Pinnatoxin A: A Toxic Amphoteric Macrocycle from the Okinawan Bivalve *Pinna muricata*

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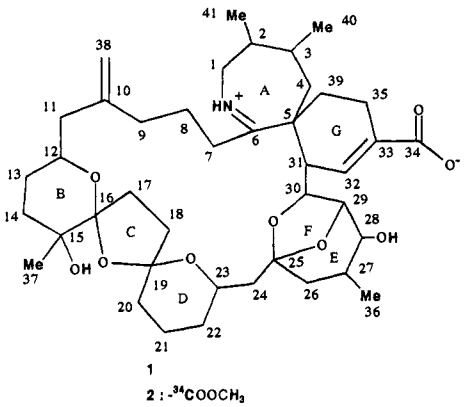
Shellfish of the genus *Pinna* live mainly in shallow areas of the temperate and tropical zones of the Indian and Pacific Oceans.[1] The adductor muscle of this bivalve is eaten in Japan and China, and food poisoning resulting from its ingestion occurs frequently.[2] Chinese investigators have reported that the toxic extract from *Pinna attenuata*, referred to as pinnatoxin, is a Ca^{2+} channel activator.[3] We report here the isolation of two toxins from *Pinna muricata*, viz. pinnatoxin A (**1**) and B,[4] and describe the gross structure of **1**, a novel polyether macrocycle with carboxylate and iminium functionalities.



1
2 : -^{34}COOCH$_3$

Viscera (45 kg) of *P. muricata* collected in Okinawa, Japan, were extracted with 75% ethanol. The extract was filtered, and the concentrated filtrate was washed with ethyl acetate and evaporated. The oily residue was successively chromatographed on TSK G-3000S polystyrene gel (Tosoh Co., Japan) (50% EtOH), Sephadex LH-20 (MeOH), DEAE Sephadex A-25 (0.02 N pH 6.9 phosphate buffer) and ODS-AQ (YMC, Inc., Japan) (50% MeOH), using a bioassay-guided (intraperitoneal mouse lethality) fractionation. Final purification was achieved by reverse-phase HPLC to give pinnatoxin A (**1**) [3.5 mg, LD_{99} 180 μg/kg; $[\alpha]_D$ 2.5° (*c* 0.32, MeOH); UV λ_{max} (EtOH) 216 nm] and B (1.2 mg, LD_{99} 22 μg/kg).

Table 1. NMR Data of Pinnatoxin A (**1**) (500 MHz, in CD_3OD)

position	δ_H	δ_C (mult)[a]	position	δ_H	δ_C (mult)[a]
1	4.27, 3.62	52.1 (t)	22	1.68, 1.26	32.5 (t)
2	1.70	39.6 (d)	23	4.04	70.8 (d)
3	1.40	35.3 (d)	24	1.99, 1.90	45.1 (t)
4	2.05, 1.63	36.0 (t)	25		110.4 (s)
5		51.8 (s)	26	1.72, 1.62	41.7 (t)
6		202.5 (s)	27	2.20	30.9 (d)
7	3.57	36.0 (t)	28	3.77	67.0 (d)
8	2.09, 1.96	21.8 (t)	29	4.58	81.6 (d)
9	2.06, 1.95	34.2 (t)	30	3.92	79.1 (d)
10		145.4 (s)	31	3.66	44.7 (d)
11	2.36, 2.18	46.5 (t)	32	6.42	134.0 (d)
12	4.09	69.6 (d)	33		135.8 (s)
13	1.68, 1.31	29.8 (t)	34		170.2 (s)
14	1.89, 1.53	35.5 (t)	35	2.55	23.0 (t)
15		71.2 (s)	36	1.03	16.9 (q)
16		113.4 (s)	37	1.23	22.9 (q)
17	2.20, 1.78	31.5 (t)	38	4.92, 4.85	112.6 (t)
18	2.04, 1.85	39.0 (t)	39	2.04, 1.91	33.7 (t)
19		109.8 (s)	40	1.07	21.0 (q)
20	1.87, 1.53	35.6 (t)	41	1.23	19.9 (q)
21	1.83, 1.66	21.4 (t)			

[a] Multiplicity determined with DEPT experiment.

