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DIARRHETIC SHELLFISH TOXINS

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Abstract—As the causative agents of a new type of shellfish poisoning, named diarrhetic shellfish poisoning, okadaic acid, 35(*S*)-methylokadaic acid, 7-O-acyl derivatives of 35(*S*)-methylokadaic acid, two novel polyether lactones named pectenotoxin-1 and -2 have been isolated and had their structures determined. Three pectenotoxin analogues were also present. In addition to the previously identified *Dinophysis fortii*, *D. acuminata* was newly suggested as a source of toxins.

In various parts of the world, normally edible fish and shellfish may become toxic as the result of feeding on toxic organisms, mostly dinoflagellates, and thus accumulating their toxins. The best known example is the paralytic shellfish poisoning (PSP) which involves highly potent toxins of the saxitoxin family produced by *Gonyaulax* (= *Protogonyaulax*) spp.[1] or *Pyrodinium bahamense* var. *compressa*.[2] Recently Yasumoto *et al.*[3] became aware of the folklore in northeastern Japan of frequent occurrence of gastroenteritis after ingestion of shellfish harvested during late spring to summer. The presence of acetone-soluble toxins in the digestive glands of the implicated shellfish, together with negative results for known pathogenic bacteria, confirmed the gastroenteritis to be an undescribed type of shellfish poisoning. The disease was thus named diarrhetic shellfish poisoning (DSP) after its predominant human symptom.[4] Although no red tide was sighted during the infestation period, the regional and seasonal variation of shellfish toxicity strongly suggested the planktonic origin of the toxins. This hypothesis was soon verified by subsequent surveys of plankton populations and shellfish toxicity, as well as by chemical analyses of dinoflagellate samples collected with a specially devised screening apparatus. The dinoflagellate *Dinophysis fortii* was identified as the organism which transmits toxins to shellfish.[4] Noteworthy is the fact that shellfish may become toxic beyond the safety level in the presence of this species at a very low cell density of 200 cells per liter, or even lower.

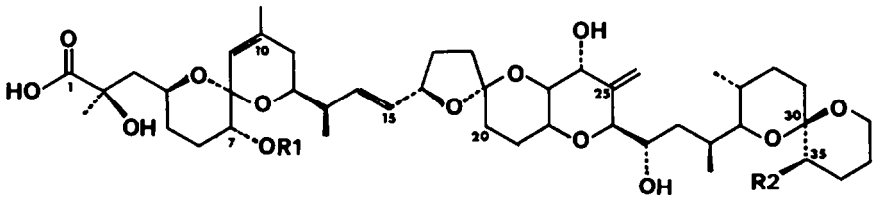
Unlike PSP, DSP does not appear to be fatal, but its high morbidity rate and worldwide occurrence have become apparent in recent years. The number of known DSP cases in Japan exceeds 1300 since 1976 despite the existence of extensive surveillance; it is reported to be 5000 in the single year of 1981 in Spain;[5] and 400 in 1983 in France.[6] Sporadic occurrence of DSP has also been known in the Netherlands[7] and in Chile.[8] Thus, the magnitude of the impact of DSP on public health and on the shellfish industries is no smaller than that of PSP, especially when the long duration of infestation period is taken into account.

The first toxic component isolated from toxic mussels, code-named dinophysistoxin-1 (DTX_1), was very similar in chromatographic properties to okadaic acid (I), a C_{38} polyether fatty acid derivative isolated first from sponges by Tachibana *et al.*[9] as a potent cytotoxic component, and then from the dinoflagellate *Prorocentrum lima* by us[10] as a possible secondary toxin in ciguateric fish. Comparison of spectral data between DTX_1 and okadaic acid confirmed the structure of DTX_1 as 35(*S*)-methylokadaic acid (II).[11] Subsequent effort revealed the presence of other okadaic acid derivatives, named dinophysistoxins (DTX), and novel polyether lactones, named pectenotoxins (PTX) after the generic name of the scallop used for toxin extraction.

We now wish to report the isolation and structural determination of dinophysistoxin-3 (DTX_3, III), pectenotoxin-1 (PTX_1, IV), and pectenotoxin-2 (PTX_2, V). Isolation of pectenotoxin-3, -4, -5 (PTX_{3-5}) and okadaic acid from toxic scallops or mussels and the assignment of *Dinophysis acuminata* as a new probable source of okadaic acid are also described.

The digestive glands (200 kg) of scallops were extracted with acetone at room temperature. After evaporation of acetone, the residue was partitioned between diethyl ether and water. Purification of toxins in the ether soluble residue was achieved by the following procedures: initial treatment on two successive columns of silicic acid, gel permeation through Sephadex LH-20 column to separate DTX_1 and PTX from DTX_3, which has a bulky fatty acid moiety; reversed phase chromatography on a Lobar column for processing a relatively large quantity; chromatography on a basic alumina column to separate DTX_1, which has a free carboxyl group, from neutral PTX; HPLC on reversed phase column; and HPLC on a silicic acid column to separate PTX_1 from PTX_3 and PTX_2 from PTX_4.

