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Identification of Pinnatoxins and Discovery of Their Fatty Acid Ester Metabolites in Mussels (*Mytilus edulis*) from Eastern Canada

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Supporting Information

ABSTRACT: Pinnatoxins are a group of fast-acting cyclic imine toxins previously identified in shellfish from Asia, the southern Pacific, and northern Europe. In this work pinnatoxins were detected in mussels from locations across the eastern coast of Canada. Pinnatoxin G (**6**) was the major structural variant present, sometimes at levels >80 μg/kg, whereas much lower levels of pinnatoxin A (**1**) were detected in some samples. Increased concentrations were observed following base hydrolysis of extracts, leading to the discovery by LC-MS of a range of fatty acid esters of **6**. Information on the structures of these acylated derivatives was provided through a series of mass spectrometric experiments, supported by partial synthesis, and it is proposed that the compounds are 28-*O*-acyl esters of **6**. Although acyl esters of a range of other phycotoxins are known to form as metabolites in shellfish, this is the first report of their existence for this particular toxin class. The occurrence of pinnatoxins in North American shellfish further highlights the international distribution of these toxins.

KEYWORDS: *pinnatoxins, shellfish, Canada, fatty acids, esters, metabolites, LC-MS*

INTRODUCTION

Pinnatoxins are a group of phycotoxins with a characteristic cyclic imine function that were initially detected in Chinese shellfish.[1] Pinnatoxin A (**1**) was characterized first,[2] followed by the structures of pinnatoxins B, C (**2**), and D (**3**).[3−5] Pinnatoxins E (**4**), F (**5**), and G (**6**) were discovered in shellfish from Australia and New Zealand and were characterized by mass spectrometry and nuclear magnetic resonance.[6] Pinnatoxins A (**1**) and G (**6**) have been detected recently in Norwegian shellfish and in Norwegian seawater using passive samplers.[7] The chemical structures of the pinnatoxins reported to date are shown in Figure 1. A variety of pinnatoxin-producing dinoflagellates have now been isolated, including a producer of **4** and **5** from New Zealand,[8] a producer of **6** from Japan,[9] and a producer of **4**, **5**, and **6** from Australia.[10] The dinoflagellate *Vulcanodinium rugosum* was recently isolated from Mediterranean water samples,[11] and through morphological comparison with the isolates from Japan, New Zealand, and Australia, it has been identified as the causative organism of pinnatoxins in those locations.[12] Pinnatoxins were responsible for a series of previously unexplained mouse bioassay positive samples from a harbor in the North Island of New Zealand, dating back to the early 1990s.[13] Like the structurally related spirolides,[14] pinnatoxins are classified as "fast-acting toxins", and studies have demonstrated that pinnatoxins have high toxicities via both intraperitoneal injection and oral gavage.[8,15] Recent studies have shown that the mode of action of pinnatoxins involves potent and selective inhibition of nicotinic acetylcholine receptors.[16,17] The available data suggest that pinnatoxins may have a higher oral toxicity than spirolides.[7,18] Spirolides were first identified in shellfish and phytoplankton from Nova Scotia[14,19] and are frequently determined at low levels in shellfish harvested around eastern Canada (data from Canadian Food Inspection Agency (CFIA) monitoring program). Up to this point pinnatoxins have not been reported in Canadian waters; however, due to the fact that they have now been found in both Atlantic and Pacific locations, the occurrence of pinnatoxins in Canadian shellfish would be expected.

A number of publications have reported on the metabolism of algal toxins in shellfish because of the need to understand the fate and occurrence of toxins and the possibility of additional toxicity associated with metabolites. The presence of 7-*O*-acyl derivates of okadaic acid and dinophysistoxins has been well documented,[20−23] and similar acyl esters of pectenotoxins,[24] brevetoxins,[25] and spirolides[26] have been reported. Because pinnatoxins also contain hydroxyl groups that could be acylated, it seems possible that similar metabolites of these compounds would occur.

In this work, studies were carried out using liquid chromatography−mass spectrometry (LC-MS) to confirm the presence of pinnatoxins in Canadian shellfish and to identify previously unreported pinnatoxin fatty acid esters.

MATERIALS AND METHODS

Chemicals and Reagents. HPLC grade acetonitrile (MeCN), glass-distilled methanol (MeOH), and ACS grade chloroform were obtained from Caledon (Georgetown, ON, Canada) or BDH Inc. (Toronto, ON, Canada). Formic acid (>98% ACS grade) was obtained from EMD (Gibbstown, NJ). Ammonium formate (99%) and palmitic acid anhydride were obtained from Sigma-Aldrich (Oakville, ON, Canada). "Pyridine Plus" reagent (pyridine with 4-dimethylaminopyridine) was obtained from Alltech Associates (Deerfield, IL). All water used in

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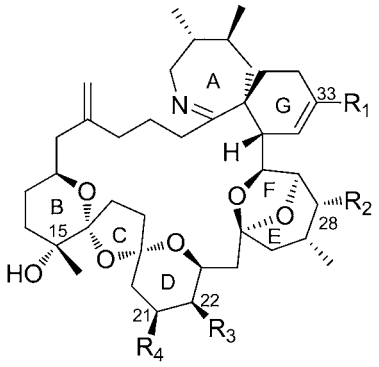

	Toxin	[M+H]+	R1	R2	R3	R4
1	Pinnatoxin A	712	—CO_2H	OH	H	H
2	Pinnatoxin B/C	741	CO_2H, 36, NH_2	OH	H	H
3	Pinnatoxin D	782	CO_2H, O	H	OH	CH_3
4	Pinnatoxin E	784	CO_2H, OH	H	OH	CH_3
5	Pinnatoxin F	766	O, O	H	OH	CH_3
6	Pinnatoxin G	694		OH	H	H

Figure 1. Structures of major pinnatoxin analogues.

these experiments was of ultrahigh purity, prepared with a Milli-Q water purification system (Millipore Ltd., Billerica, MA).

For screening work at the CFIA, instrument calibrations were performed using matrix-matched standards prepared from a sample of **6** kindly provided by the Cawthron Institute (Nelson, New Zealand).[6] More recently, the National Research Council's (NRC) Certified Reference Materials (CRM) Program (Halifax, NS, Canada), in collaboration with the Cawthron Institute, has prepared a CRM for **6**. Following purity assessment by nuclear magnetic resonance (NMR), a solution of **6** was prepared in methanol (with 0.01% acetic acid). This was then quantified by quantitative NMR.[27] A standard calibration curve for **6** was made by preparing a serial 3-fold dilution of the stock in methanol (with 0.01% acetic acid) using a Microlab diluter (Hamilton Co., Reno, NV). A standard of **1** of unknown purity was prepared in methanol (with 0.1% acetic acid) from toxin isolated at the Cawthron Institute.[6]

Sample Preparation. All mussel samples used in this study were received by the CFIA for regulatory monitoring of lipophilic toxins. Whole flesh from a minimum of 12 animals was homogenized before extraction. For some analyses the hepatopancreas tissues were carefully dissected using scalpels prior to homogenization.

