JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification of Fatty Acid Esters of Pectenotoxin-2 Seco Acid in Blue Mussels (*Mytilus edulis*) from Ireland

ALISTAIR L. WILKINS,[†,‡] NILS REHMANN,[§] TRINE TORGERSEN,[†] THOMAS RUNDBERGET,[†] MYRA KEOGH,[§] DIRK PETERSEN,[#] PHILIPP HESS,[§] FRODE RISE,[#] AND CHRISTOPHER O. MILES*[,†,⊥]

National Veterinary Institute, P.B. 8156 Dep., 0033 Oslo, Norway; Chemistry Department, The University of Waikato, Private Bag 3105, Hamilton, New Zealand; Biotoxin Chemistry, Marine Institute, Galway Technology Park, Parkmore, Galway, Ireland; Department of Chemistry, University of Oslo, P.O. Box 1033 Blindern, NO 0315 Oslo, Norway; and AgResearch Ltd., Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand

Pectenotoxins from marine dinoflagellates of the genus *Dinophysis* are rapidly hydrolyzed by many shellfish to give pectenotoxin-2 seco acid, which isomerizes to 7-*epi*-pectenotoxin-2 seco acid. Three series of fatty acid esters of pectenotoxin-2 seco acid (PTX-2 seco acid) and 7-*epi*-PTX-2 seco acid were detected by LC-MS analysis of extracts from blue mussels (*Mytilus edulis*) from Ireland. The locations of the fatty acid ester linkages were identified by a combination of LC-MS^n in positive- and negative-ion modes, LC-MS analysis of the products from reaction of the esters with sodium periodate, and NMR analysis of purified samples of the two most abundant ester derivatives. The 37-*O*-acyl esters of PTX-2 seco acid were the most abundant, followed by the corresponding 11-*O*-acyl esters, accompanied by low levels of the 33-*O*-acyl esters. The most abundant fatty acid esters in the fractionated sample were, in order, the 16:0, 22:6, 14:0, 16:1, 18:4, and 20:5 fatty acids, although a wide array of other PTX-2 seco acid fatty acid esters were also present at low levels.

KEYWORDS: ***Dinophysis*; mussel; metabolism; pectenotoxin; fatty acid; ester; NMR; LC-MS**

INTRODUCTION

Pectenotoxins (PTXs) (**Figure 1**) are a group of complex cyclic polyether lactones originating from *Dinophysis* species and having a worldwide distribution (*1*). The most commonly found PTX in algae is PTX-2 (8). It has been identified in *Dinophysis fortii* from Japan and Italy (*2*−*4*), *Dinophysis acuta* in New Zealand, Norway, Spain, and Ireland (*5*−*10*), *Dinophysis acuminata* in Norway and New Zealand (*8*, *11*), *Dinophysis caudata* from Spain (*10*), and *Dinophysis rotundata* from Norway (*8*). PTX-2 is enzymatically hydrolyzed into its seco acid form [PTX-2 seco acid (**4**)] by many shellfish species (*12*−*14*), a reaction that constitutes a detoxification mechanism (*12*, *15*). Epimerization at C-7 of **4** occurs readily and is apparently not enzyme-catalyzed (*12*, *15*), producing 7-*epi*-pectenotoxin-2 seco acid (7-*epi*-PTX-2 seco acid). Other enzyme-mediated conversions of PTX-2 include oxidation at C-43 in the scallop *Patinopecten yessoensis* to afford PTX-1 (**9**), PTX-3 (**10**), and PTX-6 (**11**) (*9*).

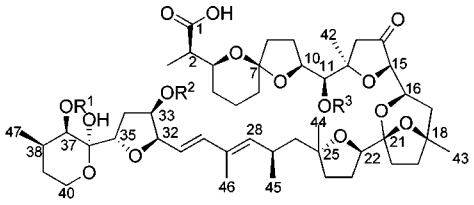
During LC-MS examination of shellfish extracts from Ireland being fractionated for isolation of dinophysistoxin-2 (DTX-2), we observed a series of late-eluting peaks with m/z ≈1050−1250. The masses and fragmentation patterns for these peaks were not consistent with fatty acid esters of DTX-2, but instead appeared to arise from previously unreported fatty acid esters of pectenotoxin-2 seco acids. We wished to confirm this proposal by detailed analysis of fragmentation patterns of $[M + NH_4]^+$, $[M + Na]^+$, and $[M - H]^-$ ions observed during LC-MS/MS analysis of the fractions, together with LC-MS analysis of periodate degradation products of the fractions and NMR analysis of the major components isolated from the mixture.

MATERIALS AND METHODS

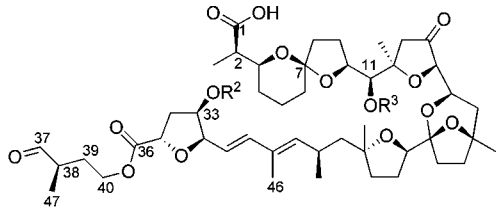
Fractionated Shellfish Extracts. Fractions containing PTX-2 seco acid fatty esters were obtained during the isolation of azaspiracids (*16*−*18*). Digestive glands (≈800 g) were dissected from blue mussels (*Mytilus edulis*) from Castletownbere, Co. Cork, Ireland (harvested August 2001), and blended with acetone (3 L) for 2 min in a Waring blender. The homogenate was filtered through filter paper on a Büchner funnel. The filter cake was placed in the blender again and extracted with MeOH (3 L) for 2 min and filtered as above. The filter cake was extracted a third time with MeOH in the same manner. The three filtrates were combined, and the solvent was evaporated in vacuo with the addition of small amounts of *n*-butanol to prevent bumping and to

* Corresponding author (telephone +47-2321-6226/+64-7-838-5041; fax +47-2321-6201/+64-7-838-5189; e-mail chris.miles@vetinst.no or chris.miles@agresearch.co.nz).
[†] National Veterinary Institute.
[‡] University of Waikato.
[§] Marine Institute.
[#] University of Oslo.
[⊥] AgResearch Ltd.