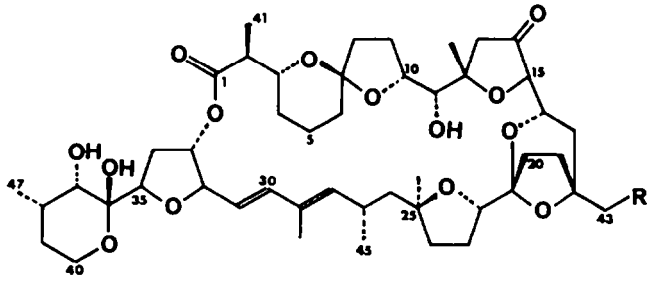
DTX_3, obtained as colorless solid, showed no absorption maximum in UV region above 220 nm. The IR spectrum of DTX_3 was very similar to that of DTX_1. The structural resemblance between DTX_1 and DTX_3 is also evident in the ^1H NMR spectra (Fig. 1). Additional signals observed in the spectrum of DTX_3 are



I okadaic acid : R1=H, R2=H

II dinophysistoxin-1 : R1=H, R2=CH_3

III dinophysistoxin-3 : R1=acyl, R2=CH_3



IV pectenotoxin-1 : R=OH

V pectenotoxin-2 : R=H

2R, 3R, 7S, 10R, 11R, 12S, 15S, 16S, 18S, 21S, 22S, 25S, 27S, 32S, 33S, 35R, 36R, 37S, 38S

attributed to the presence of an unsaturated fatty acid moiety on the basis of the following assignment: δ 0.88 (3H, t, terminal Me), 1.25 (*ca* 25H, br s, saturated methylene), 1.98 (*ca* 2H, m, methylene adjacent to a double bond), 2.15 (2H, t, methylene adjacent to ester), 2.80 (*ca* 6H, br dd, methylene between double bonds), and 5.35 (*ca* 5H, olefinic protons). Subsequent gas chromatographic analyses of hydrolysis products confirmed the presence of DTX_1 and the following fatty acids in the hydrolysate: $C_{14:0}$ (13%), $C_{16:0}$ (29%), $C_{18:3}$ (3%), $C_{18:4\omega3}$ (9%), $C_{20:5\omega3}$ (23%), and $C_{22:6\omega3}$ (23%). The number of protons estimated by the ^1H NMR spectrum of DTX_3 support a structure of DTX_3 as a mixture of DTX_1 derivatives having one of the above fatty acids as an ester. Another distinction between DTX_1 and DTX_3 in the ^1H NMR spectra is that one oxymethine proton of DTX_1 (δ 3.40) is deshielded by 1.36 ppm (δ 4.76 ppm) in DTX_3. This proton is easily assignable to C-7 H because its sharp doublet of doublet (J = 11.8, 4.2 Hz) signal coincides with the axial–axial and axial–equatorial coupling of C-7 H to C-6 methylene protons. From the close structural resemblance between DTX_1 and okadaic acid,[9] the signals at δ 4.10 (br d, 10 Hz) and δ 4.07 (t, 10 Hz) are assignable to C-24 H and C-27 H, respectively. The broadness of C-24 H caused by its long-range coupling to olefinic protons on C-42 carbon and the triplet shape of C-27 H caused by its coupling to C-26 H and C-28 2H also support the assignment. These signals remain unchanged in DTX_3, although their signal shapes are deformed by the overlapping C-4 H slightly shifted downfield from the original position of DTX_1, excluding the possibility of any modification of C-24 OH and C-27 OH. Acylation of the C-2 hydroxyl can be excluded because the singlet at δ 1.37, which is assignable to the Me on C-2 carbon, remains unchanged in DTX_3. These results allow us to assign the structure of DTX_3 to III.

DTX_3 is extremely sensitive toward acid, alkali and exposure to air. Leaving a dried sample exposed to air easily resulted in the loss of toxicity and production of multiple spots on TLC. Repurification of one of such deteriorated samples led to isolation of a compound non-lethal to mice, but indistinguishable from DTX_3 chromatographically. Field desorption mass spectrum exhibited ($M^+ + H$) at m/z 1057 for a composition of $C_{61}H_{100}O_{14}$, conforming to that of palmitoyl DTX_1. Gas chromatographic analyses of its hydrolysates proved the presence of DTX_1 and palmitic acid. The ^1H NMR spectrum had all characteristic features of DTX_3 except that signals arising from olefinic protons of the fatty acid moiety were absent in the spectrum of the newly isolated compound. These results confirm that the component is 7-O-palmitoyl DTX_1. The lack of significant toxicity of this compound (>5mg/kg, ip) indicates that toxicity of DTX_3 is related to the unsaturation of the acyl moiety.

