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Detection and Identification of Spirolides in Norwegian Shellfish and Plankton

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Mussels sampled in the spring of 2002 and 2003 from Skjer, a location in Sognefjord, Norway, tested positive in the mouse bioassay for lipophilic toxins. The symptoms, which included cramps, jumping, and short survival times (as low as 4 min), were not characteristic of toxins previously observed in Norway. A survey of the algae present at the aquaculture sites showed that the toxicity correlated with blooms of *Alexandrium ostenfeldii*. Up to 2200 cells/L were found at the peak of one bloom. In Canadian waters, this alga is known to be a producer of the cyclic imine toxins, spirolides. Analysis of mussel extracts from Skjer in the spring of 2002 and 2003, using liquid chromatography tandem mass spectrometry, revealed the presence of several new spirolides. The same compounds were also found in algal samples dominated by *A. ostenfeldii*, which had been sampled from Skjer in February 2003. A large-scale extraction of mussel digestive glands and chromatographic fractionation of the extracts allowed the isolation and structure elucidation of the main spirolide, 20-methyl spirolide G, with a molecular weight of 705.5. This is the first confirmed occurrence of spirolides in mussels and plankton from Norway.

Introduction

After the discovery in the mid 1990s of unknown fast-acting toxin(s) in the Sognefjord, Skjer location (*1*, *2*), a routine monitoring program was set up to study this occurrence. Biweekly, samples of mussels (*Mytilus edulis*) were obtained and analyzed with the mouse bioassay for diarrhetic shellfish poisoning (DSP) toxins, which uses a chloroform partitioning procedure (*3*, *4*). Fast-acting toxin(s) did not show up again until April 2002 and in late January−April 2003. In the mouse bioassay, the mussel extracts gave survival times as low as 4 min, similar to the acute toxicity of paralytic shellfish poisoning (PSP) toxins. This was not characteristic for any of the known lipophilic toxins, which had previously been found to be present in the samples. The symptoms in the mice included jumping on all four legs, arching of the back so that the tail almost touched the nose, followed by death within minutes.

Analysis of lipophilic toxins during the episode in 2002 showed the presence of only minor amounts of okadaic acid (OA), dinophysistoxin-1 (DTX1), and yessotoxin (YTX) (<50 μg/kg shellfish meat), while azaspiracids (AZA) and pectenotoxins (PTX) were not found. At the time the shellfish grounds in this area had been closed due to PSP toxin levels slightly above regulatory levels (0.8 mg STX equivalents per kg shellfish meat). In 2003, DTX1 and PTX2 were present in amounts above the regulatory limit (*5*) in the mussels, but the survival time upon intraperitoneal (IP) injection in mice for these toxins is usually not less than 40−50 min for DTX1 and for PTX-2 more than 4 h (*1*, *6*). No people were reported ill on either occasion, but the location had already been closed due to presence of PSP or DSP toxins.

The occurrence of the fast-acting toxins in mussels correlated with the bloom of algae in this area, which was comprised mainly of *Alexandrium* spp., along with *Dinophysis* spp. and *Protoceratium reticulatum*. *Alexandrium ostenfeldii* was the main species peaking at 2200 cells/L on April 8, 2003. Since the known lipophilic toxins at this location could not explain the toxicity, the presence of *A. ostenfeldii* was regarded as a possible source of the major toxic component in the mussels. In Canada, *A. ostenfeldii* is known to produce the fast-acting toxins **1**−**6** (Figure 1) known as spirolides (*7*, *8*), and the reported symptoms in mice were quite similar to those observed in the current event. Besides Canada, *A. ostenfeldii* have been found in the North Sea and in Denmark to produce spirolides, including **7** (*9*, *10*). Initial toxicological studies have shown that spirolides are highly toxic in mice through IP injections, while their oral toxicity is more than 1 order of magnitude lower (*11*). Furthermore, one of the toxins in the spirolide family, 13-desmethyl spirolide C **5**, has been shown to induce dose-dependent neurotoxicity toward mice and rats upon IP injections (*12*).

