1.51−1.57
H-32	3.57, 3.40	3.58, 3.39

[a] Assignments derived from a combination of ^1H, COSY, HMBC, and HSQC NMR data. [b] Chemical shifts correspond closely to those determined by Stewart (1997). [c] Tentative assignments derived from analyses of partly overlapping COSY and/or HOHAHA correlation peaks. [d] Determined with resolution enhancement (LB = −1.5, GB = 0.33). [e] Assignments are interchangeable.

Table 2. HMBC NMR Correlations Observed for Gymnodimine B (1) and Gymnodimine (2) in $CDCl_3$

^1H signal (δ)	correlated ^{13}C signals[a] (δ)
	Gymnodimine B (**1**)
1.96 (H-25)	174.7 (C-1), 130.3 (C-2), 147.1 (C-3)
1.71 (H-26)	125.2 (C-5), 132.8 (C-6)
1.91 (H-27)	125.9 (C-8), 140.4 (C-9), 80.0 (C-10)
0.96 (H-28)	34.8 (C-14), 41.1 (C-15), 90.9 (C-16)
5.19 (H-29_Z)	90.9 (C-16), 81.7 (C-18)
	Gymnodimine (**2**)
1.96 (H-25)	174.8 (C-1), 130.3 (C-2), 147.1 (C-3)
1.69 (H-26)	124.8 (C-5), 132.9 (C-6), 46.1 (C-7)
1.81 (H-27)	127.0 (C-8), 139.7 (C-9), 79.5 (C-10)
1.09 (H-28)	37.4 (C-14 and C-15), 89.7 (C-16)
1.52 (H-29)	89.7 (C-16), 134.4 (C-17), 124.5 (C-18)

[a] Chemical shift resolution ±0.4 ppm.

RESULTS AND DISCUSSION

As part of a study to evaluate the toxicity of gymnodimine (**2**), cells were recovered by filtration from a dinoflagellate culture, and residual **2** in the filtrate was adsorbed onto HP-20. The isolate used for the production of **2** was *Gymnodinium* sp. nov. obtained from Foveaux Strait, New Zealand, in 1994 and is associated with gymnodimine contamination of New Zealand shellfish (MacKenzie et al., 1996). A formal description of this novel species is currently in preparation, but the isolate carries the Cawthron Institute Micro-algae Culture Collection designation CAWD79 (Ponikla, 1999).

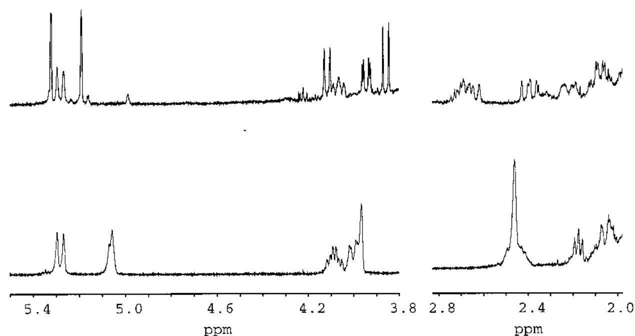

Figure 3. Selected regions of the ^{1}H NMR spectra of gymnodimine B (**1**) (top) and gymnodimine (**2**) (bottom), showing characteristic spectral differences between the two compounds. The pair of peaks at 4.99 and 5.16 ppm in the ^{1}H NMR spectrum of **1** is consistent with the presence of a second, unidentified, gymnodimine analogue containing an exocyclic methylene at C-17.

During the purification of gymnodimine-containing material extracted from the cells of the culture, an early-eluting peak was observed in the reverse-phase HPLC chromatogram that exhibited a UV absorbance spectrum very similar to that of **2** (Figure 2), consistent with the presence of a butenolide moiety. The compound responsible was isolated by semipreparative HPLC and examined by mass spectrometry, which revealed an apparent molecular ion consistent with the addition of an oxygen atom to **2**. Although constrained by the small amount of material (≤200 μg) and its partial decomposition during NMR data acquisition, detailed analysis of ^{1}H, NOE difference, COSY, HOHAHA, and HMBC NMR spectral data identified the compound as gymnodimine B (**1**) (Figure 1).