The molecular formula of pinnatoxin A was found to be $C_{41}H_{61}NO_9$ (MH^+, *m/z* 712.4444 Δ 2.0 mmu) by HRFABMS. Detailed analysis of ^{1}H-NMR, ^{13}C-NMR, DEPT, and HSQC spectra (Table 1)[5] showed that **1** contained four methyl groups, 18 methylenes, 10 methines, nine quaternary carbons, and three protons on heteroatoms.[6] Isotope shifts in ^{13}C-NMR signals, as observed by the chemical shift differences in CD_3OD and CD_3OH solutions, led to the identification of hydroxyl-bearing carbons; significant shifts were observed for C15 (quaternary carbon) ($\Delta\delta$ = 0.13 ppm) and C28 ($\Delta\delta$ = 0.10 ppm),[7] indicating the presence of two hydroxyl groups. One group was in a secondary alcohol, and this assignment was supported by a marked downfield shift (H-28, δ_H 3.64 to δ_H 5.15 ppm) in *p*-bromobenzoate, which was obtained by treatment of methyl ester **2** (vide infra) with *p*-bromobenzoyl chloride/DMAP. The third exchangeable proton was in an iminium group. In addition to supporting the presence of the hydroxyl groups (3420 cm^{-1}), the IR (KBr) spectrum of **1** showed absorption bands for carboxylate (1590 cm^{-1}) and iminium (1680 cm^{-1}) groups. ^{13}C signals at 170.2 (C34) and 202.5 ppm (C6)[8] were consistent with the presence of carboxylate and iminium functionalities in this highly water-soluble compound. The simplification of the HPLC peak pattern with the addition of trifluoroacetic acid to the eluant suggested that **1** may be amphoteric. More rigorous proof was obtained by treatment of **1** with diazomethane, which gave methyl ester **2** (EIMS, *m/z* 725, M^+). ^{13}C signals were found at 166.0 (C34) and 172.7 ppm (C6) for the methyl ester carbonyl and imine carbons, respectively, in **2**.

The proton connectivities of the components described above were elucidated by detailed analyses of data obtained from DQF-

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(1) Rosewater, J. *Indo-Pac. Mollusca* **1961**, *1*, 53/501−53/632.

(2) Human intoxication due to ingestion of *Atrina pectinata* (or *Pinna pectinata*) occurred at Fukuoka, Japan, in October 1975; 1730 people were affected. The poisoning was believed to have originated with *Vibrio* sp. However, some of the symptoms of the patients suggested the presence of a neurotoxin. Despite preventive measures, 950 people were affected by food poisoning of the same origin in October 1980. (a) Otofuji, T.; Ogo, A.; Koishi, J.; Matsuo, K.; Tokiwa, H.; Yasumoto, T.; Nishihara, K.; Yamamoto, E.; Saisho, M.; Kurihara, Y.; Hayashida, K. *Food Sanit. Res.* **1981**, *31*, 76−83. (b) Department of Public Health, Fukuoka Prefecture. *Food Sanit. Res.* **1976**, *26*, 11−20.

(3) Human intoxication resulting from *P. attenuata* occurred at Guangdong, China, in 1980 and February 1989. Chinese investigators have conjectured that the disease may be attributable to neurotoxins contained in *Pinna* shellfish, based on its predominant human symptoms, i.e., diarrhea, paralysis, and convulsion. Zheng, S. Z.; Huang, F. L.; Chen, S. C.; Tan, X. F.; Zuo, J. B.; Peng, J.; Xie, R. W. *Zhongguo Haiyang Yaowu (Chin. J. Mar. Drugs)* **1990**, *33*, 33−35.

(4) We will report our finding regarding pinnatoxin B very soon elsewhere.

(5) The numbering system will take into consideration the biogenesis of the final structure **1** described later.

(6) Three protons (δ_{NH} 11.70, δ_{OH} 4.78, δ_{OH} 3.72 ppm) exchangeable with D_2O were observed in the ^{1}H-NMR (400 MHz, DMSO-d_6) spectrum of **1**.

(7) ^{13}C-NMR signals of the carbinol carbon showed significant shifts ($\Delta\delta$ = 0.10−0.13 ppm) between CD_3OD and CD_3OH solutions, while the other ether-bearing carbon signals were superimposable within 0.04 ppm. Pfeffer, P. E.; Valentine, K. M.; Parrish, F. W. *J. Am. Chem. Soc.* **1979**, *101*, 1265−1274.

(8) In the ^{13}C-NMR spectrum of the Schiff base between camphor and benzylamine, the carbon signal of the imine group was observed at 183.6 ppm, whereas this signal in the corresponding iminium salt was shifted to 202.8 ppm.

