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Production of 7-*epi*-Pectenotoxin-2 Seco Acid and Assessment of Its Acute Toxicity to Mice

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Pectenotoxins (PTXs) accumulate in shellfish feeding on dinoflagellates of the genus *Dinophysis*, so that humans can be exposed to these toxins through shellfish consumption. Some PTXs are toxic to experimental animals, whereas others are of much lower toxicity. Pectenotoxin-2, the most abundant PTX from most *Dinophysis* spp., is rapidly metabolized by most shellfish to a mixture of pectenotoxin-2 seco acid (**2**) and 7-*epi*-pectenotoxin-2 seco acid (**1**). A mixture of **1** and **2** was produced during purification of an extract from in vitro enzymatic hydrolysis of pectenotoxin-2. These were separated by preparative HPLC, and the structure of **1** was confirmed by one- and two-dimensional ^{1}H and ^{13}C NMR spectroscopy and LC-MS^{3} analyses. No toxic changes were recorded in mice injected intraperitoneally with **1** or **2** at a dose of 5000 μg/kg. PTX seco acids are therefore unlikely to be of consequence to human consumers at the concentrations found in contaminated shellfish.

KEYWORDS: pectenotoxin; seco acid; PTX-2 seco acid; *Dinophysis*; toxicology; mussel

INTRODUCTION

The pectenotoxin family of polyether toxins has been found in a range of shellfish worldwide (*1*−*5*). *Dinophysis* spp. species appear to be the producers of the pectenotoxins (PTXs), although the current inability to grow these algae in culture prevents testing of this hypothesis. Several PTXs have been identified in algae from many locations, including pectenotoxin-2 (PTX-2; Figure 1) (*1*, *2*, *6*−*11*), pectenotoxin-11 (PTX-11; Figure 1) (*11*−*13*), and pectenotoxin-12 (PTX-12) (*10*). When *Dinophysis* spp. are ingested by shellfish, pectenotoxins are absorbed and metabolized, and two main routes of metabolism have been identified. In the Japanese scallop *Patinopecten yessoensis*, oxidation of the C-43 methyl group of PTX-2 yields hydroxymethyl (PTX-1; Figure 1), aldehyde (PTX-3), and carboxylic acid (PTX-6) derivatives (*9*). In many other species, including green-lipped (*Perna canaliculus*) and blue (*Mytilus edulis* and *Mytilus galloprovincialis*) mussels and New Zealand scallops (*Pecten novaezelandiae*), rapid enzymatic hydrolysis of PTX-2 interconverts to give pectenotoxin-2 seco acid (PTX-2 seco acid; Figure 1) (*13*−*15*). PTX-2 seco acid appears to progressively epimerize to the thermodynamically more stable 7-*epi*-pectenotoxin-2 seco

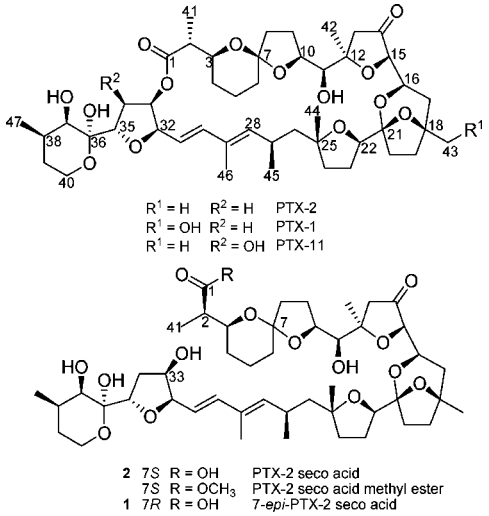

Figure 1. Structures of pectenotoxin-2 (PTX-2), pectenotoxin-1 (PTX-1), pectenotoxin-11 (PTX-11), pectenotoxin-2 seco acid (PTX-2 seco acid) (**2**), pectenotoxin-2 seco acid methyl ester (PTX-2 seco acid methyl ester), and 7-*epi*-pectenotoxin-2 seco acid (7-*epi*-PTX-2 seco acid) (**1**).

