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Full Papers

Spirolides Isolated from Danish Strains of the Toxigenic Dinoflagellate *Alexandrium ostenfeldii*

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Using LC/MS methodology, spirolides were detected in two clonal isolates of *Alexandrium ostenfeldii* isolated from Limfjorden, Denmark. Examination of the LC/MS profiles of extracts from these Danish cultures revealed the presence of two dominant peaks representing two previously unidentified spirolide components and one minor peak identified as the previously reported desmethyl spirolide C (**1**). Culturing of these clonal strains, LF 37 and LF 38, of *A. ostenfeldii* resulted in the accumulation of sufficient cell biomass to allow for the isolation and structure elucidation of two new spirolides, 13,19-didesmethylspirolide C (**2**) and spirolide G (**3**). While **2** was found to differ from **1** only in that it contained one less methyl group, **3** was the first spirolide to be isolated that contained a 5:6:6-trispiroketal ring system. The effect of this new feature on the toxicity of **3** relative to other spirolides is presently being pursued.

A class of macrocyclic imines known as spirolides was first identified in extracts of the digestive glands of mussels and scallops from the Atlantic coast of Nova Scotia, Canada, in the early 1990s.[1] The distinguishing feature of these compounds is the presence of a cyclic imine moiety, which has been found elsewhere only in the marine toxins known as pinnatoxins, pteriatoxins, spiro-prorocentrimine, and gymnodimine.[2−5] This unusual cyclic imine feature is the pharmacophore responsible for the "fast acting" symptomology observed when spirolides are assayed by intraperitoneal administration to mice.[6] The marine dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech & Tangen was identified as the cause of spirolide toxicity in Nova Scotia.[7,8] This finding was surprising because *A. ostenfeldii* has been previously known as a source of neurotoxins associated with paralytic shellfish poisoning (PSP), an unrelated toxin syndrome.

To date, seven compounds belonging to the spirolide class have been isolated and structurally characterized from shellfish extracts and cultured dinoflagellate isolates from Nova Scotia.[1,6,9] Using LC/MS methodology, spirolides have now been detected in cultures of *A. ostenfeldii* isolated from Limfjorden, Denmark. Examination of the LC/MS profiles of extracts from these Danish cultures has revealed the presence of desmethylspirolide C (**1**) and two previously unidentified spirolide components. Here we report the structure elucidation of these two novel spirolides, 13,19-didesmethylspirolide C (**2**) and spirolide G (**3**). Of particular interest is the structure of **3**, which contains a 5:6:6-trispiroketal ring system, which has not been observed in previously identified spirolides and other marine toxins.

Results and Discussion

Since the discovery of spirolides in *A. ostenfeldii* culture isolates from Nova Scotia, we have been monitoring the occurrence of these components in cultures obtained from different locations around the world. Recently, we discovered spirolide-like components in the LC/MS profiles of methanol extracts from wet cells of Danish isolates LF 37 and LF 38 of *A. ostenfeldii*. The profiles of both strains, Figure 1, showed the presence of 13-desmethylspirolide C (peak **a**) at approximately 3.3 min. Additionally, more intense peaks were observed at approximately 2.3 min (peak **b**) in the LF 37 profile and at 2.3 (peak **b**) and 3.5 min (peak **c**) in the LF 38 profile. All three components were suspected to be spirolides because the MS/MS spectra of each contained a fragment ion at *m*/*z* 164 that, from our previous studies of spirolides, indicated the presence of spirolide C analogues containing vicinal dimethyl groups in the cyclic imine ring.[9] The mass spectra and retention times of these peaks did not coincide with previously isolated spirolides.

The molecular formulas of **2** and **3** were found by HRMS to be $C_{41}H_{59}NO_7$ ($[M + H]^+$ *m*/*z* 678.4375, calc 678.4370) and $C_{42}H_{61}$-

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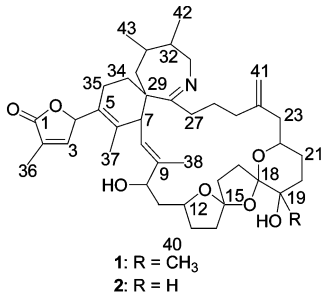
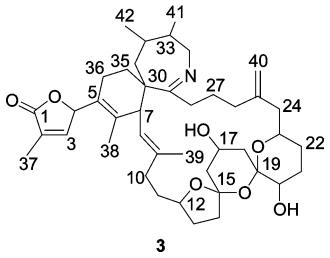


1: R = CH_3
2: R = H



3

NO_7 ([M + H]$^+$ *m*/*z* 692.4564, calc 692.4526), respectively. FTIR results supported the presence of hydroxyl groups (3470 cm^{-1}), a C═O and/or C═N group (1684 cm^{-1}), and a γ-lactone ring (1746 cm^{-1}) in both the structures. Inspection of one-dimensional proton and carbon NMR spectra and mass spectral data showed that peak **b** in *A. ostenfeldii* strain LF 37 was identical to peak **b** in strain LF 38 (Figure 1).

