New Esters of Okadaic Acid in Seawater and Blue Mussels (*Mytilus edulis*)

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Marine algal toxins of the okadaic acid (OA) group can occur as diol esters and sulfated diol esters in algae and as fatty acid esters in shellfish. Several of these ester forms have been identified, but the most common procedure for detecting OA group toxin esters is by measuring the increase in parent toxin after alkaline hydrolysis. Use of this alkaline hydrolysis method led to the discovery of high levels of conjugates of OA and dinophysistoxins-2 (DTX2) in seawater and of OA, DTX1, and DTX2 in blue mussel hepatopancreas (HP) from Flødevigen, Norway, during a bloom of *Dinophysis* spp. In the water sample, a C_8-diol ester, a C_9-diol ester, and a previously undescribed C_8-triol ester of OA were characterized using HPLC-MS[2], -MS[3], and -MS[4] in combination with various derivatization procedures. Palmitic acid (16:0) ester derivatives of these diol/triol esters were found in mussel HP and characterized using HPLC-MS[2], -MS[3], and -MS[4]. To the authors' knowledge, hybrid diol-fatty acid esters of OA have not been previously described. Mass spectral analysis showed the presence of two forms of hybrid esters: one with the fatty acid conjugated to the 7-OH of the OA moiety and the other with the fatty acid conjugated to the OH group in the "diol" moiety. In the water sample, the C_8-diol ester was the most abundant, whereas in the mussels, the 16:0-C_9-diol hybrid ester was most abundant, and only minor amounts of the 16:0-C_8-diol hybrid ester were detected, suggesting that C_8- and C_9-diol esters of OA may be metabolized differently in blue mussels. 7-*O*-acyl esters of OA, DTX1, and DTX2 are thought to contribute to shellfish toxicity by being hydrolyzed in the human stomach to the parent toxins, and the newly characterized hybrid esters are likely to contribute similarly.

KEYWORDS: Okadaic acid; hybrid ester; diol ester; fatty acid esters; *Mytilus edulis*; *Dinophysis*; blue mussel; algal toxin; DSP

INTRODUCTION

Diarrhetic shellfish poisoning (DSP) is a severe gastrointestinal illness caused by the consumption of shellfish that have accumulated toxins from dinoflagellates such as *Dinophysis* and *Prorocentrum* spp. (*1, 2*). Typical symptoms in humans include nausea, vomiting, and diarrhea. The okadaic acid (OA) group of lipophilic toxins, which includes OA (**1**) (*3*), dinophysistoxin-1 (DTX1) (*4, 5*), and dinophysistoxin-2 (DTX2) (*4, 6*) (**Figure 1**), are principally responsible. OA analogues are thought to mediate their toxic effects primarily through inhibition of protein phosphatases (*7*), and OA and DTX1 also have tumor-promoting activity (*8*).

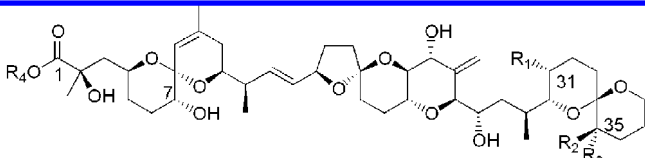A wide range of OA esters have been identified and may be detected in cultures of *Prorocentrum* spp. (**Figure 2**). The C1 acid group of okadaic acid is esterified with 4−10-carbon side chains, many of diol origin (hence the common designation "diol esters"), with various degrees of saturation and branching (*9−13*). A C_9-triol ester and a hydroperoxide derivative of a C_9-diol ester of OA have been identified in cultures of *Prorocentrum lima* (*12*). In *Dinophysis acuta*, similar diol esters were only recently reported (*14−16*). In addition, Suzuki et al. reported a series of compounds tentatively assigned to be similar esters, but they have not been unequivocally identified (*16*). The complexity of okadaic acid esters identified in *D. acuta* is much less than that of the OA esters described in *Prorocentrum* spp. However, *Prorocentrum* spp. can easily be cultured and so are more extensively studied than *Dinophysis* spp., which are difficult to maintain in culture. None of these esters have been detected in shellfish, and studies show that some diol esters may be rapidly hydrolyzed by esterases in the digestive glands of the New Zealand green-lipped mussels (*Perna canaliculus*) (*15*). In bivalve molluscs and crustaceans, other derivatives of the DSP toxins have been found in which the C7 hydroxyl

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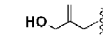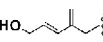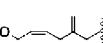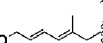§ AgResearch Ltd.
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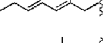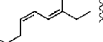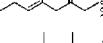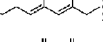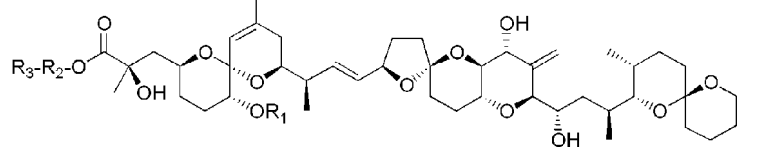
Compound	R_1	R_2	R_3	R_4	m/z [M-H]⁻	m/z [M+Na]⁺	Reference
Okadaic acid (1)	CH₃	H	H	H	803	827	(3)
DTX1	CH₃	CH₃	H	H	817	841	(4,5)
DTX2	H	H	CH₃	H	803	827	(4,5)
C₄-diol OA	CH₃	H	H	HO—		897	(9)
C₆-diol OA	CH₃	H	H	HO—		923	(12)
C₇-diol OA	CH₃	H	H	HO—		937	(13)
C₇-diol OA	CH₃	H	H	HO—		937	(10)
C₈-diol OA (2)	CH₃	H	H	HO—		951	(10)
cis-C₈-diol OA	CH₃	H	H	HO—		951	(14)
C₉-diol OA	CH₃	H	H	HO—		965	(13)
C₉-diol OA	CH₃	H	H	HO—		965	(10)
C₉-diol OA	CH₃	H	H	HO—		965	(11)
C₉-triol OA	CH₃	H	H	HO—…OH		981	(12)
C₁₀-diol OA	CH₃	H	H	HO—		977	(12)

Figure 1. Structures of OA, DTX1, DTX2, and selected diol and triol esters of OA previously identified in samples of *Prorocentrum* and/or *Dinophysis* spp. References are to the original structural identification of the ester.