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acid (7-*epi*-PTX-2 seco acid; Figure 1) (*13*−*15*), although the factors controlling the reaction are only partially understood. Thus, PTX-2 seco acid and 7-*epi*-PTX-2 seco acid are found together in shellfish that have been exposed to algae containing PTX-2, in ratios that probably depend on the time since exposure of the shellfish to the algae; the shellfish species; and the procedures used for extraction, treatment, and storage of the sample. PTX-11 and, to a lesser extent, PTX-12 are less susceptible to enzymatic hydrolysis, and seco acid forms of these compounds are therefore relatively less abundant in mussels than are the seco acids of PTX-2 (*10*).

Pectenotoxins were originally classified as diarrhetic shellfish toxins (*16*, *17*). Recent work has shown, however, that PTXs do not cause diarrhea in laboratory animals (*13*, *18*). Furthermore, whereas PTX-2 is highly toxic by intraperitoneal injection, it is of very low toxicity by the oral route (*13*). The toxicities of PTX-2 seco acid and 7-*epi*-PTX-2 seco acid were first investigated by Burgess et al. (*19*), who reported diarrhea in mice dosed with a mixture of 35% PTX-2 seco acid and 65% 7-*epi*-PTX-2 seco acid. It was suggested (*19*) that the seco acids were responsible for an outbreak of poisoning in Australia in 1997, in which nausea, vomiting, and diarrhea were reported in individuals consuming shellfish contaminated with these substances. Subsequent studies, however, indicated that the diarrhea recorded in mice was most likely due to contamination of the PTX-2 seco acids with okadaic acid esters (*20*), and no diarrhea or any other toxic effects were seen in animals dosed with a different batch of PTX-2 seco acid (*21*). In accord with the latter observation, no toxic effects were observed in mice receiving pure PTX-2 seco acid at 5000 μg/kg either by intraperitoneal injection or by gavage (*13*). Although it is unlikely that PTX-2 seco acid was responsible for the observed poisoning in Australia, the question remains as to whether 7-*epi*-PTX-2 seco acid is toxic and might be a contributor to intoxication events.

Herein, we report the isolation of 7-*epi*-PTX-2 seco acid during in vitro conversion of PTX-2 to PTX-2 seco acid and full confirmation of its structure by ^{1}H and ^{13}C NMR spectroscopy. Experiments on the acute toxicity of 7-*epi*-PTX-2 seco acid to mice are also described, together with further studies on the toxicity of PTX-2 seco acid.

MATERIALS AND METHODS

Enzymatic Hydrolysis. Ultracentrifuged homogenate of digestive gland from green-lipped mussels (*Perna canaliculus*) was prepared as described previously (*13*) and stored at −80 °C until required. PTX-2 was isolated from *D. acuta* (*13*). Ultracentrifuged homogenate (7.2 mL) was added to phosphate-buffered saline (12.0 mL), and to this with gentle stirring was added PTX-2 (4.22 mg) in MeOH (1.0 mL). After 220 min at ca. 20 °C, the reaction was quenched by addition to MeCN (80 mL), and the mixture was stored at 4 °C overnight. The supernatant was filtered, evaporated to dryness in vacuo, and extracted from water (50 mL) with dichloromethane (3 × 30 mL). The combined extracts were evaporated to dryness in vacuo and applied in 20% MeOH to a 500-mg polymeric SPE column (Phenomenex, Torrance, CA). The column was eluted with a stepwise gradient of MeOH in water (20, 30, 40, 50, 60, 70, 80, 90, and 100%; 10 mL each). PTX-2 seco acid and 7-*epi*-PTX-2 seco acid were present together in both the 80% and 90% fractions, which were combined, evaporated to dryness in vacuo, and purified by preparative HPLC as described by Miles et al. (*13*) to afford PTX-2 seco acid (2.05 mg) and 7-*epi*-PTX-2 seco acid (1.81 mg) as colorless solids.

LC-MS^3 Analysis. Purification was monitored by analytical HPLC−UV as described for PTX-2 seco acid (*13*). Purified materials were also analyzed by LC-MS^3 on an LCQ Deca ion-trap mass spectrometer fitted with an ESI interface (ThermoQuest, Finnigan, San Jose, CA) coupled to a Surveyor HPLC and PDA detector (*13*).

