Characterization of a Dispiroketal Spirolide Subclass from *Alexandrium ostenfeldii*

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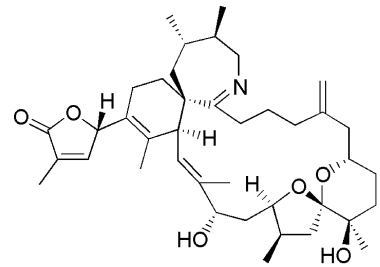
A new subclass of spirolide marine toxins, represented by spirolides H (**1**) and I (**2**), were isolated from the marine dinoflagellate *Alexandrium ostenfeldii*. Spirolides H and I are structurally distinct from other spirolides in that they contain a 5:6 dispiroketal ring system rather than the trispiroketal ring system characteristic of previously isolated spirolides. The structures were assigned using a combination of spectrometric and spectroscopic techniques. Previously isolated spirolides containing a cyclic imine moiety showed toxicity in the mouse bioassay. Spirolide H contains this cyclic imine moiety but does not show toxicity in the mouse assay, suggesting that the presence of the cyclic imine moiety is not the only structural requirement for toxicity.

Spirolides are a class of macrocycles structurally characterized by a spiro-linked cyclic iminium or keto amine functionality and a polycyclic ether moiety.[1−5] Toxicity in the mouse bioassay is the only presently known biological activity of spirolides. Intraperitoneal injection of spirolides containing a cyclic imine moiety results in neurological symptoms, including convulsions, followed by rapid death.[2] Studies on their mode of action suggest that in mammalian systems spirolides are muscarinic acetylcholine receptor antagonists and weak L-type transmembrane calcium channel activators.[6] Though toxic to mice, human illness has never been directly correlated to spirolide ingestion.[7] A cyclic imine moiety is the common structural feature of spirolides showing toxicity in the mouse assay, suggesting that this is the pharmacophore.[2] Spirolides were first identified in extracts of the digestive glands of mussels and scallops from the Atlantic coast of Nova Scotia.[1,2] Later, the marine dinoflagellate *Alexandrium ostenfeldii* was discovered to be their source.[8] Chemical investigations of various shellfish extracts and cultured dinoflagellate isolates led to the isolation and structural elucidation of spirolides A−G and several derivatives.[1−5] Cultures of a single-cell isolate of *A. ostenfeldii* (AOSH1) from Ship Harbour, Nova Scotia, yielded spirolides A, C, and 13-desmethyl C.[3] Using LC/MS methodology, spirolides H (**1**) and I (**2**) (Figure 1) were isolated from methanol extracts of wet cells of another Ship Harbour isolate of *A. ostenfeldii*, AOSH2. Unlike previously isolated spirolides, which contain a 5:5:6 or a 5:6:6 trispiroketal ring moiety,[1−5] **1** and **2** contain a 5:6 dispiroketal ring system. Here we report the structural elucidation of **1** and **2**, a new subclass of spirolide marine toxins, and discuss the toxicity of **1** to mice.

Results and Discussion

The spirolide profile of AOSH2 was investigated using LC/MS analysis. A methanolic extract of cultured dinoflagellate cells was partitioned between CH_2Cl_2 and H_2O. Separation of the components in the CH_2Cl_2 extract on a C_{18} chromatography column followed by LC/MS analysis of the fractions resulted in the identification of a spirolide-containing fraction. Examination of the LC/MS profile revealed the presence of two components with *m*/*z* 650 and 652, which had not been previously isolated and identified. Isolation of these components was undertaken using a modified isolation procedure that varied from previous spirolide isolation procedures in that an LH-20 column chromatography step was not employed. This change significantly decreased the time needed to isolate spirolide components. Characterization of **1** and **2** was achieved using a combination of spectrometric and 1D and 2D NMR spectroscopic techniques.



1 $\triangle^{2,3}$
2

Figure 1. Structures of spirolides H (**1**) and I (**2**) and relative configuration of **1**.

