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Characterization of Spirolides A, C, and 13-Desmethyl C, New Marine Toxins Isolated from Toxic Plankton and Contaminated Shellfish

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Three additional marine toxins, spirolides A (**1**), C (**3**), and 13-desmethyl-C (**7**), were isolated from contaminated scallops and phytoplankton collections obtained from a Nova Scotian aquaculture site, as well as from batch cultures of the dinoflagellate *Alexandrium ostenfeldii* obtained as a single-cell isolate from these phytoplankton assemblages. The structures of these new spirolide derivatives, characterized by mass spectrometry and NMR, indicate a close relationship with spirolides B (**2**) and D (**4**) isolated previously from contaminated shellfish in the same area. All of these compounds display "fast-acting" toxicity in the traditional bioassay used for monitoring shellfish, and this is related to the presence of a cyclic imine function in all these compounds. Those spirolides containing a vicinal dimethyl group in the seven-membered ring are resistant to oxalic acid hydrolysis, whereas those that do not are readily hydrolyzed. These observations suggest that the extra methyl group on the seven-membered imine ring of **3**, **4**, and **7** appears to block the process of imine hydrolysis perhaps by stereochemical interference.

Introduction

Since 1992 there have been brief but regular reports of unusual new mouse toxicity in various shellfish species at aquaculture sites along the Atlantic coast of Nova Scotia. These events are seasonal, usually occurring in June and July. Early attempts to isolate sufficient material for structure identification of the putative toxins were thwarted by the lack of sufficient biomass and the fact that the toxins are present in very low concentration in the polar lipid fraction obtained from the digestive glands of the shellfish. Eventually, sufficient material was obtained to permit the structure elucidation of two of these toxins, named spirolides B (**2**) and D (**4**),[1] and later two more related but biologically inactive compounds, spirolides E (**5**) and F (**6**).[2] These compounds bear a very close structural resemblance to the pinnatoxins, isolated from toxic extracts of the clam *Pinna muricata*,[3,4] which has been linked to several major poisoning events in Japan and China.

In the case of the spirolides, a seasonal pattern of six major related compounds was repeatedly observed from digestive gland extracts.[5] The structural elucidation of two remaining major congeners, spirolides A (**1**) and C (**3**) reported here, resulted from the annual accumulation of material from toxic shellfish, coupled with the isolation and culture of a toxic clone of the dinoflagellate *Alexandrium ostenfeldii* obtained from the original aquaculture site.[6] In addition, we report the structure of 13-desmethyl-C (**7**), a new spirolide congener, present in more recent extracts of shellfish as well as in cultures of *A. ostenfeldii* obtained from the same Nova Scotian aquaculture site.[7] (See Chart 1 for structures.)

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Results and Discussion

During the course of these investigations, spirolide-containing materials came from three sources: cultured scallops from aquaculture sites in Ship Harbour and Graves Shoal, Nova Scotia, that had tested positive by mouse bioassay during routine monitoring; natural phytoplankton biomass obtained from the Ship Harbour site; and laboratory cultures of a single-cell isolate of the dinoflagellate *A. ostenfeldii* obtained from the phytoplankton biomass. In shellfish extracts, the profiles of individual spirolides were similar but not identical from season to season within a site, and combined liquid chromatography−mass spectrometry (LC−MS) was used in the characterization and quantitation of a spirolide mixture in field samples.[7] This is illustrated in Figure 1, which shows the spirolide profile in a 1994 extract of scallop digestive glands. Good separation and selective detection of compounds **1** to **6** was achieved within 6 min. Also observed in this early sample was a small peak for compound **7** that has a retention time very close to that of **2**. In later samples, peak **7** was much more prominent. The same seasonal and site variability of spirolide distribution in the total complement isolated from natural phytoplankton was also observed and closely matched those obtained from scallops taken from the same aquaculture site at the time. No traces of **5** and **6** were ever found in either bulk phytoplankton from the field or in cultured *A. ostenfeldii* cells, but were present in shellfish extracts,[2,5] indicating that they are likely to be chemical or enzymatic hydrolysis products formed in the shellfish. The spirolide profile for a culture grown from a single cell isolate of *A. ostenfeldii* differed significantly from that of the bulk phytoplankton harvested concurrently from the same site. The isolate profile contained predominantly **7** (which overlaps with **2** in the chromatography), lesser amounts of **1**−**4**, and some other minor and as yet unidentified related compounds. Fortuitously, **2** was not present in these cultured algal samples, which facilitated the preparative isolation of **7** used in this study.