Extraction Method 1 (CFIA). Subsamples (2.0 ± 0.2 g) of homogenized tissues were mixed with 8.0 mL of MeOH and homogenized using a Polytron PT3000 (Brinkmann Instruments Inc., Westbury, NY). After centrifugation (10 min at 5250*g*), 2.5 mL of the supernatant was mixed in a glass tube with 0.5 mL of water. Hexane (2.5 mL) was then added, vortex mixed, and centrifuged (5 min at ≥233*g*). The hexane layer was discarded and the hexane wash repeated. Water (1.0 mL) was added to the methanolic layer before the addition of 4.0 mL of chloroform and mixing with a vortex mixer. The mixture was centrifuged (5 min at ≥233*g*) and the chloroform layer transferred to a second tube. The chloroform step was repeated, and the combined chloroform extracts were evaporated to dryness. The residue was dissolved in 0.5 mL of MeOH and filtered (0.2 μm) into an autosampler vial for LC-MS/MS.

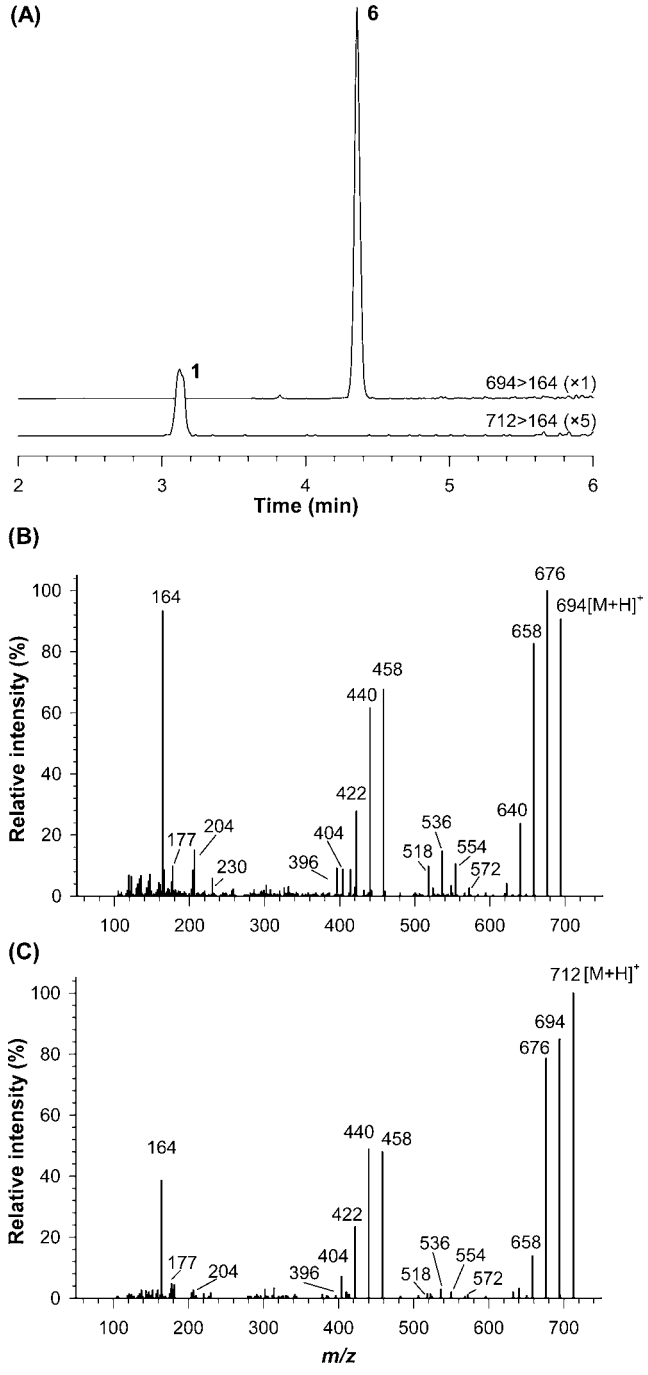

Figure 2. SRM analysis of **6** (~20 μg/kg) and low level of **1** (~1 μg/kg) in a mussel extract from Big Island, NS, Canada (November 2009) (A); product ion spectra of **6** (B) and **1** (C) acquired on the same sample. The sample was concentrated 5-fold to obtain sufficient signal for the spectrum of **1**.

Extraction Method 2 (NRC). For confirmation and quantitation work, 2 g sample portions were weighed in 50 mL polypropylene centrifuge tubes, 8 mL of MeOH was added, and samples were extracted for 1 min at 10000 rpm using a Polytron PT3000. Extracts were centrifuged at 3950*g* for 10 min at 20 °C and then syringe filtered (0.45 μm) to provide enough for direct analysis and the hydrolysis step (1−2 mL).

Extraction Method 3 (NRC). For direct acyl ester analysis mussel homogenate (5 g) was weighed into a 50 mL centrifuge tube, 20 mL of MeOH was added, and the sample was extracted at 10000 rpm for 3 min using a Polytron PT3000. During extraction, the tube was

cooled in a small beaker of ice and water to avoid any potential stability issues due to heating of the sample during the shear extraction step. After centrifugation (3950*g*, 10 min, 20 °C), the supernatant was decanted to a clean centrifuge tube. The pellet was re-extracted by vortex mixing for 1 min using 10 mL of MeOH. After centrifugation, the supernatant was combined with that of the first extraction step. Water (30 mL) was added to the methanolic extract, transferred to a 250 mL separation funnel, and partitioned twice with 100 mL portions of chloroform. The chloroform (lower phase) was collected carefully in a 500 mL round-bottom flask and reduced to near-dryness using a rotary evaporator. The residue was transferred in methanol rinses to a 15 mL glass tube and dried completely using a TurboVap LV nitrogen evaporation system (Caliper LifeSciences, Hopkinton, MA) operated at 37 °C. The residue was reconstituted in 0.5 mL of MeOH (containing 0.1% formic acid) and vortex mixed. A 200 μL portion was passed through a 0.45 μm Millex-HV PVDF spin filter (Millipore Corp., Bedford, MA) prior to analysis.

Base Hydrolysis (NRC). For hydrolysis a slight variation of a previously reported procedure was followed.[28] A 500 μL portion of extract (from extraction method 2) was placed in a 1.5 mL HPLC vial, and 65 μL of 2.5 M NaOH was then added and vortex mixed. The vials were capped tightly and heated for 40 min at 76 °C in an oven. When cool, the samples were neutralized with 65 μL of 2.5 M HCl and vortex mixed. Hydrolyzed samples were filtered (0.45 μm) prior to analysis.

Synthesis of Pinnatoxin Palmitic Esters. A palmitic acid ester of **6** was prepared following a procedure previously applied to spirolides.[26] Approximately 100 pmol of **6** was dissolved using a 100 mM solution of palmitic acid anhydride in "Pyridine Plus" reagent on a 100 μL scale (note: all glassware was dried overnight at 115 °C and cooled in a desiccator before use). The sample was reacted in an oven at 77 °C, and a 25 μL aliquot of the reaction mixture was removed at 30 min, evaporated, and redissolved in 25 μL of MeOH for LC-MS analysis.

