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Isolation of a new okadaic acid analogue from phytoplankton implicated in diarrhetic shellfish poisoning

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Abstract

A new analogue of okadaic acid (OA), the toxin mainly responsible for diarrhetic shellfish-poisoning (DSP) phenomena in Europe, has been isolated from toxic phytoplankton (*Dinophysis acuta*) collected in Irish waters. Fluorimetric LC analyses of the extracts of bulk phytoplankton samples using derivatisation with 9-anthryldiazomethane (ADAM) showed a complex toxin profile, with peaks corresponding to OA and dinophysistoxin-2 (DTX-2) as well as a third unidentified compound. This minor unidentified component was isolated by chromatographic techniques such as normal-phase chromatography, gel permeation on Sephadex, solid-phase extraction and reversed-phase separations. Ionspray mass spectrometry (MS) was used for structural investigation on this compound due to the very small amount of isolated material. Flow injection analysis (FIA)–MS of the isolated compound gave positive-ion mass spectrum dominated by the protonated molecule, $[M+H]^+$, at signal m/z 805, whereas the deprotonated molecule $[M-H]^-$ was observed in the negative-ion spectrum at signal m/z 803, thus indicating the molecular weight of 804 for the new toxin, the same as OA and its known isomers, DTX-2 and DTX-2B. Collision-induced dissociation (CID) as obtained by positive and negative tandem mass spectrometry (MS–MS) showed a fragmentation pattern for the new compound which was very similar to that of OA, DTX-2 and DTX-2B. Ionspray microLC–MS of a mixture containing the compound under investigation together with OA analogues showed the compound eluted after OA, DTX-2, DTX-2B and before DTX-1. All the chromatographic and mass spectrometric data indicated the compound to be another OA isomer and it was therefore coded DTX-2C. To the best of our knowledge this is the first report on the isolation of a new compound related to DSP toxins from natural communities of toxic phytoplankton. © 1998 Elsevier Science B.V.

Keywords: Diarrhetic shellfish poisoning; Food analysis; Okadaic acid; Toxins

1. Introduction

Diarrhetic toxins are secondary metabolites produced by toxigenic dinoflagellates, mainly belonging to the *Prorocentrum* and *Dinophysis* genera. Diarrhetic toxins can be accumulated by filter-feeding molluscs, thus causing severe intoxications to humans, the so-called diarrhetic shellfish poisoning (DSP) [1], following the ingestion of toxic seafood.

Although three classes of polyether DSP-causative toxins with distinctive chemical structures have been discovered (i.e. acidic toxins, okadaic acid—OA [1] and its derivatives, dinophisystoxins—DTXs [2,3], pectenotoxins—PTXs [3–5], yessotoxin—YTX and 45-hydroxy-yessotoxin—45-OH-YTX [5]), the OA

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group of toxins has been identified as responsible for most human DSP-related illnesses [6].

OA and DTX-1 are the only DSP toxins proven to induce diarrhoea in humans [7]. The acute toxic effect of these toxins is gastroenteritis and they are also reported to be potent tumour promoters [8].

OA is the main shellfish contaminant in Europe, whereas DTX-1 is rarely found in significant amounts [9,10] with the exception of a report on toxic shellfish from Norway [11]. Other toxins of the acidic group have however been discovered in European shellfish. 7-O-acyl derivatives of DSP toxins have been also detected in Irish mussels [12]. Moreover, an OA isomer, DTX-2, has also been identified in mussels from Ireland [13] where it continues to be the predominant toxin [14], and very recently a new OA isomer, DTX-2B, has been isolated from Irish mussels by this research group [15].

Chemical elucidation of the DSP toxins in phytoplankton are important for the study of (i) the DSP contamination/decontamination dynamics (ii) the structural modification of toxins due to the bioconversion in vector molluscs [5] and (iii) the planning of phytoplankton monitoring programmes to assure shellfish safety.

