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Liquid chromatography–mass spectrometry of spiroketal stereoisomers of pectenotoxins and the analysis of novel pectenotoxin isomers in the toxic dinoflagellate *Dinophysis acuta* from New Zealand

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Abstract

The acid-catalyzed inter-conversion of spiroketal isomers of pectenotoxins PTX1, PTX6 and PTX2 were studied by liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–MS–MS). Using a C_8-silica reversed-phase column and a mobile phase of aqueous acetonitrile containing 2 m*M* ammonium formate and 50 m*M* formic acid, the known spiroketal stereoisomers of PTX1 eluted in order of PTX1, PTX4 and PTX8, while those of PTX6 eluted in the order PTX6, PTX7 and PTX9. Acid treatment of PTX2 yielded two novel spiroketal stereoisomers, which have been named PTX2b and PTX2c. LC–MS–MS spectra obtained for the $[M+NH_4]^+$ ions of PTX2, PTX2b and PTX2c were essentially identical. As an application of the LC–MS–MS methodology, a sample of the toxic dinoflagellate *Dinophysis acuta* collected from the coast of New Zealand was analyzed for pectenotoxins. PTX2 and a new pectenotoxin, which has been named PTX11, were detected as the most predominant compounds. Novel PTX2 and PTX11 isomers were also found in the *D. acuta* although the levels of these compounds were low.

Keywords: *Dinophysis acuta*; Stereoisomer separation; Shellfish poisoning; Food analysis; Pectenotoxins; Toxins

1. Introduction

Pectenotoxins are polyether macrolide toxins frequently associated with incidents of diarrhetic shellfish poisoning (DSP) [1,2] (Fig. 1). These compounds are thought to be highly hepatotoxic and mildly diarrhetic [3,4] and have also attracted attention due to their potent cytotoxicity against several human cancer cell lines [5,6].

Pectenotoxin-2 (PTX2) is produced by the toxic dinoflagellate *Dinophysis fortii* and *D. acuta* [7–16]. It has been shown that PTX1, PTX3 and PTX6 are metabolites formed by oxidative conversion of PTX2 in Japanese scallops *Patinopecten yessoensis* [7,8,10,17]. Besides these pectenotoxin analogues, pectenotoxin-2 seco acid (PTX2sa) and its epimer 7-*epi*-pectenotoxin-2 seco acid (7-*epi*-PTX2sa) were

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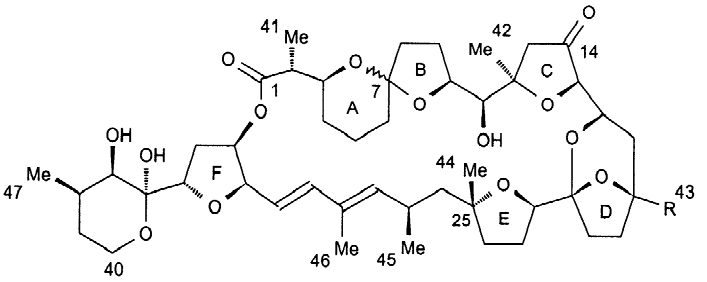
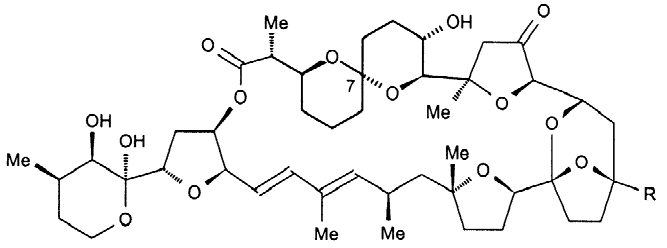


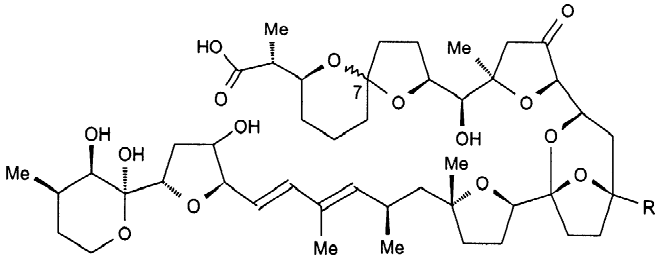
A

	R	C7	EMW
PTX2	CH_3	R	858.5
PTX1	CH_2OH	R	874.5
PTX4	CH_2OH	S	874.5
PTX3	CHO	R	872.5
PTX6	COOH	R	888.5
PTX7	COOH	S	888.5



B

	R	C7	EMW
PTX8	CH_2OH	S	874.5
PTX9	COOH	S	888.5



C

	R	C7	EMW
PTX2sa	CH_3	R	876.5
7-epi-PTX2sa	CH_3	S	876.5

Fig. 1. Structures of the known pectenotoxins.

isolated from New Zealand greenshell mussels [18]. Our previous study clarified that PTX2sa and 7-*epi*-PTX2sa are bivalve metabolites of PTX2 [19,20]. Work by Sasaki et al. [21] has also shown that PTX1 and PTX6 can undergo acid-catalyzed epimerization at the spiroketal carbon of the A/B ring system by ring opening and re-closure of the system. The epimers of PTX1 were named PTX4 and PTX8, while those of PTX6 were named PTX7 and PTX9 (see Fig. 1).