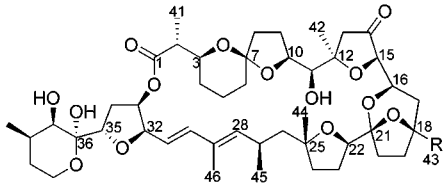
10.1021/jf060396j CCC: $33.50
Published on Web 07/01/2006



1	37-*O*-Acyl PTX-2 seco acid	R^1 = Fatty acid ester, R^2 = H, R^3 = H
2	33-*O*-Acyl PTX-2 seco acid	R^1 = H, R^2 = Fatty acid ester, R^3 = H
3	11-*O*-Acyl PTX-2 seco acid	R^1 = H, R^2 = H, R^3 = Fatty acid ester
4	PTX-2 seco acid	R^1 = H, R^2 = H, R^3 = H



5	oxidized 33-*O*-Acyl PTX-2 seco acid	R^2 = Fatty acid ester, R^3 = H
6	oxidized 11-*O*-Acyl PTX-2 seco acid	R^2 = H, R^3 = Fatty acid ester
7	oxidized PTX-2 seco acid	R^2 = H, R^3 = H



		R
8	PTX-2	CH_3
9	PTX-1	CH_2OH
10	PTX-3	CHO
11	PTX-6	CO_2H

Figure 1. Structures of PTXs and their fatty acid ester derivatives. Structures **2**, **3**, and **5**–**7** are inferred from LC-MS^n analyses.

aid evaporation of water. The residue was partitioned between EtOAc (1 L) and water (1 L). The organic layer was removed and the aqueous phase re-extracted with EtOAc (1 L). The organic extracts were combined, and the solvent was evaporated in vacuo. The residue was then partitioned between hexane (1 L) and methanol/water (4:1) (1 L). The aqueous phase was removed, and the hexane was re-extracted with methanol/water (4:1) (1 L). The aqueous−methanolic extracts were combined and evaporated in vacuo.

The weighed residue was suspended in acetone with sonication and loaded onto a 2-cm i.d. glass column slurry-packed in acetone with 5 times the sample weight of silica gel 60 (Merck). The column was eluted with acetone (3 column volumes) followed by MeOH (3 column volumes). The acetone fraction was evaporated under vacuum and transferred with methanol (10 mL) to glass ampules for storage under nitrogen at −20 °C until required for analysis or purification of **1**. The MeOH fraction was used for isolation of azaspiracids as reported previously (*16*, *17*).

Unfractionated Shellfish Extracts. Fresh whole shellfish flesh homogenate (4 g) from mussels collected in August 2004 from Castletownbere (Co. Cork) on the southwestern coast of Ireland was extracted with methanol (2 × 10 mL) and analyzed directly by LC-MS.

LC-MS and LC-MS^n Analyses. Liquid chromatography was performed on a 100 × 3.0 mm i.d., 3.5 μm, Symmetry C-18 column (Waters, Milford, MA), using a model P4000 pump and a model AS3000 autosampler (Thermo Separation Products, San Jose, CA). Separation was achieved with linear gradient elution from 40 to 100% with acetonitrile/water (containing 2 mM ammonium formate and 0.05% formic acid) over 20 min, with a 25-min hold and a flow rate of 1 mL/min. The HPLC system was coupled to an LCQ ion trap mass spectrometer operating with an ESI interface (Finningan MAT, San Jose, CA). The MS was operated in either MS full scan mode or MS^n scan mode. The ion injection time was 300 ms with a total of three microscans. Typical ESI parameters were a spray voltage of 3−4 kV, a sheath gas rate of 70 units of nitrogen (≈700 mL/min), and an auxiliary gas rate of 10 units of nitrogen (≈100 mL/min). Ionization and MS^n collision energy settings were 30% for ammonium ions and 60% for sodium ions. MS^2 spectra were obtained for the $[M + NH_4]^+$, $[M + Na]^+$, and $[M - H]^-$ ions of PTX-2 seco acid (**4**), 7-*epi*-PTX-2 seco acid, and the 14:0, 16:0, 16:1, 18:4, 20:5, and 22:6 fatty acid 11-, 33- and 37-*O*-acyl esters of PTX-2-seco acid and 7-*epi*-PTX-2 seco acid. MS^3 spectra were determined for the $[M + Na]^+ \rightarrow [M + Na - CO_2]^+$ ions of the respective compounds. Samples were dissolved in appropriate volumes of MeOH for analysis. Ratios of analogues were estimated from the relative peak areas for their $[M + Na]^+$ and $[M + NH_4]^+$ ions observed during LC-MS analysis, assuming identical response factors.

Preparative HPLC. Preparative HPLC was performed on a 250 × 10 mm i.d., 5 μm, Luna C_{18} column (Phenomenex, Torrance, CA) using a Gilson model 321 pump, a model 232 XL sampling injector, and a model 206 fraction collector. Eluents consisted of the specified mixtures of MeCN and water, each containing 0.4 mM ammonium formate and 5 mM formic acid, at 5 mL/min. Fractions were collected manually by monitoring absorbance at 235 nm with a Hewlett-Packard model 1100 series G1315A diode array detector.

Treatment of Extracts with Periodate. $NaIO_4$ (100 μg/mL in water; 500 μL) was added to a portion of the acetone fraction dissolved in methanol (500 μL), and the mixture was allowed to stand at room temperature for 12 h. The samples were analyzed by LC-MS for **1**−**4** and their oxidation products (**5**−**7**).

Isolation of the 16:0 and 22:6 *O*-Acyl Esters of PTX-2 Seco Acid. Purification of fractions was monitored by LC-MS. The stored dried acetone fraction was redissolved in MeOH and purified on a 220 × 16 mm i.d. Sephadex LH-20 column eluted with methanol (1 mL/min), and 28 3-mL fractions were collected. The ester-containing fractions (6−13) were combined, and the solvent was removed in vacuo. The sample was further purified by 200 × 16 mm i.d. flash column chromatography on Develosil 30 μm ODS (Phenomenex) with a linear gradient (2 mL/min) from 60 to 100% MeCN over 15 min with an 85-min hold, after which the column was flushed with 1-propanol (2 mL/min) for 25 min, and fractions of 6 mL were collected.

Final Purification of 16:0−***1***. LC-MS analysis showed that the 16:0 ester was present in the propanol flush, which was evaporated in vacuo. The residue was dissolved in MeOH (500 μL) and purified by preparative HPLC using buffered 100% MeCN as eluent, and six fractions were collected manually. The 16:0 *O*-acyl ester of PTX-2 seco acid was present in fractions 2−4, which were combined, and the solvent was evaporated in vacuo. The ester was further purified by preparative HPLC eluted with a linear gradient from 75 to 100% buffered MeCN over 40 min, with a 10-min hold.

Final Purification of 22:6−***1***. LC-MS analysis showed that the 22:6 ester was present in fractions 20−23 from flash chromatography, which were combined, and the solvent was removed in vacuo. The residue was purified by preparative HPLC eluted with a linear gradient from 75 to 100% buffered MeCN over 40 min, with a 10-min hold.

NMR Spectroscopy. NMR spectra were obtained from solutions of 16:0−**1** and 22:6−**1** (150−200 μg each) in 55 μL of CD_3OD (99.8 atom % D) (Cambridge Isotope Laboratories Inc., Andover, MA), using 1.7-mm NMR tubes (Kontes, Vineland, NJ) and a Bruker MATCH 1.7 mm microspinner clamp. Spectra were acquired with a Bruker Avance AV 600 MHz NMR spectrometer with a 5-mm CP-TCI ($^1H/^{13}C,^{15}N-^2H$) triple-resonance inverse cryoprobe, equipped with a Z-gradient coil. The data were processed using Bruker TOPSPIN (version 1.3). NMR assignments (**Table 1**) were inferred from ^1H, ^1H-double-solvent-suppressed (Bruker pulse program: lc1pnf2), correlation spectroscopy (COSY), and total correlation spectroscopy (TOCSY) data. Chemical shifts, determined at 298 K, are reported relative to internal CHD_2OD (3.31 ppm) and CD_3OD (49.0 ppm).