Although valuable data have been obtained from the studies on DSP-producing phytoplankton species that can be cultured in laboratory, namely those of the *Prorocentrum* genus [16–20], very little information is available on the toxin composition of *Dinophysis* species, the main phytoplankton causing DSP in humans [21,22].

This is mainly due to the inability of maintaining *Dinophysis* spp. in culture. Field studies are also frequently hampered by the sparse marine distribution of these algal species and by their low-density populations, which are often usually mixed with other phytoplankton species.

The ability of some *Dinophysis* species to produce DSP toxins has however been reported. OA was found in *D. fortii* [23–25], *D. acuta* [23,26], *D. acuminata* [25] and DTX-1 in *D. fortii, D. mitra, D. rotundata, D. tripos, D. acuta* and *D. norvegica* [23]. PTX-2 was identified in *D. fortii* [23,25] and very recently we have shown that *D. acuta* is the progenitor of DTX-2 in Ireland [26]. Interestingly, the occurrence of unknown OA-related compounds in *D. fortii* and *D. acuminata* has also been suggested [27] although these compounds were not isolated for structural investigations.

The LC–fluorimetry method, using derivatisation with 9-anthryldiazomethane (ADAM), is a highly sensitive and specific analytical tool for the investigation of DSP toxin-producing organisms [26]. The effectiveness of the LC–fluorimetry method for determining the accurate toxin algal profiles can be hampered by the lack of analytical reference standards for some DSP toxins. However, LC–fluorimetry profiles may be useful to indicate the presence of new compounds with similar chromatographic behaviour to those of known acidic DSP toxins and which can be subsequently isolated using suitable procedures [15].

On the other hand, mass spectrometric and tandem mass spectrometry, coupled to LC (LC–MS and LC–MS–MS) using an atmospheric pressure ionisation (API) source and an ionspray (ISP) interface, has proved to be an excellent technique for the direct detection of polar marine toxins in phytoplankton [18,24,25]. Unfortunately, NMR spectroscopy is seldom used on account of the small amounts of biological material available for analyses.

The aim of this research was to undertake a detailed investigation of *D. acuta*, one of the main DSP toxin-producing organisms in Ireland. Minor components related to acidic toxins, detected through LC–fluorimetry, were isolated by a combination of chromatographic techniques. MS and MS–MS investigation of one potentially toxic isolated compound was carried out.

2. Experimental

2.1. Reagents

All solvents were LC reagent grade; acetonitrile, methanol, acetone, ammonium hydroxide solution 20% and trifluoracetic acid (TFA) were purchased from Labscan (Dublin, Ireland) or Farmitalia Carlo Erba (Milan, Italy). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). ADAM was purchased from Serva Feinbiochemica (Heidelberg, Germany). OA and DTX-1 were purchased from Calbiochem (Calbiochem-Novabiochem, San Diego,

CA, USA). DTX-2 and DTX-2B were isolated from Irish contaminated mussels as described by Kelly et al. [28] and James et al. [15], respectively.

Individual standard stock solutions containing 5 μg/ml of OA, DTX-2, DTX-2B and DTX-1 were obtained from the pure toxins by dilution with methanol.

2.2. Sample collection and preparation

The phytoplankton samples were collected 5–10 km off the south-west coast of Ireland in 1996, using a large double plankton net. The outer net had a diameter of 140 cm and a length of 590 cm and the inner net had a diameter of 80 cm and a length of 460 cm. This composite plankton net had an outer net of 50 μm mesh size and an inner net of 108 μm mesh size and the collected phytoplankton samples were preserved using dilute acetic acid.

This method of collection gave a natural phytoplankton sample that was dominated by *Dinophysis acuta* (70%). The settled biomass volume collected using this method was ca. 100–300 ml/h. This phytoplankton sample (100–200 g) was repeatedly freeze-thawed and sonicated with methanol–water (80:20 v/v, 2×200 ml). After centrifugation, the combined solutions were extracted with chloroform (2×200 ml), dried and evaporated.