In our previous research, a new isomer of PTX1 was detected in *D. acuta* collected at Buller Bay on the west coast of the South Island of New Zealand [16]. The retention time of the PTX1 isomer obtained by liquid chromatography–mass spectrometry (LC–MS) was different from that of PTX1. In the present study, the LC–MS–MS analysis of spiroketal stereoisomers of various pectenotoxins was investigated and then applied to further characterization of the pectenotoxins in the New Zealand *D. acuta* sample.

2. Experimental

2.1. Materials

Authentic PTX1, PTX2 and PTX6 were provided by Japan Food Research Labs (Tama, Tokyo, Japan) [22]. Phytoplankton cell concentrates were collected from various depths within the water column, approximately 3–4 kilometers offshore in Buller Bay on the northwestern coast of the South Island of New Zealand, on 15 February 2002. Details of the sam-

pling method are described in our previous paper [16].

2.2. Extraction of toxins from plankton cell concentrates

Extraction of pectenotoxins from the condensed dinoflagellate sample was carried out essentially as reported previously [10,19,20,23]. A condensed phytoplankton sample in seawater was filtered through a GF/C filter. The cells on the filter were rinsed with 5 ml of methanol, which was combined with the aqueous filtrate. The filtrate of the condensed phytoplankton samples was transferred (150 ml each time) to a 500 mg C_{18}-silica solid-phase extraction (SPE) cartridge (Strata, Phenomenex, Torrance, CA, USA), previously conditioned with 10 ml of methanol and 10 ml methanol–water (1:9). The cartridge was subsequently washed with 15 ml methanol–water (3:7) and 15 ml methanol–water (6:4), and then pectenotoxins were eluted with 15 ml methanol–water (8:2). The eluates were combined and washed once with hexane (1:1, v/v). After adding water to reduce the methanol concentration to 60%, the toxins were extracted twice with chloroform (1:1, v/v). The combined chloroform extracts were evaporated on a rotary evaporator and then dissolved in a small volume of methanol.

2.3. Acid-catalyzed inter-conversion of pectenotoxins

Inter-conversion of pectenotoxins was carried out according to published procedures [21], with slight modifications for microscale operations to conserve material. One μg of PTX1, PTX2, and PTX6 were separately dissolved in 500 μl of acetonitrile–water (70:30) with 0.1% (v/v) trifluoroacetic acid (TFA) at room temperature. An aliquot (3 μl) of the reaction solution was analyzed directly by LC–MS at intervals to monitor reaction progress.

2.4. LC–MS and LC–MS–MS analysis of pectenotoxins

LC–MS was performed using an Agilent 1100 liquid chromatograph coupled to a PE-SCIEX API-165 mass spectrometer (PE-SCIEX, Thornhill, Canada). Separation of pectenotoxins was achieved on a Quicksilver cartridge column (50 mm×2 mm I.D.) packed with 3 μm Hypersil-BDS-C8 (Keystone Scientific, Bellefonte, PA, USA) maintained at 20 °C. Eluent A was water and B was acetonitrile–water (95:5), both containing 2 m*M* ammonium formate and 50 m*M* formic acid [24]. The mobile phase consisted of 50% B isocratic. The flow-rate was 0.2 ml/min and the injection volume was 3 μl. The LC flow was introduced into an IonSpray (pneumatically-assisted electrospray) interface with splitting using a low dead volume coaxial splitter to provide a 30 μl/min flow to the MS system. Full scan spectra were collected in positive mode from *m/z* 850 to 950. Selected ion monitoring (SIM) for ammonium adduct ions $[M+NH_4]^+$ of pectenotoxins (PTX1: *m/z* 892.5; PTX2: *m/z* 876.5; PTX6: *m/z* 906.5) was carried out in positive ion mode. High-purity nitrogen was used as a nebulizing gas.

LC–MS–MS was performed using an Agilent liquid chromatograph coupled to a PE-SCIEX API-4000 mass spectrometer. The LC conditions for the separation of pectenotoxins were the same as described above. The LC effluent was introduced into a Turbo IonSpray interface without splitting. High-purity air heated to 275 °C was used as a nebulizing gas. Product ion mass spectra were acquired in positive mode by colliding the Q1 selected precursor ion for $[M+NH_4]^+$ of pectenotoxins with nitrogen in Q2 operated in radiofrequency (rf)-only mode and scanning the second quadrupole mass spectrometer, Q3, from *m/z* 50 to 940. Collision energies of 45 eV for collision-induced dissociation (CID) experiments were used.