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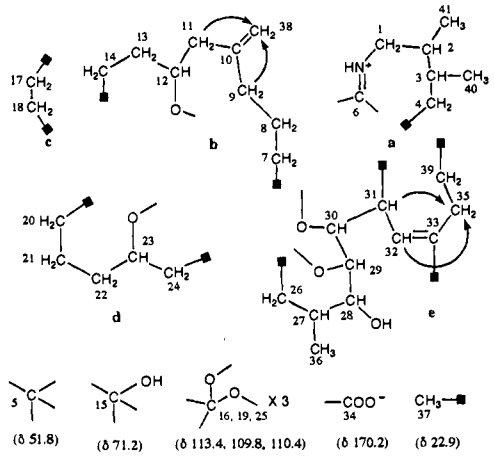

Figure 1. Segments of pinnatoxin A including five partial structures (**a**–**e**) determined by 2D-NMR (DQF-COSY, HOHAHA, HSQC-HOHAHA) spectroscopy (arrows, allylic or homoallylic coupling; ■, quaternary carbon).

COSY, HOHAHA, and HOHAHA-HSQC[9] experiments. Eventually, five partial structures (**a**–**e**) were obtained, as shown in Figure 1. Cross peaks due to long-range coupling via sp^2 carbons included H-38/H-9, H-38/H-11, H-32/H-35, and H-31/H-35.

Furthermore, HMBC techniques revealed that these fragments were linked through cross peaks due to $^2J_{CH}$, $^3J_{CH}$ long-range coupling with quaternary carbons.[10,11] The nitrogen-bearing methylene carbon was considered C1 because of its reasonable chemical shift (δ_C 52.1, δ_H 4.28, 3.62). The HMBC NMR correlation between H-1/C6, H-4/C6, and H-4/C5 indicated that a seven-membered ring (A-ring) included C1–C6 and the nitrogen atom. In addition, the A-ring was linked to the G-ring to make a 6,7-spiro ring, as suggested by the HMBC NMR correlation of H-30/C5, H-32/C5, H-39/C5, and H-4/C31. Long-range coupling between H-32/C34 was consistent with the presence of an α,β-unsaturated carboxylate, which was also supported by UV absorption at 216 nm. Moreover, methyl ester **2** showed homoallylic coupling between H-1/H-7 and long-range coupling between H-7/C6, H-4/C6, and H-1/C6.[12] This observation confirmed the assignment of C6 as the carbon (C=N) of the imine group. Consequently, connectivity among partial structures **a**, **b**, and **e** was clarified. The HMBC data also suggested that the tertiary methyl group (C37) was linked to the carbon (C15) which bore the tertiary hydroxyl group. This two-carbon unit correlated to partial structures **b** and **c** through the quaternary carbon (C16) assigned to 113.4 ppm, as suggested by the cross peak in the HMBC spectrum.[11] Partial structures **c** and **d** were also connected through a quaternary carbon (109.8 ppm, C19). The final quaternary carbon (110.4 ppm, C25) was incorporated between partial structures **d** and **e**.[11] The entire carbon chain was assembled as shown in structure **1**, and all protons and carbons were assigned as shown in Table 1.

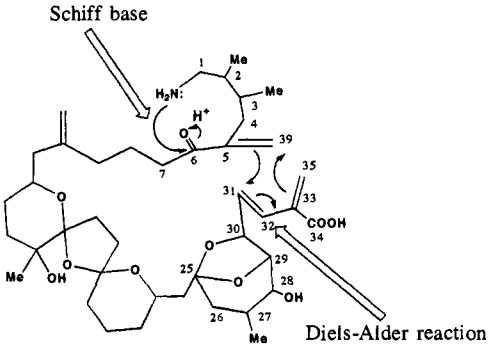

Figure 2. Plausible biogenetic pathway for the carbon framework of pinnatoxin A.

The degree of unsaturation based on the molecular formula and the structural features (two CC double bonds, a carboxylate, an iminium group, and three rings) described above suggested the presence of five ether rings. The three ketal carbons assigned as C16 (113.4 ppm), C19 (109.8 ppm), and C25 (110.4 ppm) were relatively downfield, which suggested that they may be involved in these five-membered ether structures.[13] Two ether rings were considered a 5,6-bicyclo system formed by rings E and F, based on the long-range coupling correlation of H-29/C25 and the coupling constants (J_{26a-27} = 10.0 Hz, J_{27-28} = 4.0 Hz, J_{28-29} = 2.0 Hz, J_{29-30} = 4.6 Hz) of the ring protons. The remaining three ether rings were necessarily recognized as a 6,5,6-trispiroketal moiety formed by the six-membered B ring (J_{12-13a} = 10.0 Hz, $J_{13a-14a}$ = 10.0 Hz), the five-membered C ring (J_{17-17} = −13.0 Hz, J_{17-18} = 3.0, 6.5 Hz),[14] and the D ring.

