Characterisation of okadaic acid related toxins by liquid chromatography coupled with mass spectrometry

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Abstract

In the Diarrhetic Shellfish Poisoning (DSP) phenomena, the parent toxins, namely okadaic acid (OA) and/or dinophysistoxin-2 (DTX2), are predominantly found esterified. Therefore, a toxicity assessment of a sample can only be performed after an alkaline hydrolysis step in order to recover the parent molecules in their free form. The presence of several OA diol esters has already been confirmed in \textit{Prorocentrum lima} and \textit{Prorocentrum belizeanum} cultures. This paper reports on the analysis of OA diol esters using liquid chromatography coupled with mass spectrometry (LC–MS/MS), and establishes a method for their detection and identification based upon their retention times (RT) and the fragmentation patterns of their mass spectra.

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1. Introduction

Okadaic acid (OA) and dinophysistoxins (DTXs) (Fig. 1) have been reported as the main cause of the worldwide DSP syndrome (Diarrhetic Shellfish Poisoning) (Yasumoto and Murata, 1993). In order to prevent human intoxication, many monitoring programmes of shellfish toxicity have been implemented on the basis of mouse bioassays (Fernández et al., 2003a). These analyses have been gradually complemented with the use of liquid chromatography (LC)–fluorometry methods as proposed by Lee et al. (1987) or enzymatic protocols based on the inhibition of protein phosphatase type 2A (PP2A) (Cembella et al., 2003). Both methodologies are only applicable where the carboxylic acid of these toxins is free, and normally when OA ester derivatives are present the problem is solved by a previous alkaline hydrolysis reaction to obtain a real estimation of toxin abundance (Quilliam, 2003). However, using this method no information about the toxin profile of the sample is obtained. In the
last decade, the use of liquid chromatography coupled with mass spectrometry (LC-MS) has been implemented as a standard analytical procedure. The data obtained by this combination allow more information to be obtained on variable toxin content profile present in a microalgal outbreak (Ardrey, 2003). In fact, detailed profiles of 7-O-acyl esters for OA and DTXs in plankton and shellfish collected along the Portuguese coast and in brown crabs from Norway, by direct analysis using LC–MS have been published recently (Quilliam et al., 2003; Marr et al., 1992; Vale, 2006; Torgensen et al., 2005). However, the number of reported DSP toxin derivatives have been increasing over the last few years due to these advances in spectroscopic and chromatographic techniques, as well as the use of

Fig. 1. Structures of okadaic acid (1), DTX1 (2) and OA (3-13) congeners isolated from P. lima and P. belizeanum cultures.
artificial cultures of dinoflagellates as a source of toxic material (Norte et al., 1994; Hu et al., 1992, 1995a, b; Daranas et al., 2001; Miles et al., 2006). Thus, a representative group of extremely variable toxic profile is observed in the OA diol-ester derivatives found in different strains of identical species (Bravo et al., 2001; Nascimento et al., 2005) and, OA diol esters have been isolated from the toxic dinoflagellate Dinophysis acuta bloom harvested off the coast of New Zealand (Suzuki et al., 2005; Miles et al., 2006).

Recently, we have isolated several new OA derivatives from cultures of dinoflagellates belonging to the genus Prorocentrum and subsequently their structures were determined by interpretation of their spectroscopic data (Fig. 1) (Suárez-Gómez et al., 2001, 2005; Fernández et al., 2003b). As an extension of this work, and using pure samples of each derivative, we present here a LC–MS/MS study where chromatographic conditions and fragmentation patterns allow for easy identification of these metabolites, which have large variations in their ester side-chain length.

2. Material and methods

2.1. Toxin preparation

The dinoflagellate Prorocentrum lima, PL2V strain, was isolated by Dr. Isabel Bravo from the lagoon of the Cies Islands (Vigo). The dinoflagellate Prorocentrum belizeanum, PBMA01 strain, (Morton et al., 1998) was obtained from the IEO (Vigo) collection by courtesy of Santiago Fraga. Cell cultures of P. lima and P. belizeanum (700 and 560 L, respectively) were conducted using conditions already published (Fernández et al., 2003b; Suárez-Gómez et al., 2001, 2005).

