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Liquid chromatographic methods for the isolation and identification of new pectenotoxin-2 analogues from marine phytoplankton and shellfish

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Abstract

Two acidic analogues of the polyether marine toxin, pectenotoxin-2 (PTX-2), responsible for diarrhetic shellfish poisoning (DSP), have been isolated from the toxic marine phytoplankton (*Dinophysis acuta*), collected in Irish waters. Liquid chromatography with fluorimetric detection (LC–FLD) analyses of the extracts of bulk phytoplankton samples, following derivatisation with 9-anthryldiazomethane (ADAM) or 1-bromoacetylpyrene (BAP), showed a complex toxin profile with peaks corresponding to okadaic acid (OA) and its isomers, dinophysistoxin-2 (DTX-2) and DTX-2C, as well as other unidentified lipophilic acids. LC–UV analysis showed the presence of a diene moiety in these new compounds and two acids have been isolated. LC coupled with mass spectrometry (MS) and tandem mass spectrometry (LC–MS–MS) were used to gain structural information. Through flow injection analysis (FIA)–MS, both in positive and negative ion modes, the molecular weight of 876 for both compounds was determined. Collision Induced Dissociation (CID) from each parent ion, as performed both in positive and negative ion mode, produced mass spectra which were very similar to those obtained for authentic PTX-2 (mw 858). These new compounds have been confirmed to be pectenotoxin-2 seco acids (PTX-2SAs) and they are closely related to PTX-2 except that they contain an open chain carboxylic acid rather than a lactone ring. Toxic mussels also contained these pectenotoxin-2 analogues.

Keywords: Marine algae; *Dinophysis*; *Mytilus galloprovincialis*; Seafood toxins; Pectenotoxin-2; Toxins

1. Introduction

Dinoflagellates of the *Dinophysis* genera are known to be responsible for producing toxic metabolites that can be accumulated by filter-feeding shellfish and transmitted to human consumers of contaminated seafood. A well-known and widespread human toxic syndrome is called diarrhetic shellfish poisoning (DSP) on account of its predominant gastrointestinal symptoms [1,2].

A number of chemically and toxicologically different compounds are currently included among the

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DSP toxins, okadaic acid (OA) and dinophysistoxins (DTXs) [1–10] (Fig. 1A), pectenotoxins (PTXs) [2,6,11–13] and yessotoxins (YTXs) [14–16], but only OA and DTXs have been confirmed to induce diarrhea in humans [14,17,18]. Besides OA, DTXs [19,20] and YTXs [16,21–24], which have repeatedly been reported in European shellfish, PTXs have only recently been identified in European waters [25].

Seven pectenotoxins (Fig. 1B) were isolated from Japanese shellfish [2,6,11,26] and the absolute configurations of some of these toxins have recently been established (Fig. 1B) [12,13]. There was insufficient material available for the full structural elucidation of PTX-5. Of the PTXs that are found in shellfish, only PTX-2 has been identified in phytoplankton [11,27], suggesting that an oxidation occurs in the hepatopancreas of shellfish producing the other PTXs. The site of oxidation is at C-43 where all stages, from methyl to carboxylic acid, are observed (R=CH_3: PTX-2; R=CH_2OH: PTX-1; R=CHO: PTX-3; R=COOH: PTX-6) [11].

Only limited data are available on the mechanism of action and toxicological effects of PTXs. The hepatotoxicity of PTX-1 is similar to that provoked by the mycotoxins, phalloidin and cyclochlorotine [17,28] and severe mucosal injuries of the small intestine were also reported in mice after oral administration of OA and PTX-2 [29]. PTXs have attracted renewed attention due to their selective and potent cytotoxicity against several human cancer cell lines [28,30]. Studies on the PTXs in marine biological material are therefore considered important and a better knowledge of PTX-producing organisms is required in order to support both basic scientific research, human health risk assessment and seafood monitoring.

The liquid chromatography with fluorimetric detection (LC–FLD) method, using derivatisation with 9-anthryldiazomethane (ADAM), is a highly sensitive and specific analytical tool for the investigation of organisms producing acidic DSP toxins [9,27,31–33]. However, mass spectrometry and tandem mass spectrometry, coupled to LC (LC–MS and LC–MS–MS) using an atmospheric pressure ionization (API) source and an ionspray (ISP) interface are valuable techniques for the direct detection of marine toxins [8,9,25,32,34–37]. Moreover, the LC–MS and LC–MS–MS approach is frequently the only method for obtaining structural information due to the small amounts of marine natural products available for analysis.

We now report the application of multiple chromatographic methods for the isolation and identification of PTX-2 analogues, named pectentoxin-2 seco acids (PTX-2SAs), in a wild phytoplankton sample which comprised predominantly *D. acuta*. LC–MS was also applied for the analysis of PTXs in toxic shellfish.

