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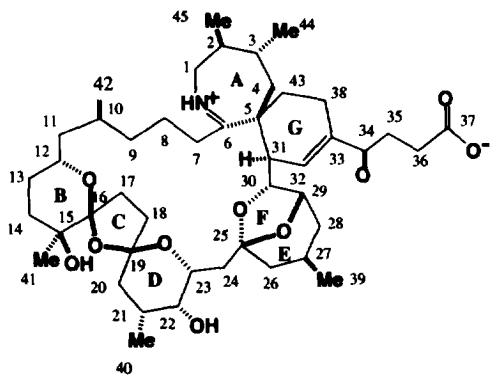
Isolation and Structure of Pinnatoxin D, a New Shellfish Poison from the Okinawan Bivalve *Pinna muricata*

Tong Chou, Takeharu Haino, Makoto Kuramoto† and Daisuke Uemura*†

Sagami Chemical Research Center, Nishi-Ohnuma 4-4-1, Sagamihara, Kanagawa 229, Japan
†Department of Chemistry, Faculty of Science, Shizuoka University, Ohya, Shizuoka 422, Japan

Abstract : A new shellfish poison, pinnatoxin D, has been isolated from the Okinawan bivalve *Pinna muricata*. The planar structure was elucidated by extensive 2D NMR experiments, and the relative stereochemistry was deduced by NOESY data and $^{3}J_{H\text{-}H}$ coupling constants. Pinnatoxin D is a novel amphoteric carbocyclic compound which is composed of a 6,7-spiro ring, a 5,6-bicyclo ring and a 6,5,6-trispiro ketal involving 15 chiral centers. Copyright © 1996 Elsevier Science Ltd

Marine toxins have attracted the attention not only of chemists but also of pharmacologists and biochemists due to their novel chemical structures and potent bioactivities; e.g., brevetoxin,[1] palytoxin,[2] ciguatoxin[3] and maitotoxin.[4] In our continuing search for physiologically active substances from marine sources,[5] with particular focus on marine toxins, we have recently found a group of new potent shellfish poisons; pinnatoxins[6] from the *Pinna* genus. This bivalve is a common seafood in Japan and China, and human intoxication resulting from its ingestion occurs frequently. In this report, we describe the structural elucidation, including the relative configurations, of pinnatoxin D (**1**), one of the principal toxins in a series of pinnatoxins.[7]



1

Pinnatoxin D (**1**) was obtained as a colorless solid[8]: LD99 0.4 mg/kg (mouse ip.); $[\alpha]_D$ 42.5° (*c* 0.5, MeOH); UV λ_{max} (MeOH) 226 nm. The molecular formula, $C_{45}H_{67}NO_{10}$, was established by HRFABMS (MH^+, *m/z* 782.4845 Δ 2.0 mmu) and NMR data.[9] The ^{13}C and DEPT spectra indicated 45 distinct resonances, including 3 carbonyl equivalents (>C=N$^+$H- at 203.3 ppm,[10] >C=O at 200.0 ppm, -COO$^-$ at 176.8 ppm), 4 olefinic carbons (>C=CH_2 at 145.3 and 112.6 ppm, -CH=C< at 136.0 and 141.4 ppm), 9 oxygenated carbons (3 ketal carbons at 113.7, 110.1 and 110.6 ppm; 5 oxymethine carbons at 67-78 ppm and one quaternary carbon bearing oxygen at 71.6 ppm), and one nitrogen-bearing methylene (-CH_2-N< at 52.0 ppm,δ_H 4.28, 3.62). The remaining highfield signals were assigned to 5 methyls, 17 methylenes, 5 methines and one quaternary carbon. The significant difference in the ^{13}C chemical shifts of only the carbinol carbon in CD_3OD and CD_3OH suggested the location of two hydroxyl-bearing carbons (C15 and C22). The high field shift at C1 (0.06 ppm) in CD_3OH suggested that C1 was adjacent to the iminium group. Eventually, we determined that **1** is composed of 5 x CH_3, 19 x CH_2, 11 x CH, 10 quaternary carbons and three protons on heteroatoms (2 x OH, 1 x N$^+$H). Detailed analysis of the DQF-COSY, HOHAHA,[11] HMQC[12] and HOHAHA-HMQC spectra of **1** gave six partial structures **a-f** (Fig.1, in boldface). The HOHAHA-HMQC technique