RESULTS AND DISCUSSION

Full scan LC-MS analysis of fractionated Irish mussel extracts in positive (**Figure 2**) and negative ionization modes showed

Table 1. Selected ^{1}H NMR Chemical Shifts and Coupling Constants Observed for 16:0–**1** and 22:6–**1** in CD_3OD[a]

	PTX-2 seco acid (**4**)		16:0–**1**		22:6–**1**	
atom	^{1}H	multiplicity	^{1}H	multiplicity	^{1}H	multiplicity
10	4.26	m	4.26	m	4.26	m
11	3.81	d, J = 4.9 Hz	3.81	d, J = 5.0 Hz	3.81	d, J = 4.9 Hz
13	2.18, 2.85	2 × d, J = 17.3 Hz	2.18, 2.85	2 × d, J = 17.3 Hz	2.18, 2.85	2 × d, J = 17.3 Hz
30	6.29	d, J = 15.7 Hz	6.29	d, J = 15.7 Hz	6.29	d, J = 15.7 Hz
31	5.71	dd, J = 15.7, 8.1 Hz	5.67	dd, J = 15.7, 8.0 Hz	5.67	dd, J = 15.7, 8.0 Hz
32	4.42	dd, J = 8.1, 2.9 Hz	4.40	dd, J = 8.0, 2.2 Hz	4.39	dd, J = 8.0, 2.2 Hz
33	4.22	m	4.20	m	4.20	m
34	2.09, 2.11	2 × m	1.80, 2.03	2 × m	1.81, 2.03	2 × m
35	4.51	dd, J = 6.6, 9.8 Hz	4.26	dd, J = 6.1, 10.1 Hz	4.27	dd, J = 6.1, 10.2 Hz
37	3.29	d, J = 2.3 Hz	4.82	d, J = 2.7 Hz	4.83	d, J = 2.7 Hz
38	2.12	m	2.31	m	2.31	m
39	1.23, 1.64	2 × m	1.31, 1.60	2 × m	1.29, 1.60	2 × m
40	3.61, 3.91	2 × m	3.68, 3.99	2 × m	3.68, 3.99	2 × m
41	1.19	d, J = 7.0 Hz	1.19	d, J = 7.0 Hz	1.19	d, J = 6.9 Hz
42	1.28	s	1.28	s	1.28	s
43	1.33	s	1.33	s	1.33	s
44	1.20	s	1.20	s	1.20	s
45	0.97	d, J = 6.7 Hz	0.97	d, J = 6.7 Hz	0.97	d, J = 6.7 Hz
46	1.80	br s	1.80	br s (d, J = 0.8 Hz)	1.80	br s
47	0.94	d, J = 6.9 Hz	0.82	d, J = 6.9 Hz	0.82	d, J = 6.9 Hz

[a] Data for **4** are from Miles et al. (*12*).

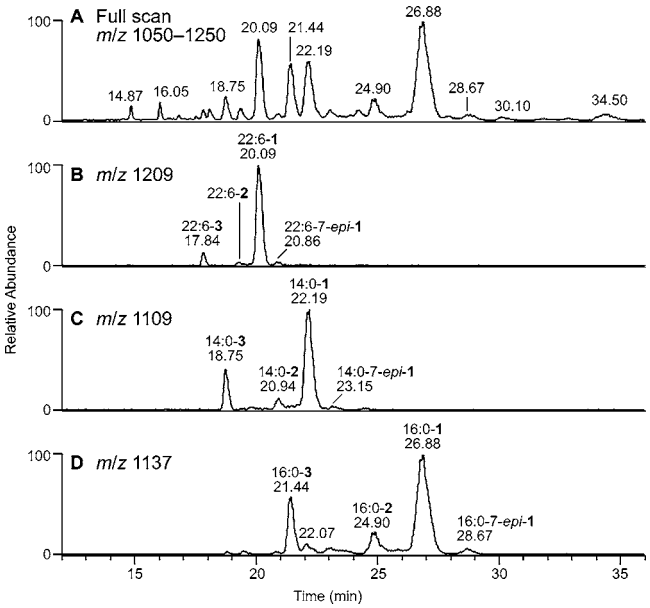


Figure 2. Extracted selected ion profiles from a typical LC-MS analysis (full scan mode, *m*/*z* 500–1500) of acetone fraction from Irish mussels. Extracted profiles correspond to fatty acid esters of PTX-2 seco acid and isomers (*m*/*z* 1050–1250), 22:6 fatty acid esters of PTX-2 seco acids ($[M + Na]^+$, *m*/*z* 1209), 14:0 fatty acid esters of PTX-2 seco acids ($[M + Na]^+$, *m*/*z* 1109), and 16:0 fatty acid esters of PTX-2 seco acids ($[M + Na]^+$, *m*/*z* 1137).

the presence of ions with masses corresponding to the $[M + NH_4]^+$, $[M + Na]^+$, and $[M - H]^-$ ions of a complex series (**1**−**3**) of what appeared to be fatty acid esters of PTX-2 seco acid (**4**). Ratios of the various fatty acids associated with **1**−**3** varied somewhat from batch to batch due to minor variations in the fractionation process. The most abundant compounds in some of the fractionated extracts showed ions attributable (in order of elution, although the 14:0 and 16:1 esters nearly coelute) to the 18:4-, 20:5-, 22:6-, 14:0, 16:1, and 16:0 *O*-acyl esters of **4** (≈1:1:1:1:1:1 ratio) and, to a lesser extent, the 7-*epi*-PTX-2 seco acid analogues of these esters. The full-scan positive-ion LC-MS profile (*m*/*z* 1050−1250 region) and selected extracted-ion profiles obtained from a sample in which the 22:6, 14:0, and 16:0 *O*-acyl esters predominated are shown in **Figure 2**.

LC-MS, -MS^2, and -MS^3 analysis of shellfish extracts and their periodate reaction products, together with NMR analyses of two isolated esters, showed that the dominant esters were 37-*O*-acyl esters (**1**) of PTX-2 seco acid and that these were accompanied by lower levels of their 11- (**3**) and 33-*O*-acyl ester (**2**) analogues. Analogous fatty acid esters of 7-*epi*-PTX-2 seco acid were also present, but at levels 2−4 times lower than that of free 7-*epi*-PTX-2 seco acid. Because the level of both free 7-*epi*-PTX-2 seco acid and fatty acid esters of 7-*epi*-PTX-2 seco acid increased with storage time and were also influenced by variations in the extraction protocol, at least part of the contribution from 7-epimers can be considered to be artifactual. Artifactual conversion of PTX-2 seco acid to 7-*epi*-PTX-2 seco acid has been observed during purification and appears to be in large part responsible for the latter compound in shellfish and algal extracts (*15*).