		R_1	R_2	R_5	m/z [M+Na]⁺	Type
1	Okadaic acid (OA)	H	H		827	
2	C₈-diol OA	H	HOCH₂C₇H₁₀-		951	
3	C₉-diol OA	H	HOCH₂C₈H₁₂-		965	
4	C₈-triol OA	H	HOCH₂CH(OH)C₆H₁₀-		969	
5	16:0-C₈-diol OA	H	-OCH₂C₇H₁₀-	H₃C(CH₂)₁₄C(=O)-	1189	2
6	16:0-C₈-diol OA	-C(=O)(CH₂)₁₄CH₃	HOCH₂C₇H₁₀-		1189	1
7	16:0-C₉-diol OA	H	-OCH₂C₈H₁₂-	H₃C(CH₂)₁₄C(=O)-	1203	2
8	16:0-C₉-diol DTX2	H	-OCH₂C₈H₁₂-	H₃C(CH₂)₁₄C(=O)-	1203	2
9	16:0-C₉-diol OA	-C(=O)(CH₂)₁₄CH₃	HOCH₂C₈H₁₂-		1203	1
10	16:0-C₈-triol OA	H	-OCH₂CH(OH)C₆H₁₀-	H₃C(CH₂)₁₄C(=O)-	1207	2
11	16:0-C₉-triol OA	-C(=O)(CH₂)₁₄CH₃	HOCH₂CH(OH)C₆H₁₀-		1207	1

Figure 2. Structures of OA (**1**) and the diol and triol esters (**2**–**4**) detected in seawater and of their hybrid esters (**5**–**11**) detected in blue mussel hepatopancreas. Compound **8** may be an ester derivative of DTX2.

group of OA, DTX1, or DTX2 is esterified with a fatty acid chain, resulting in a complex mixture of 7-*O*-acyl fatty acid esters often collectively referred to as "DTX3" (*17*–*20*). Several such esters have been described, with fatty acid chain lengths ranging from 12 to 22 carbons, and with various degrees of saturation. In blue mussels and scallops, palmitic acid (16:0) appears to be the most abundant fatty acid moiety of OA esters (*17, 20*–*22*). Recently, low levels of these 7-*O*-acyl esters of OA and DTX2 were reported in plankton samples from Portugal (*22*), but the levels were far too low to account for the high abundance of the same esters in shellfish. Because of their virtual absence in phytoplankton, 7-*O*-acyl esters are thought primarily to be metabolic products of the transformation of OA homologues in the midgut glands of bivalves (*13, 18*).

When extracts of blue mussel hepatopancreas (HP) and seawater were screened by HPLC-MS, unknown peaks with fragmentation patterns corresponding to OA derivatives were

observed, but the peaks had retention times and masses that did not correspond to any of the known OA analogues. The present study was undertaken to examine the identities of these unknown peaks.

MATERIALS AND METHODS

Chemicals. HP20-resin (DIAION HP-20, Mitsubishi Chemical Corp., Tokyo, Japan) was activated with methanol and washed with water prior to use according to the manufacturer's instructions. Pyridine, NaOH, HCl, and formic acid (98−100%) (analytical quality) were from Merck KGaA (Darmstadt, Germany). Ammonium formate (99.995%), sodium periodate, and acetic anhydride (analytical quality) were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Acetonitrile (HPLC grade S) was from Rathburn Chemicals Ltd. (Walkerburn, Scotland), and gradient quality methanol was purchased from Romil Ltd. (Cambridge, U.K.). Deionized water (Milli-Q) was used. Standards of purified OA (**23**), DTX1, and DTX2 (**4**), and C8 diol-OA (**14**) were obtained as described earlier.

Sampling and Sample Preparation. Water samples and blue mussels were collected from Flødevigen on the south coast of Norway (58° 25.7′ N, 8° 45.2′ E) on August 13, 2007. Seawater (100 L) was pumped using an AMA-DRAINER 301 SE submersible lifting pump (KSB, Frankenthal, Germany) from a 3 m depth at 100 L/min through a 20 μm plankton net hung vertically in the water at the surface. The algal concentrate (ca. 50 mL) was passed through a cartridge containing HP20-resin (5 g) at about 2 mL/min, and the cartridge was washed with 50 mL of freshwater. Toxins were eluted with 50 mL of methanol at approximately 1 mL/min. The eluate was evaporated on a rotary evaporator and reconstituted in 5 mL of methanol for analysis. Blue mussels (*Mytilus edulis*) were collected from the same site and kept chilled (4 °C) for about 6 h before they were steamed for 5 min. The mussels were removed from the shell and separated into two sub-samples. One was homogenized directly, whereas the other was separated into hepatopancreas (HP) and "residual tissue" (mussel meat without HP). The subsamples were homogenized for 5 min (Ultra-Turrax T25, Janke & Kunkel, Staufen, Germany), and aliquots of the homogenate (2 g) were extracted with 6 mL of methanol by vortex mixing for 2 min and centrifuged at 2500g for 5 min. The pellet was extracted with 6 mL of methanol and the process repeated once more. The three extracts were combined in a 20 mL volumetric flask, and the volume was adjusted to 20 mL with methanol.

Triplicates of the mussel extracts and an algal extract (one replicate) were subjected to alkaline hydrolysis by mixing 200 μL of 5 M NaOH with 800 μL of the methanolic extract. The mixtures were left to react at 37 °C for 45 min, followed by the addition of 210 μL of 5 M HCl. All extracts were filtered through 0.2 μm Spin-X centrifugal filters (Costar, Corning, NY) prior to chromatographic analysis.