This paper presents the results of our investigation, which confirmed that spirolides were present in both Norwegian mussel tissue and algal biomass collected concurrently and that they account for the observed

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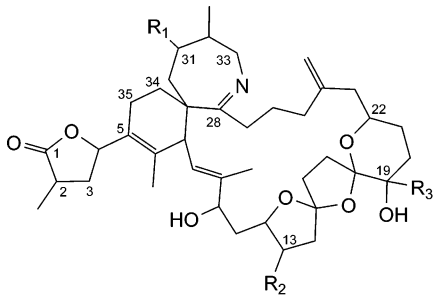
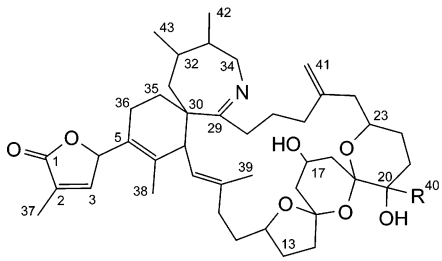


R_1	R_2	R_3	$\Delta^{2,3}$	MW		
H	CH_3	CH_3	✓	691.5	**1**	A
H	CH_3	CH_3	--	693.5	**2**	B
CH_3	CH_3	CH_3	✓	705.5	**3**	C
CH_3	CH_3	CH_3	--	707.5	**4**	D
CH_3	H	CH_3	✓	691.5	**5**	13-desMe-C
CH_3	H	H	✓	677.5	**6**	13,19-didesMe-C



R	MW		
H	691.5	**7**	G
CH_3	705.5	**8**	20-Me-G

Figure 1. Structures of some known cyclic imine toxins.

toxicity. The structural elucidation of a new spirolide in the samples, 20-methyl spirolide G **8**, is also reported.

Materials and Methods

Chemicals. Acetonitrile (high-performance liquid chromatography (HPLC) grade), methanol (HPLC grade), chloroform (HPLC grade), dichloromethane, formic acid, hexane, and ammonium formate were purchased from Fisher Scientific. Deionized water from a Milli-Q system (Millipore) was used in all preparations and manipulations requiring aqueous solutions.

Mussel Material. More than 75 kg of blue mussels (*Mytilus edulis*) were collected from Skjer, Norway in April 2002. The mussels were immediately steamed and shucked to yield 20 kg of whole mussel meat. Dissection of whole mussel material yielded 3.8 kg of digestive glands, which were then stored at −20 °C.

Algal Material. Algal material was obtained via filtration of seawater containing an algal bloom, first through a 90-μm filter and next through a 20-μm filter. The resulting algal mass used for this study contained organisms falling within a 20−90-μm size range. Samples were collected over several days resulting in the collection of $3-10 \times 10^6$ cells of *A. ostenfeldii* each day. The cells were harvested from the 20-μm filter using methanol containing 0.1% formic acid and the resultant algal material was stored in this methanol solution.

Mouse Bioassay. NMRI Outbreed (Taconic, M&B, Denmark) female mice (18−20 g), were used for testing with IP injection of lipophilic extract of mussels (*3*, *4*). All animals were treated according to Norwegian law on research animals.

Preparation of Samples for Liquid Chromatography Mass Spectrometry (LC-MS). Samples for LC-MS analysis were prepared by extracting 20 g of thawed mussel digestive glands with 80 mL of 80% methanol. Homogenization (Brinkmann Polytron, Westbury, NY) of the sample was undertaken at 10 000 rpm for 3 min and was followed by centrifugation, in 50-mL plastic centrifuge tubes, at 3800 g for 10 min. The supernatant was collected and the resulting pellets re-extracted using 10 mL of 80% methanol. Water (20 mL) was added to the combined supernatants. The resultant aqueous phase was then partitioned twice against 120 mL of $CHCl_3$. The combined $CHCl_3$ layers were evaporated to dryness using a rotary evaporator equipped with a dry ice/acetone cooled coldfinger. Last, the residues were dissolved in 1 mL of methanol (containing 0.1% formic acid), and subjected to LC-MS analysis.

LC-MS/Mass Spectrometry (MS) Methods. LC-MS/MS analyses were performed using an Agilent 1100 series LC (Palo Alto, CA) connected to an API 4000 (SCIEX, Streetville, ON, Canada) triple quadrupole MS system equipped with a Turbo ion spray source. Separations were performed on a 2 mm × 150 mm reversed-phase column packed with 3 μm BDS−Hypersil C8-silica (Thermo/Keystone, Bellefonte, PA). The mobile phase consisted of two components: water (A) and acetonitrile−water (95:5) (B), both containing 50 mM formic acid and 2 mM ammonium formate. A flow rate of 200 μL/min was used, with isocratic elution at 35% B over 40 min (*13*). The MS was operated in different MS/MS scan modes: product ion scan, precursor ion scan, or selected reaction monitoring (SRM). Product ion spectra were acquired with ESI voltage and nebulizer gas temperature set at 5000 V and 275 °C, respectively. Spectra were acquired in positive ion mode by colliding the Q1 selected precursor $[M + H]^+$ ion with nitrogen in the Q2 operated in radio frequency only mode and scanning the second quadrupole mass spectrometer, Q3, from *m/z* 50−736. The collision energy was set at 55 V. Precursor ion spectra were acquired with the same settings, except Q1 was scanned from *m/z* 400−800 while Q3 monitored a set precursor ion. The SRM mode used the same settings as the product ion scan, except that Q3 monitored a selected product $[M + H]^+$ ion. Accurate mass measurements were performed with the same HPLC system and column as above coupled to a QStar Pulsar mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON). Atmospheric pressure ionization was achieved with a nebulizer-assisted electrospray source operated in the positive ion mode with a needle voltage of 3500 V. A reference reagent solution of 9 μM bradykinin and 88 μM cesium acetate in water, pumped at a flow rate of 5 μL/min with a syringe pump, was mixed with the column effluent coaxially in the sheath flow arrangement of the source. Spectra were acquired with the time-of-flight analyzer over *m/z* 100−1600.