3. Results and discussion

3.1. LC–MS–MS of pectenotoxin spiroketal stereoisomers

Fig. 2A and B shows the LC–MS chromatograms of the reaction products of PTX1 and PTX6 after 24 h in acetonitrile–water (70:30) with 0.1% TFA at room temperature. These traces were obtained by selected ion monitoring of the $[M+NH_4]^+$ ions of the pectenotoxins after LC separation on a C_8-silica reversed-phase column. Production of spiroketal

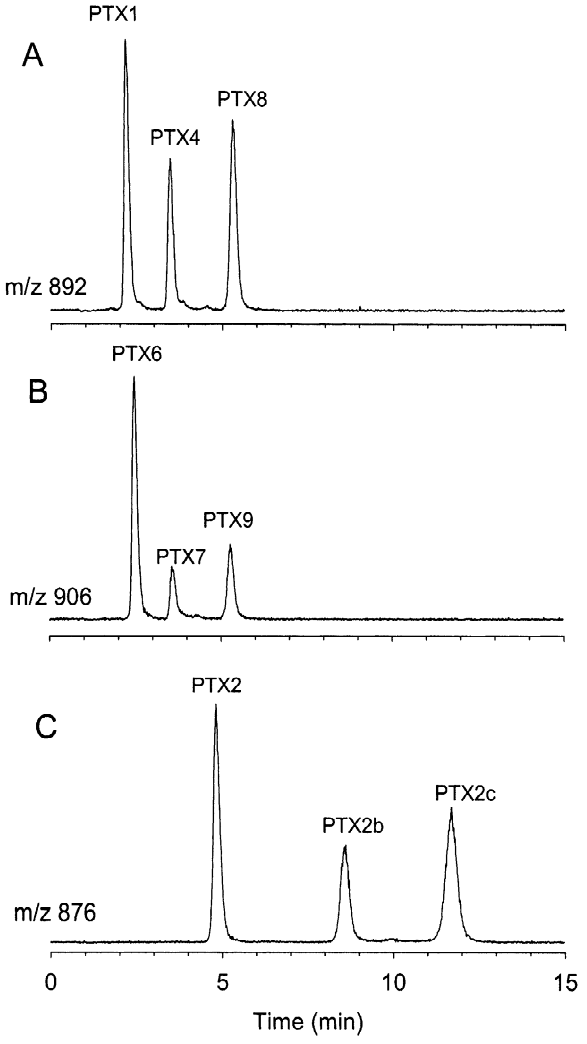


Fig. 2. LC–MS analyses of the reaction products of PTX1 (A), PTX6 (B) and PTX2 (C) after 24 h in acetonitrile–water (70:30) with 0.1% TFA at room temperature. The chromatograms were acquired by selected ion monitoring of the $[M+NH_4]^+$ ions of the pectenotoxins. Conditions: Hypersil-BDS-C8 (50 mm×2 mm I.D.) column; 0.2 ml/min mobile phase consisting of acetonitrile–water (48:52) with 2 m*M* ammonium formate and 50 m*M* formic acid; column temperature at 20 °C.

stereoisomers from PTX1 and PTX6 by acid catalyzed inter-conversion has been reported previously by Sasaki et al. [21]. The same authors also reported on the relative proportions of these isomers of PTX1 and PTX6 at equilibrium as determined by relative peak areas obtained through LC analysis with UV detection at 235 nm. Our assignment of peak identities to the specific spiroketal stereoisomers was done through a comparison of the proportion of isomers produced at equilibrium in our acid treatment experiments with those reported by Sasaki et al. [21]. The relative retention times of the PTX6 isomers are also in agreement with the reversed-phase LC retention data published by Sasaki et al. [21] on phenacyl derivatives; it is expected that the elution order should not change due to derivatization of a carboxyl function. Thus, spiroketal stereoisomers of PTX1 were eluted in the order of PTX1, PTX4 and PTX8, while those of PTX6 eluted in the order PTX6, PTX7 and PTX9. Chromatographic data for the separation of these spiroketal stereoisomers are given in Table 1. Spiroketal stereoisomers of

Table 1
Chromatographic data obtained on the separation of spiroketal stereoisomers of pectenotoxins on a Hypersil-BDS-C_8 column

	t_R[a] (min)	RRT[b]	k'[c]	α[d]	Peak area ratio[e,f]
PTX1	2.17	1.00	1.19		26 (29[f])
				2.11	
PTX4	3.48	1.60	2.52		16 (14[f])
				1.73	
PTX8	5.31	2.45	4.36		58 (57[f])
PTX6	2.48	1.00	1.51		48 (40[f])
				1.73	
PTX7	3.57	1.44	2.61		14 (16[f])
				1.66	
PTX9	5.28	2.13	4.33		38 (44[f])
PTX2	4.83	1.00	3.88		26
				1.99	
PTX2b	8.62	1.78	7.71		15
				1.40	
PTX2c	11.71	2.42	10.83		59
PTX11	4.01	1.00	3.05		31
				1.82	
PTX11b	6.49	1.62	5.56		12
				1.29	
PTX11c	8.07	2.01	7.15		57

[a] Retention times (min).
[b] Relative retention times for spiroketal stereoisomers with respect to precursors.
[c] Capacity factors.
[d] Separation factors (the ratio of capacity factors).
[e] Peak area ratio after 48 h from the start of inter-conversion.
[f] Data obtained by LC–UV analysis at 235 nm (Sasaki et al., 1998).

pectenotoxins were completely separated with separation factor (α) between 1.3 and 2.1.