High-resolution mass spectrometry experiments determined the molecular formulas of **1** and **2** to be $C_{40}H_{60}NO_6$ ($[M + H]^+$ *m*/*z* 650.4407) and $C_{40}H_{62}NO_6$ ($[M + H]^+$ *m*/*z* 652.4570), respectively. The MS/MS spectra of both components contained a fragment ion at *m*/*z* 164, which indicated the presence of a cyclic imine ring containing vicinal dimethyl groups, as seen in previously isolated spirolides.[1−5] Comparison of the ^1H and ^{13}C NMR spectral data showed that the structures of **1** and **2** were similar except that the latter compound lacked the downfield proton at δ 7.13 and a carbon at δ 149.4, but possessed two proton signals at δ 1.65 and 2.56 and a carbon signal at δ 36.2.

The ^1H, ^{13}C, DEPT 135, and HSQC NMR data showed that the carbons of **1** were distributed as 10 quaternary, 10 methine, 13 methylene, and 7 methyl carbons (Table 1). Inspection of DEPT-135 and HSQC NMR data of **2** revealed that it contained an additional methylene carbon and one less quaternary carbon, which explained the difference in molecular formula for **1** and **2**. The assignments of the γ-lactone ring at C-1 to C-4, a vinyl double bond at C-21, and an imine at C-25 in the structures of **1** and **2** were confirmed by COSY and TOCSY data (Figure 2), HMBCs, and comparison with the NMR spectral data of previously isolated spirolides.[1−5] Further analysis of COSY and TOCSY spectra established four $^1H-^1H$ spin systems corresponding to the partial structures (**a**−**d**) for both **1** and **2** (Figure 2). The most significant difference between the two compounds was observed in partial structure **a**, which included the γ-lactone ring system, C-1 through C-4. For compound **1**, the presence of unsaturation between C-2 and C-3 was indicated by the following: COSY and TOSCY

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Table 1. ^{1}H and ^{13}C Data for Spirolides H (**1**) and I (**2**) in CD_3OH[a]

	1					**2**		
position	δ_C,	mult.	δ_H (J in Hz)	HMBC[b]	ROESY[c]	δ_C,	mult.	δ_H (J in Hz)
1	176.5,	qC				182.0,	qC	
2	130.9,	qC				36.5,	CH	2.80, m
3	149.4,	CH	7.13, dq (1.7, 1.7)	1, 4	4, 34	36.2,	CH_2	2.56, m
								1.65, m
4	82.0,	CH	5.98, m	35	3, 34	79.0,	CH	5.41, dd (10.9, 5.8)
5	126.2,	qC				129.4,	qC	
6	133.2,	qC				130.7,	qC	
7	47.7,	CH	3.78, bd (10.6)		24a, 24b, 34, 35	47.5,	CH	3.76, d (10.6)
8	120.9,	CH	5.07, bd (10.6)		10, 27a, 27b, 28, 40	121.2,	CH	5.06, d (10.6)
9	149.4,	qC				149.3,	qC	
10	75.5,	CH	4.30, dd (7.3, 4.6)	8, 12	8, 11a, 11b, 36	75.4,	CH	4.29, dd (6.6, 4.7)
11a	41.7,	CH_2	1.82, m		10, 11b, 12	41.6,	CH_2	1.84, m
11b			1.74, m	10, 12	10, 11a, 12			1.74, m
12	79.3,	CH	4.27, q (6.5)	10, 14, 15	11a, 11b, 13, 14a	79.1,	CH	4.27, q (6.5)
13	38.5,	CH	2.40, m	15	12, 14b, 36	38.5,	CH	2.40, m
14a	36.9,	CH_2	2.48, dd (12.7, 7.9)	12, 13, 36	12, 14b	36.8,	CH_2	2.48, m
14b			1.60, dd (12.7, 7.1)	12, 13, 15, 36	13, 14a, 19, 36			1.60, m
15	112.9,	qC				112.8,	qC	
16	71.9,	qC				72.0,	qC	
17	36.2,	CH_2	⟨1.62⟩[d] m		18b, 37	36.1,	CH_2	⟨1.62⟩[d] m
18a	29.9,	CH_2	1.52, m		18b, 37	30.1,	CH_2	1.52, m
18b			1.43, m		17, 18a, 19			1.44, m
19	74.7,	CH	3.63, m		14b, 18b, 20a	74.5,	CH	3.63, m
20a	43.7,	CH_2	2.42, m			44.0,	CH_2	2.44, m
20b			2.18, dd (14.4, 6.7,)	19, 21, 38	19			2.18, m
21	147.6,	qC				147.6,	qC	
22a	35.4,	CH_2	2.42, m			35.3,	CH_2	2.44, m
22b			2.10, m					2.10, m
23a	22.7,	CH_2	1.90, m			22.7,	CH_2	1.92, m
23b			1.78, m					1.78, m
24a	36.9,	CH_2	3.25, m		7, 24b	36.8,	CH_2	3.25, m
24b			2.74, dt (20.9, 6.7)	25	7, 24a, 35			2.74, m
25	202.9,	qC				176.5,	qC	
26	52.0,	qC				52.0,	qC	
27a	36.5,	CH_2	2.01, m			36.7,	CH_2	2.03, m
27b			1.78, m					1.78, m
28	37.2,	CH	1.10, m		8	37.2,	CH	1.10, m
29	38.5,	CH	1.66, m		30a, 30b, 39	38.5,	CH	1.66, m
30	51.9,	CH_2	4.17, dd (12.8, 4.6)		29, 30b, 31a	52.0,	CH_2	4.20, m
			3.58, m	25, 28, 29, 39	29, 30a, 39			3.58, m
31a	31.7,	CH_2	1.98, m		30a, 32a	32.1,	CH_2	1.98, m
31b			1.70, m					1.70, m
32a	20.0,	CH_2	2.27, m		31a	20.5,	CH_2	2.27, m
32b			1.70, m					1.70, m
33	10.4,	CH_3	1.90, t (1.5)	1, 2, 3		14.9,	CH_3	1.23, d (7.0)
34	16.7,	CH_3	1.76, s	5, 6, 7, 26	3, 4, 7	16.5,	CH_3	1.65, s
35	12.7,	CH_3	1.94, s	8, 9, 10	7, 24b	12.6,	CH_3	1.93, s
36	15.7,	CH_3	1.00, d (6.9)	12, 13, 14	10, 13, 14b	15.7,	CH_3	1.00, d (7.0)
37	21.7,	CH_3	1.27, s	15, 16, 17	17, 18a	21.7,	CH_3	1.27, s
38a	113.5,	CH_2	4.90, m	20, 22		113.4,	CH_2	4.89, m
38b			4.84, m	20, 22				4.86, m
39	18.5,	CH_3	1.05, d (6.9)	28, 29, 30	29, 30b	18.5,	CH_3	1.05, d (6.8)
40	20.0,	CH_3	1.06, d (6.9)	28, 29, 30	8	20.0,	CH_3	1.06, d (6.8)