In addition to the dominant 18:4, 20:5, 22:6, 14:0, 16:1, and 16:0 *O*-acyl esters, the extracts also contained minor amounts of a variety of other fatty acid acyl esters, including the 15:0, 15:1, 17:0, 17:1, 18:0, 18:1, 18:2, 18:3, 20:2, and 20:3 fatty acid esters of PTX-2 seco acid.

Identification of 11-, 33-, and 37-*O*-Acyl Esters. The structure elucidation of 11-, 33-, and 37-*O*-acyl esters of **4** (i.e., **1**−**3**) is illustrated primarily for the 16:0 and 22:6 *O*-acyl esters. These esters were the dominant saturated and unsaturated acyl esters found in the Irish mussel extracts. Positive- and negative-ion-mode MS^2 spectra of the $[M + NH_4]^+$, $[M + Na]^+$, and $[M - H]^-$ ions of PTX-2 seco acid and its 11-, 33-, and 37-*O*-acyl 16:0 esters are presented in **Figures 3**−**5**, and TOCSY NMR data for PTX-2 seco acid (**4**) and the major 37-*O*-acyl esters (16:0−**1** and 22:6−**1**) are presented in **Figure 6**.

$[M + NH_4]^+$ MS^2 Spectra. MS^2 spectra of the $[M + NH_4]^+$ ions of the 11-, 33-, and 37-*O*-acyl esters each showed a series of ions attributable to the loss of NH_4OH followed by water losses, together with a series of $[M - NH_4OH - RCO_2H - (H_2O)_n]^+$ ions arising from the loss of the acyl moiety as the corresponding acid (RCO_2H), where R = $C_{15}H_{31}$ for the 16:0 *O*-esters (**Figure 3**) or R = $C_{21}H_{31}$ for the 22:6 *O*-esters. Irrespective of the ester chain length (16:0, 18:4, 22:6, etc.),

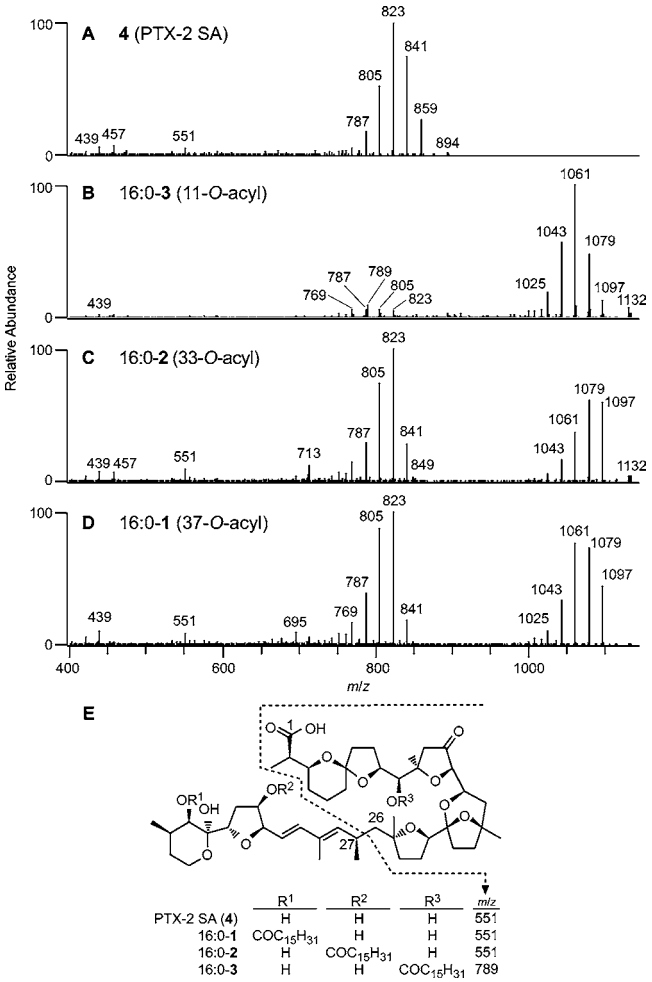


Figure 3. MS^2 spectra of $[M + NH_4]^+$ ions of (**A**) PTX-2 seco acid (*m*/*z* 894), (**B**) 16:0–**3** (*m*/*z* 1132), (**C**) 16:0–**2** (*m*/*z* 1132), (**D**) 16:0–**1** (*m*/*z* 1132), and (**E**) assigned structurally informative fragmentations observed in MS^2 spectra of $[M + NH_4]^+$ ions of 16:0 *O*-acyl esters **1**–**3**.

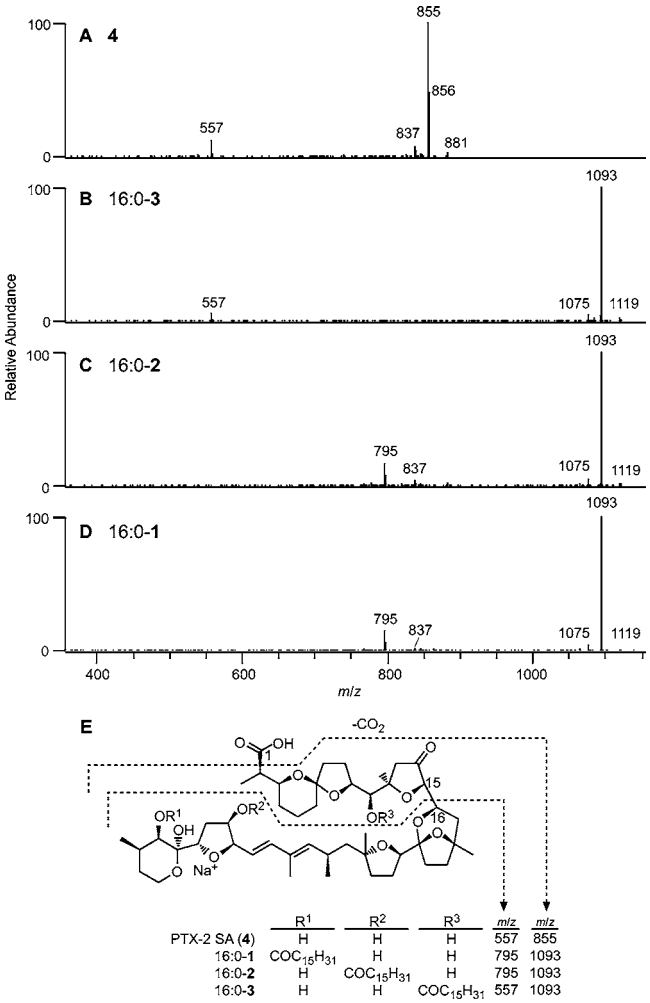


Figure 4. MS^2 spectra of $[M + Na]^+$ ions of (**A**) PTX-2 seco acid (*m*/*z* 899), (**B**) 16:0–**3** (*m*/*z* 1137), (**C**) 16:0–**2** (*m*/*z* 1137), (**D**) 16:0–**1** (*m*/*z* 1137), and (**E**) assigned structurally informative fragmentations observed in MS^2 spectra of $[M + Na]^+$ ions.