Fig. 3 shows the MS–MS product ion spectra obtained for the $[M+NH_4]^+$ ions of PTX1, PTX6 and PTX2 on a triple quadrupole mass spectrometer. The spectra show a characteristic fragment ion at m/z 213, as well as series of ions resulting from the loss of first ammonium and then water molecules from $[M+NH_4]^+$. In addition, PTX1, PTX6, and PTX2 produced characteristic fragment ions at m/z 567, 581, and 551, respectively. A proposed MS–MS fragmentation diagram is shown in Fig. 4. The

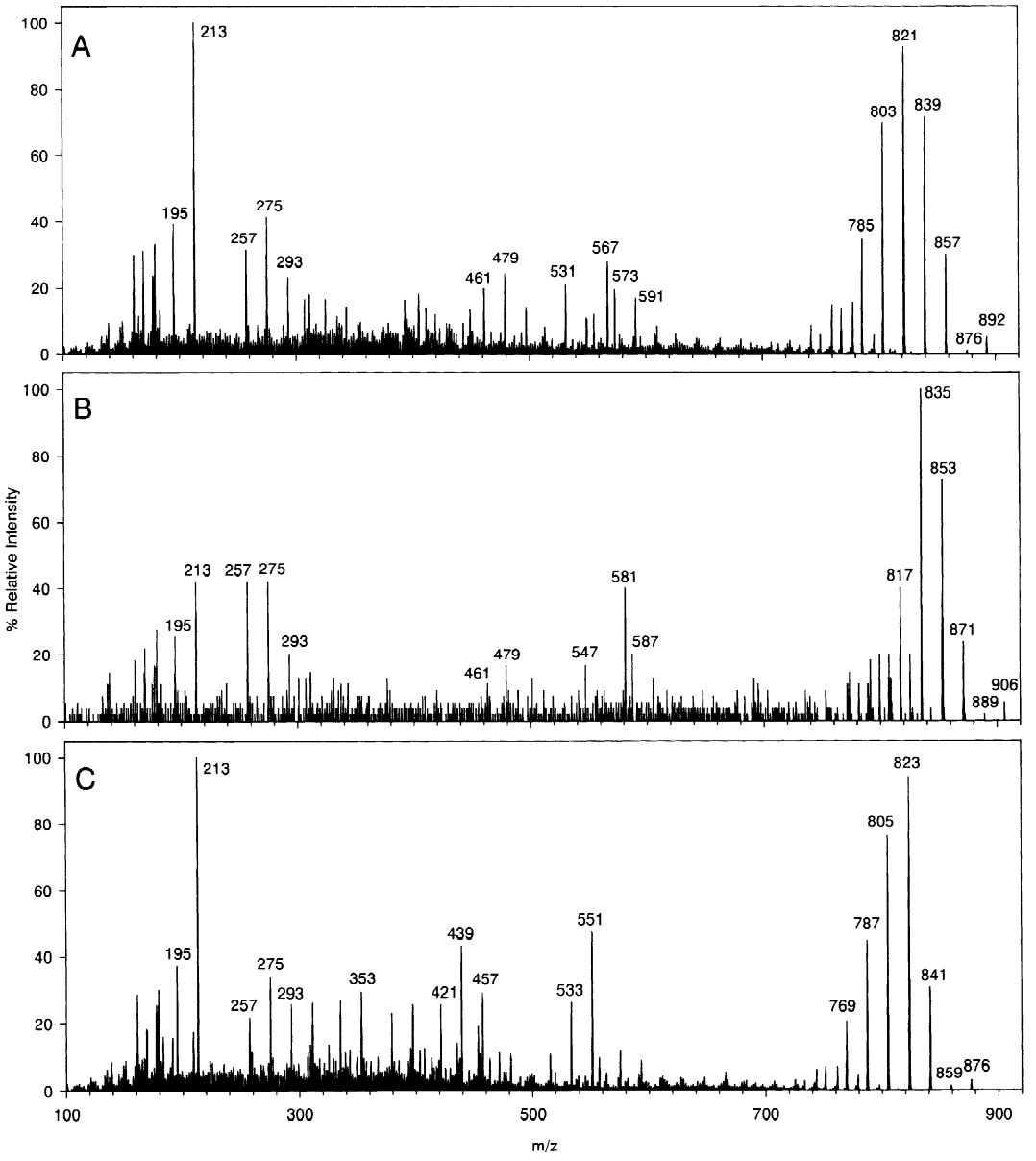


Fig. 3. MS–MS product ion spectra obtained for the $[M+NH_4]^+$ ions of PTX1 (A), PTX6 (B) and PTX2 (C). LC conditions as described in Fig. 2. Collision energy was 45 eV. All m/z values have been rounded down.