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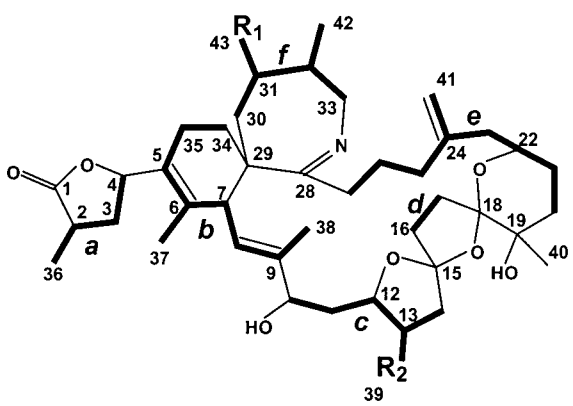
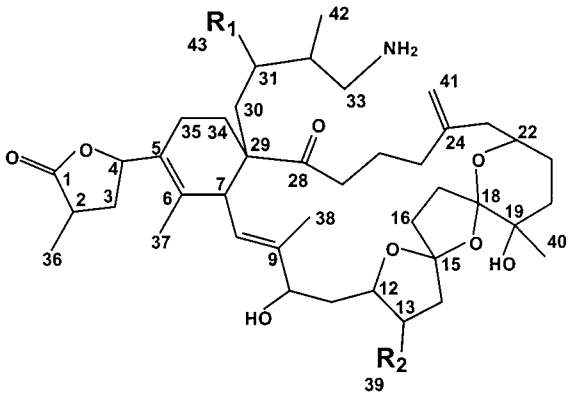
Chart 1





1	**A**	$\Delta^{2,3}$,	R_1 = H,	R_2 = Me
2	**B**		R_1 = H,	R_2 = Me
3	**C**	$\Delta^{2,3}$,	R_1 = Me,	R_2 = Me
4	**D**		R_1 = Me,	R_2 = Me
7	desMe-**C**	$\Delta^{2,3}$,	R_1 = Me,	R_2 = H

5	**E**	$\Delta^{2,3}$,	R_1 = H,	R_2 = Me
6	**F**		R_1 = H,	R_2 = Me

(Note: Following the previous style, drawings of the spirolides are reversed compared with those for the pinnatoxins)

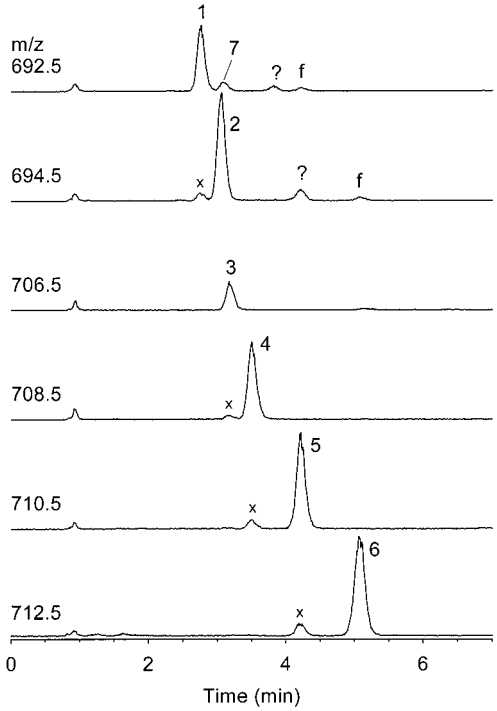


Figure 1. LC−MS analysis of an extract of scallop digestive glands, showing elution times for compounds **1**−**7**: × = signals due to interference by ^{13}C-isotope peaks from compounds with lower molecular weights; f = signals due to fragment ions of compounds **5** and **6**. Vertical scale is constant for all traces.