Isolation of 20-Methyl Spirolide G (8). The digestive glands (2.79 kg after thawing) of mussels obtained in 2002 from Skjer, Norway, were extracted with methanol to yield a dark-colored extract, which was concentrated and subjected to a spirolide isolation scheme similar to that previously reported (*13*). Briefly, the resuspended extract was partitioned against hexane and subsequently extracted with dichloromethane. The concentrated dichloromethane layer was then subjected to silica gel column chromatography with a gradient elution using dichloromethane/methanol. The spirolide-containing fractions were pooled and repetitively subjected to both flash C18 column and Sephadex LH-20 column chromatography. Final purification of 20-methyl spirolide G 7 was accomplished using a 10 mm × 250 mm column packed with 5 μm Vydac 201TP C18-silica (Mandel Scientific Company Ltd., ON, Canada), which was eluted with acetonitrile/water with 0.1% trifluoroacetic acid.

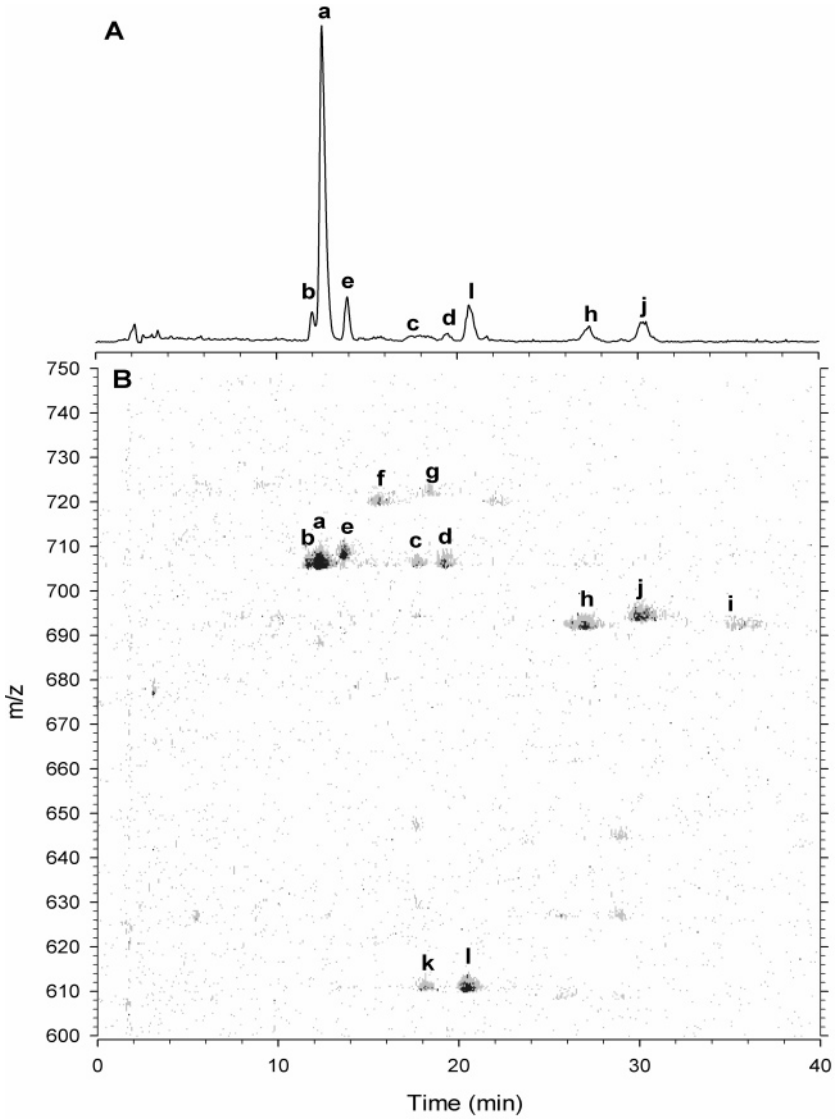


Figure 2. Precursor ion scan (product ion = *m/z* 164) presented as (A) total ion chromatogram (TIC) and (B) contour plot with intensity of spots representing abundance of ions. Peak letters are given in Table 1.