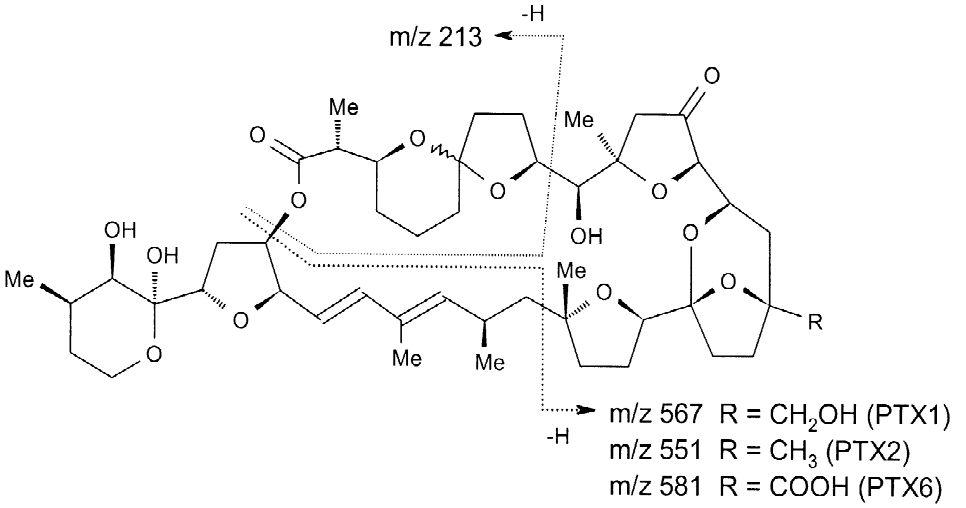


Fig. 4. Proposed MS–MS fragmentation for pectenotoxins.

product ion spectra of PTX4 and PTX8 were found to be essentially identical to that of PTX1, as were those of PTX7 and PTX9 to their precursor, PTX6.

To our knowledge, there has never been a report of spiroketal stereoisomers of PTX2. Fig. 2C shows the LC–MS analysis of the acid-induced products of PTX2 under the same reaction conditions used for PTX1 and PTX6. The chromatogram shows two new compounds, which we have assigned as PTX2 spiroketal stereoisomers and named as PTX2b and PTX2c. Chromatographic data for separation of PTX2 spiroketal stereoisomers are listed in Table 1. Relative retention times of PTX2b and PTX2c with respect to PTX2 were comparable to those of the other spiroketal isomers of pectenotoxins. The MS–MS product ion spectra of PTX2b and PTX2c were essentially identical to that of PTX2 (Fig. 3C). We propose the structures shown in Fig. 5 based on this evidence. Of course, positive confirmation will require further studies using NMR spectroscopy.

Fig. 6 shows the kinetics for the acid catalyzed inter-conversion of PTX1, PTX6 and PTX2. Spiroketal stereoisomers of pectenotoxins reached an equilibrium ratio after 48 h under acidic condition as reported previously [21]. The proportion of spiroketal stereoisomers of PTX1 and PTX6 at equilibration calculated by peak area obtained by LC–MS (SIM) were fairly similar to those previously obtained by UV detection at 235 nm (Table 1) [21]. The proportions of PTX2, PTX2b and PTX2c at equilibrium were close to those for PTX1, PTX4 and PTX8 (Fig. 6 and Table 1). The kinetics for PTX6 inter-conversion were somewhat different from those of the other pectenotoxins.

3.2. Application of LC–MS–MS to New Zealand Dinophysis acuta

Fig. 7A shows the total ion current chromatogram from the full scan positive-ion LC–MS analysis of an extract of a *D. acuta* sample collected from New Zealand coastal waters. The most abundant component gave a peak at 4.8 min in the m/z 876 extracted mass chromatogram (Fig. 7B) corresponding to $[M+NH_4]^+$ of PTX2. The retention time and MS–MS spectrum were essentially identical to those of authentic PTX2 (Fig. 3C), thus re-confirming that PTX2 is a prominent toxin in *D. acuta* in New Zealand [16,19].

A prominent peak at 4.0 min was also observed in the m/z 892 chromatogram corresponding to $[M+NH_4]^+$ of PTX1 (Fig. 7C), but the retention time of this peak was quite different from that of authentic PTX1, as well as its spiroketal stereoisomers, PTX4 and PTX8 (Table 1). In addition, the MS–MS product ion spectrum obtained for the $[M+NH_4]^+$ ion of this compound (Fig. 8) was slightly different from that of PTX1 and its stereoisomers. Instead of