Much of the ^{1}H NMR spectrum of **1** was similar of that of **2**. Correlations observed in the COSY and HOHAHA spectra of **1** identified the majority of the resonances associated with the H-25/H-3/H-4, H-26/H-7/H-8/H-27/H-10, H-11/H-12/H-13/H-14/H-15/H-16/H-28, H-18/H-19/H-20, and H-30/H-31/H-32 spin systems. The chemical shifts of the H-3, H-4, H-7, H-8, H-10, H-12, H-13, H-23, H-24, H-25, H-26, H-30, H-31, and H-32 resonances of gymnodimine B, and their multiplicities, corresponded closely to those we (Table 1) and Stewart (1997) determined for **2**. There were, however, marked differences in the chemical shifts, and in some cases signal multiplicities, of the H-11, H-14, H-15, H-16, H-18, H-19, H-20, H-27, H-28, and H-29 resonances of **1**, compared to those of **2** (Table 1). The most noticeable differences were the replacement of the three-proton olefinic methyl group (H-29, 1.52 ppm) of **2** by two one-proton signals (5.19 and 5.32 ppm) attributable to the presence of an olefinic methylene group, the appearance of H-18 (4.11 ppm) and H-16 (3.85 ppm) as well-defined doublet signals (J = 10.1 and 10.5 Hz, respectively), and the increased complexity of the region at 2.0−2.8 ppm (Figure 3). These results indicated the presence in **1** of intact butenolide (accounting for the essentially identical UV absorbance spectra of **1** and **2**) and spiroimine ring systems and that **1** differed from **2** only in the macrocyclic ring.

In gymnodimine (**2**), H-18 (5.06 ppm) arises from an olefinic proton, whereas in gymnodimine B (**1**) this signal (4.11 ppm) is attributable to the presence of a secondary hydroxyl group. The coupling constant (J = 10.5 Hz) of the H-16 signal of **1** is indicative of a *trans-anti* relationship between H-16 and H-15. In **2**, notwithstanding the broad singlet character of its H-16 signal, X-ray crystallographic analysis of a derivative revealed a *trans* relationship between H-16 and H-15 (Stewart et al., 1997). The differences in the H-15−H-16 coupling constants and the H-18 and H-29 chemical shifts of **1** and **2** are consistent with the presence in **1** of an 18-hydroxy group and a 17(29)-exocylic double bond. Because of these differences, the adjacent furanyl

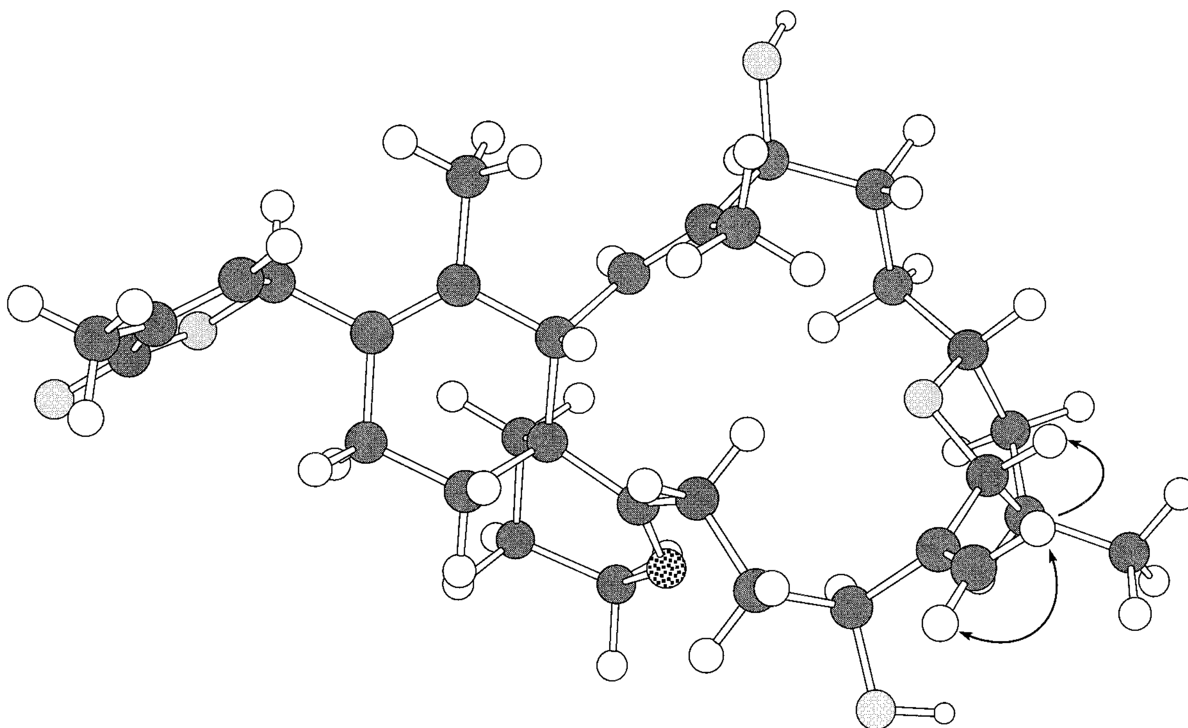

Figure 4. Preferred conformation of gymnodimine B (**1**) as predicted by molecular modeling (MM2*), with observed NOE enhancements shown.

ring system of **1** adopts a conformation different from that of **2** (Figure 4).