Tandem Quadrupole Mass Spectrometry. Quantitative determination of OA, DTX1, and DTX2 by HPLC-MS/MS was performed on a Waters 2695 Alliance Separation Module coupled to a Micromass QuattroUltima triple-quadrupole mass spectrometer (Waters Micromass, Manchester, U.K.). Negative electrospray ionization (ESI) was employed, and the quadrupoles were operated in multiple reaction monitoring (MRM) mode. Analytes were separated on a 3.5 μm Waters XTerra C18 analytical column (2.1 × 50 mm), using a linear gradient at 300 μL/min from 35% MeCN in water to 98% MeCN over 10 min, followed by a 10 min hold at 98% MeCN. Both mobile phase components contained 2 mM ammonium formate and 0.1% (v/v) formic acid. MRM chromatograms were recorded for the transitions m/z 803.5 → 255.1 (OA/DTX2) and m/z 817.5 → 255.1 (DTX1), using a collision energy of 50 eV. Aliquots (10 μL) of hydrolyzed and unhydrolyzed extracts were injected, and peak areas were compared to peak areas of calibration standards of OA, DTX1, and DTX2. The concentrations of esters of OA, DTX1, and DTX2 were calculated from the difference between the concentration of the toxins in extracts before and after hydrolysis.

Ion Trap Mass Spectrometry. HPLC-MS^2 and -MS^3 of the algal extract and one pooled sample of the triplicate extracts of HP from mussels was performed on a Surveyor HPLC system coupled to an LTQ linear ion trap mass spectrometer (Thermo Electron Corp.,

Waltham, MA). The MS was operated in positive ESI mode, with the spray voltage set at 4.5 kV, the capillary temperature at 250 °C, and the capillary voltage at 9 V. The maximum ion injection time was 30 ms with a total of three microscans, and the MS^2 and MS^3 collision energy was 55%. Prior to injection, a portion (10 mL) of each extract was concentrated in vacuo and reconstituted in methanol (2 mL). Aliquots of concentrated extracts were filtered through 0.2 μm Spin-X centrifugal filters, and 10 μL was injected onto a 3.5 μm Waters Symmetry C18 column (2.1 × 50 mm). Analytes were separated using a linear gradient at 300 μL/min from 30% MeCN in water to 98% MeCN over 10 min, followed by a 20 min hold at 98% MeCN. Both mobile phases contained ammonium formate (2 mM) and 0.1% (v/v) formic acid.

Algal Extracts. Five methods with positive ion HPLC-MS^2 scans were set up to search for [M + Na]$^+$ ions of OA diol esters in the concentrated algal extract. In each of the methods, MS^2 was conducted on five ions. This provided MS^2 data of a total of 25 ions, each ion two m/z units apart, from m/z 937 to m/z 985. Extracted ion chromatograms of m/z 827 (corresponding to [M + Na]$^+$ with loss of the diol group as the corresponding ketene) were constructed for all channels, and spectra of peaks were compared to previously acquired spectra of [M + Na]$^+$ for OA (**1**) and OA C$_8$-diol ester (**2**). This identified candidate esters of OA with [M + Na]$^+$ of m/z 951, m/z 965, and m/z 969. The concentrated algal extract was subjected to HPLC-MS^2 performed on m/z 951, m/z 965, and m/z 969 and to HPLC-MS3 on the transitions m/z 951 → 827, m/z 965 → 827, m/z 969 → 827, m/z 951 → 723, m/z 965 → 723, and m/z 969 → 723.

The concentrated algal extract (200 μL) was evaporated under nitrogen, dissolved in 100 μL of pyridine and 100 μL acetic anhydride, and left to react at room temperature for about 16 h. The solvent was evaporated in vacuo and reconstituted in 200 μL of methanol. HPLC-MS^2 analysis was performed on [M + Na]$^+$ ions at m/z 1119, m/z 1161, m/z 1203, m/z 1133, m/z 1175, m/z 1217, m/z 1137, m/z 1179, and m/z 1221, corresponding to the tetra-, penta-, and hexa-acetates of the ester ions of m/z 951, m/z 965, and m/z 969 mentioned above. HPLC-MS^3 analysis was also conducted on the transitions m/z 1119 → 953, m/z 1133 → 953, m/z 1179 → 953, and m/z 1179 → 1119.

NaIO$_4$(aq) (100 mM, 200 μL) was added to the concentrated algal extract (200 μL), and the mixture was left to react at room temperature for 2 h. HPLC-MS^2 was performed on [M + Na]$^+$ ions at m/z 951, m/z 965, m/z 969, and m/z 937.

Mussel Extracts. Six methods with positive ion HPLC-MS^2 scans, of five masses each, were set up to search for [M + Na]$^+$ ions of OA ester derivatives in the concentrated mussel extract. This provided HPLC-MS^2 chromatograms for a total of 30 ions, each two m/z units apart, from m/z 1177 to m/z 1235. Extracted ion chromatograms of m/z 827 and m/z 1065 were constructed for all channels, and spectra of peaks were compared to previously acquired spectra of [M + Na]$^+$ of OA, to identify candidate diol-fatty acid esters of OA. HPLC-MS^2 analysis was conducted on m/z 1189, m/z 1203, and m/z 1207 in the concentrated HP extract, corresponding to palmitate derivatives of one triol and two diol esters. HPLC-MS^3 was conducted on the concentrated mussel extract for transitions m/z 1189 → 827, m/z 1203 → 827, m/z 1207 → 827, m/z 1189 → 1065, m/z 1203 → 1065, and m/z 1207 → 1065, and HPLC-MS^4 was conducted on the transitions m/z 1189 → 827 → 723, m/z 1189 → 1065 → 705, m/z 1203 → 827 → 723, m/z 1203 → 1065 → 705, m/z 1207 → 827 → 723, and m/z 1207 → 1065 → 705.