Cell extracts were chromatographed using gel filtration on a Sephadex LH-20 column as a first step, eluting with a mixture of chloroform:methanol:n-hexane. (1:1:2). Then a medium pressure reversed-phase Lobar LiChroprep RP-18 column eluted with methanol:water (17:3) was used. Final purifications were achieved on a µ-Bondapak C-18 HPLC column using a gradient of two mobile phases: acetonitrile:water:acetic acid (50:50:1) (solvent A) and acetonitrile:acetic acid (100:0.1) (solvent B). The separation of fractions started with 100% of A (1 min), followed by a linear ramp of B (60 min) up to 100%. Afterwards, for each of the selected fractions, the chromatographic sequence consisting of an isocratic elution, methanol:water (17:3) with the same column was carried out (Fernández et al., 2003a, b; Suárez-Gómez et al., 2001, 2005). Purification was followed by TLC and UV when it was possible. The crude extract from P. lima cells yielded a total of 12 pure OA congeners: OA (1) (80.0 mg), DTX-1 (2) (6.0 mg), methyl okadaate (3) (0.6 mg), and a series of OA esters: norokadanone (4) (2.7 mg), 2-hydroxymethyl-allyl okadaate (5) (0.2 mg), 5-hydroxy-2-methylene-pent-3-enyl okadaate (6) (1.2 mg), 7-hydroxy-2,4-dimethyl-hepta-2,4-dienyl okadaate (7) (1.2 mg), 7-hydroxy-4-methyl-2-methylene-hepta-4-enyl okadaate (8) (14.0 mg), 5,7-dihydroxy-2,4-dimethylene-heptyl okadaate (9) (1.0 mg), 5-hydroperoxy-7-hydroxy-2,4-dimethylene-heptyl okadaate (10) (0.6 mg), 4-formyl-2-methylene-pent-4-enyl okadaate (DTX-6) (11) (0.6 mg), 7-hydroxy-2-methyl-hepta-2,4-dienyl okadaate (12) (0.6 mg). The extract from P. belizeanum resulted in the isolation of high concentration of OA (1) (80.0 mg) and the new OA diol ester 7-hydroxymethyl-2-methylene-octa-4,7-dienyl okadaate (13) (8.0 mg) (Suárez-Gómez et al., 2005) (Fig. 1).

2.2. LC–MS and LC–MS/MS analyses

LC–MS characterisation of the toxins was carried out using OA (1) and 11 OA congeners (2–13) (Fig. 1) that were previously purified from cells of either P. lima or P. belizeanum cultures, and subsequently fully identified by NMR spectroscopy (Suárez-Gómez et al., 2001, 2005; Fernández et al., 2003b).

LC–MS separations of the OA congeners were carried out on a Synergy 4μ MAX-RP 80A (Phenomenex, Torrance, CA, USA), on a X Terra C-18 5μ (Waters Corp., Milford, MA, USA) and on a XBridge C-18 5μ HPLC columns (150 x 2.1 mm) (Waters Corp.), thermostatised at 30°C in a column oven. Ammonium acetate 2 mM, pH 5.8 (solvent A) and methanol (solvent B) as mobile phases were used. Ammonia solution (25% v/v), acetic acid (96% v/v) and methanol (liquid chromatographic grade) were purchased from Merck (Darmstadt, Germany). For the Synergy and XTerra columns, the following gradient was used: the initial eluent composition 70% B (8 min), was followed by a slow ramping (30 min) up to 80% B and maintaining this condition for 35 min; finally the gradient was returned to the initial conditions at 45 min. When the XBridge column was used, the gradient was modified as indicated in Table 1. A flow rate of
200 μL/min and injection volumes of 2 μL of sample in methanol were used. LC–MS detection was performed using an ion trap mass spectrometer, LCQ-Advantage (Thermo-Finnigan, San Jose, CA, USA), equipped with an electrospray ionisation interface (ESI). ESI was performed by means of a 5.5 kV spray voltage using a capillary temperature of 200 °C and flows of 40 mL/min for sheath gas and 20 mL/min for auxiliary gas. Full scan data were acquired from m/z (500–2000), in both negative and positive ionisation modes. Selected ion chromatograms for OA and their derivatives were performed for negative ions at m/z [M+CH₃COO]⁻ and a weak ion at m/z [M–H]⁻. The acetate adduct was formed because of the use of ammonium acetate as a mobile phase. The pseudomolecular ions formed in the positive mode, and summarised in Table 2, were at m/z [M+H]⁺, [M+Na]⁺ and [M+NH₄]⁺. The most relevant ions in all cases were the ammonium adducts, formed due to the use of the described mobile phase.

