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ARTICLE

Isolation, Structural Determination and Acute Toxicity of Pinnatoxins E, F and G

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Pinnatoxins and pteriatoxins are a group of cyclic imine toxins that have hitherto only been isolated from Japanese shellfish. As with other cyclic imine shellfish toxins, pinnatoxins cause rapid death in the mouse bioassay for lipophilic shellfish toxins, but there is no evidence directly linking these compounds to human illness. We have identified the known pinnatoxins A (**1**) and D (**6**), and the novel pinnatoxins E (**7**), F (**8**) and G (**5**), in a range of shellfish and environmental samples from Australia and New Zealand using LC−MS. After isolation from the digestive glands of Pacific oysters, the structures of the novel pinnatoxins were determined by mass spectrometry and NMR spectroscopy, and their LD_{50} values were evaluated by ip administration to mice. Examination of the toxin structures, together with analysis of environmental samples, suggests that pinnatoxins F and G are produced separately in different dinoflagellates. Furthermore, it appears probable that pinnatoxin F (**8**) is the progenitor of pinnatoxins D (**6**) and E (**7**), and that pinnatoxin G (**6**) is the progenitor of both pinnatoxins A−C (**1** and **2**) and pteriatoxins A−C (**3** and **4**), via metabolic and hydrolytic transformations in shellfish.

KEYWORDS: Pinnatoxin; pteriatoxin; cyclic imine; oyster; razor fish; shellfish toxin; toxicity; LD_{50}; *Crassostrea gigas*; *Pinna bicolor*

INTRODUCTION

In 1990, Chinese investigators reported the presence of a toxic compound of unknown structure in extracts of the bivalve mollusc *Pinna attenuata*, which they designated "pinnatoxin" (*1*). Subsequently, substances named pinnatoxins A−D were isolated from the viscera of *Pinna muricata* from Okinawa, Japan. The two-dimensional structure of pinnatoxin A (**1**) was reported in 1995 (*2*), and its relative stereochemistry was subsequently ascertained by NMR analysis (*3*). The absolute stereochemistry of **1** was established by total synthesis (*4*). Structures for pinnatoxins B and C (**2**) (*5*) and D (**6**) (*6*) were reported soon after. The related pteriatoxins A (**3**) and B and C (**4**) were isolated from the Okinawan bivalve *Pteria penguin* and were assumed to have the same absolute stereochemistry as the pinnatoxins (*7*). The absolute stereochemistry of **2**, **3** and **4** was confirmed by total synthesis (*8*, *9*). Pinnatoxins and pteriatoxins are part of a group of marine toxins known as cyclic imines, which share common macrocyclic features and a cyclic imine moiety. This group also includes gymnodimine, prorocentrolides, spiro-prorocentrimine and spirolides. They are fast acting toxins which exhibit a rapid, highly potent toxic response in mice after intraperitoneal (ip) injection. The presence of pteriatoxins and pinnatoxins in extracts from the digestive glands of both *Pinna* and *Pteria* species led to the suggestion that these compounds were produced by common symbionts (*7*). However, many other cyclic imines are produced by dinoflagellates; prorocentrolides and spiro-prorocentrimine by *Prorocentrum* spp.(*10*, *11*), spirolides by *Alexandrium ostenfeldii* (*12*) and gymnodimine by *Karenia seliformis* (syn: *Gymnodinium* cf. *mikimotoi*) (*13*).

In the summer of 2007, Pacific oysters (*Crassostrea gigas*) were collected from South Australia for routine biotoxin monitoring. The mouse bioassay developed for monitoring lipophilic toxins, as described by Hannah et al. (*14*), indicated the presence of a fast-acting toxin or toxins. LC−MS analysis of extracts of the toxic oysters revealed the presence of compounds with MS characteristics consistent with novel spiroimine toxins such as spirolides or pinnatoxins.

In this paper we report the isolation of pinnatoxins A (**1**) and three novel pinnatoxins, E (**7**), F (**8**) and G (**5**) from the digestive glands of Pacific oysters and their structural elucidation by NMR spectroscopy and mass spectrometry. Pinnatoxin D was also identified in the extracts by a combination of LC−MS analysis and chemical interconversion. Pacific oysters, razor fish (*Pinna bicolor*), water and sediment samples collected from the affected area were analyzed by LC−MS in attempt to find the biogenic source of these pinnatoxins and understand their metabolism. We also report the presence of **7** and **8** in archival samples of Pacific oysters collected from New Zealand waters in 1995.

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MATERIALS AND METHODS

General. Methanol was from Burdick & Jackson (MI). Pro analysi grade ethanol, ethyl acetate and Lichrosolv-grade acetone, acetonitrile and hexane, and Suprapur-grade formic acid were from Merck (Darmstadt, Germany). Hypersolv-grade trifluoroacetic acid and AnalaR grade NaH_2PO_4 were products of BDH, (Poole, U.K.). The 13-desmethylspirolide C standard was a Certified Reference Material from The National Research Council of Canada, Institute of Marine Biosciences (Halifax, NS, Canada). Deionized water was produced with a Milli-Q system (Millipore, Nepean, ON, Canada). Evaporation of samples was performed by rotary evaporation under reduced pressure.

Samples. Digestive glands (32 kg) were excised from 24,000 Pacific oysters (*Crassostrea gigas*) collected from Franklin Harbour, South Australia, in January 2008. This material was homogenized, frozen and lyophilized. The dry material was ground to a powder (6.8 kg) and stored at −20 °C. Additional samples collected from Franklin Harbour in February and March 2008 included Pacific oysters, razor fish, sediment and water. Water samples were collected with an integrated pipe sampler 2.5 cm in diameter and 4 m in length, the sample was placed in a bucket, swirled and transferred to 500 mL PET bottles. The sediment samples were scooped from sand bars at low tide with a spatula. Pacific oysters from Rangaunu Harbour, New Zealand, collected in February 1995, were stored at −20 °C.

Sample Preparation for LC−MS Analysis. For comparative analyses, Pacific oyster and razor fish flesh was finely blended with a hand-held homogenizer. All solid samples including sediments were weighed (2 g) into 50 mL test tubes. Shellfish samples were homogenized with 18 mL of methanol−water (9:1 v/v) using an Ultra-Turrax (IKA, Guangzhou, China) at 19,000 rpm for 1 min followed by centrifugation at 3000*g* for 10 min (*15*). Sediment samples were extracted with 18 mL of methanol−water (9:1 v/v) by mixing on a vortex stirrer for 1 min followed by centrifugation at 3000*g* for 10 min. Water samples were filtered through 45 and 20 μm plankton nets, yielding size fractions >45 μm, 20−45 μm and <20 μm. Fifteen milliliters of each fraction was centrifuged at 3000*g* for 5 min. The resulting pellet was extracted with 1 mL of methanol by ultrasonication at 50 kHz for 5 min and then centrifuged at 3000*g* for 10 min.

Chemical Conversions. Reductions: $NaBH_4$ (20 μL, 1 M in H_2O) was added to methanolic solutions of **7** (180 μL, 5 μg/mL) and **6** (180 μL, 4 μg/mL). $NaBH_3CN$ (20 μL, 1 M in H_2O) was added to a solutions of **7** (180 μL, 5 μg/mL) and **6** (180 μL, 4 μg/mL) in methanol containing 0.1% formic acid. Base hydrolysis: 125 μL of 2.5 M NaOH was added to 1 mL of shellfish extract and heated at 76 °C for 40 min, cooled and neutralized with 125 μL of 2.5 M acetic acid, based on the procedure of Mountfort et al. (*16*). Reactions were monitored by LC−MS (method 1).

Solid Phase Extraction (SPE). Strata-X polymeric resin (Phenomenex, Torrance, CA) was conditioned before sample application with methanol (20 mL/g) followed by loading solvent (20 mL/g).

Semipreparative HPLC. A Shimadzu 10A HPLC system with diode array detection (Shimadzu, Kyoto, Japan) scanned from 200 to 300 nm was used for purification. The column was a Luna C18(2) 10 μm 250 × 10 mm (Phenomenex, Torrance, CA). The column was eluted at a flow rate of 4 mL/min.