[a] Spectra were recorded at 500.13 MHz (^{1}H) and 125.77 MHz (^{13}C). Chemical shifts δ_H and δ_C were referred to CHD_2OH = 3.30 ppm and $^{13}CD_3OH$ = 49.0 ppm (^{13}C), respectively. [b] HMBC correlations are from the proton stated to the indicated carbon. [c] Unambiguous ROESY correlations, recorded at 700 MHz, are from the proton stated to the indicated proton. [d] Average value for an incompletely resolved methylene group.

correlations of H-3 with H-4 and H-33 and of H-33 with H-4 (Figure 2), and HMBC correlations of H-33/C-1, C-2, C-3 and H-3/C-1, C-4. The additional methylene carbon in the γ-lactone ring of **2** was evidenced by COSY and TOCSY correlations of H-33 with H-2, H-3, H-4 and of H-2 with H-3 (Figure 2) and supported by HMBCs of H-33/C-3 and H-3b/C-5, C-6.

Chemical shifts of **1** (Table 1) were similar to those reported in the spirolide literature, except that one carbon resonance was observed at δ 112.9, characteristic of a 5:6 spiroketal carbon and corresponding to C-15. The absence of other downfield carbon signals in the range δ 112−119 indicated that there were no additional 5:6, 5:5, or 6:6 linkages. Furthermore, there were no carbon signals corresponding to the methylene carbons found at positions C-16 and C-17 in previously isolated spirolides.[1−5] These observations suggested the presence of a dispiroketal ring system, which was confirmed by HMBC connectivities of H-13/C-15 and H-37/C-13, C-15, C-16, C-17 in **1**.