The chromatographic study was focused on determining the most appropriate column and gradient conditions for OA and related compounds, for this purpose a Phenomenex Synergi, a Waters XTerra and a Waters XBridge column were tested. With the XBridge column, the shortest RTs and the same separation between peaks were obtained, compared to the other columns. Despite very close RTs being obtained for the pair of compounds 3, 4 and 9, 10, modifications of the gradient elution profile and/or the chromatographic column used did not improve the resolution of peaks (Table 3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular formula</th>
<th>[M+H]⁺</th>
<th>[M+Na]⁺</th>
<th>[M+NH₄]⁺</th>
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<tr>
<td>OA (1)</td>
<td>C₄₄H₆₈O₁₃</td>
<td>805</td>
<td>827</td>
<td>822</td>
</tr>
<tr>
<td>DTX1 (2)</td>
<td>C₄₅H₇₀O₁₃</td>
<td>819</td>
<td>841</td>
<td>836</td>
</tr>
<tr>
<td>3</td>
<td>C₄₅H₇₀O₁₃</td>
<td>819</td>
<td>841</td>
<td>836</td>
</tr>
<tr>
<td>4</td>
<td>C₄₅H₇₀O₁₁</td>
<td>759</td>
<td>781</td>
<td>776</td>
</tr>
<tr>
<td>5</td>
<td>C₄₃H₆₆O₁₁</td>
<td>875</td>
<td>897</td>
<td>892</td>
</tr>
<tr>
<td>6</td>
<td>C₄₅H₇₀O₁₄</td>
<td>901</td>
<td>923</td>
<td>918</td>
</tr>
<tr>
<td>7</td>
<td>C₄₅H₇₂O₁₄</td>
<td>943</td>
<td>965</td>
<td>960</td>
</tr>
<tr>
<td>8</td>
<td>C₄₅H₇₂O₁₄</td>
<td>943</td>
<td>965</td>
<td>960</td>
</tr>
<tr>
<td>9</td>
<td>C₄₅H₇₂O₁₅</td>
<td>959</td>
<td>981</td>
<td>976</td>
</tr>
<tr>
<td>10</td>
<td>C₅₀H₇₆O₁₄</td>
<td>975</td>
<td>997</td>
<td>992</td>
</tr>
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<td>DTX6 (11)</td>
<td>C₅₁H₇₈O₁₄</td>
<td>913</td>
<td>935</td>
<td>930</td>
</tr>
<tr>
<td>12</td>
<td>C₅₂H₈₀O₁₄</td>
<td>928</td>
<td>951</td>
<td>946</td>
</tr>
<tr>
<td>13</td>
<td>C₅₂H₈₂O₁₄</td>
<td>955</td>
<td>977</td>
<td>972</td>
</tr>
</tbody>
</table>