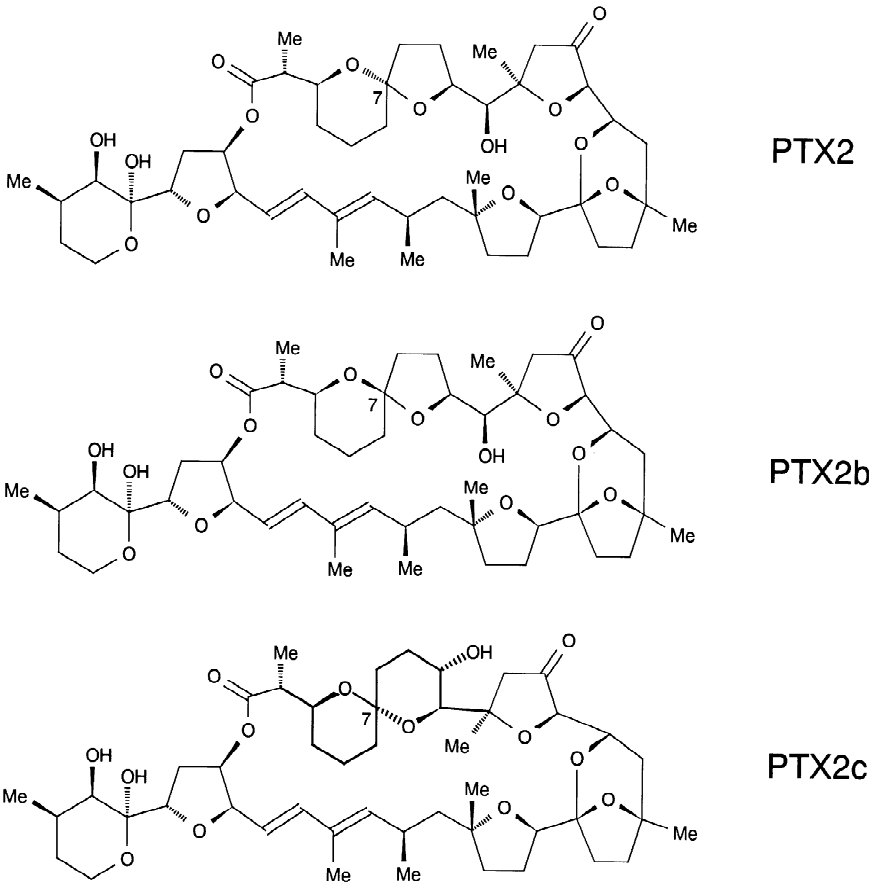


Fig. 5. Structure of PTX2 and proposed structures for PTX2b and PTX2c.

the characteristic fragment ion at m/z 567 observed for PTX1, a fragment ion at m/z 551, which is a characteristic fragment ion of PTX2, was detected from the new compound. This indicates a PTX2 base structure with a hydroxyl group placed between C-26 to C-40 or on one of C-45, C-46 or C-47 methyl groups (Fig. 4). All this suggested that the compound is a novel isomer of PTX1, which we have now named as ''PTX11''. This compound is the same as that reported in our previous paper and temporarily called ''PTX1i'' [16]. Elucidation of the detailed structure of PTX11 is being performed and will be reported elsewhere. It is interesting that PTX11 was not detected in *D. acuta* collected in Queen Charlotte Sound, New Zealand [19]. This indicates that toxin profiles are different among the same species collected in different locations.

The *D. acuta* sample was also found to contain several minor compounds, one of which has a retention time and mass spectrum that match with those of PTX2b (Fig. 7B), the spiroketal stereoisomer of PTX2. Although presence of PTX2b in the *D. acuta* is interesting, it might have been chemically converted from PTX2 during extraction and storage of the sample. This needs to be investigated further by analysis of samples immediately after collection.

Two other components, which gave small peaks in the m/z 892 chromatogram of the *D. acuta* sample, appear to be additional PTX1 isomers. These have been labeled as PTX11b and PTX11x (Fig. 7C). The MS–MS spectra of PTX11, PTX11b and PTX11x were identical indicating they are probably stereoisomers. The relationship of these minor compounds with PTX11 was investigated further by reacting

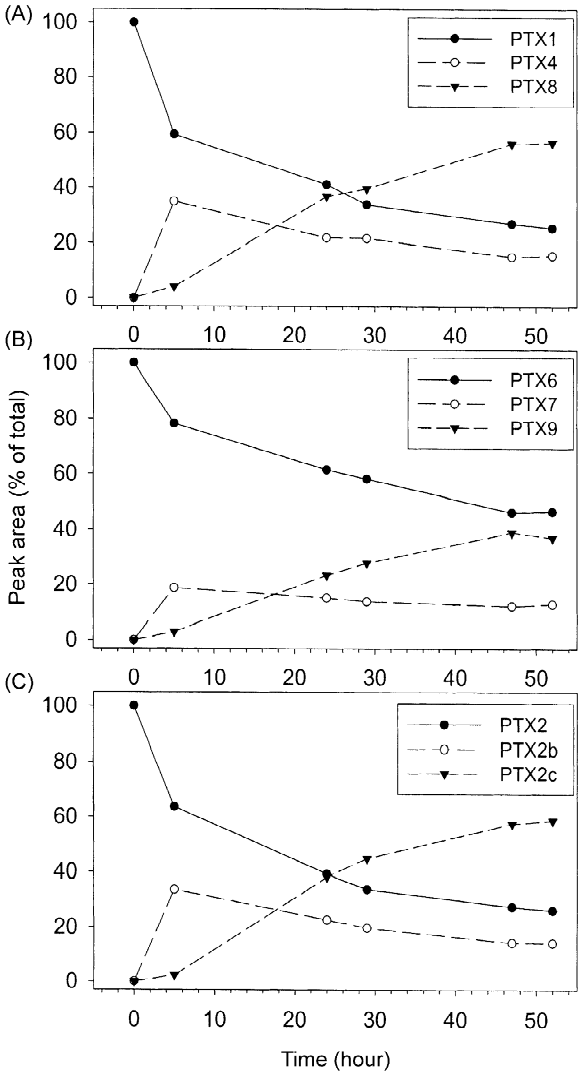


Fig. 6. Kinetics of acid catalyzed inter-conversion of PTX1 (A), PTX6 (B), and PTX2 (C). Peak areas of each pectenotoxin were obtained by LC–MS (SIM) with conditions as described in Fig. 2.