Table 1. ^{1}H and ^{13}C NMR Assignments for 7-*epi*-PTX-2 Seco Acid in CD_3OD[a]

	PTX-2 seco acid		7-*epi*-PT X-2 seco acid	
atom	^{13}C	^{1}H	^{13}C	^{1}H
1	179.0		179.0	
2	46.7	2.46	47.0	2.36
3	77.3	3.71	73.1	3.93
4	29.7	1.26, 1.60	29.6	1.30, 1.59
5	22.8	1.62, 1.85	21.6	1.68, 1.81
6	34.9	1.68, 1.68	33.8	1.62, 1.66
7	109.2		107.7	
8	33.2	1.52, 2.48	38.2	1.73, 1.85
9	26.3	1.93, 2.09	27.2	1.91, 2.02
10	81.4	4.26	78.8	3.99
11	78.3	3.81	78.3	3.70 (d, J = 5.8 Hz)
12	83.1		84.0	
13	46.4	2.18, 2.85	45.6	2.28, 2.78 (both d, J = 17.5 Hz)
14	215.2		215.1	
15	81.0	3.90	81.0	3.93 (d, J = 2.4 Hz)
16	71.8	4.23	72.0	4.25
17	37.1	1.26, 2.10	36.8	1.28, 2.10
18	81.9		82.0	
19	34.7	1.70, 1.89	34.7	1.68, 1.90
20	31.0	2.00, 2.09	31.4	2.02, 2.11
21	109.6		109.6	
22	81.1	3.87	81.2	3.86
23	28.8	1.82, 1.97	28.6	1.84, 1.97
24	38.2	1.58, 1.73	38.1	1.60, 1.73
25	85.7		85.7	
26	49.2	1.55, 1.65	49.1	1.53, 1.64
27	30.8	2.72	30.7	2.71
28	141.6	5.35	141.7	5.36 (br d, J = 9.6 Hz)
29	132.2		132.2	
30	139.7	6.29	139.7	6.31 (d, J = 15.7 Hz)
31	124.0	5.71	124.0	5.71 (dd, J = 15.7, 7.9 Hz)
32	87.1	4.42	87.0	4.43 (dd, J = 7.9, 2.6 Hz)
33	75.4	4.22	75.3	4.21
34	36.8	2.09, 2.11	36.8	2.10 (2H)
35	82.6	4.51	82.5	4.49 (dd, J = 9.9, 6.5 Hz)
36	98.9		98.9	
37	71.6	3.29	71.7	3.28 (d, J = 2.4 Hz)
38	30.6	2.12	30.6	2.10
39	28.2	1.23, 1.64	28.2	1.21, 1.63
40	61.7	3.61, 3.91	61.7	3.62, 3.93
41	13.7	1.19	13.2	1.14 (d, J = 6.9 Hz)
42	23.4	1.28	23.7	1.348
43	26.2	1.33	26.2	1.337
44	27.1	1.20	27.0	1.21
45	23.0	0.97	23.0	0.97 (d, J = 6.7 Hz)
46	12.8	1.80	12.8	1.80
47	17.9	0.94	17.9	0.94 (d, J = 6.9 Hz)

[a] Assignments for PTX-2 seco acid are from Miles et al. (*13*).

NMR Spectroscopy. NMR spectra were obtained from solutions of **1** (ca 1.81 mg) in CD_3OD (>99.8 at. % D; Aldrich, Milwaukee, WI) with a Bruker 400-MHz spectrometer. NMR assignments (**Table 1**) were obtained from examination of ^{1}H, ^{13}C, DEPT135, 1D-TOCSY, 1D-ROESY, COSY, TOCSY, g-HSQC, g-HMBC, ROESY, and NOESY NMR spectroscopic data. Chemical shifts, determined at 30 °C, are reported relative to internal C*H*D$_2$OD (3.31 ppm) and *C*D$_3$OD (49.0 ppm) (*22*).