The relative configuration of **1** (Figure 1) derived from ROESY data (Table 1) was in agreement with the relative configuration reported for spirolide D, in the area of their common structure, namely, C-7, -10, -12, and -13, in addition to C-16, -26, -28, and -29, which correspond to C-19, -29, -31, and -32, respectively, in spirolide D (Figure 2).[9] Further, ROESY correlations from H-4 to H-3 and H_3-34 and not to the methylene protons at C-32, and from H-3 to H-4 and H_3-34 only, favor an *S** configuration at C-4, relative to the rest of the spirolide structure in **1**.

One of the major fragmentation pathways for $[M + H]^+$ spirolide ions is the intramolecular retro-Diels−Alder reaction of the six-

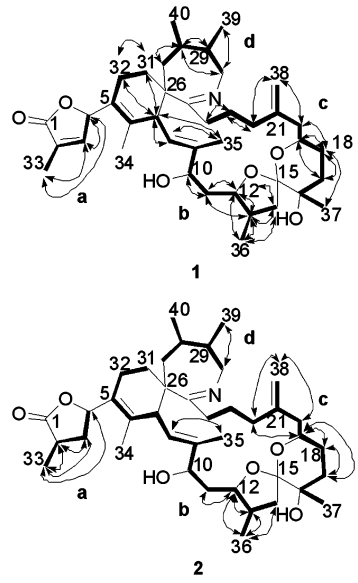

Figure 2. COSY and TOCSY correlations (curved arrows) in **1** and **2** and partial structures **a**−**d** (bold lines) representing ^{1}H−^{1}H spin systems.

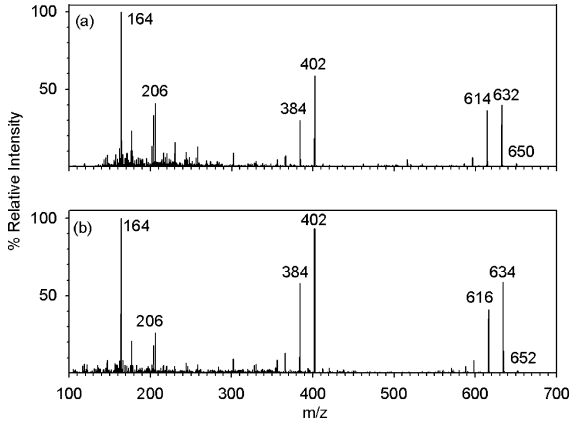

Figure 3. Product ion mass spectra of the $[M + H]^+$ ions of **1** (a), *m*/*z* 650, and **2** (b), *m*/*z* 652. Conditions: electrospray ionization; collision energy of 55 V; enhanced product ion scan on an API 4000 Qtrap mass spectrometer.

membered monounsaturated ring followed by the six-centered concerted loss of H_2O at C-10, cleaving the C-11 to C-12 bond. This produces fragment ion masses in the range *m*/*z* 408−460 for known spirolides.[7,10] On inspection, spirolide H (**1**) was determined to be most similar structurally to spirolide C.[3] The MS/MS spectrum of spirolide C gave a fragment ion at *m*/*z* 458 for the species produced from the retro Diels−Alder reaction. In comparison, the MS/MS spectra of **1** and **2** contained a prominent ion at *m*/*z* 402 (Figure 3). The mass difference between spirolide C and **1** is in agreement with the absence of the five-membered cyclic ether moiety. Fragment ions at *m*/*z* 632 and 614, and at *m*/*z* 634 and 616 for **1** and **2**, respectively, corresponded to the successive loss of H_2O at C-10 and C-16. The proposed fragmentation pattern of **1** is illustrated in Figure 4.

Toxicological investigations have shown that spirolides A, B, and C, 13-desmethylspirolide C, and 20-methylspirolide G are highly toxic to mice, with intraperitoneal LD_{50} values between 6.9 and 99 μg/kg.[11] In contrast, only transient hunching and lethargy were observed in mice injected intraperitoneally with **1** at doses up to 2000 μg/kg. Our results indicate that cyclic imine functionality is not the only requirement for toxicity of the spirolides, as suggested by earlier studies on the toxicity of these substances.