LC-MS. *CFIA Method.* Screening of samples was performed using a Waters Acquity UPLC connected to a Waters Quattro Premier XE MS/MS (Waters Inc., Milford, MA). Separation was performed on a 100 × 2.1 mm i.d., 1.7 μm, Acquity BEH Shield RP18 column (Waters Inc.) operated at 35 °C, injecting 5 μL samples. A binary mobile phase of 9% MeCN in water (A) and 81% MeCN in water (B) was used, with each containing 4 mM ammonium formate and 44 mM formic acid. A gradient was run at 400 μL/min from 35 to 95% B over 4.5 min and holding at 95% B for 1 min before re-equilibration for the next run. The MS was operated in positive ionization mode with a capillary voltage of 2.5 kV, 130 °C source temperature, 450 °C desolvation temperature, 450 L/h desolvation gas, cone gas at 40 L/h, and 50 eV collision energy. Transitions monitored were as follows: pinnatoxin A (**1**), 712.5 → 164.1; pinnatoxin E (**4**), 784.5 → 164.1; pinnatoxin F (**5**), 766.5 → 164.1; pinnatoxin G (**6**), 694.5 → 164.1.

NRC Methods. Confirmation of pinnatoxins in mussel samples was performed on an Agilent 1200 LC system (Agilent Inc., Palo Alta, CA) connected to an API4000 QTRAP mass spectrometer (AB Sciex, Concord, ON, Canada) equipped with a Turbospray ionization source. Quantitation of pinnatoxins and characterization of acyl esters was performed on an Agilent 1260 LC system connected to an API5500 QTRAP mass spectrometer equipped with a Turbospray ionization source.

NRC Method 1. For confirmation and quantitation of pinnatoxins, separations were performed on a 50 × 2.1 mm i.d., 1.9 μm, Hypersil Gold C18 column (Thermo Scientific, Mississauga, ON, Canada). A binary mobile phase of water (A) and MeCN/water (95:5) (B), each containing 50 mM formic acid and 2 mM ammonium formate, was employed, with a gradient from 25 to 75% B over 6 min at 300 μL/min and held at 100% B for 2 min before re-equilibration for the next injection. For quantitation the MS was operated in selected reaction monitoring mode (SRM) selecting the pinnatoxin $[M + H]^+$ ions (Figure 1) in Q1, fragmenting in the collision cell, and selecting m/z 164 in Q3 as a common fragment ion for all pinnatoxins. For **1** and **6** a second SRM transition was acquired by selecting m/z 572 as the fragment ion in Q3. Collision energies were set at 85 and 55 for m/z 164 and 572 transitions, respectively. Product ion spectra of **6** and **1** were acquired using a 60 eV collision energy.

NRC Method 2. For direct analysis of pinnatoxin acyl esters, separations were performed on a 50 × 2.1 mm i.d., 3 μm, BDS Hypersil C8 column (Thermo Scientific). The same binary mobile phase system described above for quantitation (see NRC Method 1) was employed. A gradient was run from 40 to 100% B over 5 min at 250 μL/min and held at 100% B for 25 min before re-equilibration for the next injection. The mass spectrometer was operated in positive ionization mode acquiring data with a variety of scan types. Typical parameters were 5500 V electrospray voltage, 400 °C nebulizer gas temperature, and 75 V declustering potential. SRM data were collected for the pinnatoxin acyl esters as described above for free pinnatoxins. Precursor ion spectra were acquired in positive ion mode using a Q1 scan range of m/z 800−1100, fragmenting with nitrogen in Q2, and monitoring the m/z 164 product ion in Q3 (85 eV collision energy). Product ion spectra of acyl esters were acquired by selecting precursor $[M + H]^+$ ions in Q1, fragmenting in Q2 (65−75 eV collision energy), and scanning from m/z 100 to 1000 in Q3.

Accurate Mass Analysis. An Accela High Speed LC was connected to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific), equipped with a heated electrospray ionization probe (HESI-II). The instrument was calibrated and operated as previously described.[29] The LC conditions described above for the direct analysis of pinnatoxin acyl esters (NRC Method 2) were used. Data were acquired in positive ion mode with a 3.3 kV spray voltage, sheath and auxiliary gas flows of 45 and 10 (arbitrary units), respectively, 250 °C capillary temperature, and 300 °C heater temperature. Separate scans with and without higher energy collisional dissociation (70 eV HCD) were obtained at the 50000 resolution instrument setting (2 Hz scan rate).

RESULTS AND DISCUSSION

Identification and Occurrence of Pinnatoxins in Canadian Mussels. Mussels (*Mytilus edulis*) harvested on the Atlantic coast of Canada were analyzed at the CFIA by LC-MS/MS as part of the Canadian Shellfish Sanitation Program. Pinnatoxin G (**6**) was detected in various samples and partially confirmed through a retention time match with a standard. Trace quantities of pinnatoxin A (**1**) were detected in some of the samples that contained **6**, but during the initial screening work it was not possible to be certain of its identity as a standard for **1** was not available at the time. The SRM transition used in the screening method selected m/z 164 as the fragment ion in Q3. The m/z 164 ion is a fragment ($C_{11}H_{18}N^+$) corresponding to a seven-membered iminium ring that is common to all reported pinnatoxins and results from a retro-Diels−Alder opening of the G-ring.[6] It is also common to the majority of spirolides,[30] so misidentification is possible due to the wide range of cyclic imine toxins known to occur.[31−33] It was found that Canadian mussel samples containing **6** frequently contained spirolides, in agreement with monitoring data from other locations.[7]

Confirmatory analyses were carried out at the NRC to unambiguously identify the toxins present. As expected, **6** eluted later than **1** due to the presence of an acidic carboxyl function on the latter (Figure 2A), and the retention times matched those of **6** and **1** standards. Product ion spectra were acquired for the proposed pinnatoxins in a mussel sample (Figure 2B,C). These spectra matched those obtained for purified standards and contained the range of fragment ions that are specific to **6** and **1**. A distinguishing fragment for pinnatoxins is m/z 572 (followed by sequential water losses to give m/z 554 and 536), resulting from a cleavage across the 5,6-bicyclo ring.[6] The m/z 572 fragment can be utilized as a pinnatoxin-specific product ion for SRM detection and confirmation of these toxins in samples.