It was determined from ^{1}H, ^{13}C DEPT, and HSQC NMR experiments that the carbons of **2** were distributed as five methyl, 16 methylene, 10 methine, and 10 quaternary carbons. Inspection of ^{13}C DEPT and HSQC NMR data of **3** revealed that its structure contained an additional methylene carbon. This coincided with the difference observed in the HRMS analysis for **2** and **3**. The assignments of a γ-lactone ring at C-1 to C-4, an imine at C-28 in **2** and C-29 in **3**, and a vinyl double bond at C-24 in **2** and C-25 in **3** were confirmed via inspection of COSY and TOCSY spectra (Figure 2), HMBCs, and comparison with NMR spectral data of **1** (Table 1). The six remaining ^{13}C resonances between 122 and 149 ppm were assigned similarly to the three double bonds at C-2/C-3, C-5/C-6, and C-8/C-9 in both **2** and **3** on the basis of the observed HMBC correlations.

Comparison of the molecular formula, and the carbon and proton NMR data, indicated that **2** and **3** contained one less methyl group than 13-desmethylspirolide C (**1**). As mentioned previously, the presence of a fragment ion at *m*/*z* 164 in their mass spectra indicated the presence of vicinal dimethyl groups in the imine-containing ring. In addition to the observed COSY and TOCSY correlations (Figure 2), the placement of the C-42 and C-43 methyl groups in **2** was supported by HMBC correlations of H-42 with C-31, C-32, and C-33 and of H-43 with C-30, C-31, and C-32. Similarly, placement of the C-43 and C-44 methyl groups in **3** was confirmed via H-43 with C-32, C-33, and C-34, and H-44 with C-32, HMBC correlations. The remaining three methyl groups at C-36, C-37, and C-38 in **2** and C-37, C-38, and C-39 in **3** were confirmed by HMBC correlations.

The COSY and TOCSY correlations outlined in Figure 2 resulted in the elucidation of four ^{1}H−^{1}H spin systems (**a** through **d**) in both **2** and **3**. Spin systems in **2** were linked via HMBC correlations similar to those observed for previously isolated spirolides. The shift magnitude of carbons C-15 and C-18 confirmed the presence of 5:5[1,10] and 5:6-spiroketal[2,10−12] carbons, respectively, and therefore the presence of the characteristic spirolide 5:5:6-trispiroketal ring system.

The mass spectrum of **2** showed a protonated molecule at *m*/*z* 678, which was followed by a series of ions at *m*/*z* 660, 642, and 624, ions representative of the multiple H_2O losses characteristically