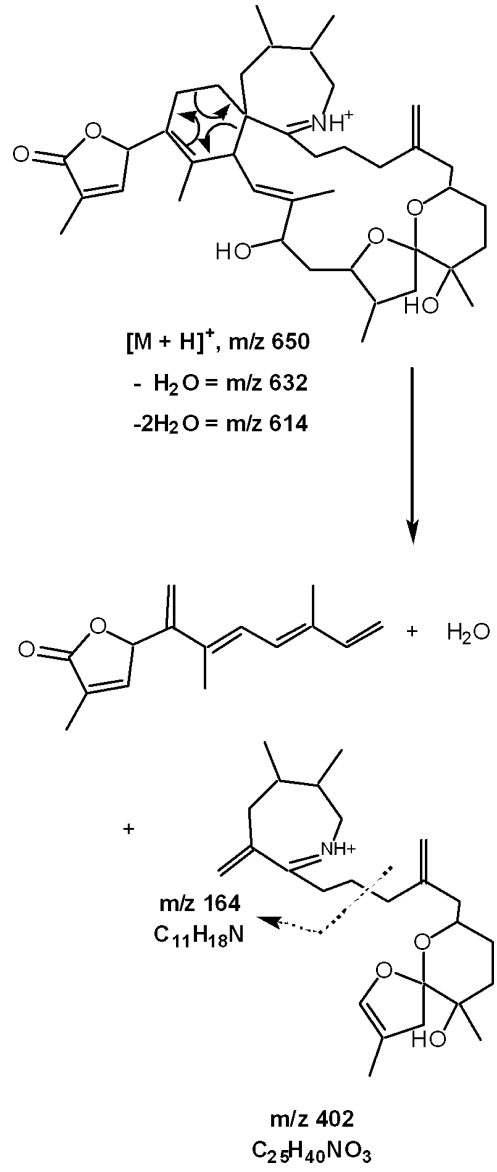

Figure 4. Key MS/MS fragment ions for **1**.

Structural studies are presently underway to investigate the conformational features that are responsible for the difference in toxicity seen between spirolide H and previously isolated spirolides.

Experimental section

General Experimental Procedures. NMR spectra were measured on a Bruker DRX-500 spectrometer (Bruker Canada Ltd.) with the following conditions: frequency 500.13 MHz (^{1}H), 125.7 MHz (^{13}C); solvents CD_3OH (referenced to ^{1}H 3.30 and ^{13}C 49.0), 5 mm tubes, temperature 20 °C; standard Bruker pulse sequences for ^{1}H single pulse, double quantum filtered COSY, TOSCY (160 ms mixing time), HSQC, HMBC (60 and 90 ms mixing time), ^{13}C DEPT 135, and ^{13}C{^{1}H}-waltz decoupled experiments. A Bruker Avance III (700 MHz) (Bruker Canada Ltd.) [frequency 700.23 MHz (^{1}H), 176.07 MHz (^{13}C); solvents CD_3OH (referenced to ^{1}H 3.30 and ^{13}C 49.0), 5 mm tubes, temperature 20 °C] was used to acquire ROESY (400 ms mixing time) data on spirolide H. The initial identification of spirolide compounds in the culture was performed on an Agilent 1100 LC/MSD single quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ionization source. Accurate mass spectra for measurements were performed on a Micromass Q-TOF Premiere (Micromass, Manchester, UK) time-of-flight mass spectrometer. Product ion MS/MS spectra were acquired on a hybrid 4000 QTrap mass spectrometer. Separations were performed on a 1.8 μm Agilent Zorbax C_{18} silica (4.6 × 50 mm) column that was eluted isocratically with 74% A (0.1% TFA) and 26% B (0.1%

TFA, CH_3CN) at a flow rate of 1 mL/min. Analyses were conducted in positive ion mode using selected ion monitoring of $[M + H]^+$ ions. The column eluent was split, with 10% going to the mass spectrometer.

Biological Material. The *A. ostenfeldii* culture (AOSH2) was initiated from a single cell isolated from plankton samples collected at Ship Harbour, NS, in 2000. The clonal isolate was identified by Nomarski contrast interference microscopy and by epifluorescence microscopy after staining the thecal plates with calcofluor.[12] The culture is maintained at the National Research Council's Institute for Marine Biosciences.

Culturing of *A. ostenfeldii* Clonal Isolates. AOSH2 was initiated in L1 growth medium diluted 1:10 with sterile seawater in multiwell tissue culture plates. Cultures were scaled-up in full-strength L1 medium by serial transfer into 15 mL culture tubes and then into 2.8 L Fernbach flasks, total culture volume 80 L. Unialgal cultures were maintained at an ambient photon flux density of 90 mmol m^{-2} s^{-1} at 14 °C and a 14:10 h light/dark photocycle in a controlled growth chamber. Cells were harvested in late exponential growth phase by gravity filtration onto a 20 mm Nitex mesh sieve and concentrated by centrifugation (2750*g*) for 20 min.