PT toxins are readily distinguishable from DT toxins by their strong UV absorption at 235 nm. On basic alumina column they were easily separable from DTX_1, which was firmly retained on the column due to the presence of the free carboxyl group. The presence of five PTX were recognized. PTX_1 was obtained as a colorless crystalline solid. The IR bands at 3400, 1760, 1740, 970 cm^{-1}, and the UV λ_{max} at 235 nm (ε 12,400 methanol) indicated hydroxyl, five-membered ring ketone, ester and conjugated diene groups. The

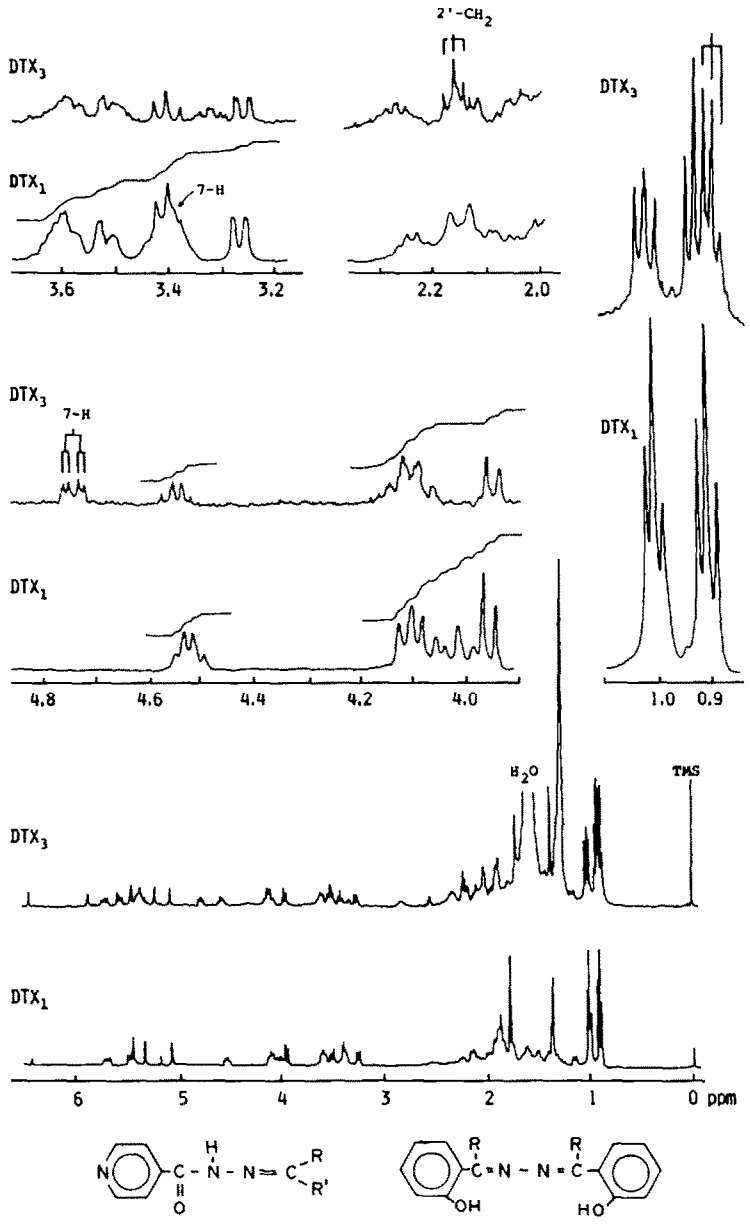


Fig. 1. Comparison of PMR spectra between dinophysistoxin-1 and dinophysistoxin-3.

^{1}H NMR spectrum exhibited 6 methyls (3 doublets, 3 singlets) and 3 olefinic protons of a *trans-trans* conjugated diene. The ^{13}C NMR spectrum indicated 47 carbons: 1 ketone singlet at δ 213.6; 1 carbonyl singlet at 173.3; 4 olefinic carbons at 140.5 (d), 135.6 (d), 130.7 (s) and 120.6 (d) ppm; 3 ketal or hemiketal singlets at 108.8, 107.6, 97.2; 15 oxycarbons (3 singlets, 10 doublets, and 2 triplets) between 85.5 and 61.3 ppm. The remaining 23 high field signals ranged from 50.3 to 12.7 ppm and included 3 methines and 6 methyls. A secondary ionization mass spectrum gave an *m/z* 875 ($M^+ + H$), indicating a formula of $C_{47}H_{70}O_{15}$. The relative stereostructure of PTX_1 was secured by single crystal X-ray diffraction techniques. A computer generated perspective drawing of the X-ray model is given in Fig. 2. This structure (IV) is completely consistent with the spectral data and an essentially complete assignment of the proton signals was achieved by *J*-correlated two-dimensional decoupling measurements (Fig. 3).