Table 1. Pinnatoxin G (6) and A (1)[a] Concentrations in a Selection of Mussel (*Mytilus edulis*) Samples from Eastern Canada before and after Hydrolysis

		before hydrolysis (μg/kg)		after hydrolysis (μg/kg)		% esterified	
harvest location	harvest date	6	1	6	1	6	1
Baie de Tracadigache, QC	June 2, 2008	0.3	<LOD	0.7	<LOD	57	
Baie de Cascapedia, QC	July 1, 2008	0.3	<LOD	0.6	<LOD	50	
Neguac, NB	July 7, 2008	13	0.3	16	0.4	19	25
Duck Island, Bull Arm, NL	Sept 17, 2010	16	0.2	31	0.3	48	33
Flat Rock Tickle, NL	Sept 20, 2010	20	0.3	22	0.4	10	25
Bear Cove, NL	Sept 20, 2010	19	0.4	34	0.7	44	42
Boughton River, PE	Sept 22, 2010	20	0.3	28	0.3	29	0
Lagune du Havre Aux Maison Centre, QC	Sept 27, 2010	34	1.2	108	1.6	69	25
Osmonton Arm, NL	Oct 7, 2010	19	0.5	48	0.8	60	38
Tea Arm, Strong Island Sound, NL	Oct 7, 2010	24	0.4	43	0.5	44	20
Lagune de la Grande Entrée Sud, QC	Oct 11, 2010	30	1.6	87	2.4	66	33
Corkum's Island, NS	Oct 12, 2010	23	0.6	26	0.7	12	12
Dildo Run Provincial Park, NL	Aug 30, 2011	21	0.6	73	0.9	71	33
Lennox Channel, PE	Sept 7, 2011	41	1.5	59	2.1	30	29
Johnstown Harbour, NS	Sept 12, 2011	19	0.4	52	0.6	64	33
Dildo Run Provincial Park, NL	Sept 19, 2011	36	1.1	90	1.5	60	27

[a]Pinnatoxin A (1) concentrations were calculated using a pinnatoxin G (6) standard.

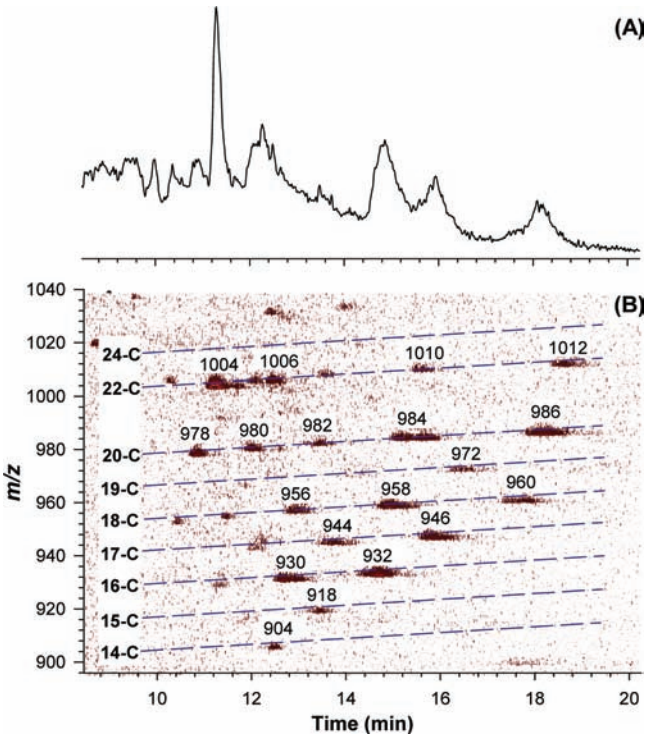

Figure 3. LC-MS precursor ion scan for m/z 164 in an extract of a mussel sample from Dildo Run Provincial Park, NL (September 2011): (A) total ion chromatogram and (B) contour plot (B) on which the $[M + H]^+$ ions are indicated for a number of potential fatty acid esters of **6** (Table 2).

Pinnatoxins were detected at various levels in a range of samples from locations in all eastern Canadian provinces: New Brunswick (NB), Newfoundland (NL), Nova Scotia (NS), Prince Edward Island (PE), and Quebec (QC). Samples were harvested during a period from January 2010 to November 2011. A total of 1010 mussel samples were analyzed from 97 harvest sites across all provinces. There was widespread geographical and seasonal occurrence of **6**, with 92% of samples containing detectable levels (LOQ ~ 1 μg/kg). Approximately 40% of samples contained levels ≥10 μg/kg. The average pinnatoxin G (**6**) concentration measured across all sites was 12 μg/kg, whereas the maximum concentration measured was 83 μg/kg for a sample harvested in Dildo Run Provincial Park on the northern NL coast (July 2011). It was noted that levels were typically high in this location; however, sampling is performed at this site only during summer and autumn months. For locations in NS (Martinique) and PE (Lennox Channel), concentrations >40 μg/kg were observed in both the summer and winter months. Therefore, from the data available, the occurrence of pinnatoxins does not appear to be seasonal. Although the standard was of high purity, the pinnatoxin concentration data reported for these Canadian mussel samples is considered to be only indicative as certified standards were not available at the time of the analysis. It is noted that the concentrations were equivalent to those observed in a survey of Norwegian mussels.[7]

Whereas other pinnatoxin analogues, such as **2**–**5**, have been detected in shellfish from China, Japan, Australia, and New Zealand,[2−4,6] only **6** and **1** were detected in the Canadian samples analyzed as part of this work. The corresponding pinnatoxin profiles for European[7] and Canadian locations in the northern Atlantic are in contrast to the broader profiles observed thus far in Pacific areas.

Quantitation of Pinnatoxins and Hydrolysis. A number of samples screened for pinnatoxins at the CFIA were selected for further analysis at the NRC. Calibration curves prepared from a certified standard (prerelease) of **6** showed good linearity of response (R^2 > 0.999) for a seven-point curve (0.1–63 ng/mL). Concentrations of **6** in the samples tested ranged from 0.3 to 41 μg/kg (Table 1), and these results correlated well with the data initially obtained for the same samples at the CFIA. In the data shown (Table 1), **1** was quantitated using the **6** standards, assuming an equivalent molar response factor. It should, however, be noted that response factors for different analogues of a toxin group can vary in electrospray ionization LC-MS, particularly in SRM mode, so the results for **1** (Table 1) are

considered estimates. For samples that did contain **1**, the concentrations were low, at approximately 3% of those measured for **6**. Pinnatoxin A (**1**) is reported to be a metabolite of **6** formed in shellfish,[6] and the results of this work correlate well with the low levels of **1** observed in Norwegian mussels.[7]

It has been reported that spirolides are generally unstable in the base hydrolysis procedure used to monitor acyl esters of the okadaic acid group;[34] however, pinnatoxins appear to be resistant.[6] This resistance to hydrolysis has been utilized as a tool to confirm pinnatoxin peak identity in shellfish samples that also contain spirolides.[7] The mussel samples shown in Table 1 were also analyzed following base hydrolysis. The stability of **6** and **1** under the hydrolysis conditions was evident from their survival during the process. However, significant increases in the concentrations of **6** were observed for all samples following the treatment, and the proportion of change varied considerably between the samples tested (Table 1). The highest level of **6** following hydrolysis was 108 μg/kg, approximately 3 times greater than before hydrolysis. Although present at much lower concentrations to begin with, increased levels of **1** following hydrolysis were also observed in many of the samples tested. These increases following base hydrolysis, particularly for **6**, were a strong indication of pinnatoxin esters. The existence of acyl esters of pinnatoxins seems to be reasonable due to the presence of hydroxyl groups on the pinnatoxin structure (Figure 1) and because of the occurrence of such derivatives for a variety of other toxin groups, which are known to form metabolites in shellfish.[20−26]