RESULTS AND DISCUSSION

Quantitative analysis before and after hydrolysis of extracts from whole mussels and mussels subdivided into HP and residual tissue revealed high amounts of DTX2 and OA and of esters of OA, DTX1, and DTX2. However, free DTX1 was not detected (**Table 1**). DTX2 was the most abundant of the three toxins in the mussels. In the HP, DTX2 esters were present in similar concentrations to free DTX2, whereas negative concentrations were obtained for the esters in the residual tissue and in whole mussels. One possible explanation for this may be

Table 1. Quantitative Analysis of OA, DTX1, DTX2, and Their Esters[a] in the Algal Concentrate and HP Extracts from *M. edulis*

	toxin concentration[b]					
	OA		DTX1		DTX2	
sample	free	esterified	free	esterified	free	esterified
seawater	3.6	2.5	0.1	nd[c]	2.1	0.6
hepatopancreas[d]	41 (16)	89 (12)	nd	68 (28)	127 (47)	127 (26)
residual tissue[d]	9.1 (1.4)	0.6 (1.0)	nd	nd	39 (4.5)	−22 (3.2)
whole mussel[d]	29 (2.0)	0.9 (2.0)	nd	13 (4.0)	82 (16)	−31 (4.7)

[a] Calculated from the change in toxin concentration after alkaline hydrolysis. [b] Toxin concentration in seawater is given in ng/L for unconcentrated water, whereas the concentration in mussels is given in ng/mL in the extract. [c] Not detected. [d] Mean (± standard deviation), $n = 3$.

degradation of toxins under the hydrolysis conditions used, although Doucet et al. reported degradation of pure OA during alkaline hydrolysis in the absence of blue mussel sample matrix and no degradation in the presence of sample matrix (*24*). For OA, the amount of conjugates in HP released by hydrolysis was approximately twice the amount of free OA. Significant levels of OA conjugates were not found in the residual tissue or the whole mussel. No free DTX1 was detected in any of the mussel samples, although substantial levels of DTX1 esters were detected in both the HP and whole mussels. The explanation of the various results with different parts of the mussels is not yet understood, but as high proportions of all three toxins were released on alkaline hydrolysis of the HP extracts, it was decided to perform detailed analyses only on HP extracts.

In the water sample, collected at the same location on the same day as the mussels, OA was the most abundant toxin, with DTX2 present at a level of about 60% of OA, and only a low level of DTX1 was detected. However, no DTX1 esters were released by hydrolysis, even though DTX2 esters were detected at a level of 30% relative to free DTX2 and OA esters were present at a level of approximately 70% relative to free OA. A bloom of *Dinophysis* spp. reached a maximum density 4 days before the water and mussel samples were collected. *D. norvegica* and *D. acuminata* dominated the bloom, but lower levels of *D. acuta* and *D. rotundatum* were also present (Lars-Johan Naustvoll, personal communication).

The above results indicate the presence of substantial levels of hydrolyzable conjugates of OA and DTX2 in the water and of OA, DTX1, and DTX2 in shellfish HP extracts. In view of the available literature, the conjugates in the algal extract were expected to consist primarily of diol esters of OA, DTX1, and DTX2 (*9, 10, 12, 15, 16*), whereas the conjugates in the mussel extracts were expected to consist of fatty acids coupled as 7-*O*-acyl esters of OA, DTX1, and DTX2 (*17, 18, 20−22*). We performed a series of HPLC-MS[n] experiments on the mussel HP and algal extracts, including samples subjected to various derivatization procedures, to test this conjecture.

HPLC-MS[2] screening of algal extracts for 25 ions from *m/z* 937 to *m/z* 985 revealed the presence of prominent OA derivatives with [M + Na]+ of *m/z* 951, *m/z* 965, and *m/z* 969. The [M + Na]+ ions of the OA analogues were examined, rather than the more commonly studied [M + NH$_4$]+ ions, because the fragment ion spectra of [M + Na]+ ions were more structurally informative than the spectra obtained from fragmentation of [M + NH$_4$]+ ions (*21*). The MS[2] spectra of all three compounds were characterized by two initial water losses (**Figure 3**), but the major fragment ion was *m/z* 827, which corresponds to [M + Na]+ of OA and DTX2. Other prominent fragments detected at *m/z* 809, *m/z* 791, *m/z* 723, and *m/z* 571 were all characteristic for [M + Na]+ of OA derivatives (*21*). HPLC-MS[3] spectra derived from [M + Na]+ → *m/z* 809 mainly afforded the *m/z* 791, *m/z* 723, and *m/z* 571 ions, but at a higher
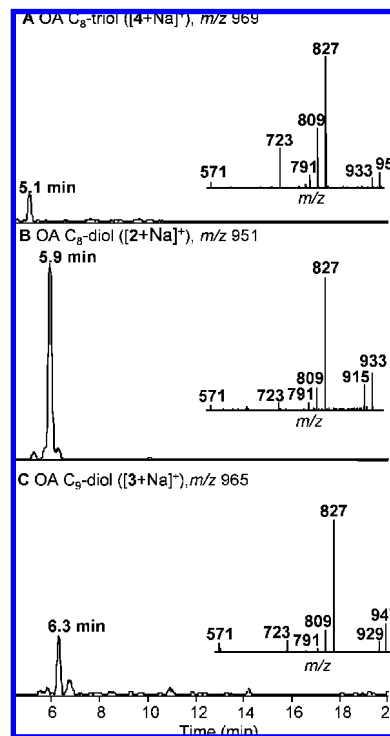

Figure 3. HPLC-MS[2] chromatograms and spectra of (**A**) a C$_8$-triol ester of OA (**4**), (**B**) a C$_8$-diol ester of OA (**2**), and (**C**) a C$_9$-diol ester of OA (**3**). Minor peaks eluting after the main peaks in traces **B** and **C** are attributable to analogues of DTX2. The origin of fragments with *m/z* 827 and lower is identical to that depicted for hybrid esters in **Figure 8**; other fragments are water losses from the [M + Na]+ ions.

