Spirolide Toxin Profile of Adriatic *Alexandrium ostenfeldii* Cultures and Structure Elucidation of 27-Hydroxy-13,19-didesmethyl Spirolide C

Patrizia Ciminiello,*[,†] Carmela Dell'Aversano,[†] Ernesto Fattorusso,*[,†] Martino Forino,[†] Laura Grauso,[†] Luciana Tartaglione,[†] Franca Guerrini,[§] and Rossella Pistocchi[§]

Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli "Federico II", via D. Montesano, 49, 80131 Napoli, Italy, and Centro Interdipartimentale per le Scienze Ambientali, Università di Bologna, via Sant'Alberto, 163, 48100 Ravenna, Italy

Received July 3, 2007

This paper reports on the determination of the toxin profile of batch cultures of *Alexandrium ostenfeldii* established from water samples collected along the Emilia-Romagna coasts (Italy) in November 2003. Combined use of LC-MS/MS and 1D and 2D NMR techniques led us to identify 13,19-didesmethylspirolide C and 13-desmethylspirolide C as products of the alga and to highlight the occurrence of a new component, 27-hydroxy-13,19-didesmethylspirolide C, whose structure was fully elucidated by NMR. MS data for a number of minor spirolides are also reported.

Toward the end of November 2003 a high concentration (15 612 cells per liter) of *Alexandrium ostenfeldii* cells was detected along the Emilia-Romagna coasts (Italy). A batch culture of the alga was prepared and its toxin profile evaluated by liquid chromatography–mass spectrometry (LC-MS) by our research group.[1] In the wake of previous reports[2,3] we investigated the occurrence of spirolides and paralytic shellfish poisoning (PSP) toxins in this Adriatic *A. ostenfeldii* culture. In fact, while Canadian strains of this dinoflagellate were proven to produce high levels of spirolides A, B, C, D, 13-desmethyl C, and 13-desmethyl D,[2] only New Zealand strains produced PSP toxins.[3] Even more complex was the toxin profile of certain *A. ostenfeldii* populations from Scandinavia, which were shown to produce both spirolides and PSP toxins.[4] Our study characterized the Adriatic *A. ostenfeldii* as an organism producing several spirolides but none of the major PSP toxins.[1]

Spirolides constitute a group of toxins (Figure 1) featuring a spiro-linked tricyclic ether ring system and an unusual seven-membered spiro-linked cyclic imine moiety.[5] The unique cyclic imine group is the pharmacophore responsible for the fast-acting syndrome induced in mice when spirolides are administered intraperitoneally (LD_{50} = 40 μg/kg).[6] Human toxicity is still unknown, although gastric distress and tachycardia were diagnosed in shellfish consumers in coincidence with the occurrence of spirolides in Canadian mollusks.[6]

Many chemical studies have been performed on strains of *A. ostenfeldii*, and a considerable number of spirolide toxins differing in slight structural details were isolated (**1**−**7**),[5,7–10] among which 13-desmethylspirolide C (**4**) represented by far the major component.[11]

The LC-MS analyses we reported[1] on the Adriatic *A. ostenfeldii* cultures also proved that **4** was the main toxin produced by the dinoflagellate among the spirolides known at the time. The presence of **4** ($[M + H]^+$ ion at *m/z* 692.5) was assessed by the presence of the following three characteristic fragment ion clusters in the MS/MS spectrum acquired on an ESI triple quadrupole MS instrument: (i) ions at *m/z* 674, 656, and 638 due to subsequent losses of three water molecules, respectively; (ii) ions at *m/z* 462, 444, 426, and 408 due to fragment A-type and associated water losses (Figure 1); (iii) an abundant ion at *m/z* 164 due to a B-type fragment (Figure 1).[11] We also reported that the *A. ostenfeldii* cultures contained a complex mixture of other, potentially new, spirolides. Unfortunately, the small amount of available material prevented us from fully assigning the structures of these new spirolides, even by MS/MS analyses. Therefore, large-scale culturing of *A. ostenfeldii* appeared to be an unavoidable step in order to isolate the new derivatives in larger amounts.

In the present paper we report on the extensive investigation of large cultures of Adriatic *A. ostenfeldii* and on preparative work that resulted in the isolation of the new 27-hydroxy-13,19-didesmethylspirolide C (**8**), whose structure was fully elucidated by LC-MS/MS and 1D and 2D NMR analyses.