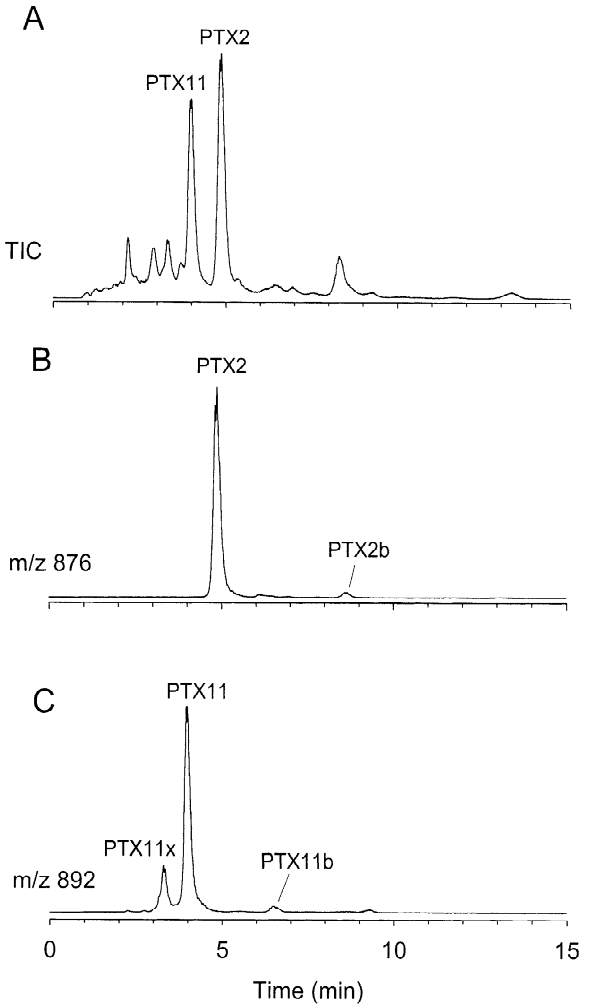


Fig. 7. Positive ion full scan LC–MS chromatograms obtained from *D. acuta* collected in New Zealand: (A) total ion chromatogram (TIC) (scan range: m/z 850–950); (B) m/z 876 extracted mass chromatogram; and (C) m/z 892 extracted mass chromatogram. LC conditions as described in Fig. 2.

with acid a small amount of PTX11 isolated by LC. The concentration of PTX11 decreased during the reaction and two isomeric compounds appeared (Fig. 9). One of these products was PTX11b, giving retention time and MS–MS spectrum identical to the compound in the *D. acuta* sample. The other acid reaction product has been called PTX11c. These products appear to be analogous to the acid-induced rearrangement products of PTX2, PTX2b and PTX2c. Their relative retention times and equilibrium ratios match those of the analogous compounds as reported in Table 1. In contrast to PTX11b and PTX11c, the intensity of the peak area of PTX11x did not increase under acid treatment of the sample, suggesting that this compound is not an acid-induced

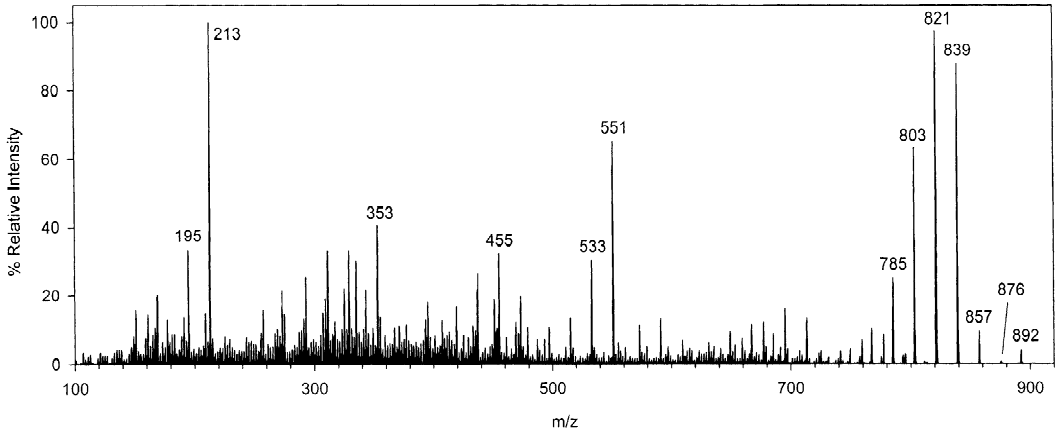


Fig. 8. MS–MS product ion spectra obtained for the $[M+NH_4]^+$ ions of PTX11 present in *D. acuta* collected in New Zealand. Conditions as described in Fig. 3.

stereoisomer of PTX11. Further work will be required to determine the nature of this compound.

4. Conclusions

In conclusion, chromatographic separation and tandem mass spectrometry of spiroketal stereoisomers of pectenotoxins were investigated in this study. Two novel spiroketal stereoisomers of PTX2 were obtained by acid catalyzed inter-conversion of PTX2. We have called these isomers PTX2b and PTX2c. LC–MS–MS analyses of *D. acuta* collected from New Zealand revealed the presence of PTX2, as the major toxin, and one of these isomers, PTX2b. It was also found that the *D. acuta* sample contained a high level of a novel isomer of PTX1, which we have now called PTX11. This is the same isomer as that reported in our previous paper, which was temporarily called ''PTX1i'' [16]. Elucidation of the structure of PTX11 will be reported elsewhere with further spectroscopic data including nuclear magnetic resonance spectrometry (NMR). Low levels of other minor isomers of PTX11 were also present in the sample. One of these, which we have called PTX11b, appears to be a spiroketal rearrangment product, analogous to PTX4, PTX7 and PTX2b. Acid-induced rearrangement of PTX11 results in the formation of both PTX11b and an additional isomer, PTX11c.