2. Materials and methods

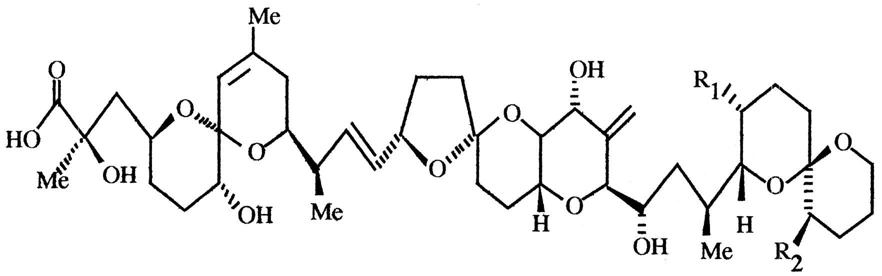
2.1. Materials

LC reagent grade solvents were used; acetonitrile, methanol, acetone, ammonium hydroxide solution 20% and trifluoroacetic acid (TFA) were purchased from Labscan (Dublin, Ireland) or Farmitalia Carlo Erba (Milan, Italy). Water was purified in a Milli-Q system (Millipore Corp., Bedford, MA, USA). Purchased chemicals included 9-anthryldiazomethane (ADAM) (Serva Feinbiochemica, Heidelberg, Germany), 1-bromoacetylpyrene (BAP) and diisopropylethylamine (Aldrich, Gillingham, UK), OA (Calbiochem-Novabiochem, San Diego, CA, USA). DTX-2 was isolated from Irish contaminated mussels as described by Kelly et al. [38] and PTX-2 was isolated from Japanese scallops [6]. Individual standard stock solutions containing 2.5 $\mu g\,ml^{-1}$ of OA, DTX-2 and PTX-2 were obtained from the pure toxins by dilution with methanol.

2.2. Sampling

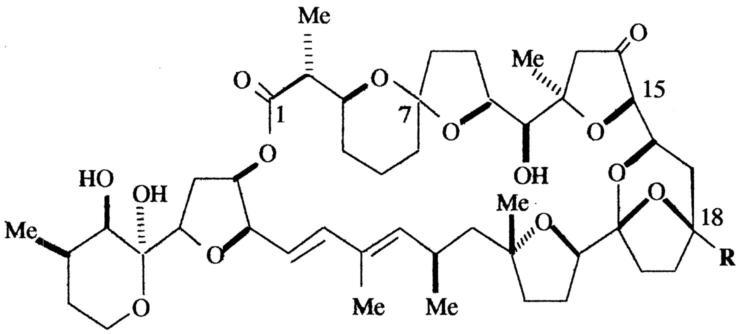
The phytoplankton samples were collected 5–10 km off the southwest coast of Ireland, using a large double plankton net. The outer net had a diameter of 140 cm and a length of 590 cm and the inner net had a diameter of 80 cm and a length of 460 cm. This mesh sizes of this composite plankton net were 50 μm (outer) and 108 μm (inner) and the collected phytoplankton samples were preserved using dilute acetic acid. This method of collection gave a natural phytoplankton sample that was dominated by *D. acuta* (>60%) and the settled biomass volume

A)

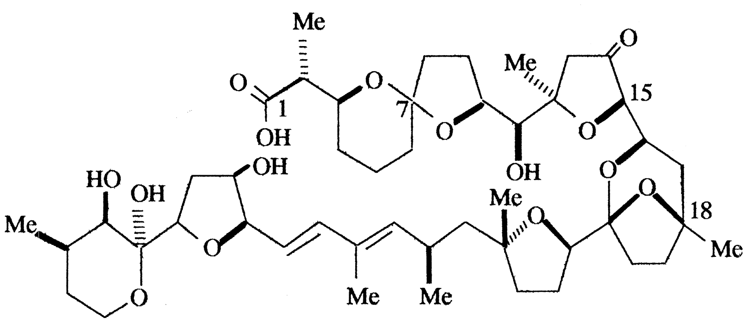


	R_1	R_2
OA	CH_3	H
DTX-1	CH_3	CH_3
DTX-2	H	CH_3

B)



C)



	R	C-7
PTX-1	CH_2OH	*R*
PTX-2	CH_3	*R*
PTX-3	CHO	*R*
PTX-4	CH_2OH	*S*
PTX-6	COOH	*R*
PTX-7	COOH	*S*
PTX-2SA		*R*
7-*epi*-PTX-2SA		*S*

Fig. 1. Structures of DSP toxins: (A) okadaic acid and analogues; (B) pectenotoxins (PTXs); and (C) pectenotoxin-2 seco acids (PTX-2SAs).

collected using this method was ca. 100–400 ml. Details of the field activities and sampling are discussed elsewhere [39]. Toxic mussels (*Mytilus galloprovincialis*) were collected in coastal areas of the North Adriatic Sea at the time in which routine control testing had shown these shellfish to be positive for DSP toxins.

2.3. Isolation of toxins from phytoplankton

The phytoplankton sample (100–200 g) obtained above 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.

In the following chromatographic steps, the isolation of polyether acids was monitored by taking an aliquot from each chromatographic fraction and determining its inhibition of protein phosphatase (PP2a) and the toxin profile using LC–FLD and LC–UV. Colourimetric PP2a assays were carried out with 4-nitrophenyl phosphate as a substrate using a procedure based on that developed by MacKintosh [40] and Tubaro et al. [41] which is described elsewhere [39].

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

Reverse-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 (Flash 40M, Biotage), using step gradients of 40–80% acetonitrile in water. The final part of the purification of AC1-3 was carried out using a Prodigy C_{18} column (250×3.2 mm, 5 μm, Phenomenex, Macclesfield, UK) at 30°C with a gradient of water–methanol (30–100% methanol) over 35 min; flow-rate=0.5 ml min^{-1}, with photodiode-array (PDA)-UV detection (Waters 994, Millipore, Milford, MA, USA) with absorbance monitored in the range 200–300 nm (AC-3, λ_{max}= 238 nm). Typically, the amounts of the polyether acids obtained in this isolation sequence were: AC1 (100–150 μg), AC2 (2–5 μg) and AC3 (100–150 μg).