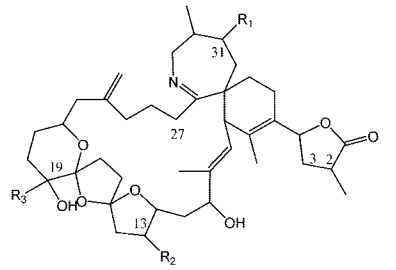
Results and Discussion

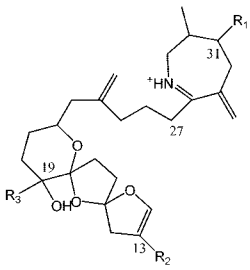
Identification of Known Spirolides in Cultured Adriatic *A. ostenfeldii.* Several batch cultures of *A. ostenfeldii* were combined and centrifuged in order to separate cell pellets from growth medium. The pellets were extracted by CH_3OH/H_2O (8:2), whereas the growth medium was concentrated by solid-phase extraction (SPE). The pellet extract and the SPE eluates were combined and directly analyzed by LC-MS. The chromatographic separation was carried out using a reversed-phase column and a buffered mobile phase, as suggested by Quilliam et al. for the analysis of various lipophilic toxins.[16] MS detection was accomplished by using a heated ESI linear ion trap MS instrument operating in data dependent (DD) scanning mode. DD experiments produce comprehensive data from a single analysis with a small amount of sample in a short time. In particular, we set the mass spectrometer to perform a full-scan MS experiment in the range *m/z* 150−1000 and subsequently an MS/MS scan on the most intense ions contained in the full-scan mass spectrum. DD experiments were initially carried out on a standard solution of **4** and showed that fragmentation of the pseudomolecular ion generated the expected water losses, A-type fragments but not the B-type fragment, which instead is formed when MS/MS experiments are performed on a triple-quadrupole MS. Table 1 reports DD results for compounds we identified as putative spirolides on the basis of their retention times, protonated ions, and fragmentation patterns.

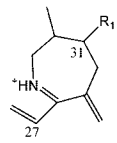
We observed a much higher number of spirolides than that reported in our previous paper[1] for this *A. ostenfeldii* extract. Some of the detected derivatives corresponded to those described by other authors on the basis of $[M + H]^+$ ions and fragmentation patterns,[9–11] while some others appeared to have never been reported so far. Particularly, a compound with a protonated ion at *m/z* 678.5 produced the most abundant LC-MS peak. Its fragmentation pattern resembled the recently described 13,19-didesmethylspirolide C (**5**)[9] in the presence of the following ion clusters in the MS/MS spectrum of the ion at *m/z* 678 recorded on a triple-quadrupole MS: (i) ions at *m/z* 660, 642, and 624 due to subsequent losses of three water molecules, respectively; (ii) ions at *m/z* 448,

* To whom correspondence should be addressed. Tel: +39 081 678507. Fax: +39 081 678552. E-mail: fattoru@unina.it and ciminiel@unina.it.
† Università di Napoli.
§ Università di Bologna.

10.1021/np0703242 CCC: $37.00
Published on Web 11/16/2007







A-type Fragments B-type Fragment

Spirolide	R_1	R_2	R_3	Δ	$[M+H]^+$ (*m/z*)	A-type Fragments (*m/z*)	B-type Fragment (*m/z*)
1 A	H	CH_3	CH_3	$\Delta^{2,3}$	692.5	462/**444**/426	150
2 B	H	CH_3	CH_3		694.5	462/**444**/426	150
3 C	CH_3	CH_3	CH_3	$\Delta^{2,3}$	706.5	476/**458**/440	164
4 13-desMeC	CH_3	H	CH_3	$\Delta^{2,3}$	692.5	462/**444**/426/408	164
5 13,19-didesMeC	CH_3	H	H	$\Delta^{2,3}$	678.5	448/**430**/412/394	164
6 D	CH_3	CH_3	CH_3		708.5	476/**458**/440	164
7 13-desMeD	CH_3	H	CH_3		694.5	462/**444**/426	164

Figure 1. Structures of major spirolides and related fragment ions contained in MS/MS spectra (boldfaced *m/z* values denote the most abundant peaks in each ion cluster).

Table 1. Protonated Molecules, Fragmentations Due to Subsequent Water Losses, Characteristic A-Type Fragments, and Associated Water Losses for a Number of Spirolides Contained in the Crude Extract of *Alexandrium ostenfeldii* Batch Cultures (boldfaced *m/z* values denote the most abundant peaks in each ion cluster)[a]

$[M + H]^+$ (*m/z*)	t_R (min)[b]	water losses (*m/z*)	A-type fragments (*m/z*)
596.5	6.95	**578**/560/542/524	448/**430**/412/394
596.5	6.95	**578**/560/542/524	**446**/428/410/392
660.5	8.85	**642**/624/606	530/**512**/494/476/458
678.5[c]	6.67	**660**/642/624	448/**430**/412/394
692.5	9.33	**674**/656/638/620	**444**/426/408
692.5[d]	7.04	**674**/656/638	462/**444**/426/408
694.5	6.43	**676**/658/640/622	464/**446**/428/410
706.5	7.25	**688**/670/652/634	**460**/442/424/406
708.5	6.91	**690**/672/654/636	478/**460**/442/424
710.5	6.17	**692**/674/656/638	**462**/444/426
710.5	5.90	**692**/674/656/638	490/**472**/454/436
734.5	7.32	**716**/698/680	504/**486**/468
734.5	7.32	**716**/698/680	476/**458**/440
750.5	6.98	**732**/714/696	520/**502**/484/466
764.5	7.53	**746**/728/710	534/**516**/498

[a] MS spectra were recorded on a Thermo Scientific heated ESI linear ion trap MS instrument LXQ operating in data-dependent scanning mode (positive ions). [b] Retention times are referred to the LC conditions used (see Experimental Section). [c] Compound identified as 13,19-didesmethylspirolide C on the basis of comparison of its MS/MS and NMR data with those reported in the literature.[9] [d] Compound identified as 13-desmethylspirolide C on the basis of comparison of its MS (retention time, fragmentation pattern, and ion ratios) and NMR data with those of an authentic sample.