confirmed the distinct connectivities of C7-C9 methylenes, whose protons resonated close to each other. The connectivity of C9 and C11 via C10 was determined by the observation of allylic coupling correlations (H9/H42, H11/H42) in DQF-COSY; while C32 and C38 were connected via C33 based on allylic or homoallylic coupling crosspeaks, H32/H38 and H31/H38.

$^{2}J_{CH}$, $^{3}J_{CH}$ long-range coupling correlations in the PFG-HMBC[13] spectrum of **1** were used to assemble the partial structural units through quaternary carbons. The HMBC crosspeaks, H1/C6, H4/C6, H4/C5 and H7/C6,[14] suggested the presence of a 7-membered ring (A-ring), consisting of C1-C6 and a nitrogen atom, and that C7 was connected to the A ring through C6 (δ_C 203.3), which in turn was assigned as the carbon of the imine group. HMBC crosspeaks, H30/C5, H32/C5, H38/C5 and H43/C5, suggested the presence of a cyclohexene ring (G ring), and revealed that the **a** and **e** units were connected via C5 to form a 6,7-azaspiro structure. HMBC crosspeaks, H32/C34, H35/C34 and H36/C34, connected the **e** and **f** units through the ketone carbonyl carbon C34 to form the α, β-unsaturated ketone, which was also supported by UV absorption at 226 nm. The location of carboxyl carbon C37 was determined by H36/C37 and H35/C37 long-range coupling correlations. Based on the HMBC correlations, H14/C15, 41-Me/C15 and 41-Me/C14, a hydoxyl-bearing singlet carbon C15 was located adjacent to C14 methylene and was also alkylated by 41-Me. The connectivities of **b** to **c**, **c** to **d** and **d** to **e** through the three singlet ketal carbons (C16, C19 and C25) were respectively clarified by the observation of HMBC crosspeaks: H14/C16, H17/C16 and 41-Me/C16; H17/C19, H18/C19 and H20/C19; H24/C25 and H26/C25. Eventually, the linkage of the entire carbon skeleton was achieved as a macrocycle, as shown in Fig. 1.

The molecular formula of **1** required 13 degrees of unsaturation. Excluding the five double bonds, a 6,7-spiro ring and the macrocycle skeleton, the remaining 5 degrees of unsaturation were accounted for by the presence of five ether rings. Larger coupling constants ($^{3}J_{26Hb\text{-}27H}$=11.0 Hz, $^{3}J_{27H\text{-}28Hb}$=10.0 Hz, $^{3}J_{12H\text{-}13Ha}$=11.0 Hz, $^{3}J_{13Ha\text{-}14Hb}$=10.0 Hz and $^{3}J_{20Ha\text{-}21H}$=9.0 Hz) suggested that B, D and E were 6-membered rings with a chair conformation. The relative downfield assignment for the three ketal carbons (approximately 110 ppm) suggested that they were involved in 5-membered ketal linkage structures. Among the five ether rings, two were composed of a 5,6-bicyclo system formed by C25-C30, and the remaining three rings were readily recognized as a 6,5,6-trispiroketal skeleton formed by C12-C23.

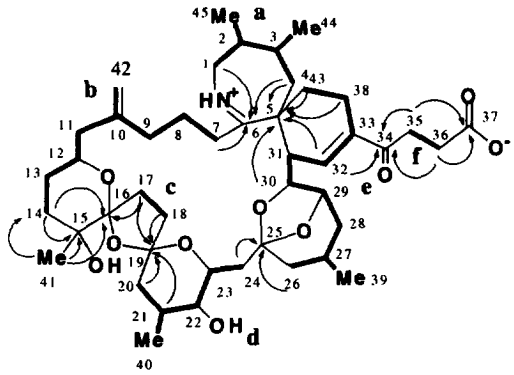


Fig. 1. Connectivities established by 2D-NMR (600 MHz) spectroscopy of **1**.
(Bold line : DQF-COSY, HOHAHA and HMQC-HOHAHA; ⟶ : HMBC.)