2.4. LC–FLD analysis of DSP toxins

The LC system consisted of an LC-10AD pump, column oven (CTO-10A), fluorescence detector (RF-551), all Shimadzu (Duisburg, Germany), with an autosampler (ISS-100, Perkin Elmer, Überlingen, Germany). Isocratic LC, flow-rate 0.5 ml min^{-1}, 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).

Derivatisation reactions with evaporated phytoplankton extracts or chromatographic eluent fractions were carried out using: (a) ADAM in methanol (200 μl, 0.2%, w/v) for 2 h at room temperature [33]; or (b) BAP in acetonitrile (500 μl, 0.1%, w/v), diisopropylethylamine (40 μl, 5%, w/v) for 20 min at 75°C [38]. Solvents were removed 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). Chloroform, stabilised with amylene, to which ethanol was added to produce a concentration of 1.25% v/v, was used. 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 were injected with fluorimetric detection: ADAM, λ_{ex}=365 nm, λ_{em}=412 nm; BAP: λ_{ex}=365 nm, λ_{em}=418 nm.

2.5. LC–MS, LC–MS–MS analysis of DSP toxins

High-pressure pump model Phoenix 20 CU (Fisons, Milano Italia), 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 and FIA–MS–MS experiments were performed on solutions of the individual DSP toxins containing 2.5 $\mu g\,ml^{-1}$ (concentrations of DTX-2 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 $\mu l\,min^{-1}$.

Extraction of DSP toxins from mussels (*Mytilus galloprovincialis*) for LC–MS analyses were performed as previously described [46].

Separation of toxins was carried out on a microcolumn packed with Vydac 218TP51 (Separation Group, Hesperia, CA, USA) (250 mm×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 $\mu l\,min^{-1}$.

All the MS experiments were performed on a model PE-SCIEX API III *plus* triple-quadrupole (PE-Sciex, Thornhill, Ontario, 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, Thornhill, Ontario, Canada) 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 thickness 300×10^{13} molecules cm^{-2}) in Q2 operated in radio frequency (rf)-only mode and scanning the second quadrupole mass spectrometer, Q3, from m/z 50 to 820. Collision energies of 30 eV and −30 eV for CID experiments in positive and negative modes were used, respectively.

Data acquisition for LC–MS analyses was performed by selected ion monitoring (SIM) on the ions corresponding to the protonated molecules, $[M+H]^+$, of the analytes, at m/z 805 for OA and DTX-2, at m/z 859, for PTX-2, at m/z 877 for PTX-2 seco acids.

3. Results and discussion

3.1. Analysis of toxins in marine phytoplankton (Dinophysis sp.)

OA, DTXs and the PTXs with a carboxyl group in the molecule, PTX-6 and PTX-7, can be all analysed by derivatisation with ADAM followed by LC–FLD [27,42]. PTX-ADAM derivatives elute before ADAM-OA and ADAM-DTX-1 in reversed-phase LC. Moreover, although the effectiveness of this method for determining the DSP toxin profiles in dinoflagellates may be hampered by the lack of standard toxins, the LC–FLD profiles may indicate the presence of new polyether acids with similar chromatographic behaviour to the known DSP toxins [9,33,43,44].

However, two serious difficulties hamper investigations aimed to define the toxin profiles of marine phytoplankton implicated in seafood contamination. Firstly, since *Dinophysis* sp. cannot be maintained in culture, confirmation of toxinogenic species and accurate definition of their toxin profiles can be achieved exclusively by investigation on natural phytoplankton populations and/or small numbers of *Dinophysis* cells, collected by a cumbersome manipulation using a microscope. Secondly, the identification of DSP toxins in marine biological material is seriously hampered by the commercial unavailability of many standard toxins.

Sensitive chromatographic methods for studies on small algal samples, collected from natural phytoplanktonic communities, have been used to identify some DSP toxins in a number of *Dinophysis* sp. Thus OA was found in *D. fortii* [25,27,32], *D. acuta* [27,33], *D. acuminata* [27] and DTX-1 in *D. fortii, D. mitra, D. rotundata, D. tripos, D. acuta* and *D. norvegica* [27] and PTX-2 in *D. fortii* [25,27]. The occurrence of unknown OA related compounds in *D. fortii* and *D. acuminata* was also suggested [45] although they were not isolated for structural investigations. Very recently, we have shown that *D. acuta* also produced OA, DTX-2 and a new OA isomer, DTX-2C [9,33].

In this study, analysis of bulk phytoplankton samples was first performed using two sensitive LC–FLD methods, specific for DSP acidic toxins

[33]. This was followed by the isolation of compounds forming fluorescent derivatives and having similar chromatographic behaviour to the known acidic DSP toxins. The ionspray FIA–MS and –MS–MS and LC–MS experiments were then employed to obtain structural information on the potentially toxic isolated compounds.

3.2. Analysis of polyether acids by LC–FLD

Bulk phytoplankton samples containing ca. 100–200 g, which comprised mainly *D. acuta,* were extracted with methanol. The analysis of DSP toxins in phytoplankton extracts and chromatographic fractions was achieved by derivatisation with ADAM, following a method [33] based on the procedure of Lee et al. [42], or with BAP [38].