Resolution enhancement of the ^{1}H NMR spectrum of **1**, in combination with a second COSY spectrum acquired under conditions which favored the detection of long-range couplings, showed that one of the H-29 methylene protons was more extensively coupled than the other. Thus, the COSY spectrum showed that H-29_E (5.32 ppm, ^2J = 1.4 Hz) coupled only with H-29_Z (5.19 ppm), whereas the latter proton was also ^4J coupled to H-16 and H-18. Strong mutual enhancements were observed between H-29_E and H-29_Z (17.4 and 17.8%, respectively) when these protons were irradiated in NOE difference experiments. Irradiation of H-29_Z (but not of H-29_E) also enhanced H-16 (6.8%) (Figure 4).

Correlations observed in the HMBC spectrum of **1** for the methyl group protons and the H-29_Z methylene proton are presented in Table 2. Both H-29_Z and H-28 exhibited correlations to the bridgehead furanyl C-16 signal at 90.9 ppm. The HMBC correlations exhibited by H-25, H-26, and H-27 of **1** corresponded closely to those of **2** (Table 2). Assignments of the ^{13}C signals of **2** were determined indirectly via two-dimensional HSQC and HMBC spectra and are in agreement with those reported in a recent Ph.D. thesis (Stewart, 1997).

A more detailed NMR examination of **1** was prevented by its partial degradation during data acquisition. The principal degradation product appeared to retain the H-29_E and H-29_Z methylene protons but exhibited upfield shifts for the correlated (COSY spectrum) H-7, H-8, and H-27 signals (to 3.43, 5.10, and 1.50 ppm, respectively).

Although the foregoing spectral data demonstrated the presence in **1** of an 18-hydroxyl group and a 17(29)-double bond, it did not define the stereochemistry at C-18. Stereochemical information leading to a tentative C-18 configuration was elicited from the COSY spectrum of **1**, which showed that H-18 (4.11 ppm) was coupled to H-19a (1.49 ppm), but not to H-19b (2.40 ppm), thereby accounting for the appearance of H-18 (4.11 ppm) as a doublet (rather than a doublet of doublets). The H-19b resonance of **1** did not exhibit COSY correlations to H-18 or to H-20a (2.23 ppm). The coupling constants of H-19b (2.40, J = 14.2 and 9.3 Hz) are attributable to a geminal ^2J coupling with H-19a along with a *trans*-diaxial ^3J coupling to H-20b (2.64 ppm). These observations are consistent with the conclusion that H-19a (1.49 ppm) exhibits a dihedral angle approaching 90° with H-20b (2.64 ppm); H-20a (2.23 ppm) exhibits a dihedral angle approaching 90° with H-19b (2.40 ppm); and H-18 (4.11 ppm) exhibits a dihedral angle approaching 90° with H-19b (2.40 ppm). The preceding NMR coupling constant analysis is, however, equally consistent with the reversal of the H-19b and H-20a assignments. Molecular modeling studies, taking into consideration both the observed NOE enhancements and the coupling constants for H-15, H-16, H-18, H-19, and H-20, were consistent with an 18β-hydroxyl with the conformation shown in Figure 4. Nevertheless, because of the limited NOE data available, the possibility that gymnodimine B possesses an 18α-hydroxyl cannot be ruled out.

Gymnodimine B contains an exocyclic methylene δ to the imine carbon, a feature it shares with the pinnatoxins and spirolides but not with gymnodimine (**2**). It appears unlikely that **1** was produced artifactually from **2** during extraction and isolation, because this would require both isomerization and oxidation. Presumably, then, gymnodimine B (**1**) originates from a precursor such as 18-deoxygymnodimine B (**3**) (Figure 1), either through biologically mediated oxidation during biosynthesis or through abiotic oxidation during extraction and isolation. If compounds such as **3** were produced by the *Gymnodinium* sp., then these could also have a role in the biogenesis of gymnodimine (**2**).

ABBREVIATIONS USED

NSP, neurotoxic shellfish poisoning; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single-quantum correlation; HMBC, heteronuclear multiple-bond correlation; COSY, correlation spectroscopy; HOHAHA, homonuclear Hartmann−Hahn; RP, resolving powers.

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Supporting Information Available: HMBC and COSY NMR spectra of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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