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NOTE

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Pinnarine, Another Member of the Halichlorine Family. Isolation and Preparation from Pinnaic Acid

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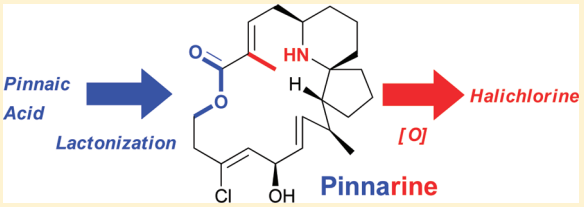
Supporting Information

ABSTRACT: Pinnarine (**1**), a new macrocyclic alkaloid, was isolated from the black marine sponge *Halichondria okadai*. The structure was elucidated on the basis of 2D NMR and comparison with the spectra of the co-isolated known halichlorine. Further confirmation of the structure and the absolute configuration was validated by a synthetic method from authentic pinnaic acid and CD analysis. The isolation of pinnarine also suggested a biogenetic pathway from pinnaic acid to halichlorine.



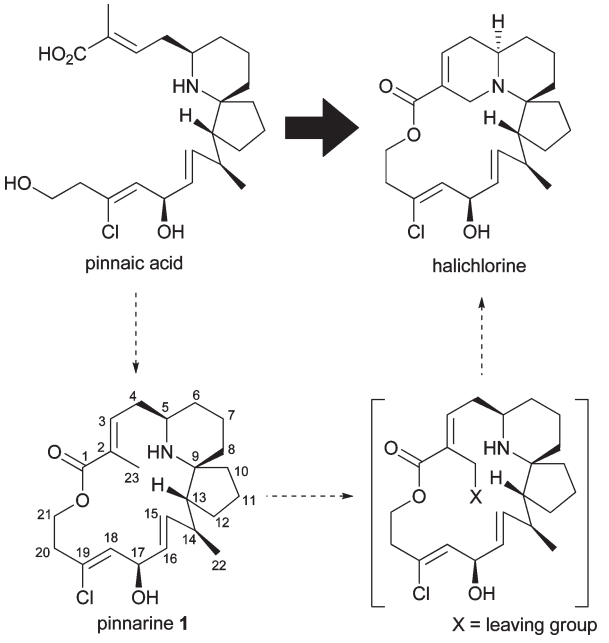
In 1996, we reported the isolation of a novel class of marine natural products represented by pinnaic acid[1] and halichlorine[2] (Scheme 1). Pinnaic acid was obtained from the Okinawan bivalve *Pinna muricata* (collected in Okinawa Island, Japan). It inhibits a cytosolic 85 kDa phospholipase ($cPLA_2$)[3] *in vitro*. Compounds that inhibit $cPLA_2$ activity have been targeted as anti-inflammatory agents. Halichlorine was obtained from the black marine sponge *Halichondria okadai* Kadota (collected at the intertidal zone in Kanagawa prefecture, Japan) and shows bioactivity for reducing the expression of vascular cell adhesion molecule-1 (VCAM-1)[4] and monocyte adhesion to endothelial cells by attenuating NF-κB activity,[5] which may be useful for treating atherosclerosis. Halichlorine also inhibits L-type Ca^{2+} channels in vascular smooth muscle cells, which makes it promising as an antihypertensive agent.[6]

Even more impressive than their bioactivities are the architectural 6-aza-spiro[4.5]decane structures of these two molecules. They have attracted considerable attention in the synthetic chemistry community. They have been the topic of a specific review[7a] and a large number of papers describing total syntheses,[7b−h] formal syntheses, and model studies.

On the basis of the obvious structural similarity of pinnaic acid and halichlorine, it was automatic for us to propose the biogenetic relationship between them.[2a,8] However, it must be noted that the two molecules were isolated from two different marine organisms from different locations (more than 1500 km away). Also, the absolute configuration of pinnaic acid is so far not confirmed[9] due to the limited amount of the isolated sample.[10]

To prove the biogenetic relationship of these two compounds, we initiated a search for the possible biosynthetic intermediates between pinnaic acid and halichlorine from various marine organisms. Because the intermediates should exist in a very limited amount, every interesting fraction was isolated one by one from a large number of extracts. After extensive work and numerous NMR analyses, to our delight, a new alkaloid, pinnarine (**1**, Scheme 1), was isolated from the black marine sponge *H. okadai* Kadota according to the following procedure. The

Scheme 1. Plausible Biosynthetic Pathway from Pinnaic Acid to Halichlorine



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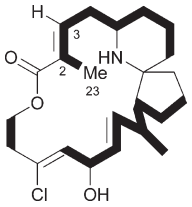


Figure 1. Gross structure of pinnarine **1**. (Bold lines: ^{1}H−^{1}H COSY correlations, $CDCl_3$, 800 MHz).