The mass spectrum of PTX_2 gave an *m/z* 859 ($M^+ + H$), suggesting that it was a deoxy PTX_1. Comparison of ^{1}H NMR spectra of PTX_1 and PTX_2 proved the absence of C-43 protons (δ 3.56, 3.61 ppm) and appearance of a new methyl signal at 1.33 ppm in the spectrum of PTX_2. A triplet at 65.8 ppm observed in the ^{13}C NMR spectrum of PTX_1 was transformed into a quartet and shifted to 26.0 ppm in the spectrum of PTX_2. Upfield shift of the resonance of a quaternary carbon at 83.7–80.7 ppm in the spectrum of PTX_2 is explainable by the removal of deshielding effect of C-43 OH on C-18. The downfield shifts of signals of PTX_1 at 27.0(t) and 31.5(t) ppm to 33.8(t) and 36.2(t) ppm in the spectrum of PTX_2 are accounted for by the loss of a γ-effect of the C-43 OH on C-17 and C-19 (C-17 and

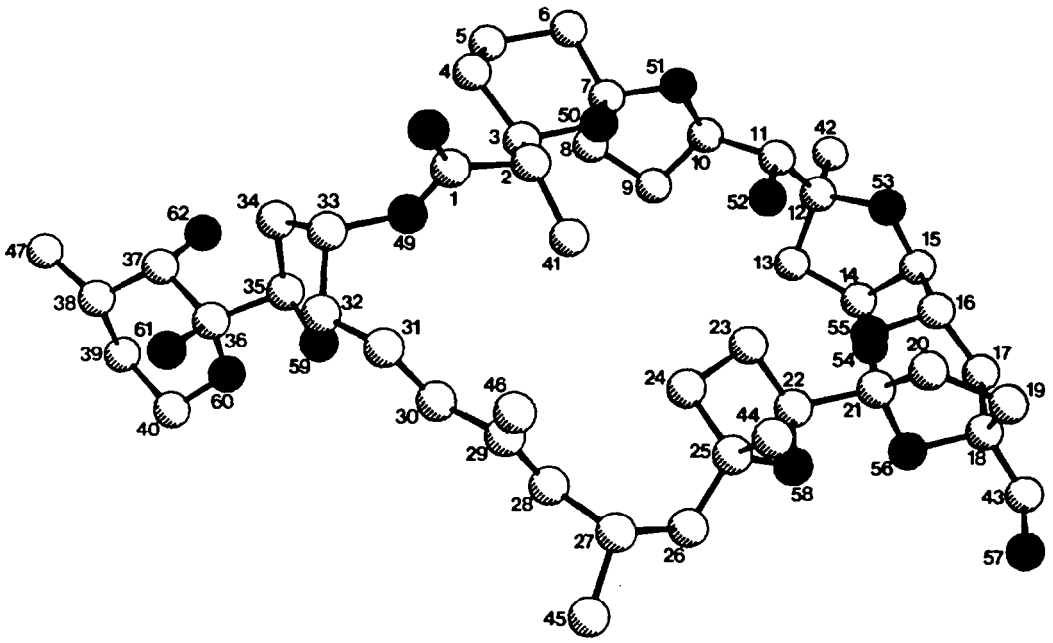


Fig. 2. Stereoview of pectenotoxin-1.

C-19 interchangeable). With all these evidences, PTX_2 was assigned to 43-deoxy PTX_1 (V).

PTX_3, PTX_4 and PTX_5 were obtained in pure form but in small amounts. Both PTX_3 and PTX_4 are indistinguishable from PTX_1 in UV and mass spectra but are distinguishable by optical rotation, suggesting that they are stereoisomers of PTX_1. The mass spectrum of PTX_5 gave a $(M^+ + H)$ ion at *m/z* 877, suggesting that this compound is a dihydro derivative of either of PTX_1, PTX_3 and PTX_4. The IR spectrum of PTX_5 confirmed the absence of the absorption at 1760 cm^{-1} due to C-14 ketone.

After our report of DSP and subsequent identification of *D. fortii* as the causative organism, extensive surveys of planktons and shellfish toxicity have been conducted by prefectural institutes. Occasionally it was noticed that mussels become toxic, though at a low level and for a brief period, in the early spring when *D. fortii* was scarce in the sea. Instead, a significant number of a related species, *Dinophysis acuminata*, were present in the sea. In order to see whether *D. acuminata* was toxic and its toxins were the same as those of *D. fortii*, a mussel specimen collected in April was extracted and the toxin was purified in a similar manner employed for purification of DTX_1. Gas chromatographic analysis of the toxic principle of the mussel proved that it was indistinguishable from okadaic acid, but clearly different from DTX_1 in retention time.