2.3. Isolation of DSP toxins

In the following chromatographic steps, the isolation of DSP toxins was monitored by analysing the toxin composition of aliquots from each chromatographic fraction using fluorimetric HPLC and determining its inhibition of protein phosphatase, PP2A [29]. The predominant toxins in *D. acuta* were OA and DTX-2, as reported recently [26].

A Flash 40M system (Biotage, Hertford, UK) with a silica cartridge and a step gradient of 0–80% methanol–ether was used. The DSP toxins (ca. 100–200 μg) eluted using 30% methanol–ether. Fractions containing toxins were transferred to a Sephadex LH-20 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) which was eluted with methanol.

Reversed-phase chromatography was carried out using a Mega Bond-Elut, C_{18} SPE cartridge, (Varian, Harbor City, CA, USA), followed by flash chromatography with a C_{18} cartridge (Biotage), using step gradients of 40–80% acetonitrile in water. Semi-preparative HPLC using an Ultremex C_{18} column (250×10 mm, 5 μm, Phenomenex) was used for the final purification. Fractions (5 ml) were collected using a gradient of acetonitrile/water (30–100%) over 35 min, with a flow-rate of 5 ml/min.

2.4. Liquid chromatography–fluorimetry

The LC system consisted of an LC-10AD pump, column oven (CTO-10A), fluorescence detector (RF-551), all Shimadzu (Duisberg, Germany), with an autosampler (ISS-100, Perkin-Elmer, Uberlingen, Germany). Isocratic LC, flow-rate 0.5 ml/min, using acetonitrile–methanol–water (80:5:15) was performed at 30°C with a Prodigy C_{18} column (250×3.2 mm, 5 μm, Phenomenex, Macclesfield, UK), equipped with a precolumn (Prodigy C_{18}, 30×3.2 mm, 5 μm). Chromatographic data handling was performed using Unipac Class-VP software (Shimadzu) and data were transferred to Microsoft Excel for graphical presentation.

ADAM solution (0.2%) was prepared by dissolving ADAM (6 mg) in acetone (50 μl) which was made up to 3 ml with methanol and filtered (0.45-μm membrane) for immediate use. Chromatographic fractions, or DSP toxin standards, were evaporated under nitrogen and treated with ADAM solution (200 μl), ultrasonicated for 5 min and allowed to stand for 2 h. All solutions containing ADAM were protected from light. Solvent was removed under nitrogen and the residue was reconstituted in chloroform–hexane (50:50, 1 ml) for clean-up using silica solid-phase extraction (SPE) (Supelclean LC-Si, 3 ml, Supelco, Poole, UK). The efficiency of the SPE clean-up depends on the amount of ethanol (approximately 1%) used as a stabiliser in commercial chloroform but this can be variable. Therefore, chloroform, stabilised with amylene, was used to which ethanol was added to produce a concentration of 1.25% (v/v). The SPE cartridge was conditioned with chloroform–hexane (50:50 v/v, 3 ml) prior to application of the sample. After washing with the conditioning solvent (5 ml), followed by chloroform (5 ml), the toxin derivatives were eluted with chloroform–methanol (95:5 v/v, 5 ml). Following evaporation to dryness under nitrogen (40°C, Turbo

Vap LV, Zymark), the residue was reconstituted in methanol (200 μl) and 20 μl was injected into the HPLC with detection parameters (λ_{ex} 365 nm, λ_{em} 412 nm).