High-resolution LSIMS experiments determined the molecular formula of spirolide A (**1**) to be $C_{42}H_{61}NO_7$ (MH^+ 692.4503 ± 0.0013 (n = 5), actual 692.4526, diff = 2.3 mDa/3.3 ppm). ^{13}C NMR confirmed the number of carbons, which from additional NMR data (^{1}H, ^{13}C DEPT, and HSQC) were distributed as 11 quaternary, 9 methine, 16 methylene, and 6 methyl carbons (Table 1). Compound **1** has two protons fewer than spirolide B (**2**) (molecular formula $C_{42}H_{63}NO_7$), suggesting an additional double bond in **1**. This was confirmed by the occurrence of two more ^{13}C resonances between 100 and 150 ppm in **1** (Table 1). The strong IR absorption bands at 3477, 1761, and 1641 cm^{-1} suggested the presence of hydroxyl groups and a γ-lactone ring, as well as a C=O and /or C=N group, the latter possibilities being supported by ^{13}C resonances at 177.1 and 179.3 ppm.

Analysis of the TOCSY and COSY spectra for **1** established six ^{1}H spin systems corresponding to the partial structures (**a**−**f**), five of which (**b**−**f**) were identical in connectivity and multiplicity to those in **2**.[1] Chemical shifts (Table 1) of systems **b**−**f** also showed a close correspondence to those of **2**. The most significant difference in the NMR data between **1** and **2** was observed in partial structure **a**, which includes the γ-lactone ring system and carbons C-1 through C-4. In **1**, the connectivities of a carbonyl group (C-1) to olefinic carbons at C-2 and C-3 were established by HMBCs of H-36/C-1, C-2, C-3. The connectivities of the γ-lactone ring were completed by HMBCs of H-3/C-1, C-4 and H-4/C-3 and by the shifts of C-4 (82.5 ppm) and H-4 (5.94 ppm) that were consistent with an oxygenated carbon bearing an acyl group. The increased shielding of the C-1 carbonyl in **1** (177.1 ppm) compared with **2** (182.3 ppm) was also consistent with the presence of a conjugated carbonyl group. As observed with previous spirolides, HMBCs of H-4/C-5, C-6 and NOE correlations between H-37 and H-3 in spirolide A linked this γ-lactone ring to partial structure **b** at position C-5. These data not only were consistent with those for the previously reported spirolides recorded under the same conditions[1] but were also consistent with the NMR data reported for the pinnatoxins,[3,4] which contain some similar structural fragments.

Tandem mass spectrometry (MS/MS) proved valuable for the partial characterization of spirolides and provided strong supporting evidence for each of the proposed structures (vide infra). For example, the MS/MS data for **1** (Figure 2a) confirmed the position of the additional double bond: Fragmentation within the pendant γ-lactone ring, with retention of C-4 and an oxygen, results in a fragment ion at m/z 624 for **1**, consistent with that for **2**, although the molecular formula for **1** indicates an extra double bond

Table 1. ^{1}H and ^{13}C Assignments[a] for Spirolides A (**1**), C (**3**), and Desmethyl Spirolide C (**7**)