LC−MS Analysis. *Method 1.* A Waters Acquity ultraperformance liquid chromatography system (Waters, Milford, MA) was coupled to a Waters−Micromass Quattro Premier triple quadrupole mass spectrometer (Manchester, U.K.). Separation was achieved with a Waters Acquity C18 BEH 1.7 μm 50 × 1 mm column at 30 °C, eluted at 0.3 mL/min with linear gradients of water (A) and acetonitrile (B), each containing 0.1% formic acid (v/v). The gradient consisted of 0 to 50% B over 1 min, increasing to 100% B at 1.5 min and held for 0.5 min, before returning to initial conditions. The electrospray ionization source was operated in positive-ion mode at 100 °C, capillary 2 kV, cone 50 V, nitrogen gas desolvation 900 L h^{-1} (350 °C), cone gas 50 L h^{-1}, with a scan range m/z 400−1000. The collision energy was 45 eV with 3 mbar of argon for all MS/MS experiments. Daughter ion experiments for fragments of $[M+H]^+$ used a scan range m/z 50−800. MRM transitions were m/z 692 → 164, 694 → 164, 712 → 164, 766 → 164, 784 → 164 and 798 → 164.

Method 2. Samples were analyzed using a Waters 2695 HPLC system connected to a Water−Micromass Quattro Ultima triple-quadrupole mass spectrometer with Z-spray electrospray ionization. The column was a Phenomenex Luna C18(2) 5 μm 150 × 2 mm at 30 °C, and the injection volume was 10 μL. Mobile phase components were (A) acetonitrile−water (1:9 v/v); (B) acetonitrile−water (9:1 v/v); and (C) water containing 500 mM formic acid and 33 mM ammonia. Linear gradients were run at 0.2 mL/min from 90% A plus 10% C, to 60% A plus 30% B plus 10% C over 10 min (held for 5 min), then to 90% B plus 10% C over 1 min (held for 9 min), before returning to initial conditions. The electrospray source was operated at 80 °C with nitrogen gas for nebulization, desolvation 500 L/h (350 °C), cone gas 50 L/h, capillary 2.5 kV, cone 100 V, electrospray ionization positive ion mode (ESI+) with a scan range m/z 400−1000. The collision energy was 45 eV with 3 mbar of argon for all MS/MS experiments. Parent ion scans for the fragment ion m/z 164 used a scan range m/z 400−1200. Daughter ion experiments for fragments of $[M+H]^+$ used a scan range m/z 50−800. MRM transitions were m/z 694 → 164, 712 → 164, 766 → 164, 782 → 164, and 784 → 164.

HR-MS. High-resolution mass spectrometry (HR-MS) was performed in positive ion mode for pinnatoxins F and G, and in both positive and negative ion modes for pinnatoxin E, on a Bruker Daltonics MicrOTOF spectrometer (Billerica, MA). Cluster ions from sodium formate (2 mM) were used for mass calibration. Mass spectra were acquired with a time-of-flight analyzer from m/z 500 to 1500. For analysis, pinnatoxins A, E and G were dissolved in methanol, and pinnatoxin F was dissolved in acetonitrile.

NMR. NMR spectra (^{1}H, COSY, TOCSY, g-HSQC, g-HMBC, ^{13}C and DEPT135, and, in the case of **5**, a NOESY spectrum) were obtained from solutions of **5** and **7** in CD_3OD (99.8+ atom % D; Aldrich), and of **8** in $(CD_3)_2CDOD$ (99.5 atom % D; Aldrich), using a Bruker Avance DRX 400 MHz spectrometer fitted with a 5 mm, gradient shielded, inverse probe at 30 °C. NMR assignments of pinnatoxin A (**1**) (ca. 100 μg) (Supporting Information) were obtained from examination of ^{1}H, COSY, TOCSY, g-HSQC, and g-HMBC NMR spectra of **1** in CD_3OD (99.8 atom % D) (Aldrich) acquired on a Bruker Avance AV 600 MHz NMR spectrometer with a 1.7 mm inverse probe, equipped with Z-gradient coils, at 30 °C. NMR spectra were calibrated relative to internal CHD_2OD (3.31 ppm) and CD_3OD (49.0 ppm), or to internal $(CD_3)_2CHOD$ (3.89 ppm) and $(CD_3)_2CDOD$ (24.2 ppm), as appropriate, and NMR data was processed using TopSpin V1.3 (Bruker BioSpin 2005).

Extraction and Fractionation. The following extraction and fractionation was guided by LC−MS analysis (method 1). The lyophilized oyster powder was extracted in twelve batches. 600 g of powder was extracted with 1.2 L of methanol by stirring and leaving to soak for 30 min, then stirring again and filtering though a 24 cm Whatman #1 filter paper. After flushing with 0.4 L of methanol, the solid was re-extracted with 1 L of methanol, filtered through Whatman #1 filter paper, and then flushed with 0.4 L of methanol. The resulting dark-colored methanolic extract was stored at −20 °C overnight. Precipitated material was removed by filtering through a 12.5 cm Whatman #542 filter paper, after which the methanol was removed by rotary evaporation at 40 °C to afford an oily residue. This was dissolved in 450 mL of ethanol−water (1:1) and defatted twice with hexane (400 and 200 mL). NaCl (20 g) was added to the ethanol−water layer, the pinnatoxins were extracted with 500 mL of ethyl acetate, and the ethyl acetate was removed by rotary evaporation to give a dark oily residue. The crude extract was dissolved in methanol−water (1:2), loaded onto a flash column containing 10 g of Strata-X polymeric resin, and eluted with 50 mL of 40% methanol followed by 200 mL of methanol. The pinnatoxins were found in the methanol fraction. The methanol fractions from the twelve batches were combined and the methanol removed by rotary evaporation at 40 °C to afford a dark brown viscous residue (32 g). The residue was dissolved in 100 mL of dichloromethane, applied (with several rinses of dichloromethane, totaling ca. 60 mL) to a 70 g Si-1 column (Phenomenex, Torrance, CA) that had been conditioned with dichloromethane, and eluted with a stepwise gradient (500 mL per step) of acetone (0, 15, 30, 50, 70%) in dichloromethane, followed by methanol (20% and 30%) in dichloromethane, and 100 mL fractions were collected. The 30% acetone to 20% methanol contained the bulk of pinnatoxins E, F and G, and these fractions were combined and evaporated to dryness. This residue (4.8 g) was dissolved in acetone−dichloromethane (3:7 v/v) and loaded onto a 70 g Si-1 column that had been preconditioned with dichloromethane, and was eluted sequentially with acetone−dichloromethane (3:7 v/v, 800 mL) and then with methanol (4, 6, 8, 10, 12.5, 15, 20, and 30% v/v) in dichloromethane (500 mL of each), and 40 100 mL fractions were collected.

The majority of **7** was in fractions 31−34, which also contained a small amount of **1** (ca. 20% of the total pinnatoxin A; the remaining 80% was in fractions 27−30). The majority of **8** was in fractions 15−18, and the majority of **5** was in fractions 12−13.

Purification of Pinnatoxins A (1) and E (7). Fractions 31−34, containing **7** and **1**, were combined and evaporated. The residue (370 mg) was dissolved in acetonitrile−water (1:9 v/v) and applied to a 1 g Strata-X SPE column. The column was eluted sequentially with 20 mL of 12.5%, 15%, 17.5% and 20% acetonitrile in water. Fractions 3 and 4, which contained **7**, were combined and evaporated. Final purification of the residue (4.9 mg) was achieved by semipreparative HPLC using a mobile phase of 30% acetonitrile in water containing 0.1% trifluoroacetic acid, with a photodiode array detector monitoring at 205 and 254 nm. **7** eluted at 19 min and **1** at 16.3 min, while a third unidentified peak (*m*/*z* 726) eluted at 17.7 min. Fractions containing **7** were combined (80 mL) and analyzed by LC−MS, which showed that approximately 10% of the **7** had ring-closed to form **8**. Triethylamine (800 μL) was added to the pooled fractions to convert the unwanted **8** into **7** by hydrolysis of the lactone ring, which was found by LC−MS to be complete within 5 min. The fractions containing **7** were then diluted with water (120 mL) and loaded onto a 1 g Strata-X SPE column. The column was eluted with 20 mL of 15% acetonitrile and 3 × 10 mL of 25% acetonitrile, and 95% of the **7** was found in the first 10 mL 25% acetonitrile fraction. This fraction was diluted by addition of 10 mL of water, loaded onto a 1 g Strata-X SPE column and eluted with 12 mL of methanol (this second column removed excess water, which made solvent removal easier). The methanol was evaporated under a stream of nitrogen to yield **7** (1.9 mg) as a colorless solid. Preparative HPLC fractions containing **1** were combined (30 mL), diluted with water (50 mL), and loaded onto a 200 mg Strata-X SPE column (preconditioned with 6 mL of methanol, followed by 6 mL of 10% acetonitrile), and the column was eluted with 5 mL of 10% acetonitrile and 2 × 5 mL of methanol. Over 95% of the **1** was found in the first methanol fraction, which was evaporated under a stream of nitrogen to give ca. 100 μg of **1** (estimated by LC−MS). Fractions containing the unidentified pinnatoxin of *m*/*z* 726 were set aside for further purification and structure elucidation.