430, 412, and 394 due to A-type fragments; (iii) an abundant ion at *m/z* 164 due to a B-type fragment. Unambiguous identification of this major component as 13,19-didesmethylspirolide C (**5**) required a large-scale isolation as well as a successive in-depth NMR investigation. In Table 2 we report NMR data of **5** recorded in CD_3OD and CD_3OD containing 0.1% TFA, respectively. In the acidic CD_3OD solution significant ^1H and ^{13}C NMR downfield shifts along the C-25/C-33 segment were detected. As it is already reported, this can be explained taking into account the protonation of the iminic nitrogen, which affects the spectroscopic properties of all the nearby nuclei.[17]

Two further major compounds were present in the crude extract at *m/z* 692.5 and 694.5, respectively. The first was identified as 13-desmethylspirolide C by comparison of its LC-MS properties (retention time, fragmentation pattern, and ion ratios) and NMR data with those of an authentic sample.[9] The compound at *m/z* 694.5 (**8**) appeared to be a potentially new spirolide. It was isolated in pure form, and its structure determination is described below.

Structure Elucidation of the Novel 27-Hydroxy-13,19-didesmethylspirolide C (8). High-resolution ESIMS data of **8** were consistent with an elemental composition of $C_{41}H_{59}NO_8$ ($[M + H]^+$ 694.43179 ± 0.0001, calcd 694.43189). The MS/MS spectrum of the $[M + H]^+$ ion at *m/z* 694.5 (Figure 2a) contained three characteristic ion clusters: (i) ions at *m/z* 676, 658, 640, and 622 due to subsequent losses of four water molecules, respectively; (ii) ions at *m/z* 464, 446, 428, and 410 due to A-type fragment and associated water losses; (iii) a peak at *m/z* 180 due to a B-type fragment. The fragmentation pattern of **8** paralleled that of 13,19-didesmethylspirolide C (**5**), and all of the peaks differed by only 16 mass units between the two spectra. Furthermore, an additional water loss from the protonated molecular ion occurred in the MS/MS spectrum of **8** versus that of **5**. The above data indicated that **8**, compared to **5**, contained an additional oxygen atom, in full agreement with the calculated molecular formula. MS/MS data allowed us to infer the position of the oxygenated group in the part of the structure producing a B-type fragment.

Results deriving from extensive 1D and 2D NMR analysis of **8** confirmed the above structural hypothesis and allowed us to precisely locate the oxygenated functionality in this new compound. In particular, interpretation of COSY and HOHAHA spectra allowed us to identify four spin systems, represented in bold lines in Figure 3. Through an HSQC experiment all the protons were associated to their relative carbons. In order to establish the location of the oxygenated functionality at C-27, which represents the structural innovation of **8** in comparison to **5**, we focused our attention on H_2-41 resonating at 4.78 (H-41a) and 4.80 (H-41b), respectively. In particular, H-41a, long-range coupled to H_2-25, led us to single out a spin system encompassing C-25, C-26, and C-27 (Table 2), in which, in comparison to **5**, an oxymethine functionality (δ_H 4.22 and δ_C 69.2) replaced the typical methylene group at C-27 common to all of the spirolides so far characterized. Some key HMBC

Table 2. ^{1}H and ^{13}C NMR Data of 13,19-Didesmethylspirolide C (**5**) in CD_3OD and CD_3OD Containing 0.1% of TFA, and 27-Hydroxy-13,19-didesmethylspirolide C (**8**) (CD_3OD) (boldfaced chemical shift values denote the major differences between compounds **5** and **8**; ^{13}C NMR resonances of **5** and **8**, respectively, were obtained through HSQC and HMBC spectra)