2.3. Partition coefficient estimation (log P)

Multiple minimisation calculations to obtain estimates of the partition coefficient of the set of toxins between two solvents (water/octanol) were performed using the program MacroModel 8.5. Each molecule in the input file was minimised twice, once in each solvent. The results of each minimisation were used to evaluate the free energy difference for the two solvents. The minimum energy conformation for each toxin was obtained after a Monte Carlo Multiple Minimum Conformational Search of 5000 steps.
Therefore, the best chromatographic conditions were obtained using the XBridge column and the LC gradient, as summarised in Table 1. Once the RTs of the individual compounds were determined, an equilibrated mixture of all the OA derivatives was injected in order to confirm the peaks’ separation (Fig. 2a). Using this method, the reference compound OA (1) elutes with a retention time of 6.64 min and DTX1 (2) of 9.69 min (Fig. 2), while the ester derivatives show significantly longer times (Table 3 and Figs. 2–4).

The increment in RTs with the decreasing degree of saturation has been reported for liquid chromatography of fatty acid (Suzuki, 1994) or of 7-O-acyl derivatives of DSP toxins (Vale, 2006; Marr et al., 1992; Quilliam et al., 2003). However, no similar elution profiles have been obtained with the XBridge column and the experimental conditions used here. Thus, although OA presents a short RT of 6.64 min, the rest of its derivatives present RTs in the interval from 12.61 min for compound (5) (2-hydroxymethyl-allyl okadaate) to 16.70 min for compound (13) (7-hydroxymethyl-2-methylene-octa-4,7-dienyl okadaate). Within this range, diverse situations appear, such as norokadanone (4) with RT at 13.53 min versus methyl okadaate (3) with RT at 13.66 min or diol ester (10) with a peroxy moiety in the C-9 side chain with a RT at 13.47 min.

In order to establish a correlation between the RTs and lipophytic properties, log P (water/octanol) for the OA derivatives were calculated. In this case, we observed the expected general relationship between log P and RTs, both factors increasing equally (Table 3). However, small deviations were found, reinforcing the requirement of the use of experimental data for a correct analysis.

The OA derivatives were easily identified by a combination of the RTs obtained using a reference material and the mass spectrum of the chromatograms reconstructed at appropriate m/z ion values. However, equal m/z ion values do not necessarily mean that the ions are identical, as in the case of DTX1 (2) and methyl okadaate (3) or 7-hydroxy-2,4-dimethyl-hepta-2,4-dienyl okadaate (7) and 7-hydroxy-4-methyl-2-methylene-hept-4-enyl okadaate (8), and further MS/MS data or the elemental composition is required.

### 3.2. LC–MS/MS analysis

In order to confirm the structure of the OA derivatives detected in each case, a LC–MS/MS analysis of OA (1) and derivatives (2–13) was performed applying a CE of 60 eV in the positive ion mode at m/z [M+NH4]^+ (Fig. 5).

Positive mode MS/MS fragmentation of the OA (1) (Fig. 1a) perfectly matched to that of the previously reported (Quilliam, 1995). This fragmentation is characterized by the loss of the NH4^+ and the consecutive loss of H2O molecules resulting in a predominant ion at m/z 751, as well as several characteristic fragments of OA such as m/z 429, 305 and 287 (Quilliam, 1995). The MS/MS spectrum of the DTX1 (2) at m/z 836 [M+NH4]^+ was identical to that shown by OA but shifted in 14 mass units (Fig. 5b) (Quilliam, 1995). The fragmentation pattern of the OA derivative (5) at m/z 892 [M+NH4]^+ (Fig. 5c) was in part similar to that of PTX1 (Suzuki et al., 2003) and PTX11 (Suzuki et al., 2006) because it keeps the side chain, but additionally shows peaks characteristic of the OA fragmentation such as 751, 305 and 429, therefore confirming their assignment. The MS/MS spectrum of the [M+NH4]^+ ion of the OA diol esters (9) at m/z 976 (Fig. 5d), (10) at m/z 992 (Fig. 5e), (6) at m/z 918 (Fig. 5f), (12) at m/z 946 (Fig. 5i), (7) and (8) at m/z 960 (Fig. 5k), (13) at m/z 972 (Fig. 5l) was in agreement with the MS/MS fragmentation of OA showing peaks at m/z 751, 429 and 305. Moreover for compound (12) a peak at m/z 946 (Fig. 5g) the fragmentation was identical to that of OA-D8 (Suzuki et al., 2004). The MS/MS spectrum of the