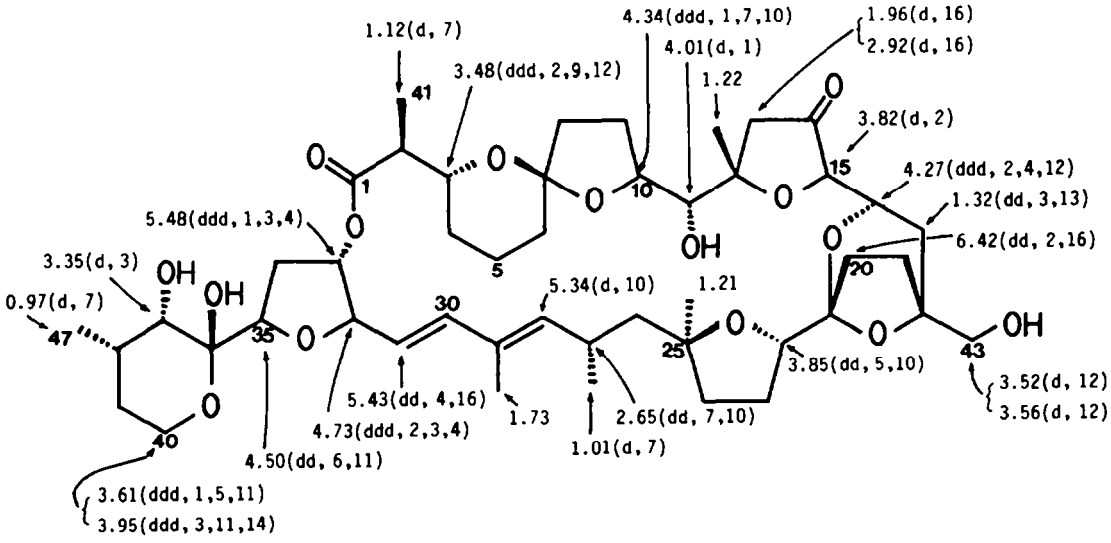


Fig. 3. Assignment of proton signals of pectenotoxin-1.

The results strongly suggest the implication of okadaic acid in DSP and the production of okadaic acid by *D. acuminata*.

DISCUSSION

Although DSP was first confirmed in Japan, its occurrence has been shown to be more frequent and widespread than suspected earlier. The absence of past records may be explained by the fact that patients often hesitate to report to health officials because the symptoms are relatively mild, or they tend to attribute the illness to other causes, especially to bacterial contamination. From health personnel point of view, lack of a proper method for detecting toxins in the past made it difficult to diagnose the suspected gastroenteritis. As the amount of toxin that induces illness in man by oral intake is very small, equivalent to 32 μg of DTX_1, assaying the toxins was not an easy task.

The present work adds to a current topic of production of bioactive polyether compounds by dinoflagellates and their involvement in fish and shellfish poisoning. Ciguatoxin, the principal toxin of ciguatera fish poisoning, is shown by Yasumoto *et al.*[12] to be a product of a benthic dinoflagellate, *Gambierdiscus toxicus*, and the polyether nature of the toxin has been confirmed by Scheuer and his colleagues on the basis of spectral analysis of a crystalline specimen.[13] Another benthic dinoflagellate, *Prorocentrum lima*, produces okadaic acid and its involvement in ciguatera, though in a limited extent, is also suspected.[10] Brevetoxins produced by *Ptychodiscus brevis*[14–16] not only cause massive fish kills but also cause neurotoxic shellfish poisoning.[17] Goniodomin, an antifungal substance isolated from *Goniodoma* sp. has been also reported to be a polyether.[18] From a chemical point of view, it is notable that PT toxins are substantially different from previously described dinoflagellate toxins. Specifically, they differ from others in having a longer carbon backbone (C_{40}), a C_{33} lactone ring rather than an acydor structure, and a novel dioxabicyclo moiety. The large oxygen rich internal cavity is grossly similar to the cavities found in the polyether ionophores from terrestrial microorganisms. Coexistence of compounds of such different skeletons raises an interesting question about the biosynthesis of polyether compounds in dinoflagellates. The occurrence of okadaic acid in Japanese mussels exposed to *D. acuminata* coincides with the occurrence of this compound in Dutch mussels collected from an area where the same species was abundant (M. Kat and T. Yasumoto, unpublished data). The incidence of gastroenteritis caused by ingestion of mussels in Chile was associated with the occurrence of *Dinophysis acuta*,[8] though details are not available. Further study seems necessary to determine production of polyether toxins by other *Dinophysis* spp.

EXPERIMENTAL

TLC was carried out on precoated silica gel 60 plates (Merck) with the solvent system benzene–acetone–MeOH–6N AcOH (150:80:19:1). Toxins were detected by heating the plates after spraying with 50% H_2SO_4. NMR spectra were measured with FX-100, FX-400 (JEOL) and NT-360 (Nicolet) instruments, mass spectra with a Hitachi M-80 mass spectrometer, IR spectra with a JASCO A-202 spectrometer, UV spectra with a Hitachi 124 spectrophotometer, and optical rotation with a JASCO ORD/UV-15 model.

Gas chromatography (GLC). GLC analyses were conducted on a Hitachi 163 instrument equipped with hydrogen flame ionization detectors. Okadaic acid and DTX_1 were trimethylsilylated with Tri-Sil "Z" either intact or after derivatization with diazomethane to methyl esters. A glass column (3 × 800 mm) packed with 2% OV-101 on 60/80 mesh Uniport HP was used for analysis of okadaic acid and DTX_1. The column temp was maintained at 315° and N_2 flow at 30 ml/min. For fatty acid analyses a glass column (3 × 200 mm) packed with 10% DEGS on Chromosorb WAW DMCS 60/80 mesh was used. Column temp was kept at 165° and the N_2 flow rate at 30 ml/min. The reference fatty acids were purchased from Wako Pure Chemicals.