	spirolide A (**1**)		spirolide C (**3**)		desmethyl spirolide C (**7**)	
C #	δ_C (mult.)	δ_H	δ_C (mult.)	δ_H	δ_C (mult.)	δ_H
1	177.1 (s)		177.1 (s)		176.8 (s)	
2	130.8 (s)		130.7 (s)		131.0 (s)	
3	150.0 (d)	7.12	149.9 (d)	7.12	149.5 (d)	7.13
4	82.5 (d)	5.94	82.5 (s)	5.95	82.0 (d)	5.98
5	125.9 (s)		126.0 (s)		126.4 (s)	
6	134.9 (s)		134.7 (s)		133.2 (s)	
7	48.8 (d)	3.57	49.1 (d)	3.56	48.1 (d)	3.78
8	124.4 (d)	5.34	124.2 (d)	5.20	122.5 (d)	5.16
9	144.5 (s)		144.6 (s)		146.0 (s)	
10	76.7 (d)	4.16	76.8 (d)	4.15	76.8 (d)	4.15
11	39.7 (t)	1.61, 2.14	39.6 (t)	1.57, 2.14	45.2 (t)	1.37, 2.25
12	81.7 (d)	4.33	81.7 (d)	4.31	79.8 (d)	4.30
13	35.3 (d)	2.42	35.4 (d)	2.41	32.8 (t)	1.70, 2.27
14	45.7 (t)	2.13, 2.26	45.8 (t)	2.14, 2.26	38.2 (t)	1.95, 2.29
15	117.3 (s)		117.4 (s)		118.1 (s)	
16	36.6 (t)	2.04, 2.19	36.5 (t)	2.04, 2.22	35.2 (t)	2.07, 2.21
17	31.5 (t)	1.76, 2.14	31.5 (t)	1.74, 2.11	32.1 (t)	1.79, 2.20
18	112.5 (s)		112.5 (s)		112.2 (s)	
19	71.2 (s)		71.1 (s)		71.1 (s)	
20	35.8 (t)	1.47, 1.84	35.8 (t)	1.49, 1.81	35.7 (t)	1.49, 1.81
21	30.2 (t)	1.23, 1.59	30.2 (t)	1.24, 1.55	29.9 (t)	1.28, 1.58
22	69.4 (d)	4.00	69.3 (d)	3.97	69.1 (d)	3.97
23	47.5 (t)	2.02, 2.34	47.6 (t)	2.01, 2.37	46.3 (t)	2.06, 2.41
24	147.8 (s)		147.8 (s)		145.6 (s)	
25	35.9 95t)	1.60, 2.12	36.0 (t)	1.58, 2.10	34.6 (t)	1.83, 2.05
26	23.7 (t)	1.39, 2.02	23.4 (t)	1.40, 2.02	21.8 (t)	1.83, 2.01
27	35.6 (t)	2.34, 2.41	35.6 (t)	2.32, 2.43	36.0 (t)	2.82, 3.10
28	179.3 (s)		178.6 (s)		201.3[b]	
29	51.4 (s)		50.8 (s)		52.4 (s)	
30	28.0 (t)	1.65, 1.90	38.3 (t)	1.55, 1.73	36.7 (t)	1.79, 2.01
31	32.1 (t)	1.06, 1.78	36.9 (d)	1.16	37.5 (d)	1.04
32	33.6 (d)	1.88	41.2 (d)	1.36	38.8 (d)	1.67
33	53.1 (t)	3.48, 3.72	53.3 (t)	3.44, 3.76	51.8 (t)	3.55, 4.18
34	32.2 (t)	1.55, 1.80	32.4 (t)	1.52, 1.80	32.4 (t)	1.67, 1.98
35	20.4 (t)	1.56, 2.11	20.3 (t)	1.51, 2.14	20.3 (t)	1.72, 2.27
36	10.4 (q)	1.88	10.4 (q)	1.86	10.5 (q)	1.90
37	17.0 (q)	1.71	17.1 (q)	1.72	16.7 (q)	1.74
38	12.2 (q)	1.85	12.3 (q)	1.87	12.9 (q)	1.91
39	15.8 (q)	1.20	15.6 (q)	1.19		
40	22.5 (q)	1.19	22.5 (q)	1.19	22.7 (q)	1.20
41	111.4 (t)	4.77, 4.75	111.3 (t)	4.75, 4.78	112.6 (t)	4.81, 4.92
42	21.0 (q)	0.92	19.4 (q)	0.98	18.9 (q)	1.05
43			21.1 (q)	0.95	20.1 (q)	1.11

[a] (s) = C, (d) = CH, (t) = CH_2, (q) = CH_3. Spectra were recorded at 500.13 MHz (^{1}H) and 125.77 MHz (^{13}C) using CD_3OH as solvent. Chemical shifts δ_H and δ_C (ppm) were referred to CHD_2OH = 3.30 ppm (^{1}H), $^{13}CD_3OH$ = 49.0 ppm (^{13}C), respectively. [b] Broadened resonance.

compared to **2**. Thus the double bond must be located between C-2 and C-3. This information, together with the similarity of the NMR data for the remaining partial structures with those for spirolide B (**2**), established the structure of spirolide A as **1**.