All of the data above conclusively led to the gross structure of pinnatoxin A (**1**), a new potent shellfish toxin. This unique structure, which includes a 6,7-spiro ring (A and G rings), can be explained by the plausible biogenetic pathway shown in Figure 2.[15] Extracts from the digestive glands of several *Pinna* sp., including *P. muricata*, *P. attenuata*, and *P. atropupurea*, and the commonly eaten shellfish *Atrina pectinata* produced the same symptoms of poisoning in mice. These data suggest that *Pinna* shellfish may become toxic as the result of feeding on toxic organisms such as dinoflagellates.[15]

Acknowledgment. We are grateful to JEOL for measuring 500 and 600 MHz NMR spectra and to Dr. H. Naoki, Suntory Institute for Bioorganic Research, for performing HRFABMS measurements. This work was financially supported by Ono Pharmaceutical Co., Fujisawa Pharmaceutical Co., and Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

Supplementary Material Available: ^1H, ^{13}C-NMR, DEPT, 2D DQF-COSY, HOHAHA, HSQC, and HMBC of **1** and **2** and HSQC-HOHAHA of **1** (34 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(9) All of the spectra were measured at 35 °C in CD_3OD and C_6D_6 using JEOL A500. In the HSQC-HOHAHA spectrum, cross peaks were successfully observed between C9/H-7, C22/H-23, C22/H-21, and C22/H-20, and the H–H connectivities of the methylenes were determined. However, it was not possible to assign each proton of the methylenes using the DQF-COSY and HOHAHA spectra. Davis, D. G.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 2820–2821.

(10) Sumners, M. F.; Marzilli, L. G.; Bax, A. *J. Am. Chem. Soc.* **1986**, *108*, 4285–4294.

(11) In the HMBC spectra of **1** (CD_3OD, $^2J_{CH}$, $^3J_{CH}$ = 8 Hz) and **2** (C_6D_6, $^2J_{CH}$, $^3J_{CH}$ = 8 Hz, JEOL A600), the connectivities around the quaternary carbons (C15, C16, C19, C25) were clarified by observation of cross peaks due to $^2J_{CH}$ and $^3J_{CH}$ between C15/H-13, C15/H-14, C15/H-37; C16/H-14, C16/H-17, C16/H-18, C16/H-37; C19/H-17, C19/H-18, C19/H-20, C19/H-21; C25/H-24, C25/H-26, and C25/H-29.

(12) Homoallylic coupling between H-1/H-7 and long-range coupling between H-7/C6 were not observed in the NMR data of pinnatoxin A (**1**). The reason may be mainly attributable to deuterium exchange on C7 in CD_3OD of **1**.

(13) The 5,6-spiroketal carbon signal is usually assigned near 110 ppm [Okadaic acid (107.8 ppm): Tachibana, K.; Scheuer, P. J.; Tsukitani, Y.; Kikuti, H.; Engen, D. V.; Clardy, J.; Gopichand, Y.; Schmitz, F. J. *J. Am. Chem. Soc.* **1988**, *110*, 7877–7878. Portmicin (109.3 ppm): Seto, H.; Furihata, K.; Saeki, K.; Otake, N.; Kusakabe, Y.; Xu, C. F.; Clardy, J. *Tetrahedron Lett.* **1987**, *28*, 3357–3360.], in constrast to that of the 6,6-spiroketal carbon, which is assigned near 100 ppm [Aplysiatoxin (98.0 ppm): (a) Kato, Y.; Scheuer, P. J. *J. Am. Chem. Soc.* **1974**, *96*, 2245. (b) Moore, R. E.; Blackman, A. J.; Cheuk, C. E.; Mynderse, J. S.; Matsumato, G. K.; Clardy, J.; Woodward, R. W.; Craig, J. C. *J. Org. Chem.* **1984**, *49*, 2484–2489.].

(14) Davies, D. B.; Danyluk, S. S. *Biochemistry* **1974**, *12*, 4417–4434.

(15) This biogenesis does not involve the sequence of oxidation steps. Interestingly, the carbon skeleton of prorocentrolide (Torigoe, K.; Murata, M.; Yasumoto, T. *J. Am. Chem. Soc.* **1988**, *110*, 7877–7878) may also be formed by the biogenetic pathway described here.