	5 (CD_3OD 0.1% TFA)		**5** (CD_3OD)		**8** (CD_3OD)	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		176.6		176.5		176.8
2		131.0		131.0		131.1
3	7.14	149.3	7.12	149.5	7.15	149.8
4	5.99	81.9	5.95	82.3	5.97	82.9
5		126.4		126.7		126.8
6		132.9		134.2		134.8
7	3.83	47.8	3.59	48.6	3.87	48.9
8	5.18	122.6	5.17	124.9	5.20	125.7
9		146.1		144.0		143.8
10	4.12	76.8	4.09	77.4	4.11	77.8
11	1.38, 2.29	45.5	1.37, 2.32	45.5	1.41, 2.33	45.7
12	4.36	79.6	4.31	81.0	4.35	80.2
13	1.70, 2.29	32.6	1.70, 2.30	32.6	1.77, 2.30	32.8
14	1.96, 2.29	37.5	1.93, 2.27	35.9	1.98, 2.29	35.1
15		118.0		117.8		118.0
16	1.95, 2.25	34.9	1.93, 2.23	35.0	1.92, 2.22	35.0
17	2.08, 2.14	35.2	2.07, 2.10	34.3	2.06, 2.12	35.6
18		109.7		111.5		114.4
19	3.37	71.6	3.33	71.7	3.37	71.7
20	1.57, 1.72	29.3	1.58, 1.68	28.6	1.56, 1.71	27.4
21	1.21, 1.72	31.0	1.19, 1.66	30.8	1.16, 1.68	31.0
22	3.86	69.5	3.91	70.6	3.94	71.7
23	2.07, 2.38	46.1	2.01, 2.32	47.0	2.06, 2.35	47.2
24		145.4		145.8		147.9
25	**1.87, 1.89**	**35.2**	**1.56, 2.12**	**35.1**	**1.66, 1.89**	**33.2**
26	**1.87, 1.96**	**22.5**	**1.38, 2.00**	**23.0**	**1.45, 2.42**	**31.5**
27	**2.89, 3.10**	**36.1**	**2.32, 2.32**	**35.3**	**4.22**	**69.2**
28		202.7		178.0		177.1
29		52.5		50.6		50.8
30	1.83, 2.05	36.4	1.58, 1.74	38.3	1.61, 1.68	38.1
31	1.05	37.4	1.11	36.9	1.06	37.0
32	1.70	38.6	1.38	41.3	1.44	41.2
33	3.56, 4.24	51.6	3.45, 3.78	53.1	3.57, 3.89	53.9
34	1.70, 1.96	32.3	1.72, 1.96	33.0	1.87, 1.96	33.4
35	1.69, 2.28	20.1	1.70, 2.27	20.2	1.55, 2.14	20.1
36	1.92	10.4	1.92	10.4	1.94	10.5
37	1.74	16.7	1.73	17.1	1.77	17.2
38	1.92	12.6	1.88	12.6	1.93	12.5
39						
40						
41	4.92, 4.95	113.1	4.74, 4.78	111.2	4.78, 4.80	111.3
42	1.07	18.7	1.05	18.7	1.05	18.6
43	1.13	20.0	1.01	20.0	1.01	19.7

correlations along with some significant ROE correlations, all depicted in Figure 3, were conclusive to connect the four spin systems, thus defining the planar structure of **8** (Figure 4).

LC-MS/MS Structural Investigation of Minor Spirolides. Most of the spirolides reported in Table 1 were obtained in too small quantity for NMR investigation; therefore, they were investigated only through MS/MS analyses. Product ion spectra were recorded on a triple-quadrupole MS by selecting the most abundant ions (*m/z* 692, 708, 710, 734, 750, and 764) as precursor ion (Figure 5). Analysis of the fragmentation pattern for each component suggested that they were potentially new spirolides.

Particularly, a compound with a protonated ion at *m/z* 692.5 (t_R 9.33 min) (Figure 5a), an isobaric analogue of **4**, eluted 2.3 min later and paralleled **4** in A-type fragments but presented a different B-type fragment (at *m/z* 178 versus *m/z* 164 in **4**). Tentatively, we could hypothesize that an extra methyl or methylene group was present in the partial structure producing the B-type fragment along with a concurrent lack of either of these two groups in the region ranging from C-12 to C-25.

A compound at *m/z* 708.5 (t_R 6.91 min) (Figure 5b) possessed the same B-type fragment as **8** but presented A-type fragments shifted to 14 amu smaller. This suggested that an extra methyl or methylene group is present in the region encompassing C-12/C-25, likely a methyl at position C-13 or C−19, referring to the most common spirolide skeletons.[11]

A compound at *m/z* 710.5 (t_R 6.17 min) (Figure 5c) showed the same B-type fragment as **8**, but its molecular weight and A-type fragments were shifted to 16 mass units smaller. Thus, it likely contains an additional oxygenated function in the region C-12/C-25.

Analysis of the MS/MS spectrum of the ion at *m/z* 734.5 (t_R 7.32 min) (Figure 5d) showed that it was actually a mixture of two compounds presenting the same $[M + H]^+$ ion and the same B-type fragment ion at *m/z* 164, but two different A-type fragment clusters at *m/z* 504/486/468 and *m/z* 476/458/440, respectively.

A compound at *m/z* 750.5 (t_R 6.98 min) (Figure 5e) presented a molecular weight that differed from that of 8 by 56 amu. The structural modification is likely in the moiety producing a B-type fragment (*m/z* 236) because it also differed by 56 amu from the corresponding ion in **8** (*m/z* 180).

A compound at *m/z* 764.5 (t_R 7.53 min) (Figure 5f) paralleled that at *m/z* 750.5 in B-type fragments but presented A-type fragments shifted to 14 amu smaller.

HRMS experiments are needed to provide better structural hypotheses for these compounds, and conclusive structural assignments might be achieved only through an NMR investigation, which requires more of each pure spirolide. To this purpose, a large-scale culturing of *A. ostenfeldii* is currently in progress in our laboratories.