Isolation of toxins. The digestive glands (200 kg) of the scallop *Patinopecten yessoensis* collected in July 1982, at Mutsu Bay, Japan, were extracted with acetone at room temp. After removal of the organic solvent, the aqueous suspension was extracted with diethyl ether. The ether soluble material was chromatographed on a silicic acid column (Wakogel C-100, Wako Pure Chemicals) with benzene–MeOH (35:1) and (9:1). The toxic residue in the last eluate was rechromatographed over the same adsorbent with diethyl ether–MeOH (40:1) and (1:1) to obtain toxins in the second eluate. To avoid the decomposition of DTX_3 by contaminant acid, the silicic acid used for the column was neutralized by dilute NaOH aq prior to activation at 110°. Subsequent gel permeation through Sephadex LH-20 column (2.8 × 120 cm) with benzene–MeOH (1:1) resolved toxins into two fractions. DTX_3 was eluted between 240 and 270 ml and DTX_1 and PTX between 280 and 320 ml. DTX_3 was successively purified on a Lobar column (LiChroprep RP-8, size B) and a LiChroprep RP-8 column (0.5 × 100 cm) with MeOH–water (20:1). The final purification was performed on a μBondapak C_{18} column (0.5 × 50 cm) with MeOH–water (97:3). The mixture of DTX_1 and PTX was chromatographed first on a Lobar column (LiChroprep RP-8, size B) with MeOH–water (17:3) and next on an alumina column (Woelm, basic, activity III) by stepwise increasing MeOH concentration in $CHCl_3$. PTX were eluted from the alumina column with $CHCl_3$ and $CHCl_3$–MeOH (9:1). DTX_1 was firmly adsorbed to the column and was not eluted until the column was washed with 50% aqueous MeOH containing 0.1% ammonia. The PTX mixture was then chromatographed on a LiChroprep RP-8 column (0.5 × 100 cm) with MeOH–water (8:2). PTX_1, PTX_3 and PTX_5 were eluted in fractions # 5–8 (5 ml each) and PTX_2 and PTX_4 in fractions # 12–16. The mixture of PTX_1, PTX_3 and PTX_5 thus obtained was further purified twice on the same column using acetonitrile–MeOH–water (2:2:3). Subsequent chromatography on Toyopal HW-40 column (Toyo Soda, 1 × 50 cm) by using MeOH as the eluent separated PTX_5 from PTX_1 and PTX_3. Further separation of PTX_1 and PTX_3 was achieved on a Develosil silicic acid column (Nomura Kagaku, 0.6 × 25 cm) by elution with CH_2Cl_2–MeOH (98:2). The mixture of PTX_2 and PTX_4 was separated on a silica gel 60 column (0.5 × 50 cm) by elution with CH_2Cl_2–MeOH (98:2). Separation of toxins was monitored with a UV spectromonitor with the wavelength set at 220 nm for DTX and at 235 nm for PTX. TLC and mouse assay were also employed for monitoring.

Dinophysistoxin-3. Five milligrams of DTX_3 judged pure by HPLC and TLC was obtained as a colorless amorphous solid: no UV maximum above 220 nm; IR 3400 (OH), 1740 (COOH), 1705 (COOR), 1600 (C=C), 1240 (COOR); minimum lethal dose to mouse 500 μg/kg (ip); R_f in TLC 0.57. Toxicity was easily lost by treatment with dilute HCl or NaOH solns. Leaving a dried sample exposed to air also resulted in the loss of toxicity and production of multiple spots in TLC. ^{1}H NMR spectrum was very similar to that of DTX_1 but showed the following additional signals: δ 0.88 (3H, t), 1.25 (*ca* 25H, br s), 1.98 (*ca* 2H, m), 2.15 (2H, t), 2.80 (*ca* 6H, br dd), and 5.35 (*ca* 5H, m) ppm. Two proton signals of DTX_1 were shifted downfield from δ 3.99 to 4.00 and from 3.35 to 4.76 ppm. DTX_3

(50 μg) was hydrolyzed by heating in 0.2 N methanolic solution of NaOH at 75° for 40 min. After addition of AcOH, the hydrolysates were extracted with diethyl ether and converted to methyl esters by treatment with diazomethane. Half of the hydrolysate was used for GLC analysis of fatty acids and a portion of the remaining half was trimethylsilylated and analyzed by GLC for DTX_1. A small portion of the methylated hydrolysate was treated with a Sep-pak cartridge (Waters, C_{18}) and subjected to mass spectral measurement. The following fatty acids were identified in the hydrolysates: $C_{14:0}$ (13%), $C_{16:0}$ (29%), $C_{18:3}$ (3%), $C_{18:4\omega3}$ (9%), $C_{20:5\omega3}$ (23%) and $C_{22:6\omega3}$ (23%). GLC analysis of trimethylsilylated product proved that its retention time (8'33") was identical with that prepared from DTX_1. The presence of the following ions in the mass spectrum also confirmed that the hydrolysis product is DTX_1: *m/z* 855 [100%, $(M^+ + Na)$], 833 [50%, $(M^+ + H)$], 815 [93%, $(M^+ + H - H_2O)$] and 797 [45%, $(M^+ + H - 2H_2O)$].