Purification of Pinnatoxin F (8). Fractions containing **8** were combined and the solvent removed by rotary evaporation. The residue (920 mg) was dissolved in *tert*-butyl methyl ether (50 mL) and partitioned against 25% methanol in water containing 0.1% trifluoroacetic acid (2 × 50 mL). The 25% methanol fractions contained **8**, and were combined and evaporated. The residue was dissolved in 12.5% methanol in water and loaded onto a 1 g Strata-X SPE column, and the column was eluted sequentially with 20, 30, 40, 50, 55, 60, 70 and 100% methanol (all containing 0.1% trifluoroacetic acid) (2 × 10 mL of each). The 50−70% methanol fractions containing **8** were combined and evaporated. The residue was dissolved in 12.5% methanol and 0.1% TFA in water, loaded onto a 1 g C18 SPE cartridge (Phenomenex, Torrance, CA) and eluted sequentially with 20, 35, 45, 50, 60, and 100% methanol containing 0.1% trifluoroacetic acid (2 × 10 mL of each). The 45 and 50% methanol fractions containing **8** were combined and evaporated. The residue (21 mg) was further purified by semipreparative HPLC using a mobile phase of 32.5% acetonitrile and 0.1% TFA in water, and monitored by UV absorption at 205 nm. Fractions containing **8** were combined (225 mL), diluted with water (375 mL), and loaded onto a 1 g Strata-X SPE column, and the column was eluted with 20% acetonitrile (4 × 10 mL), 25% acetonitrile (4 × 10 mL), 30% acetonitrile (3 × 10 mL), 40% acetonitrile (3 × 10 mL), 50% acetonitrile (4 × 10 mL) and methanol (10 mL). This fractionation did not work adequately, with **8** spread across many fractions (25−50%). The Strata-X SPE fractionation was therefore repeated on these combined fractions, but with NaH_2PO_4 (10 mM) in the eluents and inclusion of a 35% acetonitrile (4 × 10 mL) elution step. Fractions containing pinnatoxin F (30 and 35% fractions) were combined, diluted with an equal volume of water, and loaded onto a 200 mg Strata-X SPE column to remove salts and water. The column was eluted with 15% acetonitrile (5 mL) and then methanol (5 mL). The methanol fraction contained **8**, which was evaporated to dryness under a stream of nitrogen at 40 °C. During the SPE recovery, about 4% of **8** underwent methanolysis to give **10**. This contaminant was removed on a 500 mg Si-1 SPE column (Phenomenex, Torrance, CA) using a stepwise gradient consisting of 10, 20, 30, 40 and 50% acetone in dichloromethane (5 mL each). The 20 and 30% acetone fractions containing pure **8** were combined and evaporated under nitrogen to give colorless solid (2.5 mg).

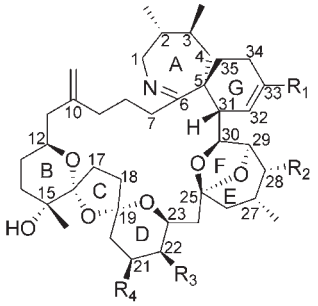
Purification of Pinnatoxin G (5). Fractions containing **5** were combined and evaporated. The residue (510 mg) was dissolved in methanol (5 mL), and HCl (3 mM, 45 mL) was added. This solution was extracted with *tert*-butyl methyl ether (100 mL). The aqueous layer, which contained **5**, was evaporated. The residue was dissolved in 15% methanol in water (20 mL) and loaded onto a 1 g Strata-X SPE column. The column was eluted using a stepwise gradient of acetonitrile (20%, 30%, 40%, and 50%) in aqueous 10 mM NaH_2PO_4 (20 mL each). The 40% acetonitrile fraction, which contained > 95% of **5**, was diluted with water (40 mL), and applied to a 500 mg Strata C18 column. The column was eluted with a stepwise gradient of acetonitrile (20%, 25%, 30%, 35%, 40%, 50%, and 60%) in aqueous NaH_2PO_4 (10 mM) (5 mL each). Fractions containing **5** (40−60% acetonitrile) were combined and diluted with an equal volume of water, then loaded onto a 200 mg Strata-X SPE column. The column was eluted with water (6 mL), 15% acetonitrile (6 mL), and then methanol (2 × 5 mL). The first methanol fraction, which contained the bulk of the **5**, was concentrated by nitrogen blowdown. The residue (2.1 mg) was purified by semipreparative HPLC with 40% acetonitrile in aqueous NaH_2PO_4 (10 mM), and monitored by UV absorbance at 230 nm. Fractions containing **5** (175 mL) were diluted with water (125 mL) and desalted by application to a 1 g Strata-X SPE column, which was then eluted with water (20 mL), 20% acetonitrile (20 mL) and methanol (2 × 10 mL). The first methanol fraction was evaporated under nitrogen to yield **5** (1.1 mg) as a colorless solid.

LD_{50} Determinations. Female Swiss albino mice, of initial body weight 18−22 g, were used in all experiments. The animals were allowed free access to food (Laboratory Chow, Sharpes Animal Feeds, Carterton, New Zealand) and tap water throughout the experimental period. Acute toxicity was determined according to the principles of OECD Guideline 425 (*17*), and LD_{50} values and 95% confidence limits were calculated using the AOT 425 Statistical Program (*18*). Pinnatoxins were dissolved in ethanol, and aliquots were diluted with 1% Tween-60 in saline. The diluted solution (1 mL), containing 5% v/v ethanol, was injected intraperitoneally. The mice were observed intensively throughout the day of dosing and then at daily intervals for a total of 14 days. Mice that survived to the end of the 14-day observation period were necropsied, and the weights of liver, kidneys, spleen, heart, lungs and gastrointestinal tract were recorded. All animal manipulations were performed under the authority of the AgResearch Ruakura Animal Ethics Committee (AEC Approval Number 11412), in accordance with the New Zealand Animal Protection (Code of Ethical Conduct) Regulations 1987, and the New Zealand Animals Protection Act (1960).

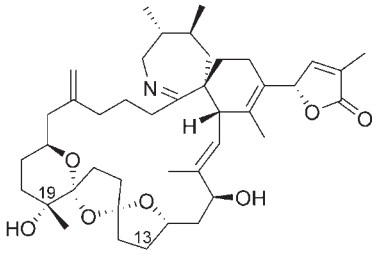
RESULTS AND DISCUSSION

An investigation was launched after Pacific oysters from Franklin Harbour, South Australia, gave a toxic response in the mouse bioassay for lipophilic biotoxins. The rapid death times in the mouse bioassay, which are characteristic of cyclic imines (*19*), suggested that these compounds might be the source of the unexplained toxicity. LC−MS analysis (method 2) found low levels of 13-desmethylspirolide C (< 5 μg/kg) in oyster flesh, but, with an LD_{50} of 6.9 μg/kg (*20*), such levels of this substance could not account for the observed toxicity. The possible presence of other cyclic imines was also investigated by LC−MS (method 2) in samples positive in the (*6*) mouse bioassay and in samples giving a negative response in this assay. Two scanning techniques were employed: an ESI+ scan, and a parent ion scan of *m*/*z* 164 (a distinctive iminium fragment ion (*21*, *22*), common to spirolides, pinnatoxins and pteriatoxins). These scans revealed the presence of three prominent peaks in the positive sample that were absent in the negative sample. Further collision-induced dissociation experiments provided putative structures for these compounds. LC−MS/MS, HR-MS and NMR spectroscopy after purification confirmed their structures as novel pinnatoxins **5**, **7** and **8**. During the purification process, other analogues were also identified including the known pinnatoxins **1** and **6**.

It should be noted that a minor modification (**Figure 1**) to the standard atom-numbering system, used previously for pinnatoxins and pteriatoxins, has been adopted in the present report.