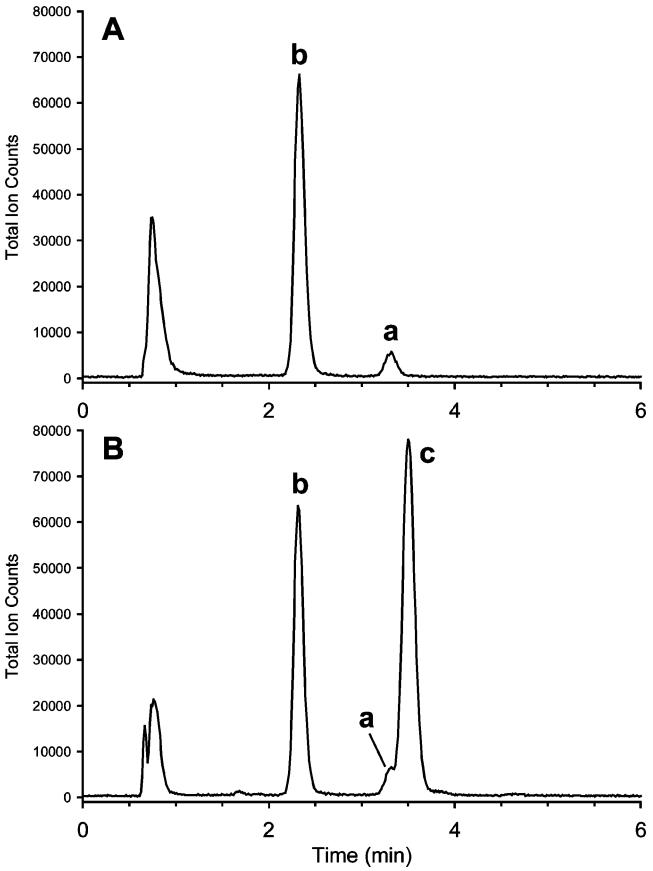
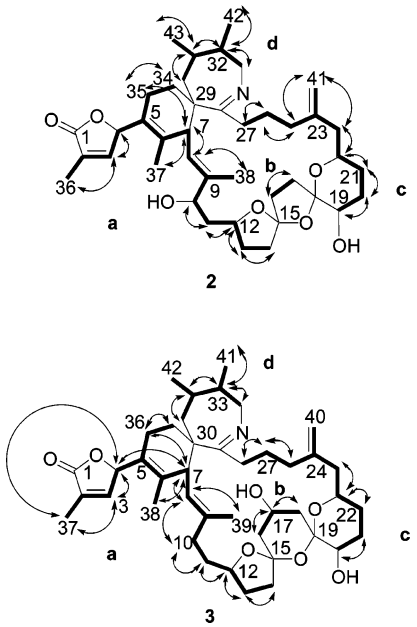


Figure 1. Total ion current chromatograms from the LC/MS analysis of a methanol extract of *A. ostenfeldii* strains LF 37 (A) and LF 38 (B).



2

3

Figure 2. Partial structures (**a**−**d**) in 13,19-didesmethylspirolide C (**2**) and spirolide G (**3**), showing COSY correlations (curved arrows) and bold lines representing ^{1}H−^{1}H spin systems identified from TOCSY and COSY spectra.

observed in polyether-containing compounds.[13−15] A prominent ion observed at *m*/*z* 430 was attributable to a retro-Diels−Alder reaction of the six-membered monounsaturated ring and a six-centered concerted loss of a water molecule at C-10, resulting in the cleavage of a bond between C11 and C-12.[9,16] In **1** this fragmentation

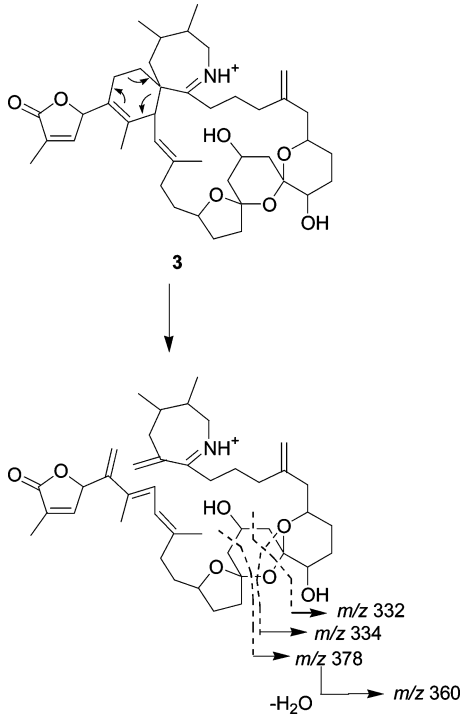


Figure 3. MS fragmentation scheme for spirolide G (**3**).

pathway resulted in an ion at *m*/*z* 444. The difference of 14 mass units between **1** and **2** of this fragment ion is in agreement with the absence of a methyl group at C-19 in **2**.

In spirolide G (**3**), linkage of the ^{1}H−^{1}H spin system **a** with that of **b** was accomplished via HMBCs between H-13, H-14, H-16, and C-15. This resulted in the assignment of C-15 to a carbon shift of 109.7 ppm, characteristic of a spiroketal carbon at a 5:6 ring junction.[2,10−12] A connectivity was established between fragment **b** and C-19 from the H-18 to C-19 HMBC, thereby assigning C-19 to the shift at 101.2 ppm, characteristic of a spiroketal carbon at a 6:6 ring junction.[17,18] These observations, along with the COSY/TOCSY data, confirmed the presence of a novel 5:6:6-trispiroketal ring system in **3** that has not been previously observed in the spirolide class of toxins. HMBC cross-peaks linked partial structure **d** with the cyclic imine ring in **3** and fragments **a** and **d** to the spirocyclic carbon at C-30.