2.5. Liquid chromatography–mass spectrometry

A high pressure pump model Phoenix 20 CU (Fisons, Milan, Italy), equipped with Valco valve (Houston, TX, USA) with interchangeable loops (1 or 0.2 μl), was used for flow injection analysis (FIA–MS), FIA–MS–MS and LC–MS experiments. FIA–MS was performed on solutions of the individual DSP toxins containing 2.5 μg/ml (concentrations of DTX-2, DTX-2B and DTX-2C are expressed as OA equivalents). The mobile phase was acetonitrile–water (90:10), containing 0.1% TFA, for positive-ion mode experiments and acetonitrile–water (90:10), containing 0.01% NH_4OH, for negative-ion mode experiments. The flow-rate was 20 μl/min.

Separation of toxins was carried out on a micro-column packed with Vydac 218TP51 (Separation Group, Hesperia, CA, USA) (250×1 mm, 5 μm) at room temperature, under isocratic conditions, with a mobile phase of acetonitrile–water (60:40, v/v) containing 0.1% TFA and a flow-rate of 40 μl/min.

All the micro-LC (μLC)–MS experiments were performed on a model PE-SCIEX API III plus triple-quadrupole (PE-Sciex, Thornhill, Canada). The mass spectrometer was equipped with an API source and an ionspray interface. Ultra-high purity (UHP) nitrogen was used as the curtain gas and nebuliser gas in the ion-spray interface. The standard software packages (PE-Sciex) were used for instrument control data acquisition and data elaboration.

Full-scan single MS mass spectra were acquired both in positive and negative modes over the mass range m/z 600–1000.

Product-ion mass spectra were acquired both in positive- and negative-ion mode by colliding the Q1 selected precursor ion with argon gas in Q2 operated in radio frequency (rf)—only mode and scanning the second quadrupole mass spectrometer, Q3, from m/z 50 to 820.

Data acquisition for LC–MS analyses was performed by selected-ion monitoring (SIM) on the ion m/z 805, corresponding to the protonated molecules, $[M+H]^+$ of OA, DTX-2, DTX-2B and DTX-2C and on the ion m/z 819, corresponding to the protonated molecules, $[M+H]^+$ of DTX-1.

3. Results and discussion

Since none of the *Dinophysis* species can be cultured under laboratory conditions, confirmation of toxigenic species, and their accurate definition of toxin profiles, can be achieved exclusively by investigation on natural phytoplankton populations and/or small numbers of *Dinophysis* cells isolated under microscopy by capillary manipulation [23,26].

In this study, a first stage analysis of bulk phytoplankton samples using the sensitive and specific LC–fluorimetry method [26] was performed. This was followed by the isolation of compounds forming fluorescent derivatives and having similar chromatographic behaviour to the known acidic toxins.

The ionspray FIA–MS and –MS–MS and LC–MS experiments were employed as the second stage analysis in order to obtain structural information on the potentially toxic isolated compound.

3.1.1. Isolation of DSP toxins and related compounds

Bulk phytoplankton samples containing ca. 100–200 g were comprised mostly of *Dinophysis acuta* and were extracted with methanol. Although the predominant DSP toxins were OA and DTX-2, unidentified acidic compounds were also observed with chromatographic behaviour similar to these known DSP toxins. Five chromatographic steps were implemented to isolate the DSP toxins, OA, DTX-2 and a new compound, DTX-2C. Flash chromatography on silica and elution from Sephadex LH-20 afforded a rapid clean-up. Gradient SPE using a Mega Bond-Elut C_{18} cartridge (Varian) gave a good preliminary separation of the DSP toxins. Final purification was achieved using a C_{18}-flash chromatography cartridge (Biotage) and semipreparative C_{18}-HPLC. The overall purification was substantially more efficient than the corresponding isolation of toxins from shellfish [28] which have a more complex matrix. These studies have demonstrated the feasibility of using wild phytoplankton as a useful source for obtaining toxin standards. DTX-2C (6 μg)

was obtained from this isolation as well as larger quantities (150–200 μg) of OA and DTX-2.