each of the 33- and 37-*O*-acyl esters showed a low-intensity fragment ion at *m*/*z* 551, analogous to that observed in the MS^2 spectrum of PTX-2 seco acid (**Figure 3A**,**C**,**D**). The proposed origin of this ion is shown in **Figure 3E**. On the other hand, the 11-*O*-16:0 and 11-*O*-22:6 acyl esters showed an ion at *m*/*z* 789 ($551 + COC_{15}H_{30} = 789$) (**Figure 3B**) or at *m*/*z* 861 ($551 + COC_{21}H_{30} = 861$), respectively, attributable to 11-*O*-acylated analogues of the *m*/*z* 551 ion. These ions, and the corresponding ions of other 11-*O*-acyl esters, were interspersed among the cluster of $[M - NH_3 - RCO_2H - (H_2O)_n]^+$ ions in the *m*/*z* 769−859 region of their MS^2 spectra. The spectra of each of the 11-*O*-acyl esters differed from those of the corresponding 33- and 37-*O*-acyl esters in that the intensity of the cluster of the $[M - NH_3 - RCO_2H - (H_2O)_n]^+$ ions was much reduced (**Figure 3B**).

$[M + Na]^+$ MS^2 and MS^3 Spectra. The dominant ion observed in the MS^2 spectra of the $[M + Na]^+$ ions of PTX-2 seco acid and all of its corresponding *O*-acyl esters, including the 16:0 esters (**Figure 4**), can be attributed to the loss of CO_2 (44 amu) from the C-1 carboxyl group. Lower intensity ions at *m*/*z* 557 also appeared in the spectra of PTX-2 seco acid (**Figure 4A**) and its 11-*O*-acyl esters (**Figure 4B**), irrespective of the chain length of the acyl ester. These *m*/*z* 557 ions can be attributed to cleavage across the C-15−C-16 bond to afford a residual $[C_{16} - C_{40} + Na]^+$ fragment ion lacking a 33- or 37-*O*-acyl substituent (**Figure 4E**). On the other hand, low-intensity ions at *m*/*z* 795 attributable to acylated analogues of the *m*/*z* 557 ion ($557 + COC_{15}H_{30} = 795$) appeared in the $[M + Na]^+$ MS^2 spectra of the 33- and 37-*O*-16:0 acyl esters (**Figure 4C**,**D**). In the case of the 33- and 37-*O*-22:6-acyl esters, the corresponding acylated ions appeared at *m*/*z* 867 ($557 + 310 = 867$). The MS^3 spectra derived from the $[M + Na - CO_2]^+$ ions of PTX-2 seco acid and its 11-, 33-, or 37-*O*-16:0 acyl esters predominantly afforded the aforementioned *m*/*z* 557 (PTX-2 seco acid and its 11-*O*-16:0 ester) or *m*/*z* 795 ions (33- or 37-*O*-16:0 esters), whereas the MS^3 spectra derived from the $[M + Na - CO_2]^+$ ions of 11-, 33-, or 37-*O*-22:6 esters predominantly afforded *m*/*z* 557 (11-*O*-22:6 ester) or *m*/*z* 867 ions (33- and 37-*O*-22:6 esters).

$[M - H]^-$ MS^2 Spectra. The negative-ion MS^2 mass spectrum of PTX-2 seco acid on the ion trap mass spectrometer (**Figure 5A**) included medium-intensity *m*/*z* 687 and 717 ions attributable to cleavages across the penultimate C-32−C-35 ring (ring F), as was observed in the collision-induced dissociation MS/MS spectrum of this compound (*5*). Although MS^2 spectra of the 11-, 33-, and 37-*O*-acyl esters were dominated by *m*/*z* 857 ions arising from the loss of the ester group as the corresponding acid, moderate- to low-intensity ions corresponding to cleavages across the F ring were also observed in the negative-ion MS^2 spectrum of some of the corresponding esters (**Figure 5**). The *m*/*z* 955 ion observed in the MS^2 spectrum of

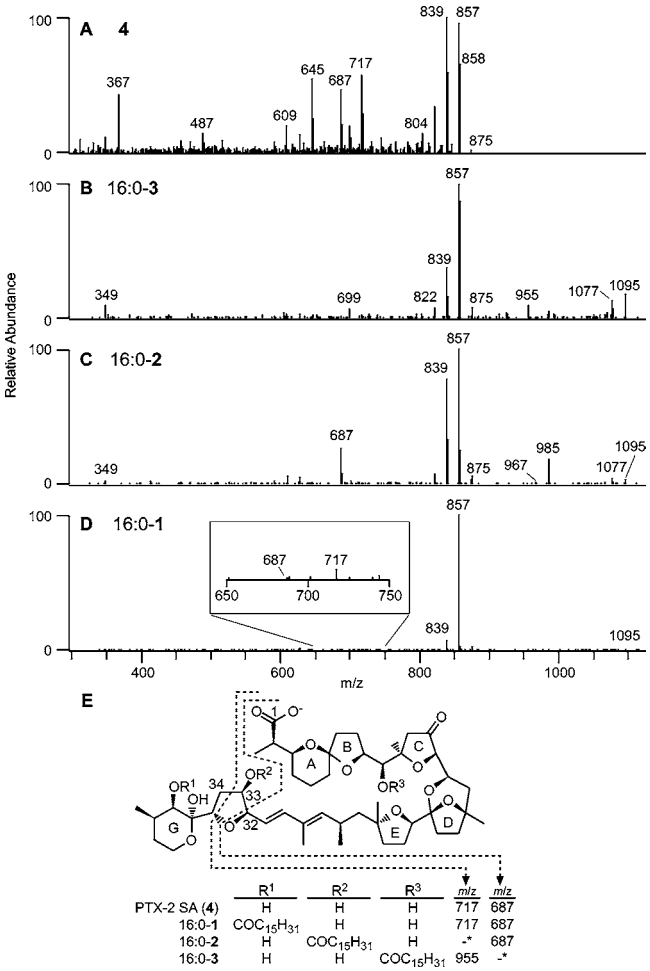


Figure 5. MS^2 spectra of $[M - H]^-$ ions of (**A**) PTX-2 seco acid (*m*/*z* 875), (**B**) 16:0–**3** (*m*/*z* 1113), (**C**) 16:0–**2** (*m*/*z* 1113), (**D**) 16:0–**1** (*m*/*z* 1113), and (**E**) assigned structurally informative fragmentations observed in MS^2 spectra of $[M - H]^-$ ions of 16:0–*O*-acyl esters **1**–**3**. * Corresponding ion not observed for this analogue.