Approximately 504 μg of pure compound was isolated for spectroscopic examination.

NMR Spectroscopy. All NMR measurements were collected on a Bruker DRX-500 spectrometer (Bruker, Canada) at 500.13 MHz (^{1}H) and 125.77 MHz (^{13}C). The sample, 20-methyl spirolide G, was dissolved in 0.5 mL of CD_3OH and run at 20 °C. Spectra were referred to CHD_2OH at 3.3 ppm (^{1}H) or $^{13}CD_3$-OH at 49.0 ppm (^{13}C). Standard Bruker pulse sequences were used with solvent suppression by presaturation where appropriate. The ^{1}H and ^{13}C NMR signal assignments were based on chemical shift comparisons with known spirolides together with data from 2D ^{1}H−^{1}H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and ^{13}C−^{1}H heteronuclear single-quantum coherence experiments (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments.

Results and Discussion

The mouse bioassay performed on the samples from Skjer in 2002 indicated the presence of fast acting toxin(s). Rapid death times in the lipophilic mouse bioassay and neurotoxic symptoms led to further investigation of this occurrence. At this time, no other lipophilic toxins were present in amounts above regulatory limits (by LC-MS) or in amounts that could explain the toxicity found in the mouse bioassay. The first chemical data indicating the possible presence of spirolides were obtained in 2002 (unpublished results), when Norwegian mussel extracts revealed the possible presence of several new spirolides after LC-MS/MS analyses using two multi-toxin methods (*14*, *15*). This work was continued with preparative isolation in 2003 and thorough MS/MS elucidation of all possible spirolides in the sample.

The known spirolides can be divided into two classes, A/B and C/D (Figure 1), with the difference being the presence of one or two methyl substituents, respectively, on the cyclic imine ring. The two classes show characteristic fragment ions in their product ion spectra, either at *m/z* 150 for the A/B class or at *m/z* 164 for the C/D class (*16*). These ions were therefore used as indicators of the presence of spirolides using the precursor ion scanning method. The results of one analysis (precursors of *m/z* 164) are shown in Figure 2 where the data are presented in both a contour plot and a total ion chromatogram (TIC). The presence of possible spirolides of the C/D class can be observed as spots of variable intensity corresponding to ion abundance on the X/Y plane. There were no observable precursors of *m/z* 150 in the sample, thus indicating the absence of spirolides of the A/B class.

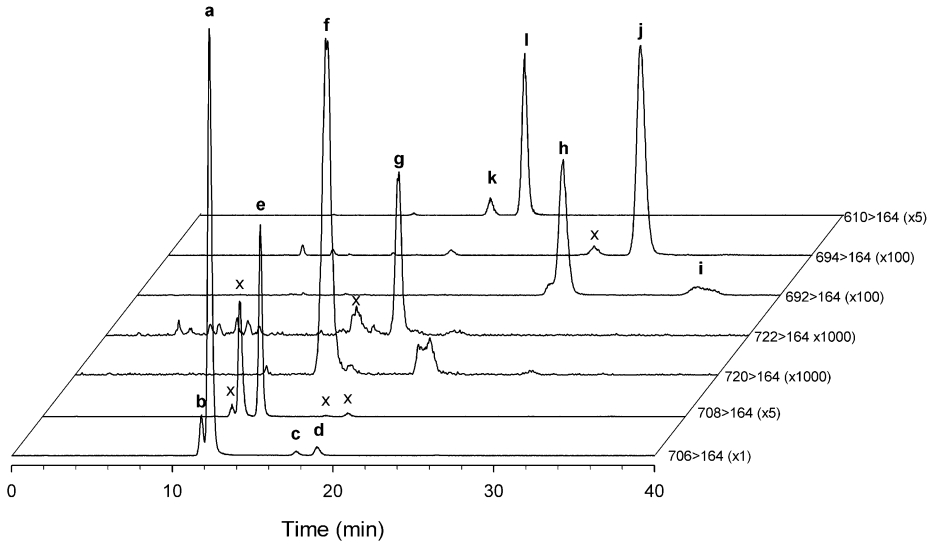


Figure 3. Chromatogram for LC-MS analysis of mussel tissue extract using SRM. Peak letters are given in Table 1. *x* = signals from ^{13}C isotopes of other compounds.