The relative stereochemistry of the 5,6-bicyclo, 6,7-azaspiro and 6,5,6-trispiroketal moieties involving 15 chiral centers was established by $^{3}J_{H\text{-}H}$ coupling constants and NOEs correlations from NOESY data. Only one possible stereochemistry of **1** (Fig. 2) can explain the NOE data observed in this high-performance NOESY spectrum.[15] The large $^{3}J_{H26b\text{-}H27}$ and $^{3}J_{H27\text{-}H28b}$ values and NOESY crosspeaks between 39-Me/H26b and between 39-Me/H28b indicated that 39-Me at C27 is in an equatorial position. In support of a chair conformation for ring E ($^{3}J_{H26b\text{-}H27}$=10.0 Hz, $^{3}J_{H26a\text{-}H27}$=5.5 Hz, $^{3}J_{H27\text{-}H28b}$=10.0 Hz, $^{3}J_{H27\text{-}H28a}$=4.0 Hz),

H-29 was considered to be equatorial ($^3J_{H28a-H29}$=2.3 Hz, $^3J_{H28b-H29}$=3.0 Hz). The obvious double doublet of H30 with $^3J_{H29-H30}$=4.5 Hz and $^3J_{H30-H31}$=11.0 Hz suggested that H30 is an equatorial proton at C30 and that there is an anti-relationship between H30 and H31. The significant NOEs between H28/H32, H29/H32, H30/H32; H27/H31 and H31/H43a deduced the relationship between F ring and G ring though C30 and C31. Since rings E and F are in a bicyclo system, the relative stereochemistry of C25 is necessarily suggested. In addition, the configuration at C5 was assigned based on NOEs correlations, H4b/H30, H3/H30, 44-Me/H30 and 7-CH_2/H31. The large $^3J_{H2-H3}$ value and NOESY crosspeaks, H2/H1a, H2/H1b, H2/44-Me, H1a/45-Me, H3/H4b, H3/45-Me, H4b/44-Me and H1b/H4a suggested a trans configuration between H2 and H3.

In the 6,5,6-trispiro portion, H12 was considered axial because of its large $^3J_{H12-H13a}$[16] values and NOEs, H12/H14b. 41-Me was considered to be axial at C15 based on NOESY crosspeaks, 41-Me/H13a and 41-Me/H14a. The NOEs correlations between the 41-Me group and the methylene at C17 (H17a and H17b) revealed that the oxygen atom of the 5-membered ether ring (ring C) was located in an axial position at C16 to form a thermodynamically stable spiro ketal. On the other hand, the large coupling constant ($^3J_{H20a-H21}$=9.0 Hz) suggested that H21 is an axial proton and that 40-Me is equatorial at C21. The broad singlet attributed to H22 at δ 3.40 ppm ($^3J_{H21-H22}$=0.5 Hz, $^3J_{H22-H23}$=1.5 Hz) and NOEs H23/H21 suggested that H23 is axial and that H22 is equatorial. The oxygen in ring C at C19 was considered to have an axial configuration based on the observation of NOESY crosspeaks between 18-CH_2 and H20a. The configuration at C16 and C19 were also supported by the fact that significant NOEs were not observed between H12 and 17-CH_2 or between H23 and 18-CH_2.

The stereochemical connectivities between the 5,6-bicyclo, 6,7-azaspiro moiety (rings E, F, A and G) and the 6,5,6-trispiroketal moiety(rings B, C and D) were determined by NOESY crosspeaks: H12/45-Me(between rings A and B), H14b/45Me(between rings A and B), H22/44Me(between rings A and D); and H22/H30 (between rings D and F). Eventually, all of the relative configurations of **1**, which is a rigid carbomacrocycle involving 15 chiral centers, were elucidated, as shown in Fig. 2.