Fig. 2A shows the LC–FLD chromatogram from the bulk phytoplankton sample, after derivatisation with ADAM. Although the predominant DSP toxins were OA and DTX-2, a small amount of an OA isomer, DTX-2C, was also detected as well as three unidentified compounds which behaved chromatographically like the known acidic DSP toxins. They were code-named AC1-AC3, numbered according to their elution order. Fig. 2B is the LC–FLD chromatogram of the pyrenacyl derivatised phytoplankton extract obtained using BAP and a reversed elution

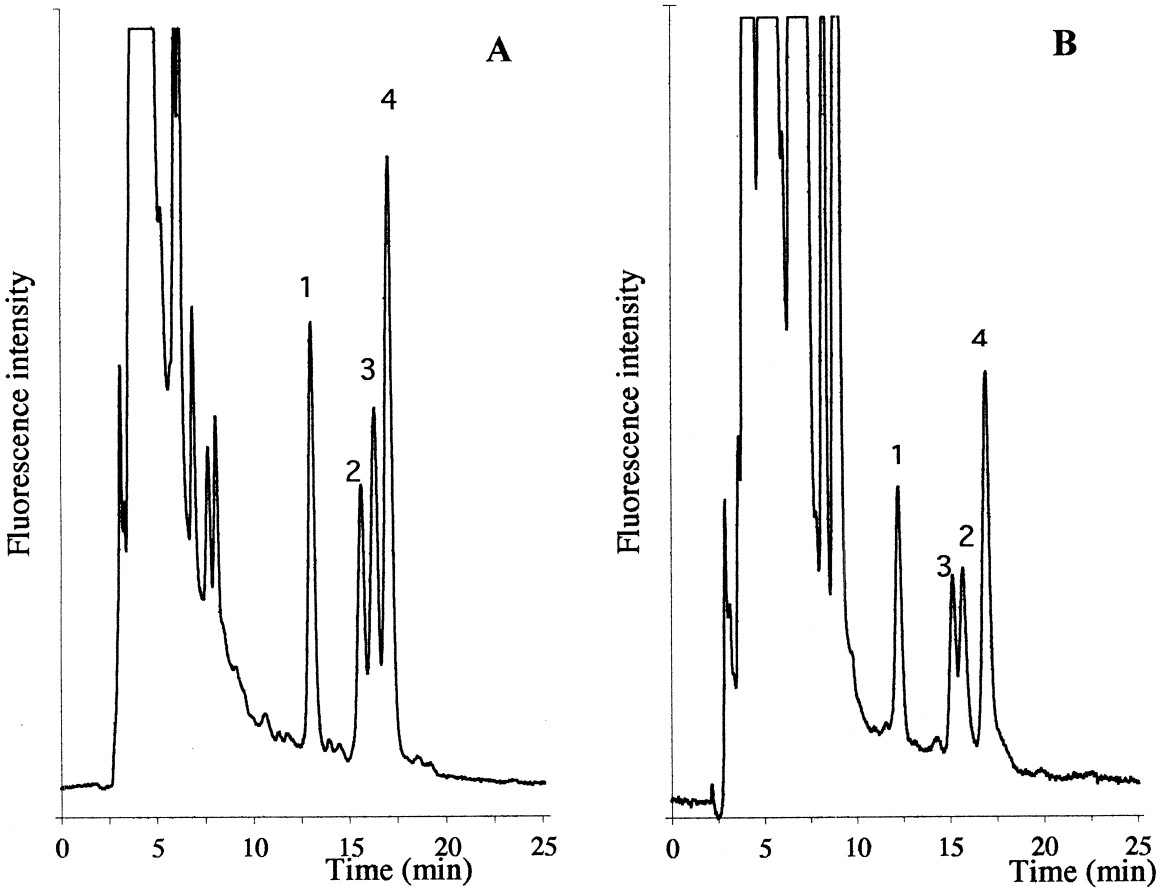


Fig. 2. Chromatograms from the LC–FLD of an extract from a phytoplankton sample (*D. acuta*): (A) the 9-anthrylmethyl esters after derivatisation with ADAM; and (B) the pyrenacyl esters after derivatisation with BAP; peak identities: 1, AC1; 2, OA; 3, AC3; and 4, DTX-2. Conditions: Prodigy C_{18} column (250×3.2 mm, 5 μm) at 30°C; mobile phase: acetonitrile–methanol–water (80:5:15); flow-rate: 0.5 ml min^{-1}; detection: λ_{ex} 365 nm, λ_{em} 412 nm.

order for OA (No. 2) and AC3 (No. 3) was observed when compared with their anthrylmethyl derivatives (Fig. 2A).

3.3. Isolation of polyether acids from marine phytoplankton

Five chromatographic steps were then implemented to isolate the unidentified compounds, AC1-AC3, as well as OA, DTX-2 and DTX-2C. The isolation procedure was essentially the same as the one previously described [9]. Flash chromatography on silica and elution from Sephadex LH-20 afforded a rapid clean-up. A step gradient elution using a Mega Bond-Elut C_{18} SPE cartridge (Varian) gave a good preliminary separation of the DSP toxins. Final purification was achieved using a C_{18}-flash chromatography cartridge (Biotage) with photodiode-array ultra-violet (PDA-UV) detection and Fig. 3 shows a chromatogram from the last step in the purification of AC3.

Although the isolation of these toxins was difficult due to their similar chromatographic behaviour, two properties of these compounds were exploited to distinguish between them in eluent fractions. Firstly, OA and its analogues are potent inhibitors of protein phosphatases (PP) but AC1-3 do not inhibit. Thus, rapid screening, using a colourimetric PP-assay, was effective in targeting eluents containing OA and DTXs. Secondly, using LC–PDA-UV analysis, PTX-2 and the AC1-3 compounds all exhibited similar UV absorptions (λ_{max}=238 nm) due to the conjugated double bonds but this structural feature is absent in OA analogues.