Direct Determination of Pinnatoxin Esters by LC-MS. In the initial screening and confirmation work done on free pinnatoxins (Table 1), analyses were performed on crude extracts prepared with a single methanol extraction step (extraction method 1 or 2). Due to the relatively low concentrations of the potential pinnatoxin acyl esters, a modified extraction, partitioning, and concentration procedure was used to examine samples for the presence of pinnatoxin acyl esters (extraction method 3). When mussel samples were extracted, it was found that 90% MeOH and 100% MeOH provided equivalent recoveries for free pinnatoxins: approximately 18 μg/kg for a mussel homogenate from Lennox Chanel, PE (July 2011). However, analysis of these extracts following base hydrolysis showed that 100% MeOH was much more effective for the extraction of pinnatoxin acyl esters, giving 52 μg/kg for total **6**, whereas the concentration with 90% MeOH was only 29 μg/kg. This difference corresponds to the increased lipophilicity of the acyl esters. Therefore, 100% MeOH is a more appropriate extraction solvent for analysis of pinnatoxin esters.

Precursor Ion Scanning. A precursor ion scan was run using the m/z 164 ion, which is structurally common to all pinnatoxins, with a scan range of m/z 800−1100. Any compounds detected could potentially correspond to pinnatoxin acyl esters. Figure 3 shows a contour plot from the analysis of a mussel extract from Dildo Run Provincial Park (NL) using this method. A pattern is discernible showing spots arranged in diagonal lines, with each spot increasing by 2 Da along the retention time axis. A number of these diagonal lines are visible, separated by 14 or 28 Da, which is consistent with what would be expected for **6** acylated with a variety of long-chain fatty acids. From the contour plot, acyl esters with carbon chain lengths of 14, 15, 16, 17, 18, 19, 20, and 22 are most clearly visible.

SRM. As the samples had potentially low concentrations of the proposed pinnatoxin acyl esters, a comprehensive analysis was performed using a sensitive SRM technique. An SRM method was constructed using specific SRM transitions for masses of theoretical pinnatoxin ester derivatives. The protonated molecules ($[M + H]^+$) of the ester derivatives were selected in Q1 and fragmented in the collision cell, and m/z 164 was selected in Q3. Increased sensitivity for the pinnatoxin acyl esters was found using m/z 164 as the Q3 ion when compared to using the ion resulting from the loss of the fatty acid chain $[M + H - RCOOH]^+$ (m/z 676 and 694 for **6** and **1**, respectively). Transitions were included for acyl esters with chain lengths of C12−C24, each containing up to six double bonds. Figure 4 shows data for a selection of **6** esters detected by SRM. Here the retention profile of the acyl esters is apparent, with shorter chain acyl derivatives and compounds with increasing numbers of double bonds eluting earlier, whereas compounds with longer chains and higher degrees of saturation elute later. The SRM analysis provided an estimate of the relative abundance of the acyl esters. Table 2A summarizes retention times, relative abundances, and proposed identities of the major esters detected by SRM. More than 20 acyl derivatives of **6** were observed in this sample, comprising compounds with chain lengths ranging from C14 to C24, which included variable numbers of double bonds. The major acyl esters of **6** were 22:6, 16:0, 16:1, 20:1, 17:0, 18:1, and 20:5. When the samples were subjected to base hydrolysis and reanalyzed using the SRM method, the acylated compounds were no longer present and the peak area of **6** had increased (Figure 5). In some of the SRM transitions multiple peaks were observed (e.g., Figure 4: 20:2 ester of **6**). Whereas some of these peaks were determined to be matrix interferences, some did show response in a second (confirmatory) SRM transition included in the analysis method, suggesting that some of the additional peaks were **6** esters. Multiple peaks have been observed previously for fatty acid esters of diarrhetic shellfish poisoning toxins,[23] so it is probable that the additional pinnatoxin ester peaks result from isomeric forms of fatty acids in shellfish tissues.[35] Due to the increases in peak areas of **1** following hydrolysis, the sample was also screened by SRM for the presence of acyl esters of **1**. It was not possible to detect any significant ester peaks for **1** with the samples available, which was not surprising considering that this analyte was only a minor constituent in comparison to **6**. On the basis of the data obtained for the acyl esters of **6**, it is possible that acyl esters of **1** could be determined in more highly concentrated samples.

Product Ion Spectra. Product ion spectra were acquired to help elucidate the proposed structure of the presumed acyl esters of **6**. Shown in Figure 6 are spectra for the 16:0, 17:0, and 20:5 esters. In addition to ions corresponding to the loss of water molecules, $[M + H - nH_2O]^+$, from the $[M + H]^+$ ions of the acyl esters, an abundant ion at m/z 676 observed in all spectra was assigned as $[M + H - RCOOH]^+$, corresponding to the elimination of a fatty acid molecule. The spectra of the esters below m/z 676 are a close match to that of **6** (Figure 2B). There are two hydroxyl groups in **6** that could feasibly be acylated: a tertiary hydroxyl located at C15 and a secondary hydroxyl at C28. Acylation of either of these hydroxyls would result in two different ester structures and possibly even a series of doubly acylated compounds. In this work only one peak was detected for the majority of esters (Figure 4) and no doubly acylated analogues were observed. As discussed above, there were additional peaks in some SRM traces, but these were most probably due to isomeric fatty acids. The product ion spectra of the esters suggested that the secondary