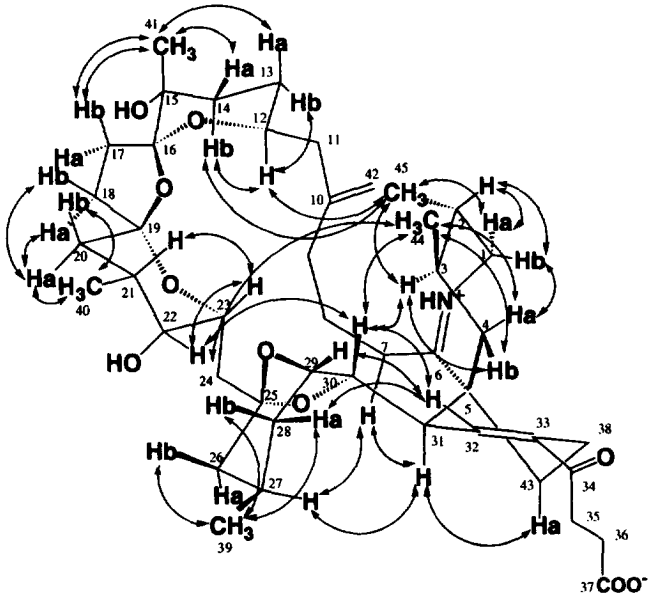


Fig. 2 Stereoview of pinnatoxin D (**1**).

Like pinnatoxin A,[6] the major toxin from *Pinna muricata*, pinnatoxin D is a novel amphoteric macrocyclic compound composed of 6,7-azaspiro, 5,6-bicyclo and 6,5,6-trispiro ketal rings. However, in

contrast with pinnatoxin A, pinnatoxin D has three more carbon units (C34-C36) and different functionalities at C28, C22 and C21. The structure-activity relationships of pinnatoxin A and D remain to be elucidated.