Table 1. List of Compounds Observed in Mussel Sample with Codes, Retention Times, Concentrations, Molecular Weights, and MS/MS Data

peak no.	RT (min)	concentration (μg/ kg)[a]	mass (Da)	$[M + H]^+$ ions observed in product ion spectra[b] *m/z* (%RI)[c]									
a	12.1	44	705.5	706 (20)	688 (100)	670 (75)	652 (20)	392 (60)	374 (40)	346 (80)	258 (15)	164 (80)	
b	11.8	1	705.5	706 (20)	688 (100)	670 (75)	652 (20)	392 (60)	374 (40)	346 (80)	258 (15)	164 (80)	
c	17.6	9	705.5	706 (90)	688 (100)	670 (40)	652 (25)	478 (20)	392 (15)	374 (15)	346 (15)	164 (20)	
d	19.0	14	705.5	706 (90)	688 (100)	670 (40)	652 (25)	478 (10)	392 (10)	374 (5)	346 (10)	164 (15)	
e	13.5	4	707.5	708 (20)	690 (100)	672 (60)	654 (20)	392 (45)	374 (30)	346 (60)	258 (10)	164 (60)	
f	15.6	2	719.5	720 (50)	702 (100)	684 (45)	666 (15)	472 (30)	454 (25)	358 (15)	346 (10)	164 (85)	
g	18.2	1	721.5	722 (50)	704 (100)	686 (45)	668 (15)	472 (30)	454 (25)	358 (15)	346 (10)	164 (85)	
h	26.5	5	691.5	692 (20)	674 (75)	656 (100)	638 (75)	426 (65)	292 (60)	274 (15)	248 (40)	230 (50)	164 (75)
i	35.1	9	691.5	692 (80)	674 (100)	656 (70)	648 (10)	638 (25)	630 (15)	292 (15)	164 (15)		
j	29.4	11	693.5	694 (20)	676 (75)	658 (100)	640 (75)	426 (65)	292 (60)	274 (15)	248 (40)	230 (50)	164 (75)
k	18.0	1	609.5	610 (20)	592 (35)	574 (20)	358 (65)	164 (100)					
l	20.2	2	609.5	610 (15)	592 (20)	574 (15)	556 (5)	358 (80)	164 (100)				

[a] Concentration in whole mussel tissue used for prep isolation work. [b] Masses are truncated. [c] Percent relative intensity.

The masses discovered in the precursor ion scan were then monitored in a separate LC-MS run using the SRM mode, which is more sensitive and useful for detection of minor isomeric peaks. The chromatogram from that analysis, shown in Figure 3, revealed the presence of 12 putative spirolides (peaks **a**−**l**) of nominal molecular weights of 609, 691, 693, 705, 707, 719, and 721. Analyses were also performed using product ion scanning of the different $[M + H]^+$ ions (Table 1). The spectra of the major peaks are presented in Figure 4**b**−**e**.

Analysis of the MS/MS fragmentation patterns for each component suggested the presence of several possible spirolide structures. In Figure 4b, the product ion spectrum of *m/z* 706.5 of the main compound, **8** (peak **a**), indicated a structural difference from most of the previously reported spirolides. The fragmentation pattern (Figure 4a) however was very similar to that of spirolide G **7**, reported recently in a Danish strain of *A. ostenfeldii*, which contains a novel 5:6:6 trispiroketal ring system (*10*). Analysis of the product ion spectra of **7** (Figure 4a) and **8** (Figure 4b) revealed a shift of 14 Da between the following **7** → **8** ions at *m/z* 332 → 346, 334 → 348, 360 → 374, 378 → 392, 656 → 670, 674 → 688, and 692 → 706. This difference suggested that **8** was a spirolide G analogue containing an additional methyl group. Possible placement of this methyl group was postulated based on the established fragmentation pattern of **7** (Figure 5) (*10*). First, the methyl group was known not to be present on the cyclic imine fragment of **8** because of the presence of the *m/z* 164 fragment ion which confirmed the presence of a cyclic imine fragment containing 2 vicinal methyl groups. The difference of 14 Da first appeared in fragments of *m/z* 346 and 348 in **8**. This occurrence strongly suggested that the extra methyl group must therefore be present between C-20 and C-26. Comparisons of other spirolide structures and analysis of the location of methyl