Table 2. Body-Weight Change and Relative Organ Weights[a] of Control Mice and of Mice Injected Intraperitoneally with PTX-2 Seco Acid or 7-*epi*-PTX-2 Seco Acid at a Dose of 5000 μg/kg. Experiment 1: Animals Killed 24 Hours after Dosing

treatment group	body-weight change (g)[b]	relative organ weight (g/100 g of body weight)					
		liver	kidneys	spleen	heart	lungs	intestine[c]
control	–0.86 ± 0.23	5.57 ± 0.33	1.69 ± 0.03	0.499 ± 0.057	0.578 ± 0.026	0.752 ± 0.057	10.39 ± 0.61
PTX-2 seco acid	–0.40 ± 0.32	5.34 ± 0.23	1.53 ± 0.075	0.338 ± 0.038	0.552 ± 0.039	0.733 ± 0.028	9.87 ± 0.34
7-*epi*-PTX-2 seco acid	–0.54 ± 0.55	5.49 ± 0.36	1.56 ± 0.045	0.375 ± 0.047	0.583 ± 0.047	0.693 ± 0.060	10.60 ± 1.04

[a] Results shown are the means ± standard errors of values for the five animals in each treatment group. [b] Body-weight change from the time of dosing to 24 h after dosing. [c] Whole intestine, from pylorus to anus.

Toxicity of PTX-2 Seco Acid and 7-*epi*-PTX-2 Seco Acid. *Experiment 1*. PTX-2 seco acid and 7-*epi*-PTX-2 seco acid were dissolved in 95% ethanol and diluted with 1% Tween 60 in saline to give a solution containing 100 μg/mL of the test compounds and 2% v/v ethanol. Fifteen female Swiss mice, of initial body weight between 18 and 22 g, were randomly allocated to three treatment groups, each containing five mice. The first group was injected intraperitoneally with the solution of PTX-2 seco acid, the injection volume (approximately 1 mL) being adjusted according to the body weight of the mouse to give a dose of 5000 μg/kg. The second group of mice was similarly injected with the 7-*epi*-PTX-2 seco acid solution, and the third (control) group was injected with the vehicle alone. After 24 h, the animals were weighed and then killed by injection of a lethal dose of pentobarbitone. At necropsy, the liver, kidneys, spleen, lungs, heart, and intestinal tract (from pylorus to anus) of each animal were weighed. The organ weights were expressed as a percentage of body weight and analyzed using Student's *t*-test (GraphPad Inc., San Diego, CA). Differences were taken to be statistically significant when $P < 0.05$. Portions of the liver, diaphragm, duodenum, jejunum, colon, and gastrocnemius, together with the kidneys, adrenals, lungs, thyroid, trachea, heart, spleen, ovary, uterus, tongue, thymus, brain, pancreas, and urinary bladder were preserved in 4% buffered formaldehyde for histological examination. The tissues were processed by standard techniques and embedded in paraffin wax. Sections were stained with haematoxylin and eosin and examined without reference to treatment group.

Experiment 2. A second group of 15 mice was randomized into groups and dosed with PTX-2 seco acid, 7-*epi*-PTX-2 seco acid, or vehicle as in the first experiment. They were examined and weighed each day for 14 days after administration of the test compounds. They were killed on the 15th day, and tissues were weighed, preserved, and examined histologically as described above. Animal experiments were approved by the AgResearch Ruakura Animal Ethics Committee.

RESULTS AND DISCUSSION

Preparation of PTX-2 seco acid by the method of Miles et al. (*13*) proceeded smoothly, with HPLC analysis revealing essentially complete conversion of PTX-2 into PTX-2 seco acid contaminated with traces of 7-*epi*-PTX-2 seco acid (<5%). LC-MS analysis indicated that quenching of the enzymatic hydrolysis with MeCN, instead of MeOH (*13*), was successful in preventing formation of PTX-2 seco acid methyl ester (Figure 1). However, modification to the purification procedure involving use of a preliminary SPE cleanup of the crude extract resulted in conversion of approximately one-half of the PTX-2 seco acid into 7-*epi*-PTX-2 seco acid. Purification of both isomers proceeded smoothly, with a high overall yield of seco acids and without further epimerization at C-7. The PTX-2 seco acid and 7-*epi*-PTX-2 seco acid were each ≥95% by HPLC−UV and NMR spectroscopy, with each containing small amounts of the other epimer as the major impurity. Our findings confirm that PTX-2 seco acid is the initial product from the enzymatic hydrolysis of PTX-2 and that this compound is readily epimerized to 7-*epi*-PTX-2 seco acid without enzymatic catalysis. No epimerization of PTX-2 seco acid or 7-*epi*-PTX-2 seco acid was detected during the acquisition of the NMR spectra in CD_3OD.