3.1.2. Analysis of DSP toxins by LC–fluorimetry

The analysis of DSP toxins in phytoplankton extracts and chromatographic fractions was achieved by derivatisation with ADAM, following a modified procedure of Lee et al. [30] and this has recently been described [28]. Calibrations using ADAM were linear for solutions prepared from okadaic acid standards (0.26–1.30 μg OA/ml, $r=0.998$) and the detection limit for OA (S/N 3:1) was 0.1 ng for 20-μl injections. This procedure is specific for the determination of acidic toxins and the preliminary analysis of the phytoplankton extracts showed that the predominant toxins present were OA and DTX-2 [26]. The five stage isolation procedure produced OA, DTX-2 and a new compound, named DTX-2C, in high purity. Fig. 1A is a chromatogram from the final step in the purification of DTX-2C after derivatisation with ADAM. Interestingly, although DTX-2C was easily separated from the known standard toxins, OA, DTX-2 and DTX-1, as their 9-anthrylmethyl derivatives (Fig. 1B), the underivatised DTX-2C was difficult to separate from DTX-1. Also, the 9-anthrylmethyl esters of DTX-2B [15] and DTX-2C were not resolved under the conditions used in this study (see LC retention data in Table 1).

Table 1
HPLC retention data for diarrhetic shellfish toxins and their 9-anthrylmethyl derivatives

Diarrhetic shellfish toxin	Retention time (min)[a] (free toxins)	Retention time (min)[b] (9-anthrylmethyl esters)
OA	6.1	22.2
DTX-2	6.6	25.2
DTX-2B	7.5	27.4
DTX-2C	8.3	27.4
DTX-1	8.5	35.7

[a] LC conditions: Vydac 218TP51 column (250×1 mm, 5 μm); room temperature; acetonitrile–water (60:40, v/v) containing 0.1% TFA; flow-rate, 40 μl/min.
[b] LC conditions: Prodigy C_{18} column (250×3.2 mm, 5 μm); temperature, 30°C; eluent, acetonitrile–methanol–water (80:5:15, v/v); flow-rate, 0.5 ml/min.

3.1.3. Mass spectrometry and tandem mass spectrometry analyses

Previous reports have demonstrated the suitability of LC–MS, using an API source and an ionspray interface, to identify and determine DSP toxins in plankton and shellfish [18,25].

The mass spectrometric strategy in this work was to use a soft ionisation LC–MS technique to obtain