7-O-*Palmitoyl DTX*$_1$. DTX_3 fraction kept dry in a freezer for a month remarkably lost its toxicity. Repurification of the deteriorated sample by chromatographic procedures described for DTX_3 yielded 2.5 mg of a colorless amorphous solid judged pure by HPLC and TLC: m.p. 78° $[\alpha]_D^{20}$ +5.9 (*c* 0.041, $CHCl_3$), R_f in TLC 0.57, non-lethal to mouse at 5 mg/kg (ip). FD mass spectrum gave an *m/z* 1057 $(M^+ + H)$, pointing to the formula of palmitoyl DTX_1, $C_{61}H_{100}O_{14}$. GLC analysis of the fatty acid moiety proved the presence of only palmitic acid and the trimethylsilylated derivative of the hydrolysis product gave only one peak corresponding to DTX_1. The ^1H NMR spectrum was very similar to that of DTX_1, but contained the following additional signals: δ 0.88 (3H, t), 1.3 (25H, br s), 2.15 (2H, t) ppm. One proton signal of DTX_1 (δ 3.4 ppm) was deshielded to δ 4.76 ppm (J = 11.8, 4.2 Hz) in the palmitoyl DTX_1. CMR spectrum of palmitoyl DTX_1 had the following signals in addition to 44 carbon signals of DTX_1: δ 173.1 (C-1'), 31.5 (C-2'), 29.1–29.7 (poorly resolved signals of more than 8 carbons, C-3'–C-13'), 26.5 (C-14'), 22.9 (C-15'), 14.1 (C-16'). One oxycarbon signal (δ 76.0 ppm) of DTX_1 was deshielded to 76.6 ppm in the spectrum of palmitoyl DTX_1, suggesting that the signal arises from C-7.

Pectenotoxin-1. Crystallization of PTX_1 from acetonitrile–water furnished 20 mg of a white cyrstalline solid: m.p. 208–209°; $[\alpha]_D^{20}$ +17.1 (*c* 0.41, MeOH); λ_{max} 235 nm (ε 12,400, MeOH); IR 3400, 1760, 1740, 970 cm^{-1}; minimum lethal dose to mouse 250 μg/kg (ip); R_f in TLC 0.43. A secondary ionization mass spectrum gave an *m/z* 875 $(M^+ + H)$, indicating a formula $C_{47}H_{70}O_{15}$. ^{13}C NMR spectrum was measured in $CDCl_3$–CD_3OD (1:1) solution: δ 12.7(q), 15.2(q), 17.5(q), 22.0(t), 22.2(t), 23.5(q), 23.7(q), 26.7(q), 27.0(t), 27.2(t), 28.1(t), 29.1(t), 29.7(t), 29.7(d), 30.6(d), 31.5(t), 32.8(t), 33.4(t), 34.3(t), 37.1(t), 43.9(t), 48.2(d), 50.3(t), 61.3(t), 65.8(t), 70.7(d), 71.2(d), 74.6(d), 74.7(d), 76.5(d), 79.0(d), 79.0(d), 80.6(d), 82.0(s), 82.2(d), 83.3(d), 83.7(s), 85.5(s), 97.2(s), 107.6(s), 108.8(s), 120.6(d), 130.7(d), 135.6(d), 140.5(d), 173.3(s), 213.6(s) ppm. ^1H NMR spectrum was measured in acetone-d_6 solution. Assignment of protons was based on the results of *J*-correlated two-dimensional decoupling measurements: [chemical shifts (δ), multiplicity, *J*(Hz), assignment)]; 0.97 (3H, d, 7, H-47), 1.01 (3H, d, 7, H-45), 1.12 (3H, d, 7, H-41), 1.21 (3H, s, H-44), 1.22 (3H, s, H-42), 1.32 (dd, 3, 13, H-17), 1.73 (3H, s, H-46), 1.96 (d, 16, H-13), 2.65 (dd, 7, 10, H-27), 2.92 (d, 16, H-13), 3.35 (d, 3, H-37), 3.48 (ddd, 1, 5, 11, H-40), 3.52 (d, 12, H-43), 3.56 (d, 12, H-43), 3.61 (ddd, 1, 5, 11, H-40), 3.82 (d, 2, H-15), 3.85 (dd, 5, 10, H-22), 3.95 (ddd, 3, 11, 14, H-40), 4.01 (d, 1, H-11), 4.27 (ddd, 2, 4, 12, H-16), 4.34 (ddd, 1, 7, 10, H-10), 4.50 (dd, 6, 11, H-35), 4.73 (ddd, 2, 3, 4, H-32), 5.34 (d, 10, H-28), 5.43 (dd, 4, 16, H-31), 5.48 (ddd, 1, 3, 4, H-33), 6.42 (dd, 2, 16, H-20) ppm. In addition to these resonances, the spectrum contained complex overlapping multiplets in the range 1.2 to 2.7 ppm.