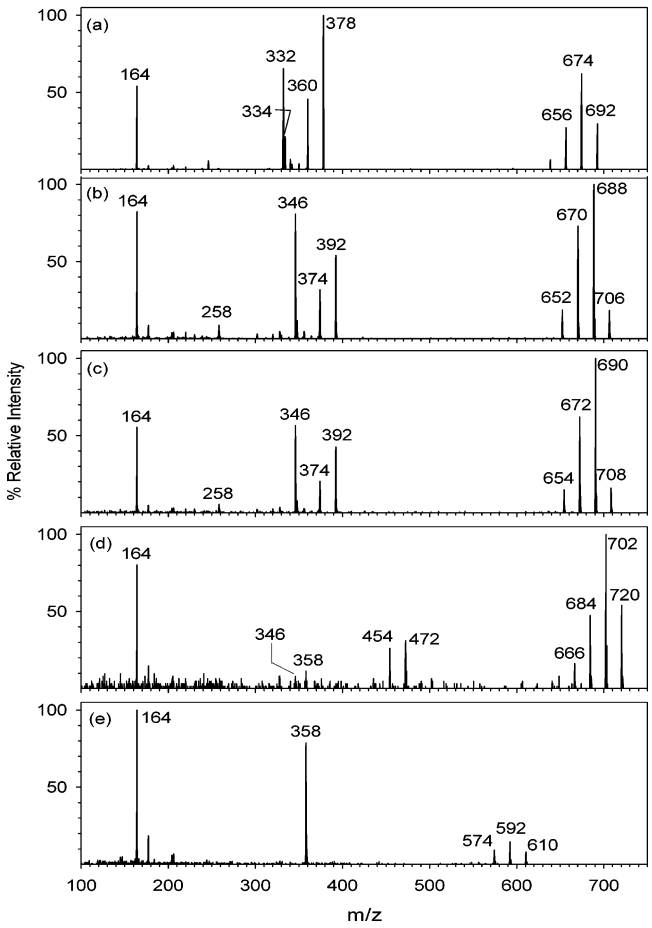


Figure 4. MS/MS product ion spectra of: (a) spirolide G **7**; (b) 20 methyl-spirolide G **8**; (c) peak **e**; (d) peak **f**; (e) peak **l**.

groups in them suggested that the extra methyl group in **8** was located at C-20. Further MS support for the structure was provided by accurate mass measurements for the $[M + H]^+$ of **8**, which was determined to be *m/z* 706.465, indicating a composition of $C_{43}H_{64}NO_7$ (expected mass 706.468, $\Delta = -4$ ppm).

The extraction of the digestive glands obtained from contaminated mussels and subsequent isolation of **8** was therefore undertaken to yield sufficient quantities to permit its structural confirmation using NMR spectroscopy. The isolation of **8** from a digestive gland extract involved additional chromatography steps compared to the isolation of similar spirolide compounds from phytoplankton biomass extracts. Many of the chromatography steps needed to be conducted repetitively. The isolated yield of **8** from 2.79 kg of the prepared digestive glands was 504 μg.

Comparison of ^{1}H and ^{13}C NMR spectra (Table 2) with those of other spirolides confirmed the structure of **8** to be 20-methyl spirolide G. ^{13}C chemical shifts of many backbone carbons of **8** (C1−C17 and C24−C36) agreed with those of **7** within 0.2 ppm, as did the methyl and exomethylene carbons C37−C39 and C41−C43). Shifts of protons bonded to these carbons, determined from HSQC spectra, agreed to within 0.03 ppm with the corresponding protons of **7**. Connectivities of ^{1}H spin systems were determined from 2D TOCSY and COSY spectra to be identical with those of **7**, apart from the loss of one ^{1}H in the H-20 to H-24 spin system, on a carbon (C-20) bearing oxygen. Chemical shifts of both ^{1}H and ^{13}C corresponding to remaining positions 17−23