Isolation of Spirolides from Cultured *A. ostenfeldii.* The wet cell pellets of AOSH2 (47.4 g) were extracted four times with CH_3OH (250 mL) followed by sonication. After centrifugation, the methanolic supernatants were pooled and evaporated to dryness. The residue was dissolved in H_2O (300 mL) and partitioned with CH_2Cl_2 (3 × 300 mL). The CH_2Cl_2 extract (892 mg) was dissolved in 30% CH_3OH/H_2O and subjected to a C_{18} flash chromatography column, which was conditioned and eluted with 50% CH_3OH/H_2O. Fractions containing spirolides were combined, evaporated to dryness, and purified using an Agilent Zorbax SB C_{18} HPLC column, which was eluted isocratically with 26% CH_3CN/H_2O (0.1% TFA) and monitored at 210 nm. The yields of **1** and **2** were determined by NMR quantitation to be 1.03 and 0.20 mg, respectively.

Spirolide H: white solid; ^1H and ^{13}C NMR (Table 1); HRMS ($[M + H]^+$ *m/z* 650.4407 (calcd for $C_{40}H_{60}NO_6$, 650.4421).

Spirolide I: white solid; ^1H and ^{13}C NMR (Table 1); HRMS ($[M + H]^+$ *m/z* 652.4570 (calcd for $C_{40}H_{62}NO_6$, 652.4577).

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Supporting Information Available: ^1H and ^{13}C NMR spectra for **1** and the ^1H NMR spectrum of **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Hu, T.; Curtis, J. M.; Oshima, Y.; Quilliam, M. A.; Walter, J. A.; Watson-Wright, W. M.; Wright, J. L. C. *J. Chem. Soc., Chem. Commun.* **1995**, 2159–2161.
(2) Hu, T.; Curtis, J. M.; Oshima, Y.; Walter, J. A.; Wright, J. L. C. *Tetrahedron Lett.* **1996**, *37*, 7671–7674.
(3) Hu, T.; Burton, I. W.; Cembella, A. D.; Curtis, J. M.; Quilliam, M. A.; Walter, J. A.; Wright, J. L. C. *J. Nat. Prod.* **2001**, *64*, 308–312.
(4) MacKinnon, S. L.; Walter, J. A.; Quilliam, M. A.; Cembella, A. D.; LeBlanc, P.; Burton, I. W. *J. Nat. Prod.* **2006**, *69*, 983–987.
(5) Cimineiello, P.; Dell'Aversano, C.; Fattorusso, E.; Forino, M.; Grauso, L.; Tartaglione, L.; Guerrini, F.; Pistocchi, R. *J. Nat. Prod.* **2007**, *70*, 1878–1883.
(6) Gill, S.; Murphy, M.; Clausen, J.; Richard, D.; Quilliam, M.; MacKinnon, S.; LeBlanc, P.; Mueller, R.; Pulido, O. *Neurotoxicology* **2003**, *24*, 593–604.
(7) Sleno, L.; Windhust, A. J.; Volmer, D. A. *Anal. Bioanal. Chem.* **2004**, *378*, 969–976.
(8) Cembella, A. D.; Bauder, A. G.; Lewis, N. I.; Quilliam, M. A. *J. Plankton Res.* **2001**, *23*, 1413–1419.
(9) Falk, M.; Burton, I. W.; Hu, T.; Walter, J. A.; Wright, J. L. C. *Tetrahedron* **2001**, *57*, 8659–8665.
(10) Sleno, L.; Chalmers, M. J.; Volmer, D. A. *Anal. Bioanal. Chem.* **2004**, *378*, 977–986.
(11) Quilliam, M.; *Spirolides: Discovery, Toxicity and Pharmacological Potential*; Presented at the 122nd AOAC Annual Meeting & Exposition, Dallas, TX, Sept 2008.
(12) Fritz, L.; Triemer, R. E. In *Toxic Dinoflagellates*; Anderson, D. M., White, A. W., Baden, D. G., Eds.; Elsevier-North Holland: New York, 1985; pp 117−120.

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