intensity. HPLC-MS[3] spectra derived from [M + Na]+ → *m/z* 723 and HPLC-MS[4] spectra derived from [M + Na]+ → *m/z* 827 → 723 produced only weak signals for the compound with [M + Na]+ at *m/z* 951. Both of these gave *m/z* 595 as the most abundant product ion, with a weak ion at *m/z* 571. The latter derives from retro-Diels−Alder (RDA) rearrangement in ring B of the OA skeleton, whereas the former appears to derive from cleavage in ring F with the 31-methyl group present in the fragment ion because DTX2 showed an equivalent ion at *m/z* 581. The proposition that the major compounds were esters of OA rather than of DTX2 was supported by the finding that an authentic specimen of **2** gave an essentially identical retention time and mass spectrum as the OA ester with [M + Na]+ at *m/z* 951 during LC-MS. However, the *m/z* 951 and *m/z* 965 ion traces also showed minor peaks eluting after the main peaks (**Figure 3**), attributable to analogius C$_8$- and C$_9$-diol esters of DTX2, as DTX2 esters were found to be present at 25% the level of OA esters by alkaline hydrolysis.

The identity of the ester of OA with [M + Na]+ at *m/z* 951 (**Figure 2**) was confirmed as **2** by its matching retention time and mass spectrum with an authentic standard of **2** (*14*). This

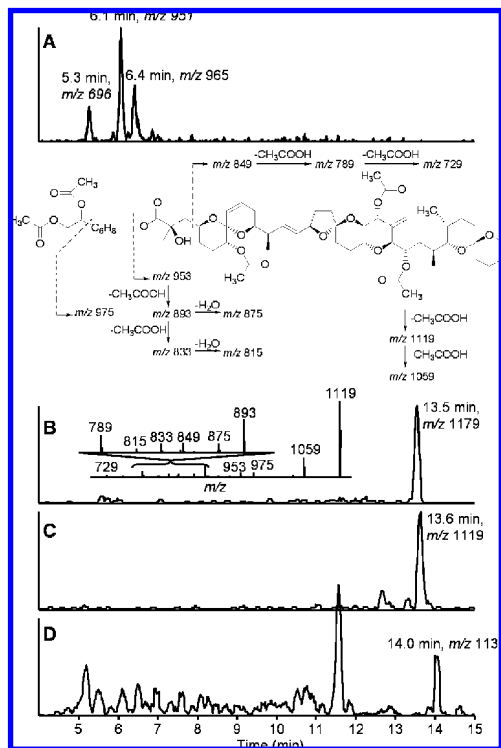**9632** *J. Agric. Food Chem.,* Vol. 56, No. 20, 2008

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Figure 4. HPLC-MS chromatograms of concentrated algal extracts before and after acetylation: (**A**) *m/z* 951, 965, and 969 in the untreated algal extract; (**B**) *m/z* 1179 in the acetylated extract, showing the MS^2 spectrum and suggested fragmentation for the peak eluting at 13.5 min (inset); (**C**) *m/z* 1119 in the acetylated extract; (**D**) *m/z* 1133 in the acetylated extract. The peak at 11.6 min in chromatogram **D** arises from a contaminating compound in the sample with *m/z* 1133 but displaying different fragments in MS^2 compared to the OA derivatives.

compound has previously been identified in both *D. acuta* and *Prorocentrum* spp. (*10, 14, 16*). The OA ester with [M + Na]$^+$ at *m/z* 965 (**3**) (**Figure 2**) was likely similar to previously described C$_9$-diol esters of OA (**Figure 1**) from *Prorocentrum* spp. (*10, 13*), but no standards were available for confirmation. The third OA ester, with [M + Na]$^+$ at *m/z* 969 (**4**) (**Figure 2**), has to our knowledge not been previously described. Its mass spectrum indicated that it was a similar ester conjugated at the acid group of OA, and its mass suggested it to be a novel C$_8$ ester containing one extra oxygen atom and one less double bond than the known C$_8$-diol esters of OA (**Figure 1**). The reduced retention time for this compound compared to **2** was also consistent with an oxidized C$_8$-diol ester analogue.

The algal extract was subjected to microscale reactions and monitored by HPLC-MS^n to obtain further structural information about the unidentified esters. Treatment of the sample with pyridine and acetic anhydride was performed to acetylate reactive primary and secondary (but not tertiary) hydroxyl groups. The OA skeleton itself contains one tertiary and three secondary OH groups, and HPLC-MS^2 experiments were set up for the acetylated derivatives of the three OA esters at *m/z* values that assumed the presence of one, two, or three reactive OH groups in the side chain. In the chromatogram of the acetylated algal extract, the original OA ester peaks with [M + Na]$^+$ at *m/z* 951, 965, and 969, previously shown to elute at 5−6.5 min (**Figure 4A**), were absent. Instead, peaks affording [M + Na]$^+$ at *m/z* 1119, 1133, and 1179 eluted at 13.5−14 min (**Figure 4B−D**). The peak at 13.5 min with *m/z* 1179 displayed a mass spectrum consistent with a penta-acetate of the *m/z* 969 compound (inset, **Figure 4B**). The dominant ion
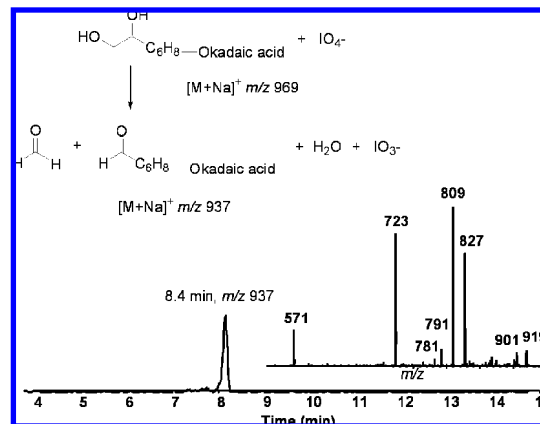