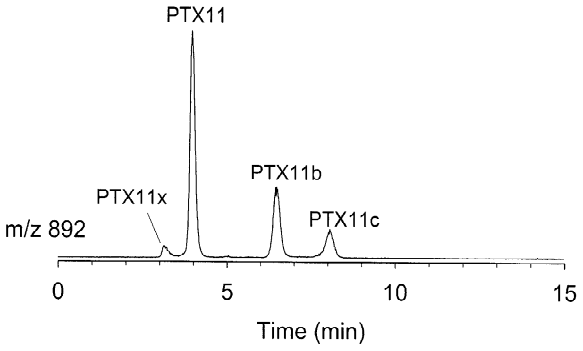


Fig. 9. LC–MS analysis of the reaction products of PTX11 after 4 h in acetonitrile–water (70:30) with 0.1% TFA at room temperature. The chromatograms were acquired by selected ion monitoring of the $[M+NH_4]^+$ ions of PTX11. LC conditions as described in Fig. 2.

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References

[1] T. Yasumoto, M. Murata, Y. Oshima, M. Sano, G.K. Matsumoto, J. Clardy, Tetrahedron 41 (1985) 1019.
[2] T. Yasumoto, M. Murata, Chem. Rev. 93 (1993) 1897.
[3] K. Terao, E. Ito, T. Yanagi, T. Yasumoto, Toxicon 24 (1986) 1141.
[4] M. Ishige, N. Satoh, T. Yasumoto, Bull. Hokkaido Inst. Public Health 38 (1988) 15.
[5] Z.H. Zhou, M. Komiyama, K. Terao, Y. Shimada, Nat. Toxins 2 (1994) 132.
[6] J.H. Jung, C. J Sim, C.O. Lee, J. Nat. Prod. 58 (1995) 1722.
[7] J.S. Lee, M. Murata, T. Yasumoto, in: S. Natori, K. Hashimoto, Y. Ueno (Eds.), Mycotoxins and Phycotoxins ’88, Elsevier, Amsterdam, 1989, p. 327.
[8] J.S. Lee, T. Igarashi, S. Fraga, E. Dahl, P. Hovgaard, T. Yasumoto, J. Appl. Phycol. 1 (1989) 147.
[9] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, R. Poletti, Toxicon 8 (1996) 923.
[10] T. Suzuki, T. Mitsuya, H. Matsubara, M. Yamasaki, J. Chromatogr. A 815 (1998) 155.
[11] K.J. James, A.G. Bishop, R. Draisci, L. Palleschi, C. Marchiafava, E. Ferretti, M. Satake, T. Yasumoto, J. Chromatogr. A 844 (1999) 53.
[12] R. Draisci, L. Palleschi, L. Giannetti, L. Lucentini, K.J. James, A.G. Bishop, M. Satake, T. Yasumoto, J. Chromatogr. A 847 (1999) 213.
[13] K. Sasaki, A. Takizawa, A. Tubaro, L. Sidari, R.D. Loggia, T. Yasumoto, Nat. Toxins 7 (1999) 241.
[14] M. Pavela-Vrancic, V. Mestrovic, I. Marasovic, M. Gillman, A. Furey, K.J. James, Toxicon 39 (2001) 771.
[15] P. Vale, M.A. Sampayo, Toxicon 40 (2002) 979.
[16] L. MacKenzie, P. Holland, P. McNabb, V. Beuzenberg, A. Selwood, T. Suzuki, Toxicon 40 (2002) 1321.
[17] T. Yasumoto, M. Murata, J.S. Lee, K. Torigoe, in: S. Natori, K. Hashimoto, Y. Ueno (Eds.), Mycotoxins and Phycotoxins ’88, Elsevier, Amsterdam, 1989, p. 375.
[18] M. Daiguji, M. Satake, K.J. James, A. Bishop, L. Mackenzie, H. Naoki, T. Yasumoto, Chem. Lett. 7 (1998) 653.
[19] T. Suzuki, L. Mackenzie, D. Stirling, J. Adamson, Toxicon 39 (2001) 507.
[20] T. Suzuki, L. Mackenzie, D. Stirling, J. Adamson, Fish. Sci. 67 (2001) 506.
[21] K. Sasaki, J.L. Wright, T. Yasumoto, J. Org. Chem. 63 (1998) 2475.
[22] H. Goto, T. Igarashi, R. Sekiguchi, K. Tanno, M. Satake, Y. Oshima, T. Yasumoto, in: B. Reguera, J. Blanco, M.L. Fernández, T. Wyatt (Eds.), Harmful Algae, Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Spain, 1998, p. 216.
[23] T. Suzuki, T. Mitsuya, M. Imai, M. Yamasaki, J. Appl. Phycol. 8 (1997) 509.
[24] M.A. Quilliam, P. Hess, C. Dell’Aversano, in: W.J. deKoe, R.A. Samson, H.P. van Egmond, J. Gilbert, M. Sabino (Eds.), Mycotoxins and Phycotoxins in Perspective at the Turn of the Century, W.J. deKoe, Wageningen, The Netherlands, 2001, p. 383.