Pinnatoxins and Pteriatoxins



13-Desmethylspirolide C

	R_1	R_2	R_3	R_4		m/z $[M+H]^+$
1	$-^{36}CO_2H$	OH	H	H	Pinnatoxin A	712
2	$^{37}CO_2H$, $^{36}NH_2$	OH	H	H	Pinnatoxins B (36*S*), C (36*R*)	741
3	^{37}S, NH_2, ^{36}OH, CO_2H	OH	H	H	Pteriatoxin A	831
4	S, ^{36}, CO_2H, $^{37}NH_2$, HO	OH	H	H	Pteriatoxins B (36*R*), C (36*S*)	831
5	^{37}, ^{36}	OH	H	H	Pinnatoxin G	694
6	^{37} ^{38}, ^{36} $^{39}CO_2H$, O	H	OH	CH_3	Pinnatoxin D	782
7	^{37} ^{38}, ^{36} $^{39}CO_2H$, OH	H	OH	CH_3	Pinnatoxin E	784
8	^{37} ^{38}, ^{36}, ^{39}, O, O	H	OH	CH_3	Pinnatoxin F	766
9	^{37} ^{38}, ^{36} $^{39}CO_2CH_3$, OH	H	OH	CH_3	**7**-methyl ester	798

Figure 1. Pinnatoxin and pteriatoxin structures, with 13-desmethylspirolide C shown for comparison. Pinnatoxins B and C (**2**), and pteriatoxins B and C (**4**), are pairs of diastereoisomers at C-36. Note that a nonstandard atom-numbering has been used here to facilitate comparison of NMR chemical shifts for all pinnatoxin/pteriatoxin analogues, and that this numbering system differs also from that used for spirolides. Analogues **7**−**9** and 13-desmethylspirolide C are depicted with arbitrary stereochemistry at C-36 based on that established for the butyrolactone moiety of gymnodimine by X-ray crystallography (*23*).

Table 1. Composition (%) of Pinnatoxin Analogues in Samples Collected from Franklin Harbour

	1	**5**	**6**	**7**	**8**
sediment	1	46	0	24	26
20−45 μm	0	59	0	6	29
C. gigas	2	31	0.4	29	32
P. bicolor	27	8	48	9	5

This system allows direct comparison of NMR chemical shifts for atoms 1−36 and their substituents in all the known pinnatoxins and pteriatoxins (Table **2**). Although C-36 of **7**−**9** is arbitrarily depicted with the same stereochemistry as that established for the lactone ring of gymnodimine by X-ray crystallography (*23*), the stereochemistry of this atom could not be experimentally determined by NMR for **7** or **8**. It should be noted, however, that the opposite stereochemistry has recently been proposed for the equivalent atom in the related 13,19-didesmethylspirolide C, based on NOE and molecular modeling studies (*24*).

Pinnatoxin G (5). 5 displayed UV absorbance with λ_{max} 227 nm during HPLC purification, consistent with the presence of a 1,3-diene. HR-MS $[M+H]^+$ m/z 694.4683 was consistent with a molecular formula of $C_{42}H_{63}NO_7$ (calcd m/z 694.4683 for $C_{42}H_{64}NO_7$). The ESI-MS/MS spectrum was almost identical to that of **1** (**Figure 2**), suggesting the compound may be an analogue of **1**−**4** differing only in the side chain at C-33. If so, the molecular formula requires that the side chain be an ethenyl group, which would create a conjugated diene and thus be consistent with the observed UV absorbance spectrum.

The DEPT135, ^{13}C and g-HSQC NMR spectra of **5** showed the presence of 4 methyl, 18 methylene, 12 methine and 7 quaternary carbon atoms. In the g-HMBC NMR spectrum, an additional carbon signal was observed, at 179.5 ppm (C-6), which showed strong correlations to signals at 3.88, 3.53, and 1.46 ppm (H-1, H-1, and H-4) and a weak correlation to 3.13 ppm (H-7). The methine signal at 3.13 (H-7) showed COSY correlations to H-8 (2.16, 1.45 ppm), TOCSY correlations to H-8 (2.16, 1.45 ppm), H-9 (2.39, 1.66 ppm), and 10-CH_2 (4.75, 4.77 ppm), and NOESY

Table 2. NMR Assignments for Pinnatoxins E (**7**), F (**8**) and G (**5**), together with Published Assignments for Pinnatoxins A (**1**) (*2*) and D (**6**) (*6*)

	pinnatoxin A (**1**)		pinnatoxin G (**5**)		pinnatoxin D (**6**)		pinnatoxin E (**7**)		pinnatoxin F (**8**)	
atom	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	52.1	4.27, 3.62	53.6	3.88, 3.53	52.0	3.62, 4.22	52.6	3.56, 4.10	53.0	3.79, 3.61
2	39.6	1.70	42.2	1.43	39.5	1.68	40.7	1.57	41.5	1.39
2-Me	19.9	1.23	20.9	1.22	20.0	1.19	20.3	1.18	20.4	1.16
3	35.3	1.40	35.1	1.37	35.7	1.34	35.51	1.30	34.6	1.28
3-Me	21.0	1.07	21.5	0.96	21.4	1.08	21.7	1.03	21.7	0.95
4	36.0	2.05, 1.63	37.8	1.79, 1.46	36.3	2.06, 1.62	36.5	1.93, 1.66	37.1	1.76, 1.44
5	51.8		51.5		52.1		51.9		50.0	
6	202.5		179.5[c]		203.3		nd[a]		175.6	
7	36.0	3.57	34.5[c]	3.13	35.3	3.68, 3.68	35.1[b]	3.53	33.5[a,b]	3.13
8	21.8	2.09, 1.96	22.7	2.16, 1.45	21.6	2.14, 1.96	21.8	2.15, 1.74	21.8	1.39, 2.29
9	34.2	2.06, 1.95	34.5	2.39, 1.66	33.6	1.98, 2.08	33.8	1.82, 2.17	33.7	1.58, 2.37
10	145.4		147.9		145.3		146.5		147.1	
10-CH_2	112.6	4.92, 4.85	110.2	4.75, 4.77	112.6	4.85, 4.95	111.3	4.81, 4.86	109.6	4.71
11	46.5	2.36, 2.18	47.3	2.33, 2.11	46.5	2.18, 2.38	46.9	2.15, 2.37	47.0	2.07, 2.32
12	69.6	4.09	69.7	4.14	69.6	4.05	69.5	4.06	68.8	4.01
13	29.8	1.68, 1.31	29.8	1.64, 1.24	29.8	1.32, 1.68	29.8	1.27,1.64	29.3	1.17, 1.60
14	35.5	1.89, 1.53	35.6	1.95, 1.48	35.6	1.52, 1.88	35.54	1.50, 1.90	35.3	1.48, 1.84
15	71.2		71.3		71.6		71.1		70.6	
15-Me	22.9	1.23	22.8	1.21	23.1	1.25	22.9	1.22	22.6	1.21
16	113.4		113.6		113.7		113.7		112.9	
17	31.5	2.20, 1.78	31.5	2.20, 1.78	32.6	2.22, 1.80	31.4	2.20, 1.78	30.9	1.78, 2.18
18	39.0	2.04, 1.85	39.1	2.07, 1.83	38.8	2.14, 1.88	38.7	2.15, 1.85	38.2	1.79, 2.17
19	109.8		109.9		110.1		110.1		109.1	
20	35.6	1.87, 1.53	35.9	1.87, 1.53	37.6	1.67, 1.67	37.7	1.66, 1.66	37.4	1.58, 1.69
21	21.4	1.83, 1.66	21.6	1.89, 1.62	32.4	2.08	32.1	2.09	31.0	2.09
21-Me					18.4	0.98	18.3	0.97	18.2	0.99
22	32.5	1.68, 1.26	32.2	1.75, 1.21	69.9	3.04	69.4	3.44	68.4	3.41
23	70.8	4.04	71.0	4.15	73.2	4.09	73.1	4.09	72.1	4.11
24	45.1	1.99, 1.90	44.8	2.01, 1.90	40.5	1.95, 2.24	40.1	1.92, 2.21	39.3	1.94, 2.19
25	110.4		109.3		110.6		109.6		108.4	
26	41.7	1.72, 1.62	42.5	1.65, 1.59	45.0	1.94, 1.44	45.4	1.87, 1.41	45.1	1.37, 1.85
27	30.9	2.20	30.8	2.33	26.0	2.25	26.0	2.29	25.2	2.33
27-Me	16.9	1.03	16.9	1.01	22.7	1.01	22.7	0.99	22.6	0.98
28	67.0	3.77	67.5	3.73	33.6	2.08, 1.64	33.8	2.04, 1.55	33.5	1.95, 1.53
29	81.6	4.58	81.9	4.47	77.1	4.80	77.4	4.62	76.7	4.65
30	79.1	3.92	80.4	3.89	80.1	3.98	81.1	3.86	80.4	3.83
31	44.7	3.66	45.0	3.43	45.6	3.78	44.7	3.52	44.1	3.40
32	134.0	6.42	127.9	5.25	136.0	6.42	121.0	5.21	123.2	5.28
33	135.8		140.2		141.4		144.3		139.2	
34	23.0	2.55, 2.55	22.1	2.29, 2.29	33.2	2.46, 2.56	21.8	2.26, 2.26	20.9	2.04, 2.25
35	33.7	2.04, 1.91	33.6	1.89, 1.76	33.8	1.92, 2.08	34.1	1.82, 1.92	32.8	1.72, 1.85
36	170.2		140.0	6.37	200.0		75.7	4.02	83.3	4.97
37			112.6	5.17, 5.01	33.2	2.94, 3.00	32.1	1.84	27.3	2.39, 2.05
38					22.7	2.62, 2.62	33.7	2.25	29.2	2.57, 2.57
39					176.8		180.3[c]		178.1	