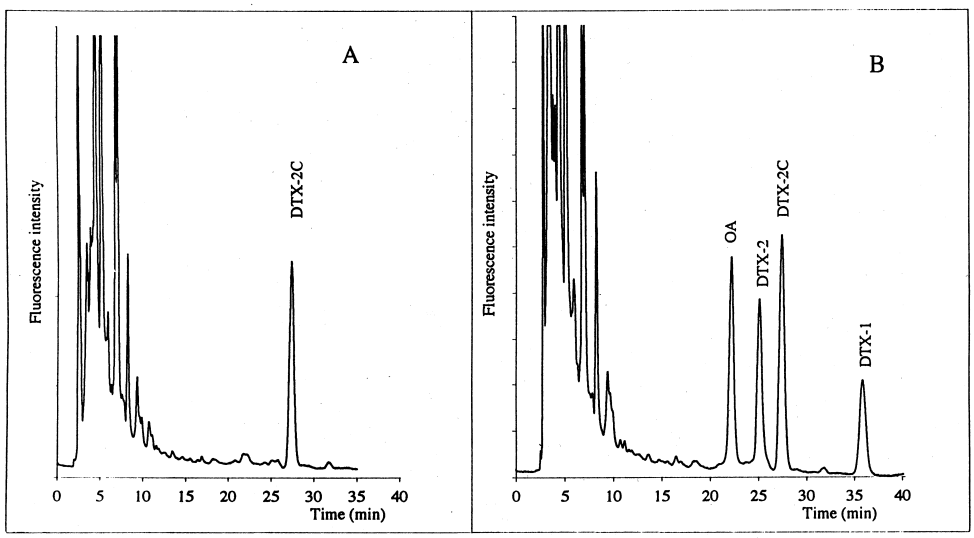

Fig. 1. Chromatograms from LC–fluorimetry of the 9-anthrylmethyl esters of (A) the new compound isolated from *Dinophysis acuta* and (B) a standard mixture of four DSP toxins (2–4 ng each on-column). LC conditions: Prodigy C_{18} column (250×3.2 mm, 5 μm); temperature, 30°C; eluent, CH_3CN–CH_3OH–water (80:5:15); flow-rate, 0.5 ml/min; detection wavelengths, λ_{ex} 365 nm, λ_{em} 412 nm.

intact protonated molecules [31] in order to determine the molecular weight, followed by collision-induced dissociation (CID) using MS–MS to gather other structural information by characteristic product ions.

FIA–MS experiments were firstly performed on the solution of the purified compound under investigation. In order to determine the molecular weight of the compound, these experiments were carried out both in positive- and in negative-ion mode under the suitable ionisation process parameters which had previously been found to favour the exclusive formation of the protonated molecules, $[M+H]^+$ and deprotonated molecule $[M-H]^-$ for DSP polyether toxins belonging to the OA group [32–35].

Fig. 2A shows the positive ionspray mass spectrum for the compound under investigation. This spectrum is simple, showing the abundant peak due to the protonated molecule, $[M+H]^+$, at m/z 805, although a small signal due to the ammonium adduct, $[M+NH_4]^+$, at m/z 822 was also noticed. The negative ionspray mass spectrum was also acquired (Fig. 2B) and here only the deprotonated molecule, $[M-H]^-$, at m/z 803, was observed.

These results indicated a molecular weight of 804 for the isolated compound which is the same as that of OA [36], DTX-2 [13] and DTX-2B [34].

The power of tandem mass spectrometry for structural investigation on acidic toxins has been previously demonstrated. Positive fragment ion mass spectra has been reported for OA [17,18,32,35,37] DTX-1 [35,37], DTX-2 [32,35] and the fragmentation pattern for underivatised OA was also proposed by some authors [32,37]. It is important to note that the use of tandem mass spectrometry in this research was the only practical approach to obtain structural information on the isolated compound, on account of the limited quantity of the biological material obtained from phytoplankton.

CID using MS–MS was implemented by FIA, both in positive- and in negative-ion mode, in order to gather other structural information on the compound, from its characteristic product ions.

Fig. 3 shows the positive (Fig. 3A) full-scan FIA–MS–MS spectrum for the compound using the