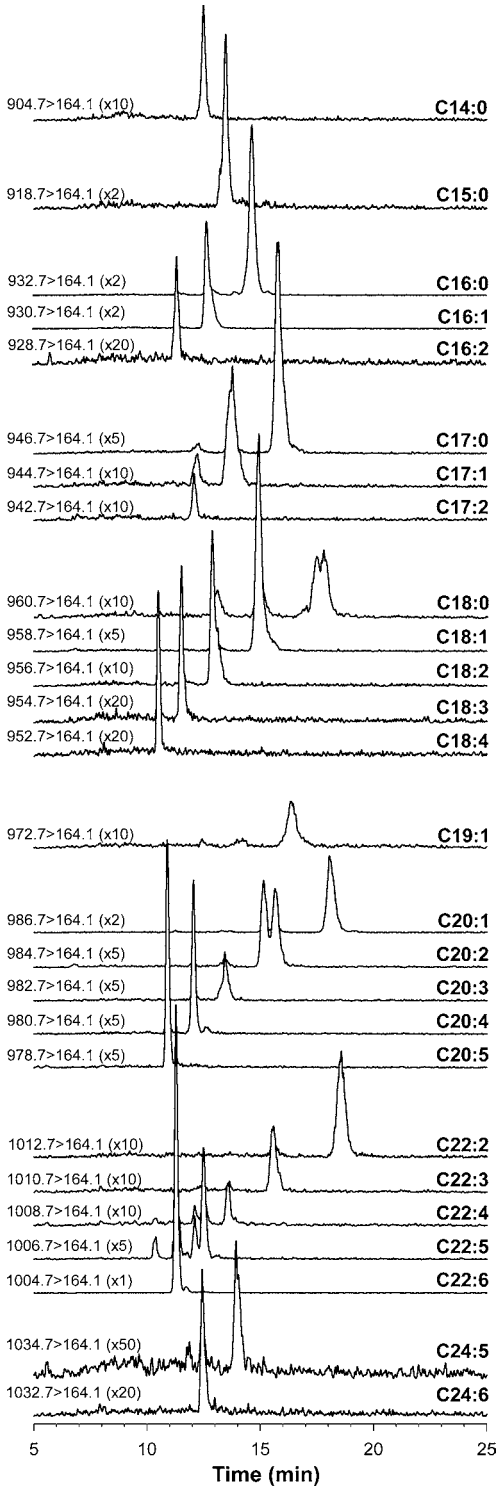

Figure 4. LC-MS/MS (SRM) analysis of a variety of fatty acid esters of **6** in a mussel sample from Dildo Run Provincial Park, NL (September 2011).

hydroxyl at C28 is the most probable location for acylation. A proposed fragmentation of the 28-*O*-palmitoyl ester of **6** is shown in Figure 7. Key ions in the spectrum of this compound (Figure 6A) are explained by specific cleavages following a retro-Diels–Alder opening of the ring adjacent to the imine ring, as reported previously for **6**.[6] Specific ions at *m*/*z* 572 and 458 would not be observed if the hydroxyl at C15 were acylated.

Table 2. (A) Retention Times and Relative Peak Areas for Esters of Pinnatoxin G (6) Detected in a Mussel Sample from Dildo Run Provincial Park, NL (September 2011); (B) Measured Accurate Masses and Calculated Exact Masses

(A) SRM analysis on QTRAP 5500			(B) accurate mass analysis on exactive		
ester	RT (min)	peak area relative to 22:6	measured accurate mass	calculated exact mass	Δ ppm
14:0	12.5	4.9	904.6647	904.6661	−1.6
15:0	13.5	6.8	918.6795	918.6817	−2.5
16:2	11.4	2.2	928.6624	928.6661	−4.0
16:1	12.7	32.1	930.6794	930.6817	−2.6
16:0	14.6	55.3	932.6947	932.6974	−2.9
17:2	12.1	1.9	942.6783	942.6817	−3.6
17:1	13.8	10.9	944.6950	944.6974	−2.5
17:0	15.8	32.7	946.7104	946.7130	−2.8
18:4	10.5	2.8	952.6630	952.6661	−3.3
18:3	11.5	3.1	954.6786	954.6817	−3.3
18:2	12.9	8.8	956.6949	956.6974	−2.6
18:1	15.0	29.0	958.7103	958.7130	−2.9
18:0	17.7	9.5	960.7262	960.7287	−2.6
19:0	16.3	4.7	972.7263	972.7287	−2.4
20:5	10.9	16.5	978.6790	978.6817	−2.8
20:4	12.0	11.7	980.6946	980.6974	−2.9
20:3	13.4	5.3	982.7103	982.7130	−2.8
20:2[a]	15.2, 15.6	11.3, 10.6	984.7259	984.7287	−2.8
20:1	18.0	31.2	986.7414	986.7443	−3.0
22:6	11.3	100.0	1004.6946	1004.6974	−2.8
22:5[a]	12.1, 12.5	3.2, 9.9	1006.7103	1006.7130	−2.7
22:4	13.5	2.1	1008.7259	1008.7287	−2.8
22:3	15.6	4.5	1010.7418	1010.7443	−2.5
22:2	18.6	9.1	1012.7570	1012.7510	−3.0
24:6	12.4	3.0	1032.7261	1032.7287	−2.5
24:5	13.9	1.5	1034.7413	1034.7443	−2.9

[a]Two peaks observed in both SRM and accurate mass analysis. Data reported for first peak, Δ <3 ppm for second peak.

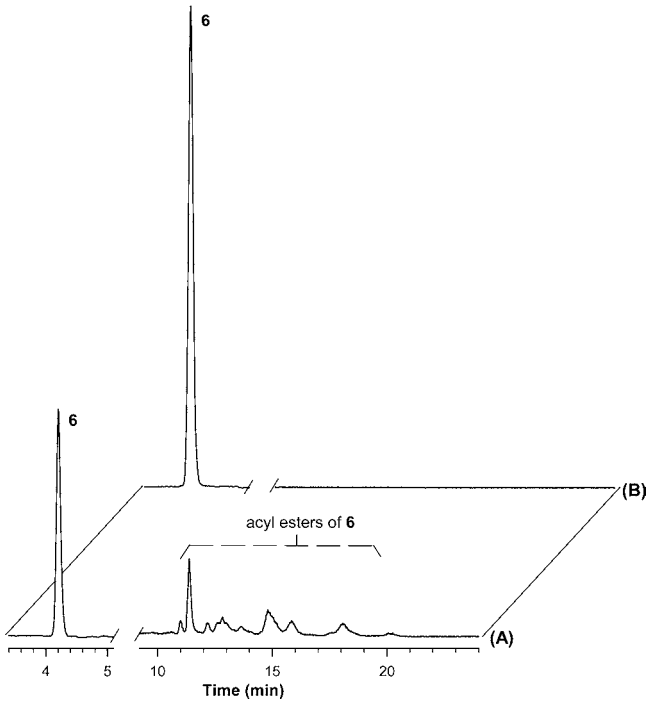

Figure 5. Total ion chromatogram of **6** and its acyl esters in mussel extract from Dildo Run Provincial Park, NL (September 2011), before (A) and after (B) base hydrolysis.