[a] Not detected in ^{13}C or g-HMBC spectra. [b] Br m −*C*HD− due to deuterium exchange. [c] Detected only in g-HMBC, value is ±0.5 ppm.

correlations to 3.43 (H-31), 2.33 (H-27), 1.66 (H-9), and 1.45 (H-8) ppm. These correlations define this methine resonance at 3.13 ppm, which integrated for 1 proton, as H-7, and shows that one of the H-7 protons had completely exchanged with deuterium from the solvent. Analysis of NOESY correlations involving H-7 (Supporting Information) were consistent with retention of the 7-*pro*-*R* proton, in accord with molecular modeling data (Supporting Information) suggesting that the 7-*pro*-*S* methylene proton would be more readily exchanged due to steric hindrance. Isotopic exchange of H-7 in CD_3OD for pinnatoxin A (*2*), and of the equivalent protons in gymnodimine (*13*) and spiro-prorocentrimine (*11*), attributable to imine−enamine tautomerism has been reported; furthermore, stereospecific exchange of one of the H-7 protons in CD_3OD has been described in the related spirolides B and D (*25*, *26*) and attributed to steric factors (*26*). Analysis of correlations observed in the COSY, TOCSY, g-HSQC, g-HMBC and NOESY NMR spectra of **5** led to the ^{1}H and ^{13}C chemical shift assignments presented in **Table 2**, which matched closely the published assignments for C-1−C-35 of **1**−**4** (*2*, *5*−*7*). Correlations observed in the NOESY NMR spectrum, as well as chemical shifts and multiplicities of protons observed in the ^{1}H and proton-correlated NMR spectra, were consistent with **5** having the same stereochemistry as that previously established for C-1−C-35 of **1**−**4** (*2*, *5*−*7*). A notable feature of the NMR spectra of **5** was the presence of olefinic resonances attributable to an ethenyl group (C-36 and C37). H-36 (6.37 ppm) showed g-HMBC correlations to C-32, -33, and -34 (127.9, 140.2, and 22.1 ppm, respectively), and NOESY correlations to H-32 (5.25 ppm) and H-37$_E$ (5.01 ppm), while the COSY spectrum of **5** included mutual correlations between H-36 (6.37 ppm) and H-37 (5.01 and 5.17 ppm). The resonance at 5.17 ppm (H-37$_Z$) showed NOESY correlations to 2.29 (H-34) and 5.01 ppm (H-37$_E$), and H-36 (6.37 ppm) showed NOESY correlations to 5.25 (H-32) and 5.01 (H-37$_E$) ppm. These correlations define the attachment of the ethenyl group as shown

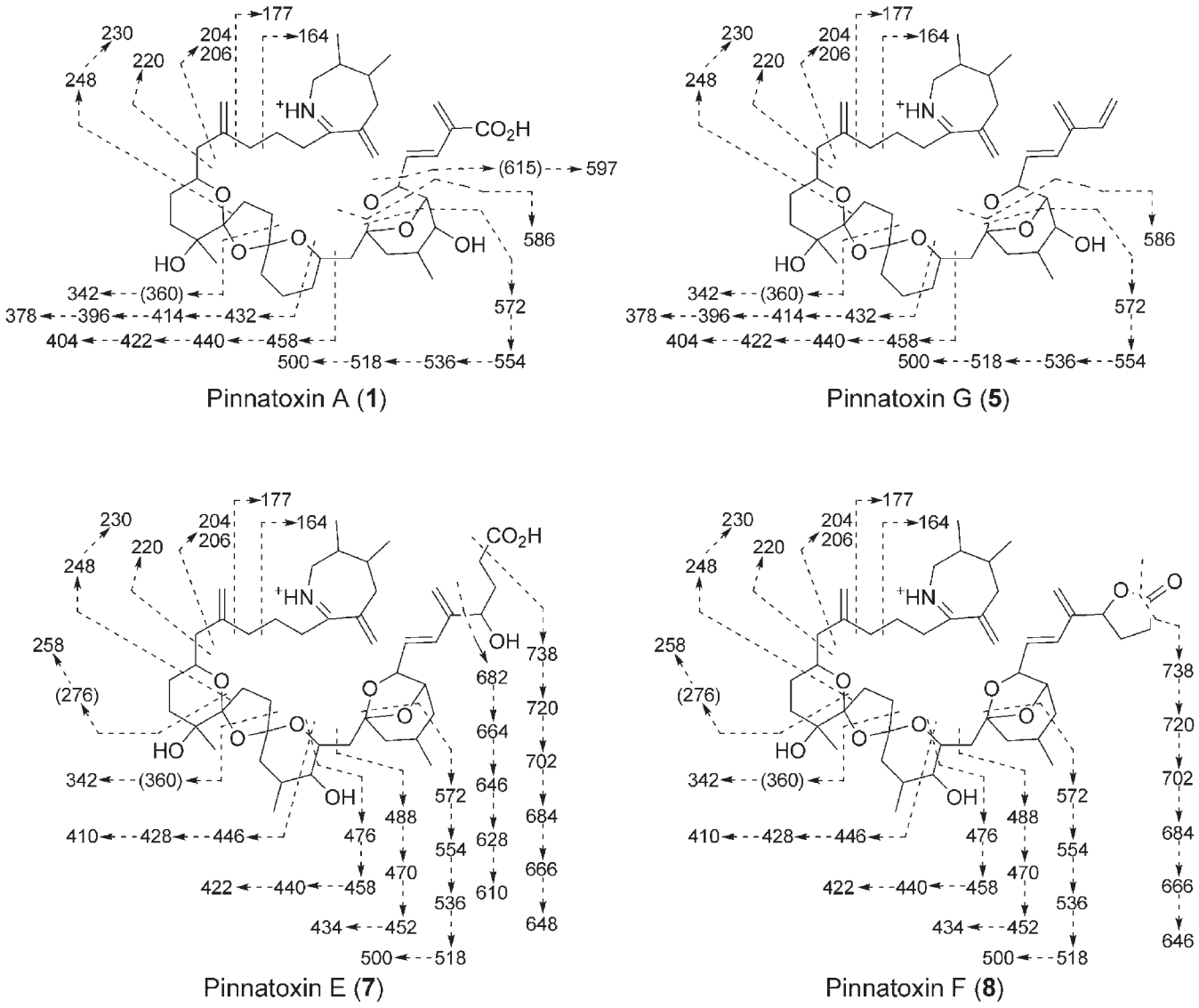


Figure 2. Proposed fragmentation after retro Diels−Alder reaction of **1** $[M + H]^+$, *m/z* 712; **5** $[M + H]^+$, *m/z* 694; **7** $[M + H]^+$, *m/z* 784; and **8** $[M + H]^+$, *m/z* 766.

in **Figure 1**, consistent with the observed UV spectrum, MS fragmentation and HR-MS data, and establish the structure of pinnatoxin G as **5**.

Pinnatoxin E (7). **7** did not display significant UV absorbance during HPLC purification, but displayed HR-MS $[M + H]^+$ *m/z* 784.5008 (calcd for $C_{45}H_{70}NO_{10}$, 784.5000), $[M + Na]^+$ *m/z* 806.4833 (calcd *m/z* 806.4819 for $C_{45}H_{69}NO_{10}Na$), and $[M - H]^-$ *m/z* 782.4815 (calcd *m/z* 782.4843 for $C_{45}H_{68}NO_{10}$) which suggested a compound with a composition of $C_{45}H_{69}NO_{10}$ containing a carboxylic acid.