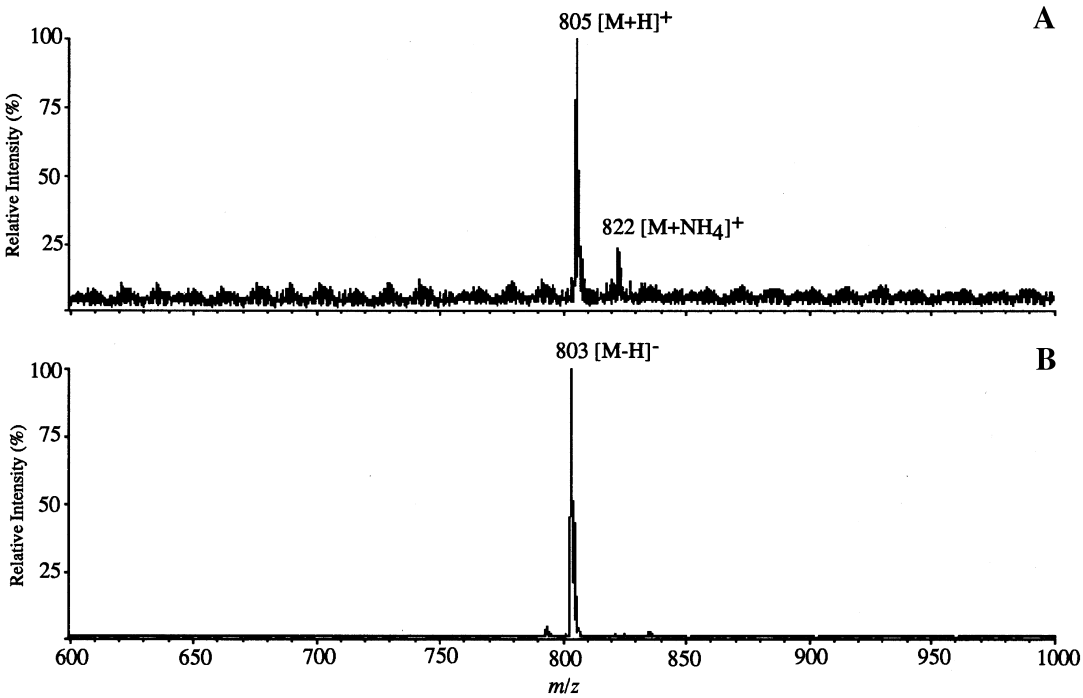

Fig. 2. Ion-spray mass spectra [(A) positive and (B) negative] of the new compound isolated from *Dinophysis acuta*.

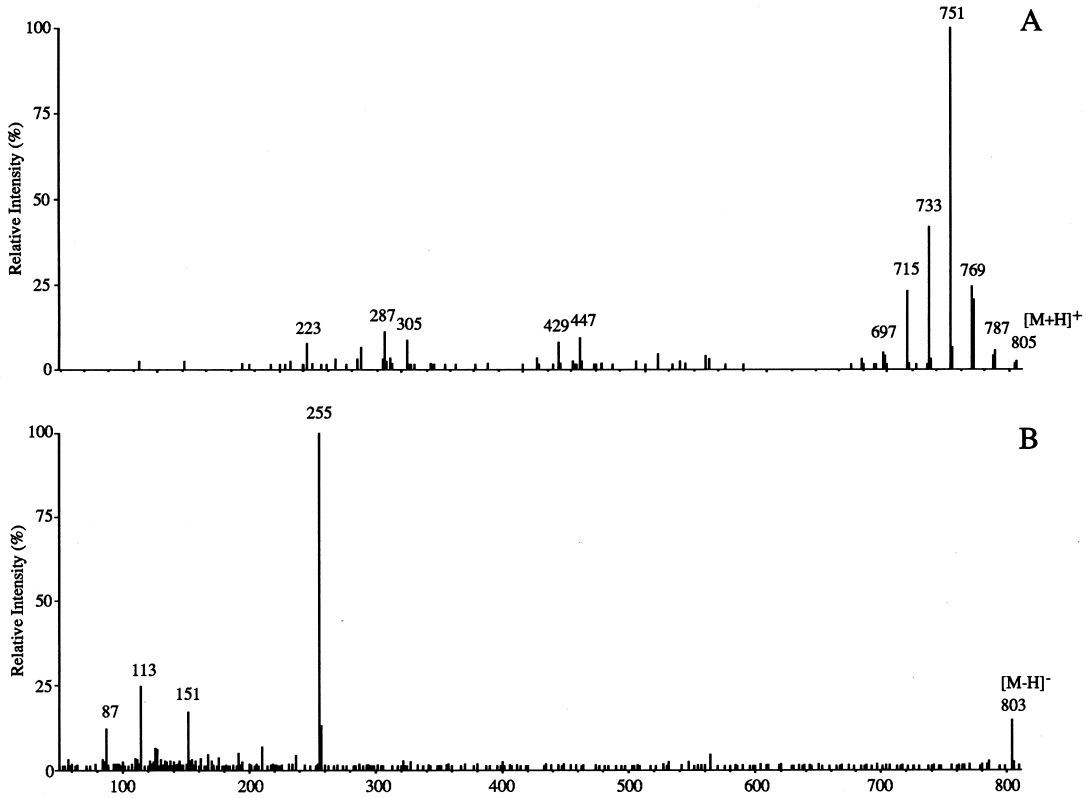

Fig. 3. (A) Positive and (B) negative product ion mass spectra of the new compound isolated from *Dinophysis acuta*.