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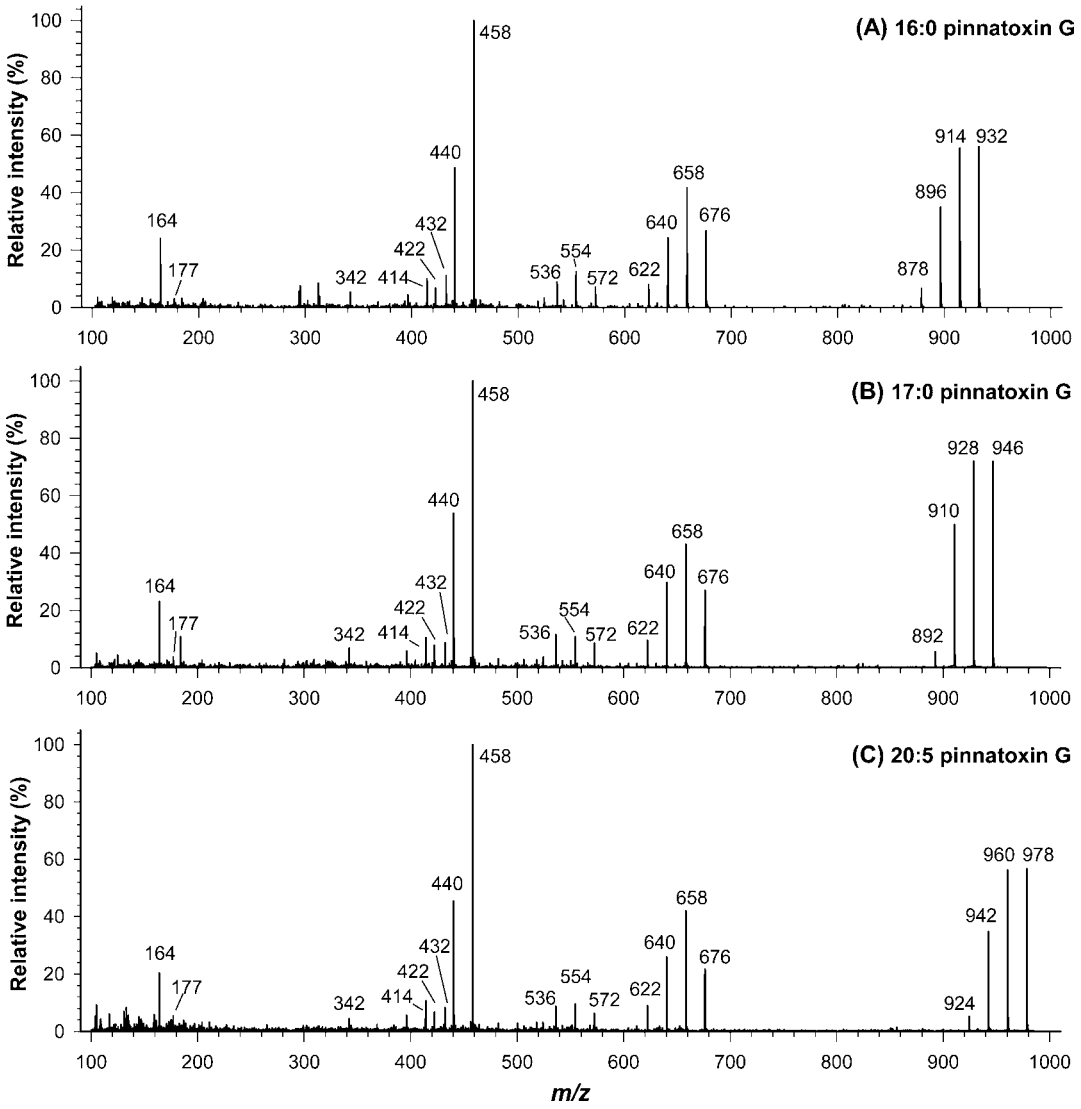

Figure 6. Product ion spectra of fatty acid esters of **6**: (A) 16:0 pinnatoxin G ($[M + H]^+$, *m/z* 932); (B) 17:0 pinnatoxin G ($[M + H]^+$, *m/z* 946); (C) 20:5 pinnatoxin G ($[M + H]^+$, *m/z* 978).

Accurate Mass Analysis. Accurate mass data were acquired to supplement information already obtained. The accurate mass observed for **6** was 694.4662 ± 0.0015 (Δ −2.1 ppm for $C_{42}H_{64}NO_7$) and for **1** was 712.4404 ± 0.0015 (Δ −2.2 ppm for $C_{41}H_{62}NO_9$). Accurate mass analysis was also used to confirm the elemental composition of the **6** acyl esters detected by SRM. Table 2B shows the measured accurate masses, calculated exact masses, and errors associated with the analysis. In general, masses for the various acyl esters of **6** were measured within 3 ppm error of the exact mass values, whereas errors were slightly higher for some of the trace level compounds. Accurate masses matched for the isomeric esters that were observed (e.g., 20:2, 22:5). This information also helped to clarify from which pinnatoxin analogue some of the esters originated. A number of potential acyl esters of **1** and **6** share the same $[M + H]^+$ ion that would be used for detection in low-resolution mass spectrometry. As an example, the *m/z* 946.7 ion could correspond to the 16:2 acyl ester of **1** or the 17:0 acyl ester of **6**. However, the exact masses of these compounds differ significantly, and from the high-resolution LC-MS analysis, it was determined that all peaks detected in SRM transitions were in fact the acyl derivatives of **6**.

Synthetic Confirmation of Pinnatoxin Fatty Acid Esters. A partial synthesis was performed for final confirmation that the compounds identified are fatty acid esters of **6** and to further demonstrate the rationale for the proposed location of the acyl function. Pinnatoxin G (**6**) was reacted with a large excess of palmitic acid (16:0) anyhydride (Figure 8). The reaction went to completion (>99% conversion) under the relatively mild conditions used, and the palmitoyl derivative of **6** was clearly observed. The retention time of the derivative matched that of the naturally formed putative 16:0 ester of **6** in the mussel sample (Figure 4). The product ion spectrum (Figure 8C) was also a match for that obtained from the mussel sample (Figure 6A). Only a single product was observed in this derivatization experiment (Figure 8B). Because acylation of tertiary alcohols with acyl anhydrides is very slow,[36] it is most reasonable to assume that only the C28 secondary hydroxyl was acylated. These observations, along with the rationalized fragmentation, provide strong evidence for the natural metabolites of **6** being acylated at C28.

Although no acyl esters of **1** were directly determined in this work, increases were observed for **1** following base hydrolysis (Table 1). The partially synthetic acylation approach was applied

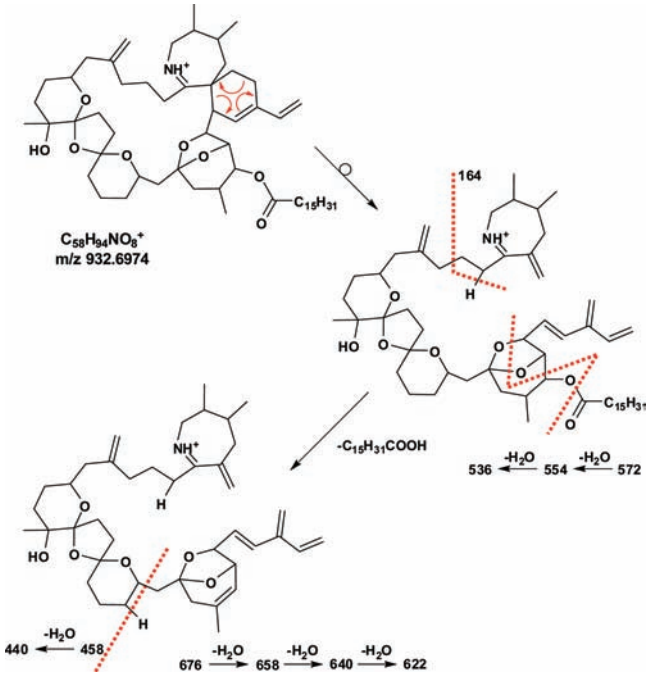

Figure 7. Proposed fragmentation of the 28-*O*-palmitoyl ester of **6** that is consistent with the product ion spectra of the observed compound (Figure 6A).

to a small amount of purified **1** to produce the 16:0 ester (see the Supporting Information). The product eluted earlier than the equivalent ester of **6** and, as for the **6** esters, the fragmentation pattern observed in the product ion spectrum was similar to that observed for **1** following the water loss from the $[M + H]^+$ (Figure 2C). This suggested that esters of **1** are acylated at the same location (C28) as the esters of **6**.