Pectenotoxin-2. PTX_2 (40 mg) was obtained as a colorless amorphous solid: $[\alpha]_D^{20}$ +16.2 (*c* 0.105, MeOH); λ_{max} 235 nm (ε 16,000, MeOH); IR 3400, 1760, 1740, 970 cm^{-1}; minimum lethal dose to mouse 260 μg/kg (ip); R_f in TLC 0.71. A secondary ionization mass spectrum of PTX_2 gave an *m/z* 859 $(M^+ + H)$, indicating a formula $C_{47}H_{70}O_{14}$. The ^{13}C NMR spectrum of PTX_2 was measured under the same condition as for PTX_1. The resonances at δ 65.8(t), 83.7(s), 27.0(t), and 31.5(t) ppm observed in the spectrum of PTX_1 were transformed or shifted to the following signals in the spectrum of PTX_2: δ 26.0(q), 80.7(s), 33.8(t) and 36.2(t) ppm. All other signals showed a good agreement within a range of 0.3 ppm. PMR spectrum of PTX_2 differed from that of PTX_1 only in the presence of a new methyl signal at δ 1.37(s) ppm and in the absence of signals assigned to C-43 protons (δ 3.52 and 3.56 ppm).

Pectenotoxin-3, -4 *and* -5. PTX_3 (10 mg), PTX_4 (7 mg) and PTX_5 (0.5 mg) were obtained as colorless solids. PTX_3: $[\alpha]_D^{20}$ +2.22 (*c* 0.135, MeOH); λ_{max} 235 nm (ε 11,000, MeOH), R_f in TLC 0.49. The secondary ionization mass spectrum gave an *m/z* 875 $(M^+ + H)$, indicating a formula $C_{47}H_{70}O_{15}$. PTX_4: $[\alpha]_D^{20}$ +2.07 (*c* 0.193, MeOH); λ_{max} 235 nm (ε 12,280, MeOH); R_f in TLC 0.53. The mass spectrum gave an *m/z* 875 $(M^+ + H)$, indicating a formula $C_{47}H_{70}O_{15}$. The mass spectrum of PTX_5 gave an *m/z* 877 $(M^+ + H)$, indicating a formula $C_{47}H_{72}O_{15}$. The IR spectrum lacked the absorption band at 1760 cm^{-1} attributable to C-14 ketone of PTX_1. The UV spectrum showed a maximum at 235 nm. The R_f value in TLC was 0.41.

Crystallographic analysis of pectenotoxin-1. The relative stereostructure of PTX_1 (IV) was secured by single crystal X-ray diffraction techniques. The crystals belonged to the monoclinic system with a = 31.551(5), b = 6.219(7), c = 25.350(3) Å and β = 97.78(1)°. Systematic extinctions, the presence of chirality and crystal density indicated space group C2 with one molecule of PTX_1 (IV) forming the asymmetric unit. All of the unique diffraction maxima with $2\theta \leq 114°$ were collected on an automated four circle diffractometer using a variable speed 1° ω-scan and graphite monochromated CuKα̅ radiation (1.54178Å). Of the 2581 reflections surveyed, 1905 (74%) were judged observed after correction for Lorentz, polarization and background effects. A phasing model was achieved using direct methods and a 26 atom partial structure was extended using tangent formula refinement. Block diagonal least squares refinements with the 62 anisotropic nonhydrogen atoms of PTX_1, 3 solvent oxygens from H_2O and 70 isotropic hydrogens have converged to the present residual of 0.113 for the observed reflections.

All crystallographic calculations were done on a PRIME 850 computer operated by the Cornell Chemistry Computing Facility. Principal programs employed were: REDUCE and UNIQUE, data reduction programs by M. E. Leonowicz, Cornell University, 1978; MULTAN 78, a system of computer programs for the automatic solution of crystal structures from X-ray diffraction data (locally modified to perform all Fourier calculations including Patterson syntheses) written by P. Main, S. E. Hull, L. Lessinger, G. Germain, J. P. Declercq and M. M. Woolfson, University of York, England, BLS78A, and anisotropic block diagonal least squares refinement written by K. Hirotsu and E. Arnold, Cornell University, 1980; 1970; PLUTO78, a crystallographic illustration program by W. D. S. Motherwell, Cambridge Crystallographic Data Centre, 1978; and BOND, a program to calculate molecular parameters and prepare tables written by K. Hirotsu, Cornell University, 1978. Additional crystallographic details are available.[19]

Okadaic acid in mussels. The mussel *Mytilus edulis* was collected at Kesennuma, Miyagi Prefecture, Japan, on 25 April 1981, when *D. acuminata* was relatively abundant in the sea (532 cells/L) but *D. fortii* was scarce (2.9 cells/L). The acetone extract from 165 g of the digestive glands was purified by the procedures employed for purification of DTX_1. Toxic residue (9.8 mg, > 8 mouse units) was dissolved in diethyl ether and methylated with diazomethane, trimethylsilylated with Tri-Sil "Z" for 30 min at 70°, and immediately analyzed by GLC as described previously, with reference TMS-derivatives of okadaic acid and DTX_1. The retention time of the

mussel toxin derivative was identical with that of okadaic acid derivative (7'54") but clearly differed from that of DTX_1 derivative (8'47").

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[19]Archival crystallographic data have been deposited with the Cambridge Crystallographic Data Centre and are available from them.