Experimental Section

Chemicals. All organic solvents were of distilled-in-glass grade (Carlo Erba, Milan, Italy). Water was distilled and passed through a MilliQ H_2O purification system (Millipore Ltd., Bedford, MA). Formic acid (95–97%, laboratory grade) and ammonium formate (AR grade) were purchased from Sigma Aldrich (Steinheim, Germany). Spirolide 13-desMeC standard solution was kindly provided by Dr. Michael A. Quilliam (Institute for Marine Biosciences, National Research Council of Canada, Halifax, NS, Canada).

Cultures of *Alexandrium ostenfeldii*. *Alexandrium ostenfeldii* (Paulsen) Balech et Tangen (1985)[12] was collected in the northwestern Adriatic Sea along the Emilia-Romagna coasts (Italy) in November 2003. The dinoflagellate was isolated by the capillary pipet method,[13] and after an initial growth in microplates, unialgal cultures were grown in sterile Erlenmeyer flasks sealed with cotton plugs at 20 °C under a 16:8 h L/D cycle (ca. 90 μmol m^{-2} s^{-1} from cool white lamps); nutrients were added at the f/2 concentration,[14] and H_2O salinity was adjusted to 30 practical salinity units (psu, which corresponds to grams of salts per liter of solution). For toxicity studies, *A. ostenfeldii* was cultured in 2 L Erlenmeyer flasks each containing 1.5 L of medium. Cells from a total culture volume of 20 L were collected at stationary phase on the 30th day of growth first by gravity filtration through 0.45 μm Millipore filters and then by centrifugation at 3000*g* for 15 min at 10 °C. Both cell pellets and growth mediums were saved for analyses. Cell counts were made every other day in settling chambers by the Utermöhl method.[15]

Sample Extraction and Cleanup. Cultured cell pellets (18×10^7 cells) were suspended in a MeOH/H_2O (8:2, v/v) solution (20 mL) and sonicated for 5 min in pulse mode while cooling in an ice bath. The mixture was centrifuged at 5000 rpm for 10 min, and the pellet was washed twice with 20 mL of MeOH/H_2O (8:2, v/v). The supernatants were combined, and the volume was adjusted to 60 mL with the extraction solvent. The growth medium fraction (20 L) was separately filtered through 0.45 μm filters (Millipore, Molsheim, France). The filtrate was loaded on Sep-Pak C-18 plus cartridges (Waters Corporation, Milford, MA) equilibrated with H_2O. The columns were eluted each with 10 mL of H_2O, 10 mL of H_2O/CH_3CN (7:3, v/v), 10 mL of H_2O/CH_3CN (1:1, v/v), and 10 mL of CH_3CN. The pellet extract and all the SPE eluates were combined up to a total volume of 500 mL. A 1 mL aliquot was saved for LC-MS analyses, and the remaining part was evaporated to dryness. The residue was dissolved in 4 mL of H_2O/CH_3CN (9:1, v/v), and the solution was loaded on a ODS column equilibrated with the same solution. The column was sequentially eluted with 150 mL of H_2O/CH_3CN (9:1, 7:3, 1:1, v/v) solutions and CH_3CN