The PTX-2 seco acid was identical by LC-MS^3 and HPLC−UV to authentic material (*13*). The 7-*epi*-PTX-2 seco acid displayed the same MS fragmentations (during LC-MS^3) and UV spectroscopic characteristics (during HPLC−UV) as PTX-2 seco acid. The structure of 7-*epi*-PTX-2 seco acid was confirmed by analysis of a series of one- and two-dimensional ^1H and ^{13}C NMR spectra. ^{13}C NMR data for 7-*epi*-PTX-2 seco acid have not previously been reported. Detailed analyses of ^1H, ^{13}C, and two-dimensional COSY, TOCSY, NOESY, HSQC, and HMBC NMR spectroscopic data led to the complete assignment of the ^1H and ^{13}C NMR signals of 7-*epi*-PTX2 seco acid (Table 1).

The ^1H NMR resonances reported in **Table 1** agree to within ±0.04 ppm with those reported by Daiguji et al. (*6*) for 7-*epi*-PTX-2 seco acid. Of note are differences in the H-8, H-10, H-11, and H-13 resonances of 7-*epi*-PTX-2 seco acid of about 0.1−0.6 ppm compared to the equivalent resonances of PTX-2 seco acid (**Table 1**). Correlations observed in the HSQC spectrum of 7-*epi*-PTX-2 seco acid facilitated the identification of the corresponding carbon atoms, especially C-1−C-10. The resonances of ^1H and ^{13}C atoms remote from rings A and B were essentially identical to those of PTX-2 seco acid and demonstrated that epimerization at C-7 had not been accompanied by epimerization at other sites such as C-15, C-21, and C-36.

The marked upfield shift from 77.3 ppm in PTX-2 seco acid to 73.1 ppm in 7-*epi*-PTX-2 seco acid exhibited by C-3, and to a lesser extent by C-5 (Table 1), is consistent with the oxygen atom of the 7(10)-oxido group being axially oriented relative to ring A in 7-*epi*-PTX2 seco acid, rather than being equatorially oriented as in PTX-2 seco acid, as is observed for hydroxyl groups on six-membered rings (*23*). Similarly, the chemical shifts of C-8 are consistent with this atom being pseudoequatorially oriented in PTX-2 seco acid but pseudoaxially oriented in 7-*epi*-PTX-2 seco acid. The resonance of C-41 (the methyl-group carbon attached to C-3) showed a modest upfield shift (−0.4 ppm) to 13.3 ppm in 7-*epi*-PTX-2 seco acid, compared to 13.7 ppm in PTX-2 seco acid. These observations confirm the proposal (*6*) that the dominant epimer of PTX-2 seco acid, corresponding to the isomer isolated in this investigation, is 7-*epi*-PTX-2 seco acid.

In addition to the dominant isomers (PTX-2 seco acid and 7-*epi*-PTX-2 seco acid), we observed several minor components by LC-MS^3, the MS and MS^2 spectra of which were consistent with isomers of PTX-2 seco acid. These compounds might correspond to epimers (e.g., at C-15, C-21, and C-36) of PTX-2 seco acid or 7-*epi*-PTX-2 seco acid.