As with **1** and **5**, the MS/MS spectrum of **7** (**Figure 2**) showed fragments at *m/z* 164, 177, 204, 220, 248, 342, and 572. However, cleavage at C-23−C-24 gave a fragment at *m/z* 488, 30 Da higher than observed in **5**, consistent with the presence of methyl and hydroxyl groups on ring D as in **6**. The MS/MS fragmentation suggested that **7** differed from **6** only in its side chain. Consideration of the molecular formula, and the probable presence of a carboxylic acid in **7**, suggested that **7** could be a C-36-reduced analogue of **6**.

The DEPT135, ^{13}C and g-HSQC NMR spectra of **7** showed the presence of 5 methyl, 18 methylene, 13 methine and 8 quaternary carbon atoms. No signal attributable to C-6 was observed in the ^{13}C or g-HMBC NMR spectra of **7**. There are several reports that this signal can be broadened and difficult to observe in NMR spectra, particularly in CD_3OD, due to deuterium exchange at C-7 and enamine−imine tautomerism (as discussed for **5**, above). The methine signal at 3.53 ppm (H-7) showed COSY correlations to H-8 (2.15, 1.74 ppm), TOCSY correlations to H-8 (2.15, 1.74 ppm), H-9 (2.17, 1.82 ppm), and 10-CH_2 (4.81, 4.86 ppm). These correlations define this methine resonance as H-7, which was part of a multiplet (3.50−3.59) that also included the H-31 and one of the H-1 methine resonances. This multiplet integrated for three protons, showing—as was the case for **5**—that one of the H-7 protons in **7** had completely exchanged with deuterium from the solvent.

Analysis of correlations observed in the COSY, TOCSY, g-HSQC, and g-HMBC NMR spectra of **7** led to the ^1H and ^{13}C chemical shift assignments presented in **Table 2**, which matched closely the published assignments for C-1−C-35 of **6** (*6*). A notable feature of the NMR spectra of **7** was the presence of an extra oxygenated methine resonance at 4.02 ppm (t, $J = 6.4$ Hz, H-36), which showed a COSY correlation to 1.84 ppm (H-37) which in turn correlated to 2.25 ppm (H-38). H-36 (4.02 ppm) showed an additional correlation to 5.21 ppm (H-32), and in the g-HMBC NMR

Table 3. LD_{50} Values and 95% Confidence Intervals for Pinnatoxin E (**7**), Pinnatoxin F (**8**), and Pinnatoxin G (**5**)

compound	LD_{50} (μg/kg)	confidence interval
5	50	35−66
7	45	32−58
8	16	12−23

spectrum it showed correlations to 144.3 (C-33), 121.0 (C-32), 33.7 (C-38), 32.1 (C-37), and 21.9 (C-34) ppm. Furthermore, H-32 (5.21 ppm) showed TOCSY correlations 4.62 (H-29), 4.02 (H-36), 3.86 (H-30), 3.52 (H-31), 2.26 (H-34), 1.92 (H-35) and 1.82 (H-35) ppm, and in the g-HMBC NMR spectrum H-32 showed correlations to 144.3 (C-33), 81.1 (C-30), 75.7 (C-36), 51.9 (C-5), 44.7 (C-31), and 21.9 (C-34) ppm. Both H-37 (1.84 ppm) and H-38 (2.25 ppm) showed g-HMBC correlations to a signal at 180.3 ppm consistent with the presence of a carboxylic acid group at C-39. These correlations, taken together with coupling constants and with the remaining assignments derived from the NMR spectra (**Table 2**), the HR-MS data, and the MS/MS fragmentation pattern (**Figure 2**) defined the structure of pinnatoxin E as **7**, as shown in **Figure 1**, except that the stereochemistry at C-36 remains undefined.

Pinnatoxin F (8). 8 did not display significant UV absorbance during HPLC purification, but displayed HR-MS $[M + H]^+$ at m/z 766.4889 which suggested a molecular formula of $C_{45}H_{67}NO_9$ (calcd for $C_{45}H_{68}NO_9$, 766.4894). Thus, the molecular formula of **8** was consistent with a dehydrated analogue of **7**. In addition, LC−MS analysis showed that **8** converted to **7** during storage in weakly basic aqueous solutions. This indicated that **8** was the lactone corresponding to **7**, and that the lactone ring of **8** is readily hydrolyzed to give hydroxy-acid **7**. Furthermore, partial conversion of **7** into **8** was observed under weakly acidic anhydrous conditions, including NMR analysis in $CDCl_3$.

NMR analysis of **8** was performed in $(CD_3)_2CDOD$, as LC−MS analysis showed that **8** underwent methanolysis when stored in CH_3OH, but was stable in 2-methylpropan-1-ol. The DEPT135, ^{13}C and HSQC NMR spectra of **8** showed the presence of 5 methyl, 18 methylene, 13 methine and 9 quaternary carbon atoms. In the g-HMBC NMR spectrum, the signal at 175.6 ppm (C-6) showed strong correlations to signals at 3.79 (H-1), 3.61 (H-1), 3.13 (H-7) and 1.44 (H-4) ppm. The methine signal at 3.13 ppm (d, J = 11.8 Hz, H-7) showed COSY correlations to H-8 (2.29, 1.39 ppm), and TOCSY correlations to H-8 (2.29, 1.39 ppm), H-9 (2.37, 1.58 ppm), and 10-CH_2 (4.71 ppm). These correlations define this methine resonance as H-7, and show that one of the H-7 protons of **8** completely exchanged with deuterium from the solvent.

Analysis of correlations observed in the COSY, TOCSY, g-HSQC, and g-HMBC NMR spectra of **8** led to the ^1H and ^{13}C chemical shift assignments presented in **Table 2**, which matched closely the published assignments for C-1−C-35 of **6** (*6*) and **7** (**Table 2**). A notable feature of the NMR spectra of **8** was the presence of an extra oxygenated methine resonance at 4.97 ppm (t, J = 7.5 ppm, H-36), which showed COSY correlations to 2.05 and 2.39 ppm (H-37), which in turn correlated to 2.57 ppm (H-38). H-36 (4.97 ppm) showed an additional correlation to 5.28 ppm (H-32) in the TOCSY NMR spectrum. Furthermore, H-32 (5.28 ppm) showed TOCSY correlations to 4.97 (H-36), 4.65 (H-29), 3.83 (H-30), 3.40 (H-31), 2.57 (weak, H-38), 2.25 and 2.04 (H-34), and 1.85 and 1.72 ppm (H-35), and in the g-HMBC NMR spectrum H-32 showed correlations to 83.3 (C-36), 50.0 (C-5). Both H-37 (weak, 2.39 and 2.05 ppm) and H-38 (2.57 ppm) showed correlations to the signal at 178.1 ppm, consistent with a carboxylic acid group at C-39. These correlations, taken together with coupling constants and the assignments derived in **Table 2**, the HR-MS data, and the MS/MS fragmentation pattern (**Figure 2**), together with the interconvertibility of **7** and **8**, defined the structure of pinnatoxin F as **8**, as shown in **Figure 1**, except that the stereochemistry at C-36 remains undefined.

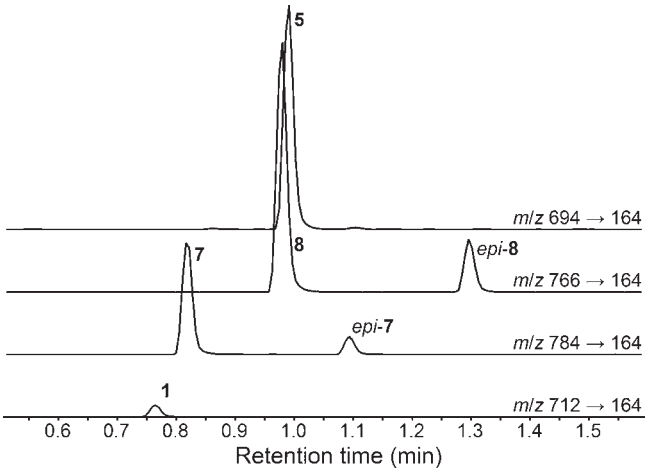


Figure 3. LC−MS MRM chromatogram of a crude Pacific oyster extract using LC−MS method 1. This chromatogram shows that compounds **1**, **5**, **7**, **8** and the epimers of **7** and **8** all elute in under 1.5 min. The speed of this method made it very useful for monitoring fractions during the isolation.