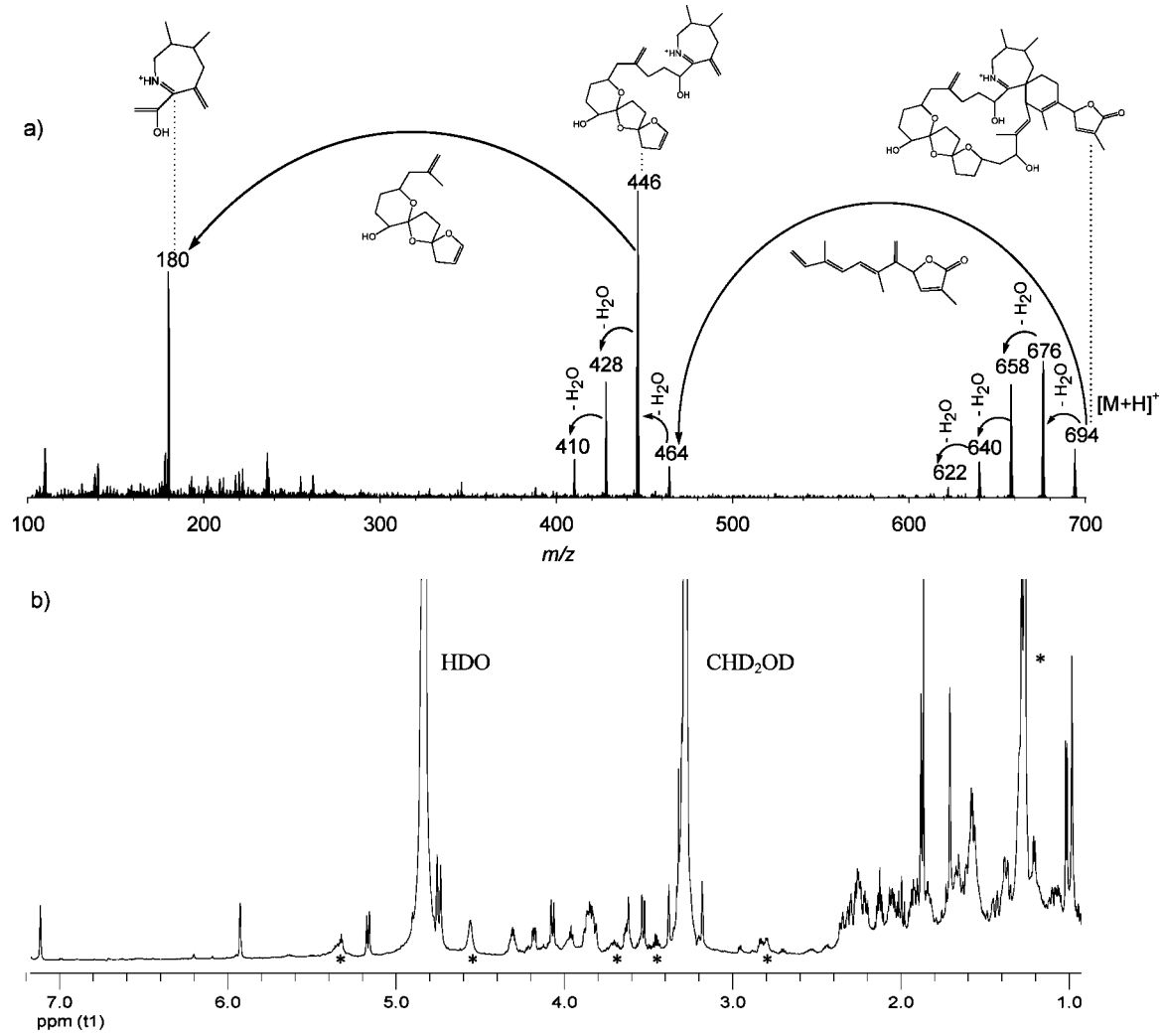


Figure 2. MS/MS spectrum (a) and ^{1}H NMR spectrum (b) of 27-hydroxy-13,19-didesmethylspirolide C (**8**). * indicates impurities.

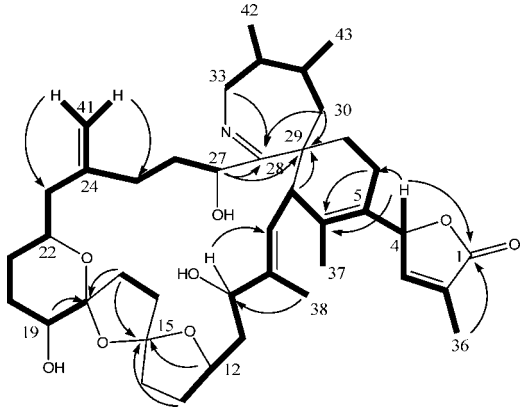


Figure 3. Bold lines represent the four spin systems detected in a HOHAHA experiment of 27-hydroxy-13,19-didesmethylspirolide C. Arrows highlight key HMBC correlations conclusive for assigning the planar structure of **8**.

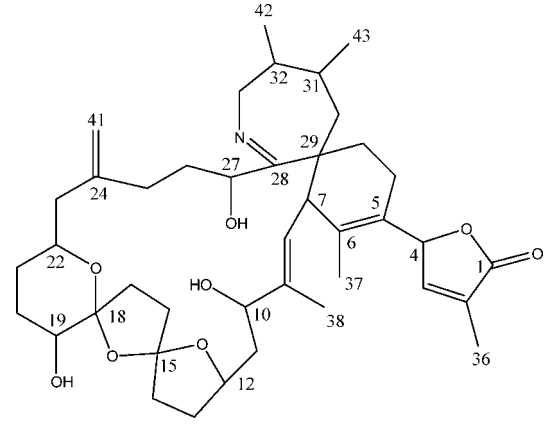


Figure 4. Planar structure of 27-hydroxy-13,19-didesmethylspirolide C (**8**).

100%. The spirolides eluted in the H_2O/CH_3CN (1:1, v/v) and CH_3CN 100% fractions. The successive cleanup step was carried out by injecting the spirolide-containing fractions on a 10 μm Luna C18 250 × 10 mm column (Phenomenex, Torrance, CA) isocratically eluted with H_2O/CH_3CN (8:2, v/v) and 0.1% trifluoroacetic acid. The flow rate was 2 mL min^{-1} (UV detector, 210 nm). Final purification was accomplished by a 3 μm Hypersil C8 BDS, 50 × 4.60 mm column (Phenomenex, Torrance, CA) eluted with H_2O (eluent A) and CH_3CN (eluent B). A gradient elution was used, namely, 10% to 30% B in 4 min, 30% to 80% B in 24 min, 80% to 100% B in 4 min, 100% B for 15 min. The flow rate was 750 μL min^{-1} (UV detector, 210 nm). Finally, 30 μg of **8**, 23 μg of **5**, and 18 μg of **4** were obtained, respectively. Quantification of these three compounds was accomplished by NMR analysis employing an internal standard (see below). LC-MS analyses were carried out throughout the purification procedure in order to check for the presence of spirolides in each fraction.