This is the first report of pinnatoxins in mussels from North America. The international occurrence of pinnatoxins is of significance to the shellfish industry and regulators alike due to the high potency of these compounds. Whereas levels of free pinnatoxins were generally low in mussel samples analyzed over almost a 2 year period (~12 μg/kg on average), concentrations in some samples were >80 μg/kg. Fatty acid acyl esters of pinnatoxins were also discovered. As some of the individual esters were present at relatively low levels, the potential of modern and sensitive LC-MS methodologies was fully utilized. Unfortunately, due to the limitations of the samples available, isolation and purification of the pinnatoxin esters was not possible to enable definitive structural elucidation by NMR. However, on the basis of extensive MS data and synthesis, it is proposed that the compounds observed are 28-*O*-acyl esters of **6**. It was shown for a selection of samples tested that upward of 50% of incurred **6** in mussels may be present as these acylated derivatives. Information on the toxicity of fatty acid esters of phycotoxins is sparse, although for diarrhetic shellfish poisoning toxins a lower toxicity has been indicated.[37] Pinnatoxin acyl esters are worthy of investigation from a toxicological point of view as they may possess toxicities similar to the nonacylated compounds. Also, hydrolysis of the esters during digestion could potentially result in exposure to significantly higher pinnatoxin levels. Information on these topics would lead to improved risk management of shellfish products contaminated with pinnatoxins and their acyl esters.

This is the first report of fatty acid acyl esters of pinnatoxins. They were not detected in a survey of mussel samples from

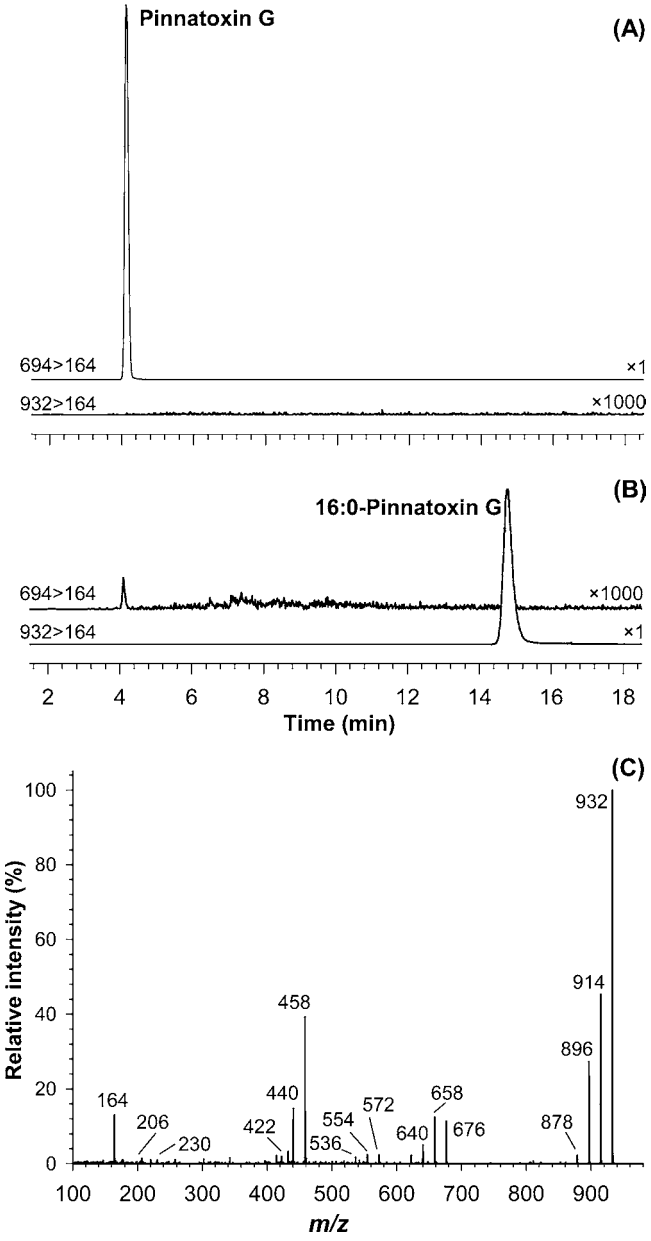

Figure 8. SRM of **6** before (A) and after (B) acylation with an excess of palmitic anhydride (>99% conversion); product ion spectrum (C) of the partially synthesized 16:0 pinnatoxin G ester product (65 eV collision energy).

Norway,[18] despite the occurrence of esters of other toxin groups in mussels harvested in similar locations.[23,26] No acyl esters of **6** were observed following base hydrolysis of contaminated oysters (*Crassostrea gigas*) from South Australia.[6] A recent study looking at the occurrence of benthic toxins from harbors in New Zealand found significant quantities of pinnatoxins and okadaic acid in a variety of marine organisms.[38] Interestingly, the majority of okadaic acid found in a species of sea hare (*Bursatella leachii*) was present in the esterified form, whereas there was no evidence of esterified pinnatoxins in the same animals. It is worth noting that the major pinnatoxins present in those samples were **3**, **4**, and **5**. These analogues share the hydroxyl at C15, but do not contain the hydroxyl present at C28 on **6**. Instead, they contain a second hydroxyl at C22. It is possible that this location may not be accessible for

enzymatic acylation, or perhaps the species in question utilizes alternative mechanisms to deal with pinnatoxins, as previously suggested.[38] Data from the Canadian shellfish toxin monitoring program has shown esters of both dinophysistoxin-1 and **6** in the same sample (W. Rourke, personal communication). The pinnatoxin-producing organism in Canadian waters has not yet been identified, but there have been no reports of pinnatoxin esters detected in producing organisms from other locations.[8−10] It is highly likely that the pinnatoxin esters are enzymatic metabolites formed in mussels, as has been suggested for other toxin groups.[24,26]

The international distribution of pinnatoxins highlights the importance of screening for these toxins as part of routine shellfish monitoring exercises to generate a data set to be used for risk assessment. The stability of pinnatoxins under base hydrolysis conditions provides an efficient method for quantitation of pinnatoxin acyl ester concentrations in samples. This feature can be easily exploited by testing laboratories that routinely perform the hydrolysis step to screen for esterified forms of okadaic group toxins in routine monitoring exercises.

■ ASSOCIATED CONTENT

Supporting Information

Summary data on the occurrence of **6** in mussel samples from eastern Canada during the period from January 2010 to November 2011 (Table S1); LC-MS/MS chromatogram of a mussel sample from eastern Canada containing pinnatoxins and spirolides (Figure S1); LC-MS/MS chromatogram and product ion spectrum of **1** derivatized with palmitic anhydride (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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