Using this isolation procedure, OA, DTX-2, DTX-2C (minor) and the three new compounds, AC1-3 were produced. Fig. 3 shows the chromatogram from the final step in the purification of AC3 using reverse-phase chromatography with gradient elution. The isolation was monitored using diode array UV detection and the insert is the UV spectrum obtained for AC3. Isolated samples were also examined by LC–FLD following fluorimetric derivatisation and Fig. 4 shows the chromatograms obtained for the ADAM derivatives of AC1 and AC3. Whereas AC3 and AC2 (minor) were isolated as pure compounds, efforts to maintain a pure sample of AC1 were unsuccessful since, despite repeated chromatography, it became apparent that AC1 was spontaneously converting to AC3.

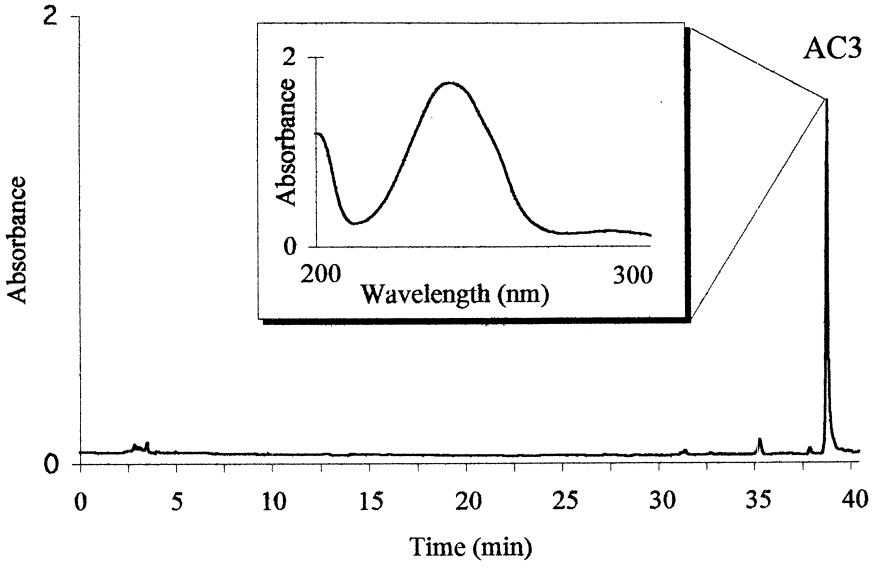


Fig. 3. Chromatogram from the final step of the isolation sequence for AC3 (7-*epi*-PTX-2SA). The insert is the UV spectrum from the photodiode-array signals produced from the AC3 peak. Conditions: Prodigy C_{18} column (250×3.2 mm, 5 μm) at 30°C using a gradient of water–methanol (30–100% methanol) over 35 min; flow-rate=0.5 ml min^{-1}; PDA-UV detection with absorbance monitored in the range 200–300 nm.

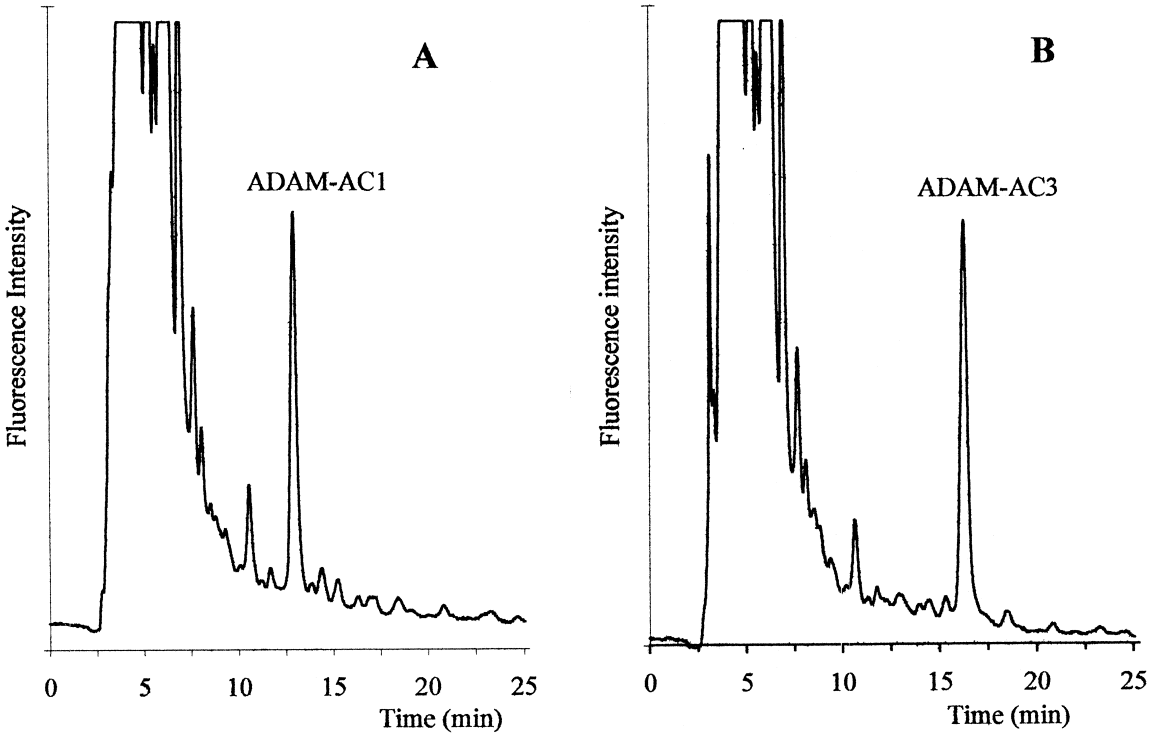


Fig. 4. Chromatograms obtained after derivatisation with ADAM from the LC–FLD of PTX-2SAs: (A) AC1; and (B) AC3, obtained from the final step in the isolation sequence. Conditions as in Fig. 2.