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7. Pinnatoxins B and C are the most potent toxins in this series. Their structures will be reported soon.
8. The crude 80% EtOH extract of viscera (45kg) of *P. muricata* (10,000) was partitioned between EtOAc and water. The water layer was purified by column chromatography on TSK G-3000S polystyrene gel (Tosoh Co., Japan) (50% EtOH), Sephadex LH-20 (MeOH), DEAE Sephadex A-25 (0.02M 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 (60% MeOH/0.1%TFA; 30 % CH_3CN/0.1% TFA) to give 2.0 mg pinnatoxin D (**1**).
9. ^{1}H-NMR data of **1** (600 MHz in CD_3OD, δppm, *J* in Hz) : 3.62 (d, J_{gem}=13.3, H-1a), 4.22 (dd, J_{gem}=13.3, J_{H1a-H2}=4.9, H-1b), 1.68 (m, J_{H2-H3}=11, H-2), 1.34 (m, H-3), 2.06 (dd, H-4a), 1.62 (d, J_{gem}=13.8, H-4b), , 3.68 (br.dd, H-7), 3.68 (br.dd, H-7), 2.14 (m, H-8a), 1.96 (m, H-8b), 1.98 (m, H-9a), 2.08 (m, H-9b), 2.18 (dd, J_{gem}=13.0, $J_{H11a-H12}$=10.0, H11a), 2.38 (dd, J_{gem}=13.0, $J_{H11b-H12}$=3.0, H-11b), 4.05 (m, $J_{H12-H13a}$=11.0, $J_{H12-H13b}$=1.6, H-12), 1.32 (m, $J_{H13a-H14b}$ =10.0, H-13a), 1.68 (m, H-13b), 1.52 (m, H14a), 1.88 (m, H-14b), 2.22 (ddd, J_{gem}=13.0, H-17a), 1.80 (ddd, $J_{H17b-H18a}$=6.5, $J_{H17b-H18b}$=3.5, H-17b), 2.14 (ddd, H-18a), 1.88 (ddd, H-18b), 1.67 (d, $J_{H20a-H21}$=9.0, H-20a), 1.67 (H-20b), 2.08 (m, $J_{H21-H22}$=0.5, H-21), 3.04 (dd, $J_{H22-H23}$=1.5, H-22), 4.09 (m, $J_{H23-H24a}$=11, $J_{H23-H24b}$=3.7, H-23), 1.95 (H-24a), 2.24 (dd, J_{gem}=14.0, H-24b), 1.94 (dd, $J_{H26a-H27}$=5.5, J_{gem}=14.0, H-26a), 1.44 (dd, J_{gem}=14.0, $J_{H26b-H27}$=10.0, H-26b), 2.25 (m, H-27), 2.08 (m, $J_{H28a-H27}$=4.0, $J_{H28a-H29}$=2.3, H-28a), 1.64 (ddd, J_{gem}=13.0, $J_{H28b-H27}$=10.0, $J_{H28b-H29}$=3.0, H-28b), 4.80 (br. ddd, H-29), 3.98 (dd, $J_{H30-H29}$= 4.5, $J_{H30-H31}$=11.0, H-30), 3.78 (br.dd, $J_{H31-H32}$=0.5, H-31), 6.42 (br. d, H-32), 2.94 (m, H-35a), 3.00 (m, H-35b), 2.62 (m, H-36a), 2.62 (m, H-36b), 2.46 (m, H-38a), 2.56 (m, H-38b), 1.01 (d, $J_{39Me-H27}$ =7.0, 39-Me), 0.98 (d, $J_{40Me-H21}$=7.0, 40-Me), 1.25 (s, 41-Me), 4.85 (s, H-42a), 4.95 (s, H-42b), 1.92 (m, H-43a), 2.08 (m, H-43b), 1.08 (d, $J_{44Me-H3}$=7.0, 44-Me), 1.19 (d, $J_{45Me-H2}$=7.0, 45-Me).
^{13}C-NMR data of **1** (125 MHz in CD_3OD, δppm) : 52.0 (t, C-1), 39.5 (d, C-2) 35.7 (d, C-3), 36.3 (t, C-4), 52.1 (s, C-5), 203.3 (s, C-6), 35.3 (t, C-7) 21.6 (t, C-8), 33.6 (t, C-9), 145.3 (s, C-10), 46.5 (t, C-11), 69.6 (d, C-12), 29.8 (t, C-13), 35.6 (t, C-14), 71.6 (s, C-15), 113.7 (s, C-16), 32.6 (t, C-17), 38.8 (t, C-18), 110.1 (s, C-19), 37.6 (t, C-20), 32.4 (d, C-21), 69.9 (d, C-22), 73.2 (d, C-23), 40.5 (t, C-24), 110.6 (s, C-25), 45.0 (t, C-26), 26.0 (d, C-27), 33.6 (t, C-28), 77.1 (d, C-29), 80.1 (d, C-30), 45.6 (d, C-31), 136.0 (d, C-32), 141.4 (s, C-33), 200.0 (s, C-34), 33.2 (t, C-35), 22.7 (t, C-36), 176.8 (s, C-37), 33.2 (t, C-38), 22.7 (q, C-39), 18.4 (q, C-40), 23.1 (q, C-41), 112.6 (t, C-42), 33.8 (t, C-43), 21.4 (q, C-44), 20.0 (q, C-45).
10. In the ^{13}C-NMR spectrum of Schiff base, the carbon signal of iminum salt was observed approximately 200 ppm downfield.
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14. As in pinnatoxin A,[6] homoallylic coupling correlation between H1/H7 and hetero long-range coupling correlation between H7/C6 were observed in the spectra of the corresponding methyl ester.
15. The NOESY spectrum of 1 was acquired in CD_3OD with mixing times of 400ms.
16. The *J* value was assigned using a triple resonance homodecoupling technique and a homodecoupling difference spectrum.

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