The molecular formula of spirolide C (**3**) was determined by high-resolution LSIMS as $C_{43}H_{63}NO_7$ (MH^+ 706.4698 ± 0.0022 (n = 3), actual 706.4683, diff = 1.5 mDa/2.2 ppm). Strong IR absorption bands at 3467, 1760, and 1642 cm^{-1} again indicated a hydroxyl group, a γ-lactone ring, and a C═O and/or C═N group. Analysis of TOCSY, COSY, HMQC, and HMBC NMR data for **3** showed that the connectivities of the spin systems corresponding to the six partial structures (**a**−**e**) in **3** were identical with those in **1** (HMBCs H-36/C-1, C-2, C-3; H-37/C-5, C-6; H-38/C-8, C-9, C-10; H-39/C-12, C-13, C-14; H-40/C-18, C-19; H-23/C-22). The associated chemical shifts for systems **a**−**e** (Table 1) were also very similar between these two compounds. However the spin system associated with partial structure **f** in **3** was identical in connectivity to system **f** in **4** and also showed chemical shifts close to that system in **4**. The extra methyl group in **3** (C-43, δ_C 21.1, δ_H 0.95) was located at position C-31, as established by HMBC of H-43/C-30, C-31, and C-32. The HMBC data H-42/C-31, C-32, and C-33, placed another methyl group (C-42, δ_C 19.4, δ_H 0.98) at C-32 in this partial structure.

Confirmation of this methyl placement came from the MS/MS data of **3**. As described previously,[1] the MS/MS spectra of the MH^+ ions of the spirolides are dominated by two major fragmentation processes. The base fragment ion is associated with the cyclic imine system, observed either at m/z 164 when C-31 is methyl-substituted (as in **4**) or at m/z 150 when C-31 is not substituted (as in **2**). Thus, it can be readily inferred from the MS/MS spectra that **3** (Figure 2b) has methyl substitution at C-31, whereas **1** does not. Finally, the fragment ion at m/z 638 in **3**, which is also observed in **4**, places the double bond between C-2 and C-3.

The IR absorption bands at 3470, 1761, 1690, and 1652 cm^{-1} observed for compound **7** were similar to those observed for the other spirolides. Indeed, the molecular formula of **7** (LSIMS; $C_{42}H_{61}NO_7$ MD^+ 693.4569 ± 0.0010, n = 4, calcd 693.4589, Δ = 2.9 ppm) indicated that it was isomeric with **1**. Furthermore, ^{1}H TOCSY, COSY, and HSQC NMR data demonstrated that partial structures **a**, **b**, **d**, **e**, and **f** were essentially identical with the equivalent