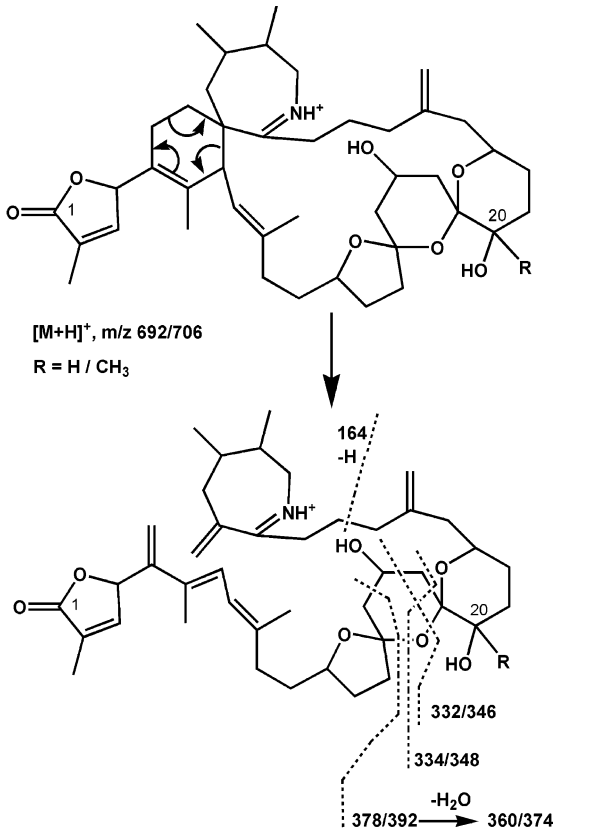


Figure 5. Proposed fragmentation of spirolide G **7** and 20-methyl spirolide G **8**.

Table 2. ^{13}C and ^{1}H Chemical Shifts for 8 in CD_3OH ($^{13}CD_3OH$ at δ_C 49.0; C^1HD_2OH at δ_H 3.30)

carbon no.	δ_C	δ_H	carbon no.	δ_C	δ_H
1	176.70		23	68.24	3.97
2	130.94		24	46.38	2.02, 2.40
3	149.38	7.12	25	145.27	
4	81.93	5.95	26	34.64	1.72, 1.79
5	126.13		27	20.36	1.91, 2.11
6	133.18		28	35.87	2.89, 3.17
7	48.49	3.74	29	202.74	
8	122.77	5.08	30	52.56	
9	143.22		31	36.56	1.81, 2.02
10	38.61	2.07, 2.16	32	37.46	1.12
11	39.41	1.19, 2.32	33	38.52	1.69
12	82.72	3.89	34	51.67	3.57, 4.21
13	32.28	1.52, 2.15	35	32.55	1.67, 1.99
14	41.29	1.90, 2.26	36	20.21	1.71, 2.25
15	109.83		37	10.35	1.89
16	45.19	1.52, 2.03	38	16.59	1.71
17	62.34	4.19	39	17.53	1.91
18	38.03	1.54, 1.90	40	22.06	1.17
19	103.53		41	112.38	4.82, 4.92
20	71.92		42	18.66	1.06
21	34.41	1.38, 1.93	43	19.89	1.13
22	29.79	1.27, 1.48			

showed changes in **8** consistent with the substitution of a methyl group (δ_H 1.17) at C-20 and the concomitant loss of one proton compared to **7**. This was confirmed by HMBC experiments showing correlations from these methyl protons to C-19, C-20, and C-21. Chemical shift changes at these and other nearby positions (Table 2) were also consistent with the substituent effect $\Delta\delta_C$ of methyl substitution, obtained from a comparison of 13-desmethyl spirolide C **5** with 13,19-didesmethyl spirolide C **6** (*10*), as follows. For **5** vs **6**, $\Delta\delta_C$ values for methyl substitution at C-19 are: −3.1 ppm at C-17, + 2.5 ppm at C-18, −0.5 ppm at C-19, +6.4 ppm at C-20, −1.1 ppm

at C-21. For **8** vs **7**, $\Delta\delta_C$ values for methyl substitution at C-20 are in close agreement: −3.2 ppm at C-18, + 2.4 ppm at C-19, −1.2 ppm at C-20, +6.4 ppm at C-21, −1.0 ppm at C-22.

The existence of the 5:6:6 trispiroketal ring system in **8** was confirmed from δ_C for C-15 and C-19 as well as TOCSY and COSY spectra showing an isolated spin system $H_2$16, H17, $H_2$19, containing the same connectivity as in **7** and slightly modified δ_H chemical shifts (Table 2) also consistent with methyl substitution at C-20. A more detailed discussion of the structure elucidation of **8** using NMR spectra will be the subject of a future paper detailing the isolation of this toxin from another *A. ostenfeldii* plankton sample isolated in Canada.

Other possible spirolides found in the precursor ion scan (Figure 2, Table 1) could not be isolated in sufficient quantities for examination by NMR. Attempts are being made to grow cultures of the *A. ostenfeldii* isolated from Norwegian waters at the time of the contamination. This may eventually produce enough material for full structure elucidation. In the meantime, some information can be gained from the MS/MS spectra of some of the compounds observed in the mussels.