3.4. FIA–MS, FIA–MS–MS and LC–MS

The need for structural information on the potentially toxic isolated compounds clearly required the use of spectroscopic techniques. Mass spectrometry was implemented, as it was the most effective approach due to the limited amounts of compounds obtained from phytoplankton.

The power of LC–MS and MS–MS, using an API source and an ionspray interface for the identification of new DSP toxins in plankton and shellfish has been unequivocally demonstrated [9,10,37,46]. Investigations on new DSP toxins are typically based on a combined approach involving single MS and MS–MS. The former gives molecular-related ions that indicate the molecular weight of the compound under investigation. In order to obtain additional structure information, collision induced dissociation (CID) of the molecular ions species can be performed in MS–MS experiments to produce fragment ions that are characteristic of the chemical structures of the molecular ions. The resulting full-scan product ion mass spectra may be interpreted by reference to model compounds and related to mass spectral behaviour from known structures.

In order to perform structural investigation on the two isolated compounds, AC2 and AC3, the soft ionisation LC–MS technique was firstly implemented to obtain the molecular weight for each analyte [47]. Subsequent investigations were performed by CID using MS–MS to gather further structural information through characteristic product ions. The same analytical approach was adopted to obtain LC–MS and MS–MS data for PTX-2, for comparative purposes.

FIA-MS experiments were first carried out, both in positive and negative ion modes, on the individual solutions of AC2, AC3 and PTX-2 under the appropriate ionisation process parameters favouring the exclusive formation of the protonated molecules, $[M+H]^+$, and deprotonated molecules, $[M-H]^-$, for DSP polyether toxins [9,10,25,36,46].