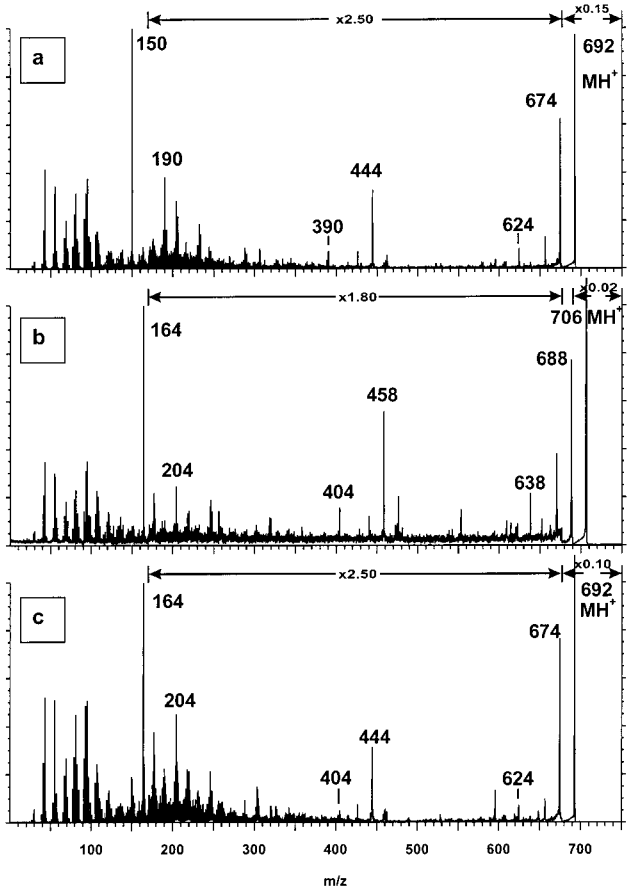


Figure 2. MS/MS spectra of compounds **1** (a), **3** (b), and **7** (c).

partial structures in **3**, with differences appearing only in partial structure **c**, around C-13 (Table 1). The TOCSY data for **7** indicated no methyl signal in the ^{1}H spin system, and COSY correlation of H-12/H-11, H-13 clearly showed that H-12 (δ 4.29) is adjacent to two methylene groups; thus the C-13 methyl group present in spirolides A−D is missing in **7**. The locations of the six methyl groups in **7** were identified by HMBC (H-36/C-1, C-2, C-3; H-37/C-5, C-6; H-38/C-8, C-9, C-10; H-40/C-18, C-19; H-42/C-31, C-32, C-33; and H-43/C-30, C-31, C-32). Other distinct HMBCs confirmed the planar connectivity of the compound: H-4/C-3, C-5; H-7/C-5, C-6, C-8, C-29; C-30; H-8/C-6, C-9, C-10; H-10/C-8, C-11, C-12; H-11/C-10, C-12, C-13; H-12/C-10, C-13; H-13/C-11, C-12, C-15; H-14/C-13, C-15, C-16; H-16/C-15, C-18; H-17/C-15, C-18; H-20/C-18, C-21, C-22; H-21/C-20, C-22; H-22/C-18, C-23, C-24; H-23/C-21, C-22, C-24; C-25, C-41; H-41/C-23, C-24, C-25; H-25/C-23, C-24, C-26; H-27/C-25, C-26; H-30/C-7, C-29, C-34, C-43; H-33/C-31, C-42; H-34/C-5, C-7, C-29, C-30, C-35; H-35/C-5. No HMBCs to C-28 were detectable in **7**, presumably because this ^{13}C resonance is greatly broadened due to a tautomeric equilibrium involving an exchangeable hydrogen at C-27.[1,2] Displacement of this equilibrium due to variations in the degree of protonation might also explain the downfield displacement of the C-28 resonance in **7** (to δ_C 201.3) compared with other spirolides (δ_C ca. 179).

As a result of the characteristic MS/MS spectra of the MH$^+$ spirolide ions, the absence of the methyl group at position 13 in **7** resulted in pronounced and interpretable differences between the MS/MS spectra of **1**, **3**, and **7** (Figure 2). From the base fragment ion data associated with the cyclic imine system, it can be readily inferred from the MS/MS spectra that **7** and **3** have methyl substitution at C-31, whereas **1** does not. The second dominant fragmentation pathway is due to opening of the macrocyclic ring adjacent to C-29 and between C-9 and C-10 to give a fragment ion at *m*/*z* 458 when $R_1 = R_2 =$ Me (as in **4**) or at *m*/*z* 444 if *either* R_1 or $R_2 =$ Me (as in **2**). Thus, the MS/MS spectra of the MH$^+$ ions of **1** and **7** are consistent with only one methyl group substituted at either C-31 or C-13, whereas both positions are methyl substituted in **3**. Fragmentation across the cyclic ether ring adjacent to C-12 and C-13 (i.e., not including R_2) is also observed, giving rise to *m*/*z* 390 for **1** and *m*/*z* 404 for **3** and **7**, consistent with the described R_1 substitution and confirming that R_2 must lie between C-11 and C-13.