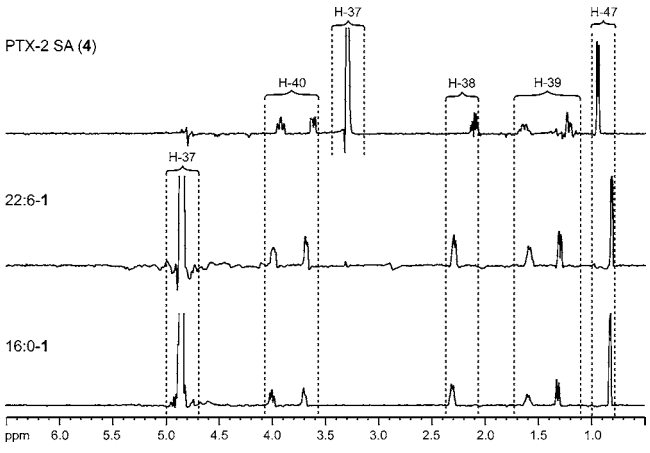


Figure 6. TOCSY NMR spectra showing correlations to H-37 of PTX-2 seco acid (**4**) (top), 22:6–**1** (middle), and 16:0–**1** (bottom).

the 11-*O*-acyl ester can be attributed an acylated variant of the *m*/*z* 717 ion (717 + 238 = 955) (**Figure 5B**), whereas the presence of a 33-*O*-acyl group is characterized by an enhanced intensity of the *m*/*z* 687 ion (**Figure 5C**) arising from cleavage across the adjacent C-33−C-32 bond (**Figure 5E**). The weak but detectable *m*/*z* 717 ion observed in the MS^2 spectrum of the 37-*O*-acyl ester (**Figure 5D**) was consistent with the presence of 11- and 33-hydroxyl groups (rather than acylated variants of these groups) in this ester (**Figure 5E**).

Treatment of Extracts with $NaIO_4$. Addition of aqueous $NaIO_4$ to subsamples of the fractionated Irish mussel extracts led to the formation of the oxidized 36,37-seco-36,37-dehydro derivatives of PTX-2 seco acid (**4**), 7-*epi*-PTX-2 seco acid, the 11- and 33-*O*-acyl esters of PTX-2 seco acid (**2** and **3**), and 7-*epi*-PTX-2 seco acid, but not of the corresponding 37-*O*-acyl esters (**1**). Full-scan LC-MS data showed that the periodate oxidation products exhibited $[M + NH_4]^+$ and $[M + Na]^+$ ions at mass values 2 units lower than for the parent compounds, whereas the $[M + Na]^+$ MS^2 spectra of oxidized products included ions attributable to the loss of a $HC(=O)CH(CH_3)$-$CH=CH_2$ fragment from the oxidized products. These data are consistent with oxidative cleavage of the terminal ring of these compounds across the 36,37 bond and its attachment to C-35 as a $HC(=O)CH(CH_3)CH_2-CH_2-O-C(=O)-$ group (i.e., **5**−**7**). This reaction was confirmed by treating a small sample of PTX-2 in an NMR tube in CD_3OD with $NaIO_4$, whereupon the expected aldehyde resonance (9.62 ppm, H-37) appeared over a period of several hours.

Notwithstanding the 1,2-*trans*-diaxial disposition of the 36- and 37-hydroxyl groups in PTX-2 seco acid and 7-*epi*-PTX-2 seco acid and the 11- or 33-*O*-acyl esters of these compounds, periodate oxidation proceeded at a reasonable rate and was typically complete within 2−6 h depending on the concentration of $NaIO_4$. The LC-MS retention times of the oxidized 36,37-seco-*O*-36,37-dehydro analogues of the 11- and 33-*O*-acyl esters (**5** and **6**) were typically 0.3−0.5 min shorter than those of the parent compounds (**3** and **2**). These results revealed the absence of a 36,37-dihydroxy moiety in **1**, but not in **2**, **3**, or **4**, and indicated that **1** is esterified on either the 36- or 37-position.

NMR Analyses of 16:0−1 and 22:6−1. Although the foregoing MS^2 and periodate oxidation data indicated the presence in some of the esters of 11- or 33-*O*-acyl groups, it could not reveal whether **1** was acylated at the 36- or 37-position. Separation of a portion of the extract afforded specimens of the dominant 16:0 and 22:6 *O*-acyl esters of PTX-2 seco acid (i.e., 16:0−**1** and 22:6−**1**, respectively) in sufficient quantity and purity for one- and two-dimensional NMR analyses. The ^1H NMR spectrum of each of the esters, when determined in CD_3OD with and without HOD and CHD_2OD peak presaturation, showed that the distinctive doublet-like H-37 resonance of PTX-2 seco acid (**4**) (3.29 ppm, $J = 2.4$ Hz) was shifted substantially downfield to 4.82 ppm (d, $J = 2.7$ Hz) and 4.83 ppm (d, $J = 2.7$ Hz) in the 16:0 and 22:6 *O*-acyl esters, respectively. In contrast, the 11-C*H*OH and 33-C*H*OH resonances of these two esters either occurred at the same positions as for **4** (H-11 signals) or were shifted slightly upfield (H-33 signals), as was also the case for some of the protons in close proximity to H-37 (e.g., the H-38, H-35, and H-34 signals) (**Table 1**). The marked downfield shift exhibited by the H-37 signals of the *O*-acyl esters (≈1.5 ppm) was consistent with 37- rather than 36-*O* substitution, whereas the moderate shifts exhibited by other nearby protons (typically those which in PTX-2 seco acid are within 2−3 Å of H-37) are consistent with the replacement of the 37-OH group by a 37-*O*-acyl group. Of particular note is the upfield shift exhibited by the H-47 protons (i.e., the protons of the secondary methyl group attached to C-38). These changes are most clearly illustrated in cross sections from the TOCSY spectra of **4**, 16:0−**1**, and 22:6−**1** (**Figure 6**) discussed below.

Two-dimensional COSY and TOCSY spectra, together with one-dimensional slices extracted from these spectra, readily

identified the resonances of the H-10−H-13, H-28−H-35, and H-37−H-40 protons (**Table 1**). The chemical shifts of H-13α (2.18 ppm, d, $J = 17.3$ Hz) and H-13β (2.85 ppm, d, $J = 17.3$ Hz) showed that the isolated esters were *O*-acyl esters of PTX-2 seco acid, rather than of 7-*epi*-PTX-2 seco acid, because the H-13 resonances of 7-*epi*-PTX-2 seco acid typically occur in the vicinity of 2.28 and 2.77 ppm, respectively, ≈0.1 ppm upfield of the corresponding resonances of these protons in **4** (*5*, *12*, *15*).