Mice injected intraperitoneally with PTX-2 seco acid or 7-*epi*-PTX-2 seco acid at a dose of 5000 μg/kg showed no signs of discomfort, toxicity or behavioral change. The appearance and consistency of fecal pellets were entirely normal. In the first experiment, in which the possibility of acute tissue damage was explored, the animals were killed 24 h after injection. A slight decrease in body weight was recorded at this time in all treatment groups; there was no significant difference between

Table 3. Body-Weight Change and Relative Organ Weights[a] of Control Mice and of Mice Injected Intraperitoneally with PTX-2 Seco Acid or 7-*epi*-PTX-2 Seco Acid at a Dose of 5000 μg/kg. Experiment 2: Animals Killed 14 Days after Dosing

treatment group	body-weight change (g)[b]	relative organ weight (g/100 g of body weight)					
		liver	kidneys	spleen	heart	lungs	intestine[c]
control	1.60 ± 0.50	5.23 ± 0.36	1.49 ± 0.05	0.405 ± 0.023	0.603 ± 0.039	0.753 ± 0.037	8.61 ± 0.22
PTX-2 seco acid	1.50 ± 0.53	5.24 ± 0.27	1.49 ± 0.06	0.396 ± 0.021	0.525 ± 0.043	0.744 ± 0.029	8.32 ± 0.40
7-*epi*-PTX-2 seco acid	1.56 ± 0.39	5.26 ± 0.22	1.50 ± 0.03	0.392 ± 0.010	0.566 ± 0.039	0.691 ± 0.019	9.18 ± 0.18

[a] Results shown are the means ± standard errors of values for the five animals in each treatment group. [b] Body-weight change from the time of dosing to 14 days after dosing. [c] Whole intestine, from pylorus to anus.

control and test animals (**Table 2**). At necropsy, mild to moderate hydrometra was observed in one control animal and in one mouse dosed with PTX-2 seco acid. Hydrometra is commonly seen in laboratory rodents, and this observation is not considered to be of any toxicological significance. No significant differences were recorded among the treatment groups with regard to relative weights of liver, kidneys, lungs, spleen, heart, or intestine (**Table 2**), although the mean splenic weight of the control animals was higher than that of the mice dosed with the seco acids, reflecting a high value in one control animal. Histological examination of tissues revealed no abnormalities attributable to treatment. Increased levels of erythropoietic tissue were observed in the spleen of the control animal showing enlargement of this organ, which could reflect increased erythrocyte loss or inadequate bone marrow erythropoiesis. Small numbers of neutrophils, accompanied by scant fibrin, were visible adherent to the hepatic capsule in one animal receiving PTX-2 seco acid. The etiology of this lesion is unknown, but because it was recorded in only a single animal, it is not considered an effect of treatment.

In the second experiment, designed to detect delayed toxic effects of the test materials, the body weight gains and relative organ weights of the animals dosed with the seco acids were not significantly different from those of control mice (**Table 3**). At necropsy, hydrometra was observed in two control mice and in one animal from each of the groups receiving the seco acids. Other tissues from animals in all treatment groups were within normal histological limits.

The acute toxicities of both PTX-2 seco acid and 7-*epi*-PTX-2 seco acid are therefore low. A dose of 5000 μg/kg, which caused no perceptible toxicity in mice after intraperitoneal injection, equates to a dose of 300 mg for a 60-kg human. The average level of seco acids recorded in a survey of 23 samples of Norwegian shellfish was 1955 μg/kg, with a range of 8−15875 μg/kg (*10*). At the average level of contamination, an individual consuming a meal of 380 g of shellfish [an intake that would encompass the 97.5th percentile consumption figures for European, North American, Australian, and New Zealand consumers (*24*)] would receive only 0.7 mg of the seco acids, and an individual consuming only shellfish with the highest reported level of contamination would receive 6 mg. Because the toxicities of PTX-2 seco acids are likely to be even lower by oral administration than by injection, as was the case with PTX-2 (*13*), it is concluded that acute toxic effects in humans resulting from ingestion of shellfish contaminated with PTX-2 seco acid or 7-*epi*-PTX-2 seco acid are most unlikely.

ABBREVIATIONS USED

PTX-2, pectenotoxin-2; PTX, pectenotoxin.

Supporting Information Available: ^{1}H, ^{13}C, DEPT, COSY, TOCSY, HSQC, and HMBC NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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