The functionalities of the five oxygens in **3**, excluding those in the γ-lactone ring, were investigated by recording a ^{13}C NMR spectrum in CD_3OH and then in CD_3OD. Both methine carbons at C-17 and C-20 showed upfield shifts of 0.11 ppm, indicating the presence of a tertiary hydroxyl group at each position.[19] An upfield shift of 0.13 and 0.3 ppm was observed for C-27 and C-28, respectively, indicating the partial exchange of protons adjacent to the imine ring, an observation that has been previously reported for spirolides.[1] These results implied that the three remaining oxygens were present in the ether linkages observed in the 5:6:6-trispiroketal ring system as no other carbons exhibited significant upfield shifts.

While the mass spectrum of **3** contained the fragment ion at *m*/*z* 164, indicative of the presence of a vicinal dimethyl group in the cyclic imine ring, it did not contain a fragment ion in the range of *m*/*z* 430−460, which would have been expected from the fragmentation pathway that produced the *m*/*z* of 430 in **2** and *m*/*z* 444 in **1**. Instead, a base peak was observed at *m*/*z* 378, which was followed in intensity by ions at *m*/*z* 360, 334, and 332. As shown in Figure 3, the ion at *m*/*z* 378 can be explained by a retro-Diels−Alder reaction followed by a rearrangement of the hydroxylated six-membered cyclic ether ring resulting in the cleavage of the bonds at C-16−C-17 and C-15−O. Subsequent loss of H_2O from this fragment ion results in the observation of an ion at *m*/*z* 360. Further rearrangement of this central spirocyclic ketal results in fragment ions at *m*/*z* 332 and 334. The fragmentation data for **3** is in agreement with the presence of a 5:6:6-trispiroketal ring system as opposed to the 5:5:6 system which is seen in all the other spirolides that have been reported to date. It also supports the absence of a methyl group at C-20 and the presence of a hydroxyl group at C-17.

A minimum lethal dose of 30 μg/kg obtained via intraperitoneal administration in mice was observed for **2** in our preliminary toxicity studies, therefore indicating that the loss of the methyl group at C-19 in 13-desmethylspirolide C results in a 5-fold loss of toxicity. Investigations into the effect that the 5:6:6-trispiroketal ring system has on the toxicity of **3** relative to other spirolides will be pursued following the isolation of more compound. Initial toxicological evaluations have indicated that spirolides are highly toxic in mice when administered intraperitoneally and 25 times less potent when administered orally to mice.[20] Further investigations involving intraperitoneal injections of **1**, using mice and rats, have shown a dose-dependent neurotoxicity.[21] More oral toxicity studies on spirolides are presently being investigated in our group as larger quantities of spirolides are made available through their isolation from cultured plankton.

To date, there is no strong evidence that links the presence of spirolides in the tissues of shellfish with human intoxication. The closely related pinnatoxins have, however, been associated with human toxic events that followed the consumption of contaminated shellfish.[2] Further toxicological studies are needed to assess the risk of spirolides to human health and for the establishment of regulatory safety limits for allowable levels of spirolides in shellfish.

Experimental Section

General Experimental Procedures. Infrared spectra were obtained on a Bio-Rad Model FTS-575C infrared spectrophotometer (Cambridge, MA) with automatic beam splitter alignment. 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 or CD_3OD (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, TOCSY(160 ms mixing time), HSQC, HMBC (60 and 90 ms mixing time), ^{13}C DEPT 135 and ^{13}C{^{1}H}-waltz decoupled experiments. The initial identification of spirolide compounds in the culture was performed on an Agilent 1100 liquid chromatograph (Palo Alto, CA) interfaced with a Perkin-Elmer-SCIEX (Thornhill, Ontario) API-165 single quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ionization source. Separations were performed on a column packed with 3 μm BDS-Hypersil C8 silica (50 × 2 mm i.d.), which was eluted isocratically with 70% A (50 mM formic acid, 2 mM ammonium formate, 0.02% trifluoroacetic acid) and 30% B (50 mM formic acid, 2 mM ammonium formate, 0.02% trifluoroacetic acid, 95% acetonitrile) at a flow rate of 200 μL/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. Accurate mass spectra for measurements were performed on a Micromass Autospec oa-TOF (Micromass, Manchester, UK) hybrid sector/time-of-flight mass spectrometer. Accelerating voltage scanning was performed using a mass resolution of approximately 7000 (10% valley definition) and PEG as calibrant. Product ion MS/MS spectra were acquired on a SCIEX API-III + mass spectrometer with a collision energy of 30 V.