The accumulation of minute amounts of spirolides from shellfish tissue is a tedious process, but the problem of supply has been considerably relieved by the occurrence of these toxins in cultures of the dinoflagellate species *A. ostenfeldii*.[6] The fact that the current laboratory culture of *A. ostenfeldii* has a different spirolide profile from harvested plankton material suggests the possibility that more than one strain of spirolide-producing organism is present in field samples. Hence variation in relative levels of these strains could account for the observed differences in toxin profiles from season to season and site to site. Since there is a question as to the relative oral toxicities of these compounds (vide infra), it is important to obtain a comprehensive understanding of the range of structural types present.

The spirolides are part of an expanding group of compounds displaying the phenomenon of "all or nothing" fast-acting toxicity following intraperitoneal injection in mice, as originally described by Tindall et al.,[8] who observed the effect with extracts of some toxic dinoflagellates. From our studies and those of others, toxins displaying this activity have grown to include the prorocentrolides,[7,8] the pinnatoxins,[3,4] and gymnodimine.[11] The pinnatoxins have been associated with human toxicity following consumption of contaminated shellfish,[3] whereas gymnodimine has been reported to be inactive following oral dosing.[12] Meanwhile, we have shown that the cyclic imine function, common to all these structures, is critical for biological activity.[2] This is based on the fact that spirolides E (**5**) and F (**6**), where the imine function has been hydrolyzed to a keto-amine, display no toxicity even at four times the dose.

The lability of the cyclic imine function to acid and/or enzymatic hydrolysis could be important with respect to any potential oral toxicity that the spirolides may possess. It may be of significance that spirolides **3** and **4**, which contain an extra methyl group on the imine ring compared to **1** and **2**, are resistant to oxalic acid hydrolysis, although the same conditions convert **1** and **2** to the inactive keto-amines **5** and **6**.[2] The pinnatoxins, which are reported as human poisons following oral consumption,[3,4] possess a vicinal dimethyl group in the cyclic imine ring, whereas the reputedly orally inactive compound[12] gymnodimine contains a six-membered imine ring with no methyl substitution. These observations suggest that the vicinal dimethyl group in the imine ring could block or hinder the process of imine hydrolysis, in which case **3** and **4** may survive acid or enzymatic hydrolysis conditions within the human digestive system and therefore may be orally toxic. Further experiments are in progress concerning the oral toxicities of the spirolides and their relative stabilities under physiological conditions.

Experimental Section

General Experimental Procedures. NMR Spectroscopy. Initial NMR experiments were undertaken with a Bruker AMX-500 spectrometer and later with a DRX-500

(Bruker Canada Ltd.). Conditions: frequency 500.13 MHz (^{1}H), 125.77 MHz (^{13}C); solvents CD_3OH or CD_3OD, 5 mm tubes, temperature 20 °C; standard Bruker pulse sequences for ^{1}H single pulse, double quantum filtered COSY, TOCSY (160 ms mixing time), NOESY and ROESY (400 ms mixing time), ^{1}H/^{13}C HMQC, HSQC, and HMBC (60 and 90 ms mixing time), ^{13}C DEPT 135 and ^{13}C{^{1}H}-waltz decoupled experiments.

LC−MS Analyses. Liquid chromatography−mass spectrometry (LC−MS) analyses were performed using a Hewlett-Packard (Palo Alto, CA) HP1100 LC system connected with a Perkin-Elmer SCIEX (Concord, Ontario) API-165 single quadrupole MS system equipped with an ion-spray source. Analyses were conducted in positive ion mode using selected ion monitoring of $[M + H]^+$ ions. Separations were performed on a 50 × 2 mm i.d. column packed with 3 μm BDS-Hypersil C8-silica with 0.25 mL min^{-1} of mobile phase composed of MeCN/H_2O (35:65, v/v) with 2 mM ammonium formate and 50 mM formic acid. The column effluent was split, with 10% going to the mass spectrometer.

Mass Spectrometry. Mass spectra for MS/MS and measurements of exact mass were obtained using a Micromass Autospec oa-TOF (Micromass, Manchester, UK) hybrid sector/time-of flight mass spectrometer. Liquid secondary ion mass spectra (LSIMS) used a matrix of 1:1 glycerol/*meta*-nitrobenzyl alcohol with 1% TFA as matrix and a beam of Cs ions at an effective incident energy of 12 keV and an accelerating potential of 8 kV. Exact mass measurements were made by accelerating voltage scanning using a mass resolution of at least 7000 (10% valley definition) and PEG as calibrant. MS/MS spectra were obtained at a collision energy of 800 eV using Xe as collision gas.