Figure 5. HPLC-MS extracted chromatogram for *m/z* 937 of the concentrated algal extract after treatment with periodate. The MS^2 spectrum of the peak eluting at 8.4 min and the periodate reaction are depicted in the inset. The origin of fragments with *m/z* 827 and lower is identical to those illustrated for hybrid esters in **Figure 8**, whereas fragments with *m/z* 919 and 901 are attributable to water losses from the *m/z* 937 [M + Na]$^+$ ion.

in the MS^2 spectrum (*m/z* 1119) is attributable to loss of acetic acid and is followed by another acetic acid loss affording *m/z* 1059. The *m/z* 975 ion is attributable to loss of (AcOCH)$_2$ from a 1,2-diacetoxy moiety in the *m/z* 1179 ion (inset, **Figure 4B**), whereas the ion at *m/z* 953 corresponds to the Na$^+$ adduct of OA-triacetate. The *m/z* 893 and *m/z* 833 ions are attributable to one and two acetic acid losses, respectively, from *m/z* 953 and the *m/z* 875 and *m/z* 815 ions to water losses from the *m/z* 893 and *m/z* 833 ions. Cleavage through C3−C4 of the OA skeleton affords daughter ions with *m/z* 849, and the ions with *m/z* 789 and 729 correspond to one and two acetic acid losses, respectively, from *m/z* 849. These observations are consistent with the proposal that the *m/z* 969 compound possesses two primary or secondary hydroxyl groups in its ester side chain. The peaks eluting at 13.6 min (**Figure 4C**) and 14.0 min (**Figure 4D**) exhibited *m/z* 1119 and 1133 ions, respectively, and showed fragmentations consistent with tetra-acetates of original compounds with [M + Na]$^+$ at *m/z* 951 and 965. These observations are consistent with the proposal that the *m/z* 951 and *m/z* 965 compounds each possess a single primary or secondary hydroxyl group in its ester side chain.

Treatment of the algal extract with aqueous NaIO$_4$ for 2 h did not affect the *m/z* 951 or *m/z* 965 compound. However, the *m/z* 969 compound disappeared and was replaced by a new compound with [M + Na]$^+$ of *m/z* 937 (**Figure 5**), indicating the presence of a 1,2-dihydroxy moiety. The new compound fragmented (inset, **Figure 5**) in a manner similar to that of the other OA derivatives (**Figure 3**) and eluted later than the C$_8$- and C$_9$-diol esters. On the basis of the foregoing observations, we propose the structure of the *m/z* 969 compound to be a C$_8$-triol ester of OA with a molecular mass of 946 Da containing a single double bond and a terminal 1,2-diol moiety (**4**). A C$_9$-triol ester of OA containing a terminal 1,3-diol moiety (**Figure 1**) has been isolated and identified from *P. lima* (*12*), and an unidentified C$_8$-triol ester with two double bonds has been reported as a biotransformation product of an OA C$_8$-diol ester in the diatom *Thalassiosira weissflogii* (*25*).

HPLC-MS^2 screening of mussel HP extract for 30 ions in the range from *m/z* 1177 to *m/z* 1235 revealed the presence of seven OA derivatives with [M + Na]$^+$ at *m/z* 1189, *m/z* 1203, and *m/z* 1207 (**Figure 6**). These *m/z* values were consistent with palmitate esters (**5−11**) of the three diol/triol esters of OA (**2−4**)

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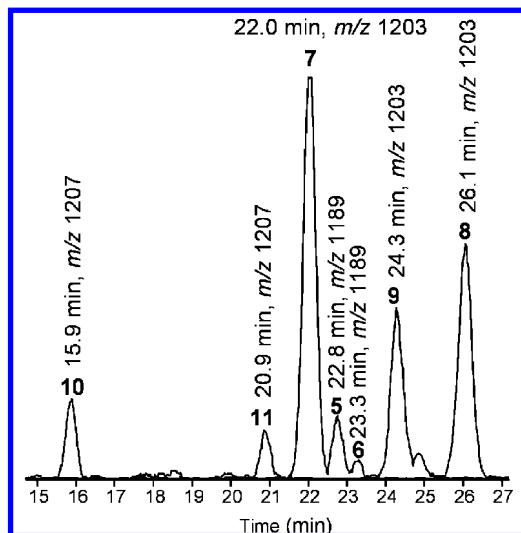*J. Agric. Food Chem.,* Vol. 56, No. 20, 2008   **9633**

Figure 6. Extracted ion chromatogram for seven 16:0 fatty acid diol/triol hybrid esters of OA and DTX2. **10** and **11** are 16:0-hybrid esters of a new C_8-triol ester of OA with $[M + Na]^+$ at *m/z* 1207. **7** and **9** are 16:0-hybrid esters of a C_9-diol ester of OA with $[M + Na]^+$ at *m/z* 1203, and **8** is presumed to be a 16:0-C_9-diol hybrid ester of DTX2. **5** and **6** are 16:0-hybrid esters of a C_8-diol ester of OA with $[M + Na]^+$ at *m/z* 1189. Compounds **6**, **9**, and **11** have the palmitic acid moiety conjugated to the 7-OH group of OA, whereas the other compounds have the palmitic acid conjugated to an OH group in the diol/triol side chain (**Figure 2**).

that were detected in the algal extract. This type of hybrid diol ester/fatty acid ester has not previously been described. Of the seven hybrid palmitate esters, two afforded $[M + Na]^+$ with *m/z* 1189 (**5** and **6**), three with *m/z* 1203 (**7**, **8**, and **9**), and two with *m/z* 1207 (**10** and **11**). The compounds with *m/z* 1203 were the most abundant.