Pinnatoxin A (1). 1 did not display a significant UV absorbance during HPLC purification, but displayed ions in HR-MS $[M + H]^+$ m/z 712.4413, consistent with the molecular formula of **1** of $C_{41}H_{61}NO_9$ (calcd for $C_{41}H_{62}NO_9$, 712.4425). ESI-MS/MS spectra showed multiple losses of H_2O and revealed G-ring-opening via a retro Diels−Alder reaction yielding a product ion at m/z 164 and prominent fragments at m/z 572, 458, 342, 230, 204, 206, and 177 across the macrocycle as shown in **Figure 2** and identical to those reported for **2**−**4** (*5*, *7*). Although this sample was much weaker than for penitrems E−G, analysis of ^1H, COSY, TOCSY, g-HSQC and g-HMBC NMR data resulted in ^1H and ^{13}C NMR assignments for almost all the resonances (Supporting Information). Chemical shifts were similar to those previously reported for **1** in CD_3OD (*2*) and, for atoms 1−30 and 35, the ^1H and ^{13}C chemical shifts were essentially identical to those of pinnatoxin G (**5**) (**Table 2**).

Toxicology. The LD_{50} of **5** by ip injection in mice was 50 μg/kg (**Table 3**). At lethal doses, mice were hyperactive for up to 10 min after injection of the test material. An abrupt decrease in activity then occurred, and the mice showed abdominal breathing, extension of the hind legs and, in some cases, slight exophthalmia. At this time, the respiration rate was normal. In the next 2−3 min, however, the respiration rate precipitately declined, and at between 22 and 26 min after dosing, the mice died. There was a brief period of running movements just before death, and the mice showed severe exophthalmia. At death, the hind legs were fully extended. At sublethal doses, mice showed abdominal breathing and became very lethargic 9−13 min after dosing. The respiration rate remained normal, however, and full recovery was achieved within 2 h. The behavior and appearance of the mice remained normal throughout the subsequent 14-day observation period. No abnormalities were noted at necropsy, and organ weights were within the normal range.

The LD_{50} of **7** was 45 μg/kg (**Table 3**), with symptoms of intoxication very similar to those induced by **5**, and with death times between 14 and 17 min after dosing. At sublethal doses, mice became very lethargic, but recovered completely within an hour. The behavior and appearance of survivors remained normal

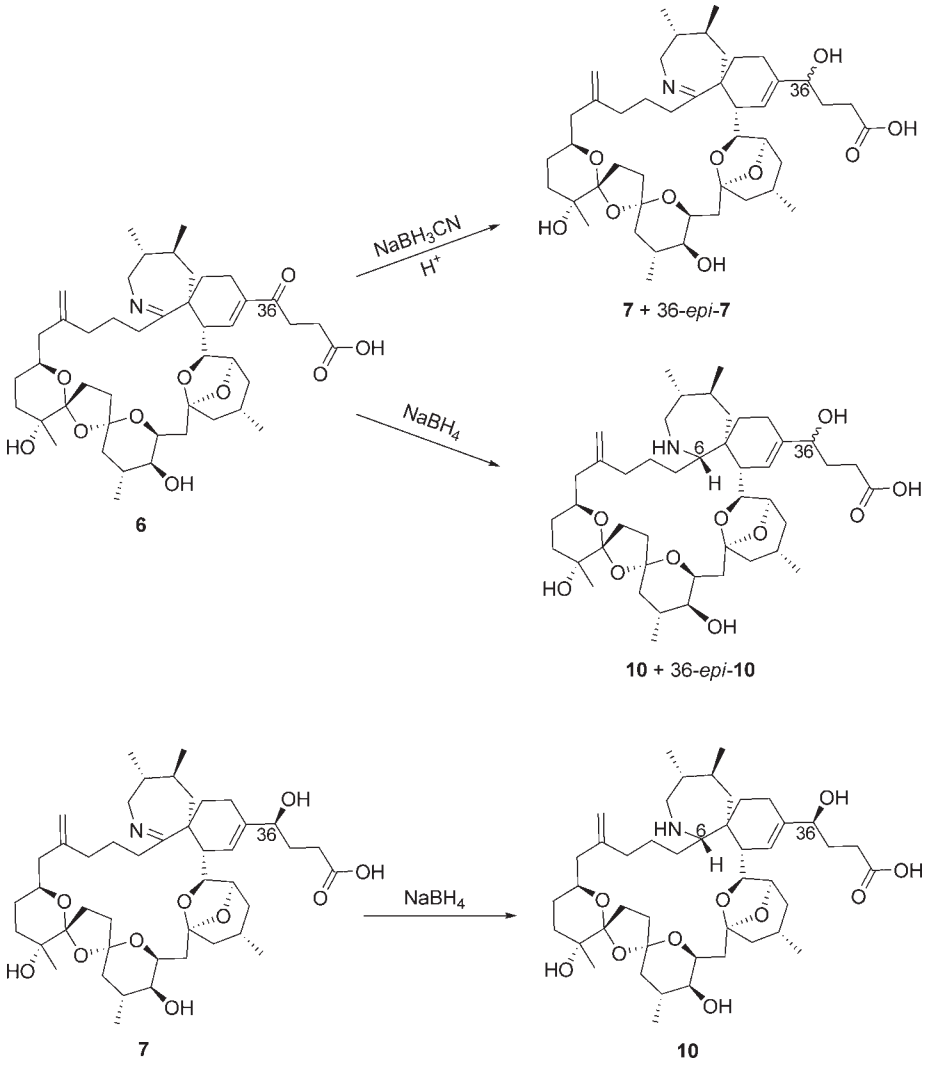


Figure 4. Reduction products of pinnatoxins D (**6**) and E (**7**). Sodium cyanoborohydride reduces the 36-keto group in **6** to two 36-OH epimers. Sodium borohydride reduces the 36-keto group in **6** to two 36-OH epimers and also reduces the imino group in both **6** and **7** to an amino group. Note that **7** and **10** are arbitrarily depicted with the same stereochemistry at C-36 as was found for the corresponding lactone in gymnodimine (*23*), and that the stereochemistry of **10** can be presumed to be 6*S* (*5*) based on steric considerations.

throughout the subsequent observation period, and no abnormalities were observed at necropsy.

The LD_{50} of **8** was 16 μg/kg (**Table 3**), and the symptoms of intoxication were very similar to those induced by **5** and **7**. Mice given sublethal doses became immobile, and did not regain full mobility until 2−3 h after dosing. After this, they appeared normal throughout the 14-day observation period, and no abnormalities were recorded at necropsy. Organ weights were within the normal range.

Chemistry of Pinnatoxins. During isolation of these pinnatoxins, it was discovered that they were more stable than the closely related spirolides. Both **5** and **7** were stable under base hydrolysis conditions, during which no release of fatty acid esters was observed in extracts from pinnatoxin-contaminated oysters. However, the lactone ring in **8** opens to form **7** in water, and is even more reactive in methanol, forming the **7**-methyl ester (**9**). The rate of ring-opening is lower in weak acid (pH < 5). Partial conversion of **7** to **8** was observed during preparative HPLC and NMR in $CDCl_3$, suggesting that cyclization to the lactone occurs readily under weakly acidic conditions. In solutions of formic and trifluoroacetic acids (>0.1%), both **7** and **8** slowly isomerized to produce a late-eluting epimer via a spiroketal rearrangement over a period of days. Because this rearrangement occurred with **7** and **8** but not with **5**, this suggested that the 22-OH group of **7** and **8** is involved in the rearrangement. These epimers were also present in natural samples at ca. 5−20% of the parent compound (**Figure 3**).

Because insufficient **6** was available for isolation and NMR analysis, its identity was established by reductive interconversions conducted on a semipurified fraction. No reaction occurred when **7** was treated with acidic $NaBH_3CN$. However, reduction of the imine group of **7** to an amine was achieved by treatment with $NaBH_4$ (**Figure 4**), as has previously been reported for **2** (*5*). This reduction of **7** yielded a single stereoisomer (presumed to be the 6*S*-isomer on steric grounds, as was presumed for the reduction product of **2** (*5*), and was demonstrated for the reduction product of the related compound gymnodimine (*23*)) of amine **10** with a molecular ion at *m*/*z* 786, 2 Da higher than the starting compound, which eluted later on LC−MS (method 1). The MS/MS spectrum of this compound was quite different from that of the corresponding imine **7**. When a semipurified extract containing putative **6** was treated with $NaBH_4$, two products (ratio ca. 1:1) with *m*/*z* 786 were obtained, with MS/MS spectra identical to that of **10**, suggestive of a pair of stereoisomers. The first-eluting stereoisomer had the same retention time as **10**, thus confirming the identity of **6** and indicating that reduction of both the imine and the 36-ketone had occurred to afford a pair of 36-hydroxy