### Table 3
Retention times (RTs) observed for OA, DTX1 and OA related compounds isolated from *Prorocentrum lima* and *Prorocentrum belizeanum* cultures, using several standard analytical columns

<table>
<thead>
<tr>
<th>Compound</th>
<th>RTs (min) in column</th>
<th>log P^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okadaic acid (OA) (1)</td>
<td>5.22</td>
<td>6.64</td>
</tr>
<tr>
<td>Dinophysistoxin-1 (DTX1) (2)</td>
<td>—</td>
<td>9.69</td>
</tr>
<tr>
<td>Methyl okadaate (3)</td>
<td>21.00</td>
<td>13.66</td>
</tr>
<tr>
<td>Norokadanone (4)</td>
<td>20.28</td>
<td>13.53</td>
</tr>
<tr>
<td>5</td>
<td>16.88</td>
<td>12.61</td>
</tr>
<tr>
<td>6</td>
<td>22.45</td>
<td>13.96</td>
</tr>
<tr>
<td>7</td>
<td>28.40</td>
<td>15.40</td>
</tr>
<tr>
<td>8</td>
<td>30.55</td>
<td>16.03</td>
</tr>
<tr>
<td>9</td>
<td>19.03</td>
<td>13.29</td>
</tr>
<tr>
<td>10</td>
<td>18.98</td>
<td>13.47</td>
</tr>
<tr>
<td>DTX6 (11)</td>
<td>17.19</td>
<td>14.61</td>
</tr>
<tr>
<td>12</td>
<td>24.85</td>
<td>14.55</td>
</tr>
<tr>
<td>13</td>
<td>32.99</td>
<td>16.70</td>
</tr>
</tbody>
</table>

^a log P (water/octanol).
OA derivative (4) at m/z 776 (Fig. 5f) yielded a prominent peak at m/z 705 due to the loss of the NH$_4^+$ and a consecutive loss of H$_2$O together with 305 and 429 ions characteristic of the OA skeleton. The fragmentation of DTX6 (11) at m/z 930 (Fig. 5j) yielded a prominent peak at m/z 859 due...
Fig. 3. Extracted ion chromatogram for [M + NH₄]⁺ (left) and mass spectrum (right) corresponding to compounds: (e) 5,7-dihydroxy-2,4-dimethylene-heptyl okadaate (9) for m/z 976, (f) 5-hydroperoxy-7-hydroxy-2,4-dimethylene-heptyl okadaate (10) for m/z 992, (g) norokadanone (4) for m/z 776, (h) methyl okadaate (3) for m/z 836 and (i) 5-hydroxy-2-methylene-pent-3-enyl okadaate (6) for m/z 918.
Fig. 4. Extracted ion chromatogram for [M + NH₄]⁺ (left) and mass spectrum (right) corresponding to compounds: (j) 7-hydroxy-2-methyl-hepta-2,4-dienyl okadaate (12) for m/z 946, (k) 4-formyl-2-methylene-pent-4-enyl okadaate (DTX6, 11) for m/z 935 (in this case the prominent ion was [M + Na]⁺), (l) 7-hydroxy-2,4-dimethyl-hepta-2,4-dienyl okadaate (7) for m/z 960, (m) 7-hydroxy-4-methyl-2-methylene-hept-4-enyl okadaate (8) for m/z 960 and (n) 7-hydroxymethyl-2-methylene-octa-4,7-dienyl okadaate (13) for m/z 972.
Fig. 5. MS² product ion mass spectra for selected peaks obtained by applying a CE of 60% for the [M + NH₄]⁺ ions: (a) m/z 822 for OA (1), (b) m/z 836 for DTX1 (2), (c) m/z 892 for 2-hydroxymethyl-allyl okadaate (5), (d) m/z 976 for 5,7-dihydroxy-2,4-dimethylene-heptyl okadaate (9), (e) m/z 992 for 5-hydroperoxy-7-hydroxy-2,4-dimethylene-heptyl okadaate (10), (f) m/z 776 for norokadanone (4), (g) m/z 836 for methyl okadaate (3), (h) m/z 918 for 5-hydroxy-2-methylene-pent-3-enyl okadaate (6), (i) m/z 946 for 7-hydroxy-2-methylene-hepta-2,4-dienyl okadaate (12), (j) m/z 930 for 4-formyl-2-methylene-pent-4-enyl okadaate (DTX6, 11), (k) m/z 960 for both 7-hydroxy-2,4-dimethyl-hepta-2,4-dienyl okadaate (7) and 7-hydroxy-4-methyl-2-methylene-hept-4-enyl okadaate (8) and (l) m/z 972 for 7-hydroxymethyl-2-methylene-octa-4,7-dienyl okadaate (13). The mass spectra range from m/z 200 to 650 is amplified 15 x in all the cases.
the loss of the NH$_4^+$ and the consecutive loss of H$_2$O molecules maintaining the side chain, and also give the characteristic daughter ions for OA-related compounds such as 751, 429 and 305. Finally, the compound (3) showed a peak at m/z 836 (Fig. 5(g)), which was the same shown by DTX1 (2) and the fragmentation of this ion yielded the 765 ion characteristic of DTX1 (2) (Fig. 5b) due to the consecutive loss of H$_2$O, but also showed the ions at m/z 305 and 287 characteristic for OA and not the ions at m/z 318 and 300 characteristic for DTX1, thus confirm their assignment as an OA derivative.