Liquid Chromatography–Mass Spectrometry. LC-MS analyses were performed by using a 3 μm Hypersil C8 BDS, 50 × 2.00 mm column (Phenomenex, Torrance, CA) at room temperature. Eluent A

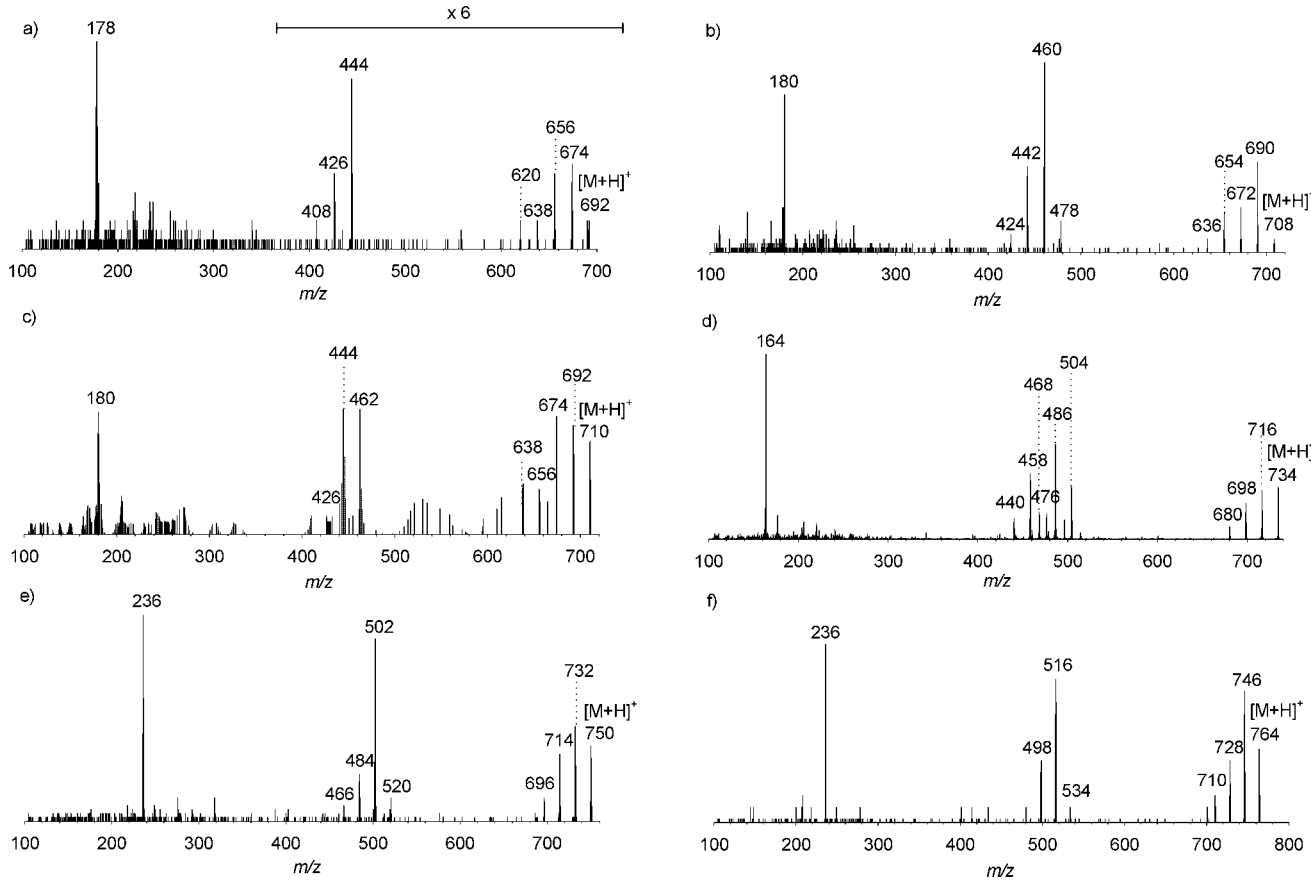


Figure 5. MS/MS spectra of minor spirolides from *A. ostenfeldii*. See text for a discussion of candidate structures.

was H_2O and B was a 95% CH_3CN/H_2O solution, both eluents containing 2 mM ammonium formate and 50 mM formic acid. The flow rate was 200 μL min^{-1}. A gradient elution (10% to 100% B in 10 min followed by 100% B for 15 min) was used. Mass spectroscopic experiments on the crude extract were performed by using an LXQ heated ESI linear ion trap MS instrument (Thermo Scientific, San Jose, CA) coupled to an Agilent (Palo Alto, CA) model 1100 LC. Data-dependent scanning experiments (positive ions) were performed in the range *m/z* 150–2000 using a capillary temperature of 300 °C, a capillary voltage of 49 V, and a collision energy of 35%. Dynamic exclusion was used. MS experiments throughout the purification procedure were carried out on an API-2000 triple-quadrupole mass spectrometer equipped with a turbo-ionspray source (Applera, Thornhill, ON, Canada) coupled to an Agilent (Palo Alto, CA) model 1100 LC. Full-scan mass spectra (positive ions) were recorded in the range *m/z* 500–800 using a temperature of 100 °C, an ionspray voltage of 5400 V, a declustering potential of 90 V, a focusing potential of 230 V, and an entrance potential of 12 V. A collision energy of 53 eV and a cell exit potential of 13 V were used in MS/MS experiments.