HPLC-MS[2] spectra of the peaks (**Figure 7**) were consistent with palmitic acid conjugated at two positions in the molecules. Compounds **6**, **9**, and **11** (type-1 hybrid esters) appeared to be OA esters with diol/triol esters conjugated at C1 together with palmitate conjugated to the 7-OH group in the same manner as previously described fatty acid esters of OA-group toxins (*17−22*). For compounds **5**, **7**, **8**, and **10** (type-2 hybrid esters), palmitic acid appeared to be conjugated to an OH group in the diol/triol moiety of an OA diol/triol ester (**Figure 2**). The dominant product ion in the MS[2] spectra of the $[M + Na]^+$ ions of all the esters was attributable to cleavage of the ester at C1. For type-1 hybrid esters (**6**, **9**, and **11**) this resulted in a palmitoyl-OA fragment ion with *m/z* 1065, but for type-2 hybrid esters (**5−8** and **10**) the resulting fragment ion was $[OA + Na]^+$ itself at *m/z* 827 (**Figure 8**). Subsequent loss of the fatty acid from C7 in type-1 hybrid esters, or of the C7-OH group in the type-2 hybrid esters, afforded *m/z* 809 fragments ions, and the *m/z* 791 ions can be attributed to loss of water from this. A characteristic fragmentation of $[M + Na]^+$ for OA and derivatives was cleavage of the C3−C4 bond of the OA skeleton (*21*). For type-2 hybrid esters, the cleavage of the C3−C4 bond afforded a fragment ion at *m/z* 723. For type-1 hybrid esters, this cleavage followed the cleavage of the ester bond at C1 and the loss of the fatty acid, affording an ion at *m/z* 705. OA and derivatives have been shown to fragment through RDA rearrangements in ring B (*21*), but this fragmentation appeared to occur only for type-2 hybrid esters, giving rise to fragment ions with *m/z* 571. Minor ions at *m/z* 773 and 1047 can be attributed to water losses from the above-mentioned ions, whereas minor ions at *m/z* 781 in the spectrum of compound **10** can be attributed to loss of formic acid from the *m/z* 809 ions (**Figure 8**). HPLC-MS[3] and
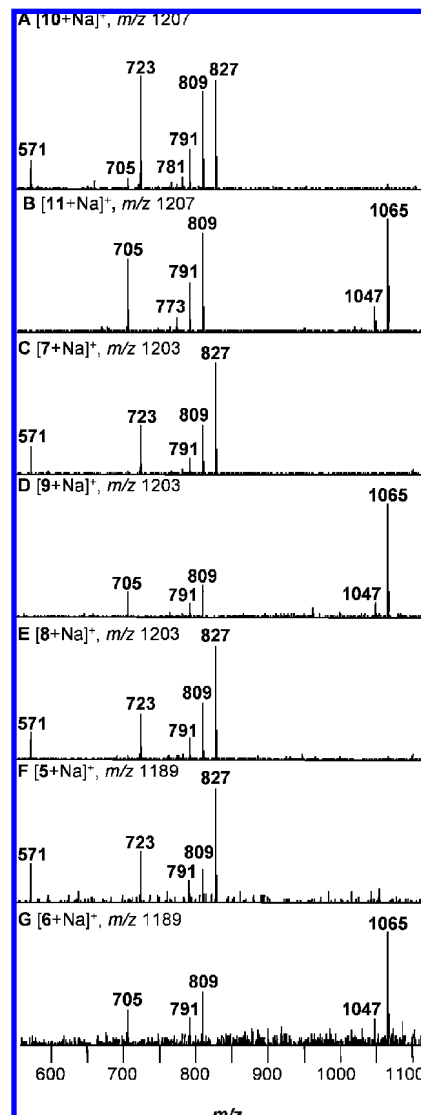

Figure 7. HPLC-MS[2] spectra of $[M + Na]^+$ of the seven 16:0 diol/triol hybrid esters of OA from the chromatogram in **Figure 5**: (**A**) 16:0-C_8-triol hybrid ester of OA with the fatty acid conjugated on the triol side chain (**10**), *m/z* 1207; (**B**) 16:0-C_8-triol hybrid ester of OA with the fatty acid conjugated on C7 (**11**), *m/z* 1207; (**C**) 16:0-C_9-diol hybrid ester of OA with the fatty acid conjugated on the diol side chain (**7**), *m/z* 1203; (**D**) 16:0-C_9-diol hybrid ester of OA with the fatty acid conjugated on C7 (**9**), *m/z* 1203; (**E**) 16:0-C_9-diol hybrid ester of DTX2 with the fatty acid conjugated on the diol side chain (**8**), *m/z* 1203; (**F**) 16:0-C_8-diol hybrid ester of OA with the fatty acid conjugated on the diol side chain (**5**), *m/z* 1189; (**G**) 16:0-C_8-diol hybrid ester of OA with the fatty acid conjugated on C7 (**6**), *m/z* 1189. Proposed fragmentation pathways are shown in **Figure 8**.

-MS[4] produced weak spectra for the most abundant of the hybrid esters (*m/z* 1203), whereas the other compounds were below the threshold intensity for fragmentation. The HPLC-MS[3] of *m/z* 1203 → 827 primarily afforded the aforementioned *m/z* 809 and *m/z* 723 ions, and HPLC-MS[3] of *m/z* 1203 → 1065 afforded *m/z* 809 and *m/z* 705 ions. However, the latter also afforded ions at *m/z* 961, indicating that the type-1 hybrid esters also fragment by a C3−C4 cleavage, whereas the fatty acid group is still conjugated. HPLC-MS[4] of *m/z* 1203 → 1065 → 961 afforded only one ion at *m/z* 705, confirming that C3−C4 cleavage preceded loss of the fatty acid group. MS[4] of *m/z* 1203 → 827 → 809 primarily afforded the *m/z* 791 ion due to another
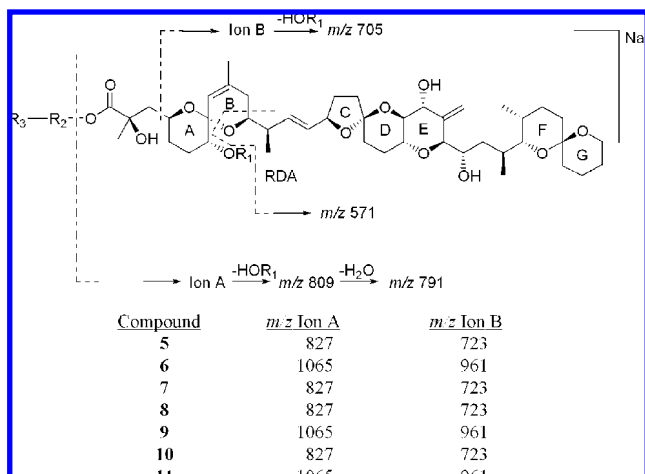
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Figure 8. Proposed fragmentation for [M + Na]$^+$ of 16:0 diol/triol hybrid esters of OA, with structures as shown in **Figure 2**.