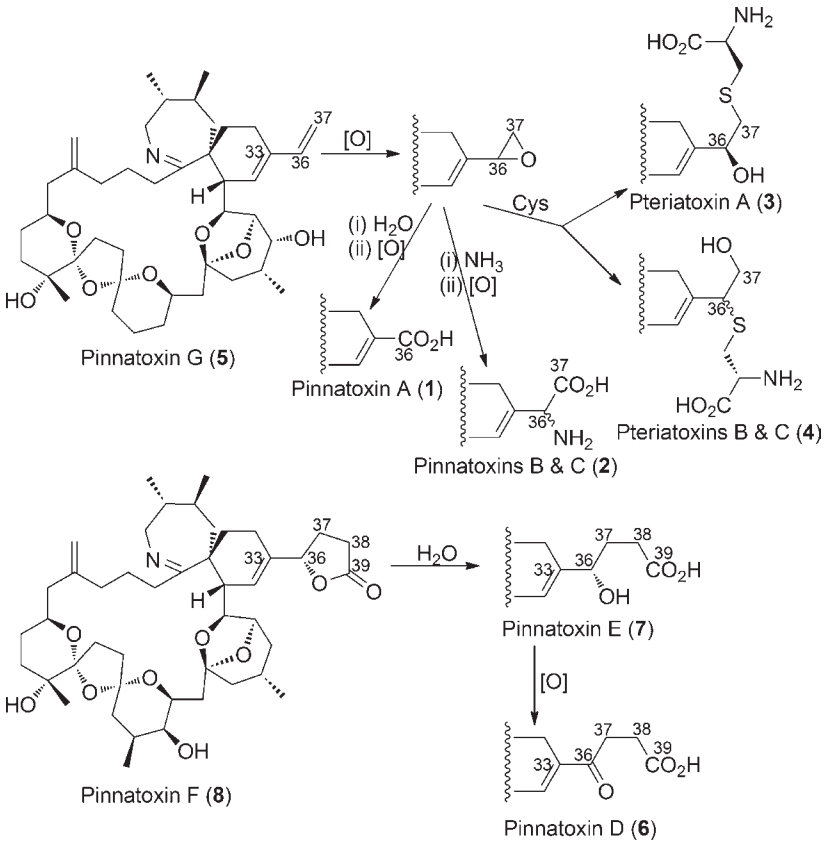


Figure 5. Proposed metabolic pathway for conversion of pinnatoxins F (**8**) and G (**5**) to form the known pinnatoxins and pteriatoxins. Note that **7** and **8** are depicted with arbitrary stereochemistry at C-36.

epimers (**Figure 4**). Further confirmation was obtained by reduction of the 36-ketone of **6** with acidic $NaBH_3CN$ to give two isomers of m/z 784, with identical MS/MS spectra to **7** and one of which coeluted with **7** in LC−MS analysis. This reduction was much slower than with $NaBH_4$, but was very selective with no sign of imine reduction (**Figure 4**).

Occurrence and Metabolism. After finding the novel pinnatoxins in Pacific oysters, it was discovered that another bivalve, the razor fish, grew in the same area. Razor fish were collected from Franklin Harbour and found to be more toxic than the Pacific oysters by mouse bioassay. LC−MS analysis of extracts from both species revealed the presence of multiple pinnatoxin analogues, total concentrations of which were higher in razor fish than in Pacific oysters. The pinnatoxin profiles of the two species were very different (**Table 1**). Some putative pinnatoxins were observed in both species which appear to be metabolites of **5** and are plausible intermediates to **1**−**4**. In razor fish, **5** appears to be readily metabolized to **1**, and **7** and **8** are metabolized to **6**. The same metabolic processes appear to occur in Pacific oysters, but at a much lower rate. The difference in toxin profiles suggests that metabolic transformations occur within the shellfish, consistent with the earlier finding of **6** and the related compounds **1**−**4** in two shellfish species (*7*).

Because pinnatoxins were detected in both species of filter-feeding shellfish, it seemed highly likely that they were being produced by one or more organisms ingested from the water column. Various environmental samples including sediment and water samples were collected from Franklin Harbour to try to identify the source of these compounds. Both sediment and water samples contained **5**, **7** and **8** but did not contain **1** or **6**. This is evidence that **5**, **7** and **8** are produced by organisms outside the shellfish and not by a symbiont. It also suggests that **5**, **7** and **8** are precursors of other pinnatoxins and pteriatoxins via shellfish metabolism, possibly via a pathway similar to that shown in **Figure 5**. The involvement of an epoxide or its equivalent has previously been proposed as a precursor to the pteriatoxins (**3**, **4**) (*9*), and pinnatoxin G (**5**) could conceivably be oxidized to a 36,37-epoxide and converted to pinnatoxins A−C (**1**, **2**) and pteriatoxins A−C (**3**, **4**) by shellfish metabolism. Similarly, hydrolysis of pinnatoxin F (**8**) and metabolic oxidation at C-36 could account for pinnatoxins E (**7**) and D (**6**).

A size-fractionated water sample revealed that only 10% of the total pinnatoxins were in the $>45\,\mu$m fraction and that 90% were in the 20−45 μm fraction, and no pinnatoxins were in the $<20\,\mu$m, consistent with a microalgal source such as a dinoflagellate. The finding that **5**, **7** and **8** were also detected in high concentrations (400−2000 μg/kg) in sediment samples suggests that the producing organism could be a benthic species.

Pacific oysters from Rangaunu Harbour in New Zealand, which had caused unexplained deaths in the lipophilic mouse bioassay in 1995, were also analyzed by LC−MS (method 2) for pinnatoxins. Both **7** and **8** were detected, but not **5**. This result suggests that **5** and **8**, which appear to be the progenitors of all the known pinnatoxins and pteriatoxins, are each produced by different species or strains of algae—as might be expected from examination of their structures (**Figure 1**) and biosynthetic considerations. Information and standards obtained in the present study have very recently allowed isolation of a dinoflagellate from Rangaunu Harbour that produces **7** and **8** in culture(*27*), and formal identification of this alga is underway (L. Rhodes, Cawthron Institute, personal commununication). It is therefore likely that this or other species of dinoflagellates are also responsible for the occurrence of pinnatoxins in Australia and Japan.

Thus, the discovery of **5**, **7** and **8** sheds new light on the origin of pinnatoxins and their metabolism in shellfish, and analysis of

environmental samples suggested an algal origin for these toxins. There are a number of minor pinnatoxin metabolites present in shellfish with unknown structures, but the tentative identities of some of these metabolites, based on LC−MS analysis, are consistent with metabolic intermediates in the pathway shown in **Figure 5**. All three pinnatoxins examined in the present experiments were toxic to mice by intraperitoneal injection, with LD_{50} values between 16 and 50 μg/kg. These substances are therefore more toxic by injection than gymodimine (*19*), but less toxic than spirolide C and 13-desmethylspirolide C (*20*). Like other cyclic imines, the pinnatoxins caused respiratory paralysis in mice, and it is likely that they target the nicotinic acetylcholine receptor at the neuromuscular junction, as demonstrated for gymnodimine and 13-desmethylspirolide C (*28*, *29*). It should be noted, however, that high toxicity to mice by intraperitoneal injection does not necessarily indicate risk to humans from consumption of contaminated shellfish (*20*), and there are no reports of human illness associated with consumption of shellfish contaminated with pinnatoxins in Australia, New Zealand or elsewhere.

ABBREVIATIONS USED

HR-MS, high-resolution mass spectrometry; ip, intraperitoneal; LC−MS, liquid chromatography−mass spectrometry; MRM, multiple reaction monitoring.

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Supporting Information Available: Table comparing NMR assignments of pinnatoxin A (**1**) isolated in this study with NMR assignments for pinnatoxin G (**5**) and published NMR assignments for **1**; information on NMR analysis of pinnatoxin A (**1**), and rationale for the modified atom-numbering system; table of dose rates and death times obtained during estimation of LD_{50} values; UV, mass and NMR spectra of pinnatoxin G (**5**); NOE correlations observed in NOESY NMR spectrum of pinnatoxin G (**5**); MS/MS spectrum of pinnatoxin A (**1**); mass and NMR spectra of pinnatoxin E (**7**) and pinnatoxin F (**8**); mass spectra of pinnatoxin D (**6**), pinnatoxin E amine (**10**), and pinnatoxin E methyl ester (**9**); rationale for stereospecific deuterium exchange of only one of the H-7 methylene protons in CD_3OD; rationale for stereospecificity of the reduction of pinnatoxin E (**7**) at C-6. This material is available free of charge via the Internet at http://pubs.acs.org.

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