NMR. NMR spectra were measured on a Varian Unity Inova 700 spectrometer equipped with a 13C Enhanced HCN cold probe. Shigemi 5 mm NMR tubes and CD_3OD as an internal standard (δ_H 3.31 and δ 49.0) were used. Standard Varian pulse sequences were employed for the respective classes of spectra; solvent signal suppression by presaturation was used when required. Assignments were checked from 2D ^{1}H COSY, HOHAHA, ^{1}H/^{13}C HSQC, and HMBC spectra. Quantification of **4**, **5**, and **8** was carried out by running an ^{1}H NMR spectrum for each of the three spirolides employing pyridine as an internal standard. The ^{1}H NMR spectrum for each spirolide was obtained setting a d1 value at 7.0 s in order to allow a complete relaxation of the pyridine standard to equilibrium. The T_1 (0.70 s) of pyridine was evaluated through the inversion–recovery T_1 experiment. The amount of each spirolide was then quantified utilizing the area per proton (determined by integration). In particular, quantification of each spirolide was carried out measuring the area of the well-isolated olefinic protons at C-3.

27-Hydroxy-13,19-didesmethylspirolide C (8). ^{1}H NMR data are reported in Table 2. HR-ESIMS (positive mode) *m/z* 694.43179, calcd for $C_{41}H_{59}NO_8$ $[M + H]^+$ 694.43189. The degree of purity of **8** (>90%) was evaluated on the basis of both HPLC and NMR analysis.

Acknowledgment. This work is a result of research supported by the EU project BIOTOX marin 2005. LC-MS experiments were performed at "Centro di Servizi Interdipartimentale di Analisi Strumentale", Università degli Studi di Napoli "Federico II". The assistance of the staff is gratefully appreciated.

References and Notes

(1) Ciminiello, P.; Dell'Aversano, C.; Fattorusso, E.; Magno, S.; Tartaglione, L.; Cangini, M.; Pompei, M.; Guerrini, F.; Boni, L.; Pistocchi, R. *Toxicon* **2006**, *47*, 597–604.
(2) Cembella, A. D.; Lewis, N. I.; Quilliam, M. A. *Nat. Toxins* **1999**, *7*, 197–206.
(3) Mackenzie, L.; White, D.; Oshima, Y.; Kapa, J. *Phycologia* **1996**, *35*, 148–155.
(4) Cembella, A. D.; Quilliam, M. A.; Lewis, N. I. *Phycologia* **2000**, *39*, 67–74.
(5) 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**, *20*, 2159–2161.
(6) Richard, D.; Arsenault, E.; Cembella, A.; Quilliam, M. A. In *Intergovernmental Oceanographic Commission of UNESCO: Harmful Algal Blooms*; Hallegraeff, G. M., Blackburn, S. I., Bolch, C. J., Lewis, R. J., Eds.; 2000; pp 383–386.
(7) 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.
(8) Sleno, L.; Chalmers, M. J.; Volmer, D. A. *Anal. Bioanal. Chem.* **2004**, *378*, 977–986.
(9) MacKinnon, S. L.; Walter, J. A.; Quilliam, M., A.; Cembella, A. D.; LeBlanc, P.; Burton, I. W.; Hardstaff, W. R.; Lewis, N. I. *J. Nat. Prod.* **2006**, *69*, 983–987.
(10) Aasen, J.; MacKinnon, S. L.; LeBlanc, P.; Walter, J. A.; Hovgaard, P.; Aune, T.; Quilliam, M. A. *Chem. Res. Toxicol.* **2005**, *18*, 509–515.

(11) Sleno, L.; Windust, A. J.; Volmer, D. A. *Anal. Bioanal. Chem.* **2004**, *378*, 969–976.
(12) Balech, E.; Tangen, K. *Sarsia* **1985**, *70*, 333–343.
(13) Hoshaw, R. W.; Rosowski, J. R. In *Handbook of Phycological Methods. Culture Methods and Growth Measurements*; Stein, J. R., Ed.; Cambridge University Press: New York, 1973; pp 53–67.
(14) Guillard, R. R. L. In Smith, W. L., Chanley, M. H., Eds. *Culture of Marine Invertebrates Animals*; Plenum Press: New York, 1975; pp 26–60.
(15) Utermöhl, H. *Mitt. Int. Verein. Limnol.* **1958**, *9*, 1–38.
(16) Quilliam, M. A.; Hess, P.; Dell'Aversano, C. In *Mycotoxins and Phycotoxins in Perspective at the Turn of the Millenium*; deKoe, W. J., Sampson, R. A., van Egmond, H. P., Gilbert J., Sabino, M., de Koe, W. J., Eds.; Wageningen: The Neatherlands, 2001; pp 383–391.
(17) 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.

NP0703242