It is interesting to note that, while compound **8** and peaks **e**−**l** were also found in the algal samples taken from the same location as the mussels, peaks **b**−**d** were found only in mussels. These three peaks have the same fragment ions as **8**, but the longer-eluting peaks **c** and **d** have a reduced degree of fragmentation to the lower mass ions, with highest abundance ions due to consecutive water losses from the molecular ion. It is possible that peaks **c** and **d** are epimers of **8** formed in the mussel, due either to metabolism or the steaming step used in the preparation of the mussels. These possibilities are being investigated further with appropriate experiments.

Peak **e**, with a molecular weight of 707.5, probably corresponds to an analogue of compound **8**, in which the double bond at the C-2 position in the butenolide ring has been reduced. This would be similar to the relationship between spirolides A **1** and B **2** and between C **3** and D **4** (*13*). The retention time (slightly longer than **8**) and the product ion spectrum of *m/z* 708.5 (Figure 4c) support this proposition. Only ions at the higher masses in the MS/MS spectra for peak **e** shift by 2 Da relative to ions in the spectrum of **8** (Figure 4c vs Figure 4b). Ions at *m/z* 346, 348, 374, and 392 remain the same, while ions at *m/z* 652, 670, 688, and 706 are shifted to *m/z* 654, 672, 690, and 708, respectively.

Peak **f** is 14 Da higher in molecular weight than **8**, suggesting the presence of an additional methyl group. However, the compound does not appear to have the same ring structure as **8**. The product ion spectrum of *m/z* 720.5 from peak **f** (Figure 4d) shows ions at *m/z* 454 and 472. These are 14 Da higher than the fragment ions observed in **3** (*13*). This suggests that peak **f** is a methyl homologue of **3**. We have observed this same compound in one of our *A. ostenfeldii* cultures and structure elucidation work is proceeding.

Peak **g** has a molecular weight of 721.5, 2 Da higher than peak **f**, has the same fragment ions below *m/z* 600, and has a slightly longer retention time (Table 1). These all indicate the double bond at the C-2 position in the butenolide ring has been reduced, similar to peak **e**.

Peaks **h** and **i** have molecular weights of 691.5, while that of peak **j** is 693.5. These are the same as **1**, **5**, and **2**, respectively, but the fragmentation patterns are not the same, and their retention times are much longer. Further conclusions about the structures of these compounds cannot be made at this time.

Peaks **k** and **l** are very unusual, as they both have a molecular weight of 609.5, considerably lower than all the known spirolides. This could correspond to compound **8** missing the butenolide group at C-5. However, the product ion spectrum of the $[M + H]^+$ ion at *m/z* 610.5 (Figure 4e) does not show the same midrange fragment ions as **8**. The fragment ion at *m/z* 358 is difficult to explain. More detailed structure elucidation is required.

An important final step is to determine if the concentration of spirolides present in the mussels could account for the observed mouse deaths. In the lipophilic mouse bioassay, a 20-g mouse is normally injected intraperitoneally with an extract containing the equivalent of 5 g of digestive glands. This corresponds to 25 g of whole flesh, assuming 20% of the whole flesh is digestive gland. The amount of a spirolide mixture with predominantly **5** needed to kill mice has been reported as 40 μg/kg bodyweight (*11*) or 0.8 μg per 20 g mouse. Therefore the level of this mixture in shellfish required to kill a mouse is 32 μg/kg mussel meat. The total amount of the spirolides in the Norwegian sample was estimated at 103 μg/kg mussel meat (Table 1). Assuming that all spirolides found in the Norwegian sample have similar specific toxicity, this could easily explain the toxicity of the Norwegian mussels in the mouse assay. Even the concentration of **8** alone (44 μg/kg) could be lethal to a mouse in this assay.

Conclusion

Spirolides have for the first time been conclusively identified in Norwegian mussels and algae. As the producing alga *A. ostenfeldii* is present in Norway, the presence of the spirolide toxins peaks **a**−**l** is not surprising, although they are not the same as those spirolides previously identified in Canada. As this is a reasonably new toxin group (first described in 1995) and the mussel-farming site was closed for harvesting due to presence of PSP toxins, any human toxic implications of this toxin are at present unknown. However, an initial toxicological evaluation indicated *oral* toxicity of **5**, even though about 25 times less potent than via the IP route to mice (*11*). Therefore it would prudent to monitor for spirolides and, if they reappear at levels toxic to mice, to close the site as a precaution. Further oral toxicity studies on spirolides are urgently needed to yield the information necessary to undertake a risk assessment. The outcome of the risk assessment may form the basis for establishing tolerance levels for spirolides in seafood.

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Supporting Information Available: ^{1}H and ^{13}C spectra of compound **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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