Culturing of *A. ostenfeldii* Clonal Isolates. Two isolates of *A. ostenfeldii* (LF 37-CCMP1772 and LF 38-CCMP1773) obtained and isolated from Limfjorden, Denmark, by P. Juel Hansen, were incubated in 2 L Fernbach flasks at 16 °C under a photon flux density of 120 μmol m^{-2} s^{-1} on a 14:10 h light/dark photocycle in a controlled environment growth chamber. Cells were harvested in late exponential growth phase by gravity filtration on a 20 μm Nitex mesh sieve. The cells were concentrated by centrifugation at 4000*g* for 20 min at 5 °C in 15 mL Falcon centrifuge tubes.

Isolation of Spirolides from Cultured *A. ostenfeldii*. The two new spirolide toxins were isolated from *A. ostenfeldii* biomass using a

Table 1. ^{1}H and ^{13}C Data for Compounds **1**, **2**, and **3** in CD_3OH[a]

	13-desmethylspirolide C (**1**)		13,19-didesmethylspirolide C (**2**)		spirolide G (**3**)	
	δ_C (mult.)	δ_H	δ_C (mult.)	δ_H	δ_C (mult.)	δ_H
1	176.8 ($_q$C)		176.6 ($_q$C)		176.7 ($_q$C)	
2	131.0 ($_q$C)		131.0 ($_q$C)		130.9 ($_q$C)	
3	149.5 (CH)	7.13	149.3 (CH)	7.12	149.4 (CH)	7.12
4	82.0 (CH)	5.98	81.9 (CH)	5.97	81.9 (CH)	5.95
5	126.4 ($_q$C)		126.4 ($_q$C)		126.1 ($_q$C)	
6	133.2 ($_q$C)		132.9 ($_q$C)		133.2 ($_q$C)	
7	48.1 (CH)	3.78	47.8 (CH)	3.81	48.4 (CH)	3.74
8	122.5 (CH)	5.16	122.6 (CH)	5.16	122.8 (CH)	5.06
9	146.0 ($_q$C)		146.1 ($_q$C)		143.1 ($_q$C)	
10	76.8 (CH)	4.15	76.8 (CH)	4.11	38.7 (CH_2)	2.07, 2.16
11	45.2 (CH_2)	1.37, 2.25	45.5 (CH_2)	1.33, 2.27	39.5 (CH_2)	1.17, 2.35
12	79.8 (CH)	4.30	79.6 (CH)	4.34	82.8 (CH)	3.89
13	32.8 (CH_2)	1.70, 2.27	32.6 (CH_2)	1.67, 2.28	32.3 (CH_2)	1.52, 2.15
14	38.2 (CH_2)	1.95, 2.29	37.5 (CH_2)	1.93, 2.24	41.3 (CH_2)	1.90, 2.28
15	118.1 ($_q$C)		118.0 ($_q$C)		109.7 ($_q$C)	
16	35.2 (CH_2)	2.07, 2.21	34.9 (CH_2)	1.92, 2.22	45.4 (CH_2)	1.54, 2.03
17	32.1 (CH_2)	1.79, 2.20	35.2 (CH_2)	2.07, 2.10	62.0 (CH)	4.24
18	112.2 ($_q$C)		109.7 ($_q$C)		41.2 (CH)	1.78, 1.78
19	71.1 ($_q$C)		71.6 (CH)	3.36	101.2 ($_q$C)	
20	35.7 (CH_2)	1.49, 1.81	29.3 (CH_2)	1.56, 1.72	73.1 (CH)	3.18
21	29.9 (CH_2)	1.28, 1.58	31.0 (CH_2)	1.19, 1.68	28.0 (CH_2)	1.65, 1.65
22	69.1 (CH)	3.97	69.5 (CH)	3.84	30.8 (CH_2)	1.16, 1.64
23	46.3 (CH_2)	2.06, 2.41	46.1 (CH_2)	2.06, 2.36	68.2 (CH)	3.87
24	145.6 ($_q$C)		145.4 ($_q$C)		46.3 (CH_2)	1.98, 2.38
25	34.6 (CH_2)	1.83, 2.05	35.2 (CH_2)	1.86, 1.89	145.3 ($_q$C)	
26	21.8 (CH_2)	1.83, 2.01	22.5 (CH_2)	1.86, 1.95	34.4 (CH_2)	1.70, 1.82
27	36.0 (CH_2)	2.82, 3.10	36.1 (CH_2)	2.86, 3.13	20.4 (CH_2)	1.91, 2.11
28	201.3 ($_q$C)		202.7 ($_q$C)		35.9 (CH_2)	2.90, 3.17
29	52.4 ($_q$C)		52.5 ($_q$C)		202.7 ($_q$C)	
30	36.7 (CH_2)	1.79, 2.01	36.4 (CH_2)	1.80, 2.02	52.6 ($_q$C)	
31	37.5 (CH)	1.04	37.4 (CH)	1.03	36.5 (CH_2)	1.81, 2.01
32	38.8 (CH)	1.67	38.6 (CH)	1.68	37.5 (CH)	1.12
33	51.8 (CH_2)	3.55, 4.18	51.6 (CH_2)	3.54, 4.21	38.5 (CH)	1.69
34	32.4 (CH_2)	1.67, 1.98	32.3 (CH_2)	1.67, 1.96	51.7 (CH_2)	3.56, 4.21
35	20.3 (CH_2)	1.72, 2.27	20.1 (CH_2)	1.65, 2.25	32.5 (CH_2)	1.67, 1.99
36	10.5 (CH_3)	1.90	10.4 (CH_3)	1.89	20.2 (CH_2)	1.71, 2.25
37	16.7 (CH_3)	1.74	16.7 (CH_3)	1.74	10.4 (CH_3)	1.88
38	12.9 (CH_3)	1.91	12.6 (CH_3)	1.91	16.6 (CH_3)	1.70
39					17.5 (CH_3)	1.91
40	22.7 (CH_3)	1.20				
41	112.6 (CH_2)	4.81, 4.92	113.1 (CH_2)	4.84, 4.92		
42	18.9 (CH_3)	1.05	18.7 (CH_3)	1.05	112.3 (CH_2)	4.81, 4.92
43	20.1 (CH_3)	1.11	20.0 (CH_3)	1.13	18.7 (CH_3)	1.05
44					19.9 (CH_3)	1.12