Isolation of Spirolides from Shellfish. Spirolides were isolated from MeOH extracts of contaminated scallop digestive glands (total 6.6 kg) following procedures closely parallel to those described previously.[1,2] The digestive glands were extracted three times with MeOH, and after evaporation the residue was dissolved in 70% MeOH/30% H_2O and partitioned against C_6H_{12}. Subsequently the MeOH layer was diluted to 25% MeOH and further partitioned against CH_2Cl_2. The CH_2Cl_2 fraction was subjected to Si gel chromatography (50 g, 100−200 mesh) followed by reversed-phase C_{18} HPLC (MeCN/H_2O/TFA, 30:70:0.1, UV detection 210 nm), yielding four peaks. Peaks I, III, and IV were collected and were determined by co-injection with authentic samples, confirmed by LC−MS and NMR analysis, to be the same spirolides A, C, and D (**1**, **3**, and **4**) as those isolated previously. Peak II was also collected (480 μg), its ^{1}H NMR spectrum showing that it was a mixture of two spirolide compounds. The minor component (ca. 40% of the fraction weight) was identified as **2** by LC−MS and NMR analysis. The major component (60%) was ultimately identified as compound **7**.

Culturing of *A. ostenfeldii* Clonal Isolate. A clonal isolate of *A. ostenfeldii* (AOSH1) from Ship Harbour, NS, was initiated in L1 growth medium diluted 1:10 with sterile seawater in multiwell tissue culture plates. Cultures were scaled-up by serial transfer into 15 mL cultures tubes, then into full-strength L1 medium in 2.8 L Fernbach flasks. Biomass for the structural elucidation was produced by inoculating cultures in late exponential growth phase in 15 L glass Bellco culture vessels containing L1 growth medium. Cultures were maintained aseptically with gentle aeration at an ambient photon flux density of 90 μmol m^{-2} s^{-1} at 15 °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 15 μm Nitex mesh sieve and concentrated by centrifugation (2750 g) for 20 min.

Isolation of Spirolides from Planktonic Material and from Cultured *A. ostenfeldii*. Planktonic material was harvested from the aquaculture site at Ship Harbour, NS, by pumping ca. 40 000 L of seawater containing a visible algal bloom dominated by dinoflagellates from 3 m depth through a plankton net (20 μm mesh) to collect 7.5 g of the 21−56 μm size fraction. The material was added to MeOH (25 mL) and sonicated, and the supernatant was collected after centrifugation. MeOH extraction of the pellet was repeated three times, and the combined supernatant evaporated to dryness, dissolved in H_2O (100 mL), and extracted against CH_2Cl_2 (2 × 100 mL). The CH_2Cl_2 extract (90 mg) was fractionated using an LH20 column (50 mL volume, MeOH eluant), and the fraction showing spirolides (53 mg) by TLC was further separated by reversed-phase chromatography (C8, MeCN/H_2O/TFA, 40:60:0.1). Finally, the spirolides (total 137 μg) were separated using HPLC (Vydac 201TP C18, MeCN/H_2O/TFA (32:68:0.1) as eluant, UV detection 210 nm). Spirolides were isolated from pellets of cultured *A. ostenfeldii* cells (9 g) using the same procedure.

Oxalic Acid Hydrolysis of the Spirolide Mixture. A purified mixture of spirolides (200 μg) was analyzed by HPLC and found to contain compounds **1**−**4**. The mixture was dissolved and heated (60 °C) in a combined solvent of THF (200 μL) and an aqueous saturated solution of oxalic acid (200 μL). Samples (5 μL) were taken every hour for direct analysis by HPLC (Vydac 201TP C18, MeCN/H_2O/TFA (40:60:0.1) as eluant). Previously identified samples of spirolides were used to calibrate the retention times. Later, a sample of **7** from cultured *A. ostenfeldii* was subjected to the same treatment.

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