Therefore, LC–MS/MS analysis provides a mean to distinguish between DTX1 (2) and methyl okadaate (3), but not between 7-hydroxy-2,4-dimethyl-hepta-2,4-dienyl okadaate (7) and 7-hydroxy-4-methyl-2-methylene-hept-4-etyl okadaate (8), which are only different in their RTs.

It should be noted that the chromatographic protocol explained in this paper has permitted the total separation of the majority of OA known diol esters and the unequivocal assignation of their corresponding RTs, using reference material with structures previously identified by NMR, assuring the easy and unmistakable detection of these toxins by the control laboratory responsible for sanitary seafood control. Analysis of the fragmentation patterns obtained by LC–MS/MS resulted in verification of their identities.

It has been proposed that the OA diol esters can be formed due to partial hydrolysis of DTX4 by an esterase in the plankton and that the conversion of DTX4 to the diol esters can be altered by the extraction protocols (Hu et al., 1995a). Therefore, one must consider the possibility that the OA diol esters detected in *P. lima* PL2V cultures have originated from DTX4 or DTX5-type OA derivatives, but the absence of these sulphated toxins from several *P. lima* PL2V batch cultures lead us away from that hypothesis, so we considered the diol esters as intermediates of the final sulphated compounds, as a consequence of an elongation of the side chain (Cruz et al., 2006). This idea has been confirmed by the high amount of DTX5c detected in *P. belizeanum*.

In conclusion, this work shows that the use of artificial cultures of dinoflagellates is a powerful tool to advance our knowledge of the real toxin production profile in toxic microalgae and one which could be immediately applied to the analysis of seafood. Accurate reference information about RTs and mass spectra of analytes is fundamental in analysis protocols, especially taking into account that LC–MS will be the leading analytical technique in the present decade. As a consequence, we envisage a high interest for the utilisation of artificial cultures as a source of toxins that can be used as reference standards in analytical laboratories (Goto et al., 2001; Uhlig et al., 2006), as well as tools for a great variety of pharmacological studies (Fernández et al., 2002).

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References


isolated from cultures of the dinoflagellates *Prorocentrum lima* and *Prorocentrum concavum*. J. Nat. Prod. 55 (11), 1631–1637.