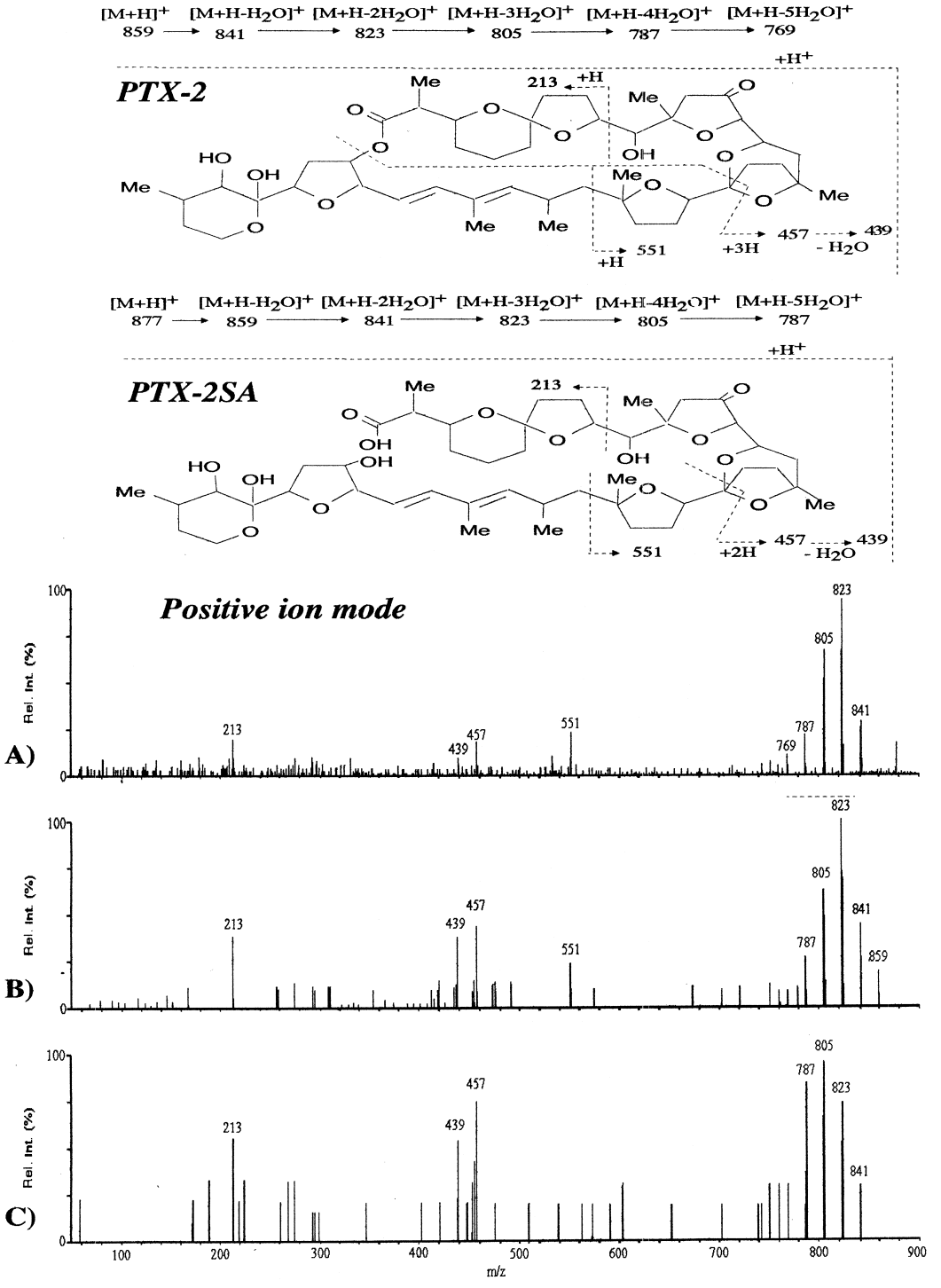


Fig. 5. Product ion mass spectra of: (A) PTX-2 and the two compounds; (B) AC2 (PTX-2SA); and (C) AC3 (7-*epi*-PTX-2SA), isolated from phytoplankton (*D. acuta*), in positive ion mode and proposed fragmentation patterns for the analytes.

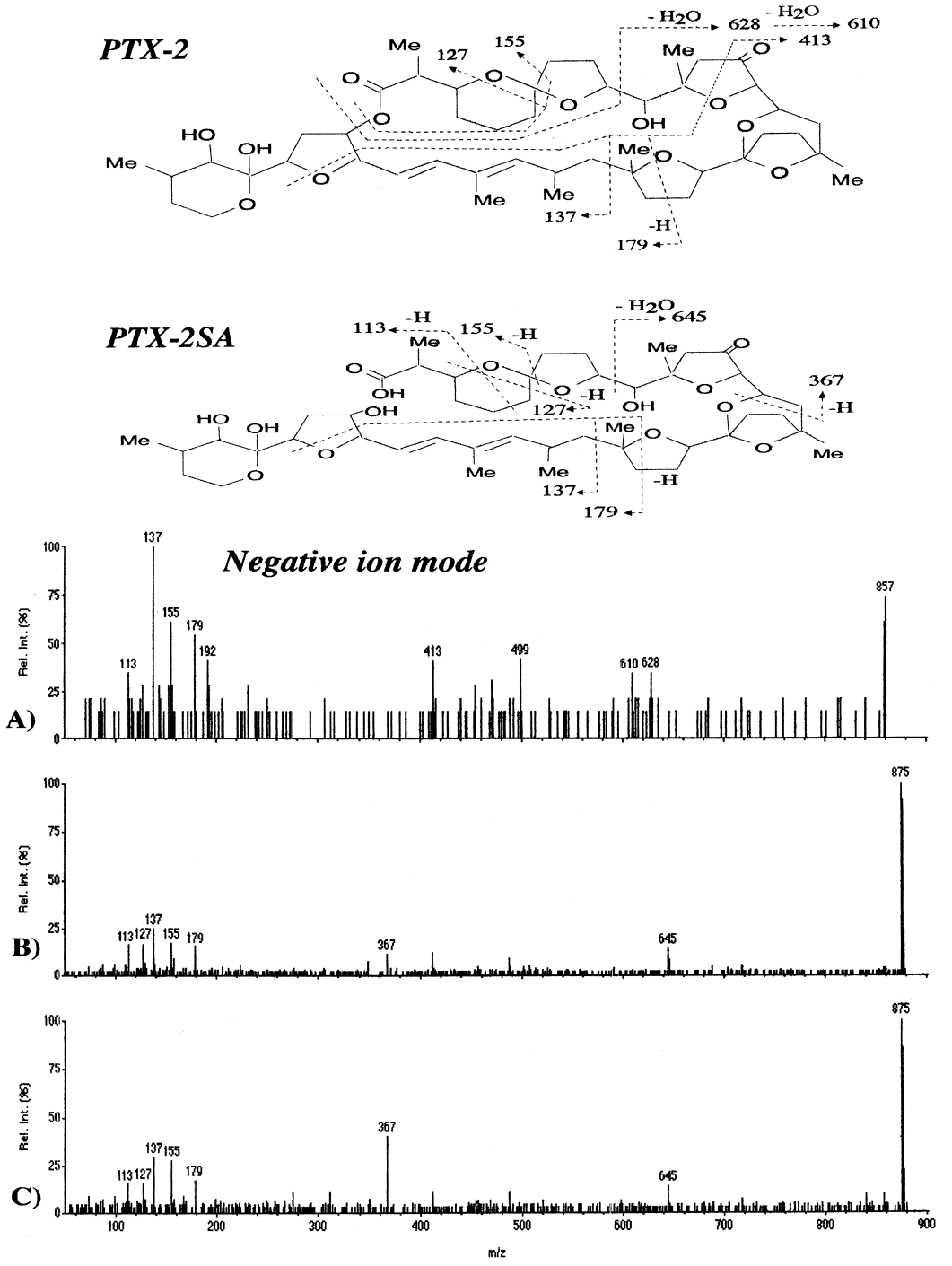


Fig. 6. Product ion mass spectra of: (A) PTX-2 and the two compounds; (B) AC2 (PTX-2SA); and (C) AC3 (7-*epi*-PTX-2SA), isolated from phytoplankton (*D. acuta*) in negative ion mode and proposed fragmentation patterns for the analytes.