$[M+H]^+$, at m/z 805, as precursor ion. This spectrum gave the same fragment ions as those obtained from CID of protonated molecules of OA and its known isomers, DTX-2 and DTX-2B [35]. Similarly, the negative product-ion mass spectrum of the compound (Fig. 3B) showed the same fragment ions obtained from CID of the deprotonated molecules of OA and its known isomers. The data obtained from repeated analyses under different collision energies, in both positive- and negative-ion mode, supports this view of the structural similarities between the compound under investigation and OA, DTX-2 and DTX-2B.

Chromatographic separation of known underivatized acidic toxins (OA, DTX-2, DTX-2B, DTX-1), together with the compound under investigation, was finally carried out using a reversed-phase column at room temperature, under isocratic conditions, with a mobile phase of acetonitrile–water, (60:40, v/v) containing 0.1% TFA and a flow-rate of 40 μl/min (Fig. 4). Under these conditions, a good separation was obtained in the SIM μLC–MS analyses (see LC retention data in Table 1).

All the above data indicated that the compound isolated from *D. acuta* to be a new OA isomer and it was therefore named DTX-2C. To the best of our knowledge, this is the first report on the isolation of a new compound related to DSP toxins from natural communities of toxic phytoplankton.

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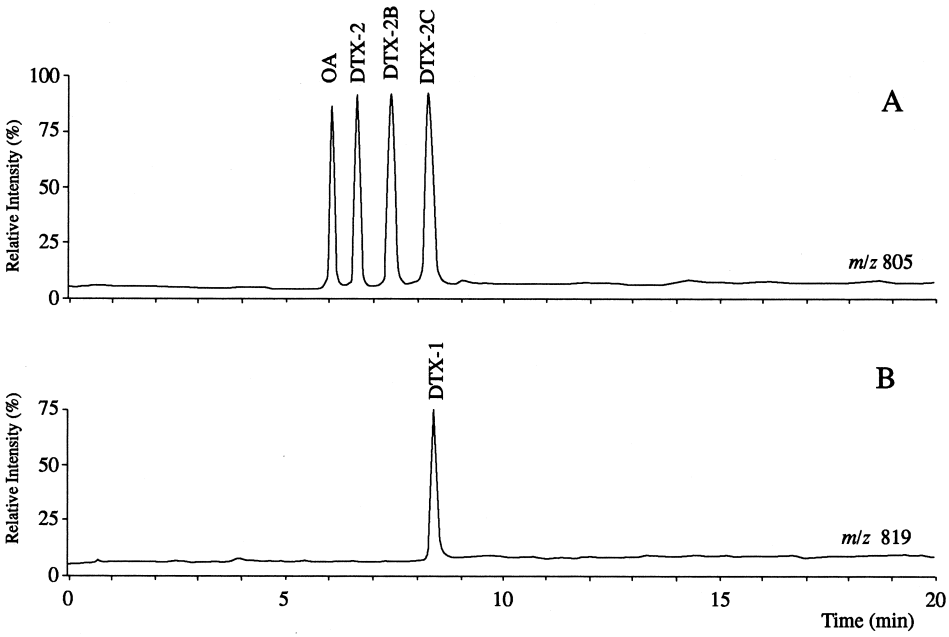

Fig. 4. SIM LC–MS chromatograms of a mixture containing underivatised DSP toxins (1 mg/ml each). A: OA, DTX-2, DTX-2B and DTX-2C at m/z 805; B: DTX-1 at m/z 819. Other conditions: see Section 2.

assistance in the acquisition of large phytoplankton samples.

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