These fatty acid derivatives of PTX-2 seco acid are similar to those reported for the okadaic acid/dinophysistoxins (*19*−*22*), brevetoxins (*23*), and spirolides (*24*). These esters all appear to be metabolites produced in the bivalves because fatty acid esters of these toxins have not been observed in phytoplankton (*24*). Presumably these esters are produced by acyl transferases, and the ability to esterify four classes of algal toxin, one of them (**4**) at three different sites, suggests that acylation of primary and secondary hydroxyl groups is to be expected in other hydroxylated lipophilic polyethers such as azaspiracids, gymnodimine, and pectenotoxins. We were not able to completely define the structures of the acyl portions of the other major ester derivatives (14:0, 16:0, 16:1, 18:4, 20:5, and 22:6 esters). However, we consider it to be likely that they correspond to myristic, palmitic, and palmitoleic acids and to those identified by gas chromatography (18:4ω3, 20:5ω3, and 22:6ω3) in a series of esters of okadaic acid (i.e., DTX-3) isolated from Japanese *P. yessoensis* (*20*).

As yet there is little definitive information as to the toxicity of fatty acid esters of algal toxins, although fatty acid esters of okadaic acid and DTX-1 appear to be somewhat less toxic than their parent congeners (*25*, *26*). In any case, PTX seco acids appear to be of very low acute toxicity (*5*, *12*, *15*, *27*, *28*) so hydrolysis of **1**−**3** in the gut is unlikely to be hazardous to consumers of shellfish, and the toxicity of the PTXs themselves appears to be relatively low by the oral route (*12*, *15*, *29*).

LC-MS analysis of an unfractionated methanolic extract whole flesh from freshly collected Irish mussels suggested that the amount of fatty acid esters **1**−**3** present was ≈20-fold higher than the amount of unesterified PTX-2 seco acid (**4**) and 7-*epi*-PTX-2 seco acid, assuming identical response factors and extraction efficiencies, and 18:4−, 20:5−, 22:6−, 14:0−, 16:1−, and 16:0−**1** in this sample were present in a ≈2:1:4:3:3:6 ratio. This ratio is significantly different from that in the material used for the structural studies, presumably due at least in part to the processing involved in the fractionation of the azaspiracids in the original sample. Nevertheless, the finding of such high levels of **1**−**3** in freshly collected unfractionated Irish mussels shows that these compounds are not storage artifacts and that they are readily formed in these shellfish. Preliminary examination of extracts from Norwegian blue mussels also showed the presence of **1**−**3**. Work is under way in our laboratories to investigate the chemistry, occurrence, and distribution of these compounds more closely.

Because PTX-2 appears to be rapidly hydrolyzed to PTX-2 seco acid in mussels (*12*, *13*, *29*), significant levels of PTX-2 fatty acid esters would not be expected even if esterification (especially at C-37) hindered the enzymatic lactone hydrolysis in the mussel. However, the situation with respect to fatty acid ester formation of PTXs that are less readily hydrolyzed, such as PTX-11 (*29*) and PTX-12 (*8*), and with shellfish such as *P. yessoensis* that do not hydrolyze PTXs into seco acids could be quite different and needs to be clarified.

ACKNOWLEDGMENT

The mussel material was donated by the Marine Institute, Ireland [ASTOX project, funded by the National Development Plan (NDP) (ST/02/02 2003−2006) and the Marine Institute].

Supporting Information Available: Postive ion LC-MS chromatogram for unfractionated methanolic extract of whole shellfish. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Draisci, R.; Lucentini, L.; Mascioni, A. Pectenotoxins and yessotoxins: chemistry, toxicology, pharmacology, and analysis. In *Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection*; Botana, L. M., Ed.; Dekker: New York, 2000; pp 289−324.

(2) Draisci, R.; Lucentini, L.; Giannetti, L.; Boria, P.; Poletti, R. First report of pectenotoxin-2 (PTX-2) in algae (*Dinophysis fortii*) related to seafood poisoning in Europe. *Toxicon* **1996**, *34*, 923−935.

(3) Lee, J.-S.; Igarashi, T.; Fraga, S.; Dahl, E.; Hovgaard, P.; Yasumoto, T. Determination of diarrhetic shellfish toxins in various dinoflagellate species. *J. Appl. Phycol.* **1989**, *1*, 147−152.

(4) Suzuki, T.; Mitsuya, T.; Matsubara, H.; Yamasaki, M. Determination of pectenotoxin-2 after solid-phase extraction from seawater and from the dinoflagellate *Dinophysis fortii* by liquid chromatography with electrospray mass spectrometry and ultraviolet detection. Evidence of oxidation of pectenotoxin-2 to pectenotoxin-6 in scallops. *J. Chromatogr. A* **1998**, *815*, 155−160.

(5) Daiguji, M.; Satake, M.; James, K. J.; Bishop, A.; MacKenzie, L.; Naoki, H.; Yasumoto, T. Structures of new pectenotoxin analogs, pectenotoxin-2 seco acid and 7-*epi*-pectenotoxin-2 seco acid, isolated from a dinoflagellate and greenshell mussels. *Chem. Lett.* **1998**, 653−654.

(6) James, K. J.; Bishop, A. G.; Draisci, R.; Palleschi, L.; Marchiafava, C.; Ferretti, E.; Satake, M.; Yasumoto, T. Liquid chromatographic methods for the isolation and identification of new pectenotoxin-2 analogues from marine phytoplankton and shellfish. *J. Chromatogr. A* **1999**, *844*, 53−65.

(7) MacKenzie, L.; Holland, P.; McNabb, P.; Beuzenberg, V.; Selwood, A.; Suzuki, T. Complex toxin profiles in phytoplankton and Greenshell mussels (*Perna canaliculus*), revealed by LC-MS/MS analysis. *Toxicon* **2002**, *40*, 1321−1330.

(8) Miles, C. O.; Wilkins, A. L.; Samdal, I. A.; Sandvik, M.; Petersen, D.; Quilliam, M. A.; Naustvoll, L. J.; Rundberget, T.; Torgersen, T.; Hovgaard, P.; Jensen, D. J.; Cooney, J. M. A novel pectenotoxin, PTX-12, in *Dinophysis* spp. and shellfish from Norway. *Chem. Res. Toxicol.* **2004**, *17*, 1423−1433.

(9) Suzuki, T.; Beuzenberg, V.; Mackenzie, L.; Quilliam, M. A. Liquid chromatography−mass spectrometry of spiroketal stereoisomers of pectenotoxins and the analysis of novel pectenotoxin isomers in the toxic dinoflagellate *Dinophysis acuta* from New Zealand. *J. Chromatogr. A* **2003**, *992*, 141−150.

(10) Fernández, M. L.; Reguera, B. First report of pectenotoxin-2 in isolated *Dinophysis caudata* cells determined by liquid chromatography−mass spectrometry. *Book of Abstracts*, 10th International Conference on Harmful Algae, St. Petersburg Beach, FL, Oct 21−25; Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO: St. Petersburg, FL, 2002; p 91.