The ionspray mass spectra showed that abundant peaks due to the protonated molecules, $[M+H]^+$, at m/z 877 for AC2 and AC3, and at m/z 859 for PTX-2 were produced in positive ion mode, together with the ammonium adducts, $[M+NH_4]^+$, at m/z 895 for AC2 and AC3 and at m/z 877 for PTX-2. On the other hand, only the deprotonated molecules, $[M-H]^-$, at m/z 875 for AC2 and AC3 and at m/z 857 for PTX-2, were observed in the negative ionspray mass spectra of the analytes. These results indicated the same molecular weight of 876 for AC2 and AC3, which is 18 mass units larger than that of PTX-2.

Fig. 5B and C show the positive full-scan FIA–MS–MS spectra for the compounds, AC2 and AC3, respectively, using the $[M+H]^+$, at m/z 877, as precursor ion for each compound. The spectra provide valuable structural information on the analytes, since AC2 and AC3 gave identical fragment ions, which were the same as those obtained from CID of the protonated molecule, $[M+H]^+$, at m/z 859, of PTX-2 (Fig. 5A).

The negative full-scan FIA–MS-MS spectra for PTX-2, AC2 and AC3, using the precursor ions, $[M-H]^-$, at m/z 857 for PTX-2 and at m/z 875, for AC2 and AC3, are shown in Fig. 6A–C, respectively.

A similar fragmentation pattern for the analytes was again observed, as identical fragment ions were produced for AC2 and AC3 with the low mass region ions matching those obtained from the CID of PTX-2. On the other hand, two significant AC1 and AC2 product ions, observed at m/z 367 and 645, were not present in the negative ion MS–MS spectrum of PTX-2. The data obtained from repeated analyses under different collision energies, in both positive and negative ion mode, support the view of the close structural similarities between AC2, AC3 and PTX-2. The MS and MS–MS data should be amalgamated with the previous observation that the new PTX-2 analogues, AC2 and AC3, give fluorescent derivatives with ADAM and BAP, showing the presence of a carboxylic acid in the molecule, and their UV absorption which is consistent with a conjugated diene moiety.

Chromatographic separation of the underivatised AC2 and AC3, together with PTX-2, was finally carried out using a reverse-phase column at room

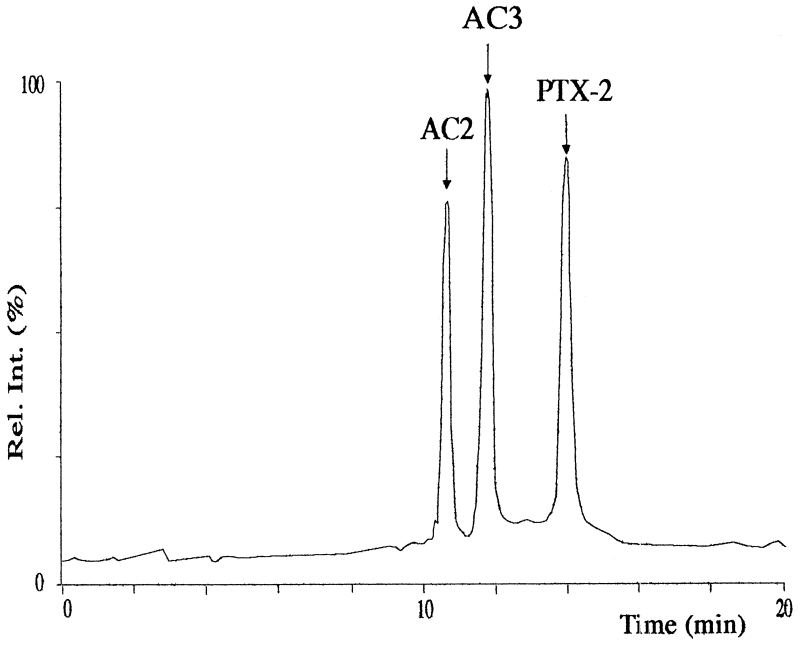


Fig. 7. SIM μLC–MS chromatogram of: (A) a mixture of underivatised compounds, AC2 (PTX-2SA), AC3 (7-*epi*-PTX-2SA), at m/z 877, and PTX-2 at m/z 859; and (B) acetone extract of toxic mussels from the North Adriatic Sea. (For conditions see text.)

temperature, under isocratic conditions, with a mobile phase of acetonitrile–water (60:40, v/v) containing 0.1% TFA. Under these conditions, a good separation was obtained in the SIM μLC–MS analyses (Fig. 7A).

All the above data indicate that the compounds, AC1-3, are isomers, related to PTX-2, and contain an open chain carboxylic acid rather than a lactone ring. These compounds have also recently been found in New Zealand shellfish and two of them have now been structurally elucidated [48]. AC1 and AC3 have been named pectenotoxin-2 seco acid (PTX-2SA) and 7-*epi*-pectenotoxins-2 seco acid (7-*epi*-PTX-2SA), respectively, and possible fragmentation patterns for PTX-2 and PTX-2SAs can be proposed (Figs. 5 and 6). The difficulty in purifying AC1 is now understandable as facile rearrangements are typically observed in the pectenotoxins, especially in solution. Interestingly, LC–MS analysis of toxic Italian shellfish showed the presence of PTX-2SAs in some samples (Fig. 7B) as well as okadaic acid (data not shown). This is the first report of the detection of PTXs in shellfish from Europe. This finding highlights the risk of contamination of European shellfish with PTXs [48] and the need for the development of routine methods to determine these compounds in marine biological materials.

Work is in progress using shellfish from different marine areas to determine the extent of contamination with PTX-2 seco acids.

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