Table 2. Proportion of Ester Moieties in the Algal and Mussel Extracts

	realative peak area^a (%)	
ester	algal extract	mussel extract^b
OA C$_8$-diol	70	6
OA C$_9$-diol	21	83
OA C$_8$-triol	9	11

^{a} Sum of areas = 100%. ^{b} In mussels, relative peak areas are of the 16:0 esters of the diols/triol.

loss of water and minor amounts of *m/z* 723 and *m/z* 571 ions (Supporting Information).

Peak areas of OA diol/triol esters in the algal sample and OA hybrid esters in the mussel extract were measured and the relative abundances of the three diol/triol-moieties compared as a proportion of the total OA/DTX ester content assuming equal molar responses on the HPLC-MS system (**Table 2**). In the algal extract, the C$_8$-diol ester (**2**) was the major ester of OA, constituting 70% of the total esters, whereas the C$_9$-diol ester (**3**) constituted 21%, and only 9% of the esters were attributable to the C$_8$-triol ester (**4**). In the mussel extracts, however, only 6% of the 16:0 hybrid esters detected were C$_8$-diol derivatives (**5** + **6**). The major 16:0 hybrid esters in the mussels were the C$_9$-diol esters (**7** + **8** + **9**), constituting 83% of the 16:0 hybrid esters detected in the extract. The remaining 11% was composed of 16:0 esters of the C$_8$-triol compound (**10** + **11**). This suggests that the fatty acid ester derivatives of C$_9$-diol esters accumulate to a much greater degree in blue mussels than do analogues of the C$_8$-diol esters. The purified C$_8$-diol ester of OA has been demonstrated to be rapidly hydrolyzed to OA by enzymes from the HP of New Zealand green-lipped mussels (*Perna canaliculus*) (*15*), but no similar study has been performed on other diol esters of OA or with enzymes from *M. edulis*. It is possible that differences in the rates of hydrolysis of the various diol/triol esters by mussel enzymes are responsible for the selective accumulation of C$_9$-diol-derived hybrid OA esters, but this hypothesis has yet to be investigated. Two peaks in the *m/z* 1203 ion trace displayed type-2 hybrid ester fragmentation, and one peak showed type-1 hybrid ester fragmentation, whereas only two peaks for each type of hybrid ester were observed in each of the two other ion traces (*m/z* 1189 and 1207). Hydrolysis of the HP extracts showed that they contained conjugates of DTX2 at a higher level than of OA, so it is probable that some of these conjugates are corresponding diol-fatty acid hybrid esters of DTX2. The

MS conditions used did not allow OA esters and DTX2 esters to be distinguished. However, it seems likely that the major hybrid esters were OA derivatives, because the diol esters in the water sample being primarily of OA origin, and that the later-eluting of the type-2 hybrid esters with *m/z* 1203 was a DTX2-derivative (**8**). Confirmation of this awaits the availability of more sample material.

OA, DTX1, and DTX2 are known to occur as 7-*O*-acyl fatty acids esters of various carbon chain lengths and unsaturation in blue mussels (*M. edulis* and *M. galloprovincialis*) and scallops (*P. yessoensis*). In blue mussels and scallops, palmitic acid (16:0) has been reported as the major fatty acid moiety present in such esters, and other major fatty acid esters reportedly include 14:0, 15:0, 16:1, 17:0, 17:1, 18:0, 18:1, 18:2, 18:3, 18:4, 20:1, 20:2, 20:4, 20:5, and 22:6 (*17, 18, 20–22*). Palmitic acid is reported to be one of the major free fatty acids present in blue mussels, together with eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6). In addition, 14:0, 16:1 18:0, 18:1, 18:2, 18:3, 18:4, 20:1, and 20:2 fatty acids are commonly present at substantial levels (*26*). It is therefore probable that there will be similar OA diol-fatty acid hybrid esters with these other fatty acid moieties in blue mussels. There is also a possibility of finding similar diol-fatty acid hybrid esters in other filter-feeding bivalve molluscs, for example, scallops, clams, and oysters, that feed by ingesting phytoplankton such as *Dinophysis* and *Prorocentrum* spp.

OA and OA analogues are thought to mediate their toxic effects primarily through inhibition of protein phosphatases, and OA, DTX1, and DTX2 are potent protein phosphatase inhibitors (*727*). For 7-*O*-fatty acid esters of OA, the intraperitoneal toxicity in mice is reported to be lower than for OA (*28*), and esterification of the carboxylic group reduces the inhibitory potency of OA analogues by 3–4 orders of magnitude (*29*). However, esterified derivatives of OA, DTX1, and DTX2 are thought to be relatively unstable (*20*) and are expected to be hydrolyzed in the stomach of humans to release the parent toxin. Hence, these newly described diol-fatty acid hybrid esters are likely to contribute to the toxicity of shellfish. The currently used alkaline hydrolysis step appears to efficiently convert the hybrid esters into their parent toxins. As long as a hydrolysis step is included in the analysis, current chemistry-based methods will provide a suitable level of protection for public health. Details of their occurrence and toxicological potency, as well as their biological origin and significance, remain to be determined.

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