(11) MacKenzie, L.; Beuzenberg, V.; Holland, P.; McNabb, P.; Suzuki, T.; Selwood, A. Pectenotoxin and okadaic acid-based toxin profiles in *Dinophysis acuta* and *Dinophysis acuminata* from New Zealand. *Harmful Algae* **2005**, *4*, 75−85.

(12) Miles, C. O.; Wilkins, A. L.; Munday, R.; Dines, M. H.; Hawkes, A. D.; Briggs, L. R.; Sandvik, M.; Jensen, D. J.; Cooney, J. M.; Holland, P. T.; Quilliam, M. A.; MacKenzie, A. L.; Beuzenberg, V.; Towers, N. R. Isolation of pectenotoxin-2 from *Dinophysis acuta* and its conversion to pectenotoxin-2 seco acid, and preliminary assessment of their acute toxicities. *Toxicon* **2004**, *43*, 1−9.

(13) Suzuki, T.; Mackenzie, L.; Stirling, D.; Adamson, J. Pectenotoxin-2 seco acid: a toxin converted from pectenotoxin-2 by the New Zealand Greenshell mussel, *Perna canaliculus*. *Toxicon* **2001**, *39*, 507−514.

(14) Suzuki, T.; MacKenzie, L.; Stirling, D.; Adamson, J. Conversion of pectenotoxin-2 to pectenotoxin-2 seco acid in the New Zealand scallop, *Pecten novaezelandiae*. *Fish. Sci.* **2001**, *67*, 506−510.

(15) Miles, C. O.; Wilkins, A. L.; Munday, J. S.; Munday, R.; Hawkes, A. D.; Jensen, D. J.; Cooney, J. M.; Beuzenberg, V. Production of 7-*epi*-pectenotoxin-2 seco acid and assessment of its acute toxicity to mice. *J. Agric. Food Chem.* **2006**, *54*, 1530−1534.

(16) Satake, M.; Ofuji, K.; Naoki, H.; James, K. J.; Furey, A.; McMahon, T.; Silke, J.; Yasumoto, T. Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, *Mytilus edulis*. *J. Am. Chem. Soc.* **1998**, *120*, 9967−9968.

(17) Ofuji, K.; Satake, M.; McMahon, T.; Silke, J.; James, K. J.; Naoki, H.; Oshima, Y.; Yasumoto, T. Two analogs of azaspiracid isolated from mussels, *Mytilus edulis*, involved in human intoxication in Ireland. *Nat. Toxins* **1999**, *7*, 99−102.

(18) Ofuji, K.; Satake, M.; McMahon, T.; James, K. J.; Naoki, H.; Oshima, Y.; Yasumoto, T. Structures of azaspiracid analogs, azaspiracid-4 and azaspiracid-5, causative toxins of azaspiracid poisoning in Europe. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 740−2.

(19) Suzuki, T.; Ota, H.; Yamasaki, M. Direct evidence of transformation of dinophysistoxin-1 to 7-*O*-acyl-dinophysistoxin-1 (dinophysistoxin-3) in the scallop *Patinopecten yessoensis*. *Toxicon* **1999**, *37*, 187−198.

(20) Yasumoto, T.; Murata, M.; Oshima, Y.; Sano, M.; Matsumoto, G. K.; Clardy, J. Diarrhetic shellfish toxins. *Tetrahedron* **1985**, *41*, 1019−1025.

(21) Marr, J. C.; Hu, T.; Pleasance, S.; Quilliam, M. A.; Wright, J. L. C. Detection of new 7-*O*-acyl derivatives of diarrhetic shellfish poisoning toxins by liquid chromatography−mass spectrometry. *Toxicon* **1992**, *30*, 1621−1630.

(22) Fernández, M. L.; Míguez, E.; Cacho, A.; Martínez, A. Detection of okadaic acid esters in the hexane extracts of spanish mussels. *Toxicon* **1996**, *34*, 381−387.

(23) Morohashi, A.; Satake, M.; Murata, K.; Naoki, H.; Kaspar, H. F.; Yasumoto, T. Brevetoxin B3, a new brevetoxin analog isolated from the greenshell mussel *Perna canaliculus* involved in neurotoxic shellfish poisoning in New Zealand. *Tetrahedron Lett.* **1995**, *36*, 8995−8998.

(24) Aasen, J. A. B.; Hardstaff, W.; Aune, T.; Quilliam, M. A. Discovery of fatty acid ester metabolites of spirolide toxins in mussels from Norway using liquid chromatography-tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1531−1537.

(25) Torgersen, T.; Aasen, J.; Aune, T. Diarrhetic shellfish poisoning by okadaic acid esters from Brown crabs (*Cancer pagurus*) in Norway. *Toxicon* **2005**, *46*, 572−578.

(26) Yanagi, T.; Murata, M.; Torigoe, K.; Yasumoto, T. Biological activities of semisynthetic analogs of dinophysistoxin-3, the major diarrhetic shellfish toxin. *Agric. Biol. Chem.* **1989**, *53*, 525−529.

(27) Burgess, V.; Shaw, G. *Investigations into the toxicology of Pectenenotoxin-2-seco Acid and 7-epi Pectenotoxin 2-seco Acid To Aid in a Health Risk Assessment for the Consumption of Shellfish Contaminated with These Shellfish Toxins in Australia*; National Research Centre for Environmental Toxicology: Archerfield, Queensland, Australia, Feb 2003; ISBN 0-9750259-1-0, p 51.

(28) Burgess, V.; Zhang, Y.; Eaglesham, G.; Tzang, C. H.; Yang, Z.; Shaw, G.; Lam, P. K. S.; Mengsu, Y.; Moore, M. R. Investigation of changes in cell cycle distribution in cultured $HepG_2$ cells with pectenotoxin-2 seco acids. *Proceedings of the 4th International Conference on Molluscan Shellfish Safety*, Santiago de Compostella, Spain, June 4−8, 2003; Villalba, A., Reguera, B., Romalde, J. L., Beiras, R., Eds.; Xunta de Galicia and IOC of UNESCO: Galicia, Spain, 2003; pp 97−105.

(29) Suzuki, T.; Walter, J. A.; LeBlanc, P.; Mackinnon, S.; Miles, C. O.; Wilkins, A. L.; Munday, R.; Beuzenberg, V.; MacKenzie, A. L.; Jensen, D. J.; Cooney, J. M.; Quilliam, M. A. Identification of pectenotoxin-11 as 34*S*-hydroxypectenotoxin-2, a new pectenotoxin analogue in the toxic dinoflagellate *Dinophysis acuta* from New Zealand. *Chem. Res. Toxicol.* **2006**, *19*, 310−318.

Received for review February 9, 2006. Revised manuscript received May 22, 2006. Accepted May 30, 2006. This study was supported by Norwegian Research Council Grant 139593/140, the BIOTOX project (partly funded by the European Commission, through 6th Framework Programme Contract 514074, topic Food Quality and Safety), and New Zealand Foundation for Research, Science and Technology Grant C10X0406 (International Investment Opportunities Fund).

JF060396J