[a] Spectra were recorded at 500.13 MHz (^{1}H) and 125.77 MHz (^{13}C). Chemical shifts δ_H and δ_C (ppm) were referred to CHD_2OH = 3.30 ppm (^{1}H) and $^{13}CD_3OH$ = 49.0 ppm (^{13}C), respectively.

procedure similar to that described by Hu et al.[9] Briefly, the wet cell pellets of *A. ostenfeldii* strain LF 38 (61.4 g) were extracted four times by adding 250 mL of methanol followed by sonication. The methanolic supernatants were pooled following centrifugation and evaporated to dryness. This residue was then dissolved in water (300 mL) and partitioned three times with 300 mL of dichloromethane to yield the toxin-containing dichloromethane fraction. This was fractionated using a Sephadex LH-20 column (2 cm × 76 cm) that was eluted with methanol. Fractions containing spirolides were identified using thin-layer chromatography, pooled, and evaporated to dryness to yield a fraction weighing 350 mg. Following dissolution in 30% methanol/water, the fraction was subjected to a C_{18} flash chromatography column, which was conditioned and eluted with 40% acetonitrile/water (0.1% trifluoroacetic acid). Fractions containing spirolides were combined, evaporated to dryness, and purified using a Vydac 201TP510 C_{18} HPLC column, which was eluted isocratically with 30% acetonitrile/water (0.1% trifluoroacetic acid) and monitored at 210 nm. The yields of spirolides **2** and **3** from the LF 38 strain were determined by proton NMR quantitation to be 5.6 and 6.3 mg, respectively. Similarly, the workup of 72.5 g of wet biomass of the LF 37 strain yielded ~0.7 mg of **2** and no detectable amount of **3**.

13,19-Didesmethylspirolide C (2): white solid; IR ν_{max} 3470, 1746, and 1684 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); HRMS $[M + H]^+$ *m*/*z* 678.4375 (calcd for C_{41} H_{59} NO_7, 678.4370).

Spirolide G (3): white solid; IR ν_{max} 3470, 1746, and 1684 cm^{-1}; ^{1}H and ^{13}C NMR (Table 1); HRMS $[M + H]^+$ *m*/*z* 692.4564 (calcd for C_{42} $H_{61}NO_7$, 692.4526).

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