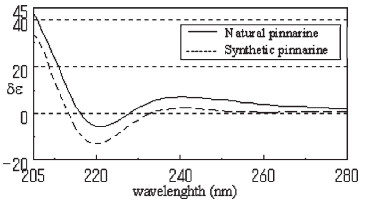
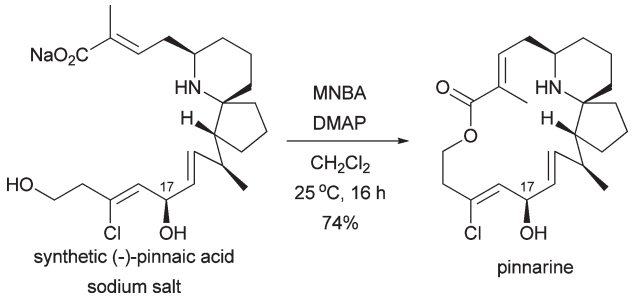


Figure 2. Comparison of CD spectra of natural and synthetic pinnarine.

Scheme 2. Synthesis of Pinnarine from Pinnaic Acid Sodium Salt[a]



[a] Conditions: 2-methyl-6-nitrobenzoic anhydride (MNBA, 3 equiv), 4-(dimethylamino)pyridine (DMAP, 8 equiv), conc = 0.0014 M.

MeOH extract of 80 kg of sponge was partitioned between EtOAc and H_2O. The EtOAc layer was fractionated by column chromatography on TSK G3000S polystyrene gel using a gradient elution of EtOH and H_2O. The fraction eluted with 40% aqueous EtOH was subjected to Develosil NH_2 silica gel column chromatography and then silica gel NH_2 F_{254S} preparative TLC to give pinnarine (0.5 mg, 6×10^{-7}% isolated yield).

The ^{1}H and ^{13}C NMR data of pinnarine resembled those for halichlorine except for the displacement of the C-23 methylene group to a methyl group adjacent to the C-2 atom (proved by a ^{1}H−^{1}H COSY correlation with H-3, Figure 1).[11] The molecular formula of pinnarine was deduced to be $C_{23}H_{34}ClNO_3$ by HRESIMS (m/z 408.2305, $[M + H]^+$, $C_{23}H_{35}{}^{35}ClNO_3$), which is 2 H more than halichlorine. Moreover, from the extract, we also isolated halichlorine (3.8 mg), which is completely identical to our previously isolated sample.[2] Thus, the structure of pinnarine was assigned as **1**.

Because of the limited amount of pinnarine sample, further NMR studies did not provide additional proof of the structure. Therefore, synthesis of structure **1** was carried out by macrolactonization of the authentic chiral pinnaic acid sodium salt, which was prepared according to our previous total synthesis route.[7f,12] Under the MNBA-DMAP condition[13] (Scheme 2), the macrolactonization proceeded smoothly at room temperature to give **1** in 74% yield. Neither dimer nor macrolactone at the C17 hydroxy group was detected. The ^{1}H and ^{13}C NMR data and the chromatographic behavior of synthetic **1** fully matched those of naturally occurring pinnarine, which confirms our proposed structure.

With chiral pinnarine in hand ($[\alpha]^{12}_D$ = +27.7 (c 0.065, MeOH)), we compared the CD spectra of both natural and synthetic pinnarine (Figure 2). The shapes and signs of both samples also matched each other. Thus, the naturally occurring pinnarine was determined to contain the same absolute configuration as halichlorine.

From a biosynthetic point of view, halichlorine should exist at a later stage on the biosynthetic pathway than pinnaic acid. There must be at least three steps between them (Scheme 1): (1) macrolactonization, (2) oxidation of the C-23 atom, and (3) bond formation between C-23 and the nitrogen atom. However, the sequence of those biosynthetic steps is not clear. The isolation of pinnarine **1** and the facile macrolactonization of pinnaic acid imply that the macrolactonization is more likely to be the first transformation on the biosynthetic pathway.

In summary, we succeeded in the isolation, structure elucidation, and synthesis of pinnarine **1**, which is a key intermediate allowing us to propose the biogenetic hypothesis of this class of natural products. It is also a further hint for the undetermined absolute configuration of naturally occurring pinnaic acid.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 polarimeter. UV spectra were recorded on a JASCO V-560 UV−vis spectrometer. CD spectra were recorded on a JASCO J-725 polarimeter. IR spectra were recorded on a JASCO FT/IR-680plus spectrometer with samples prepared as a thin film on NaCl plates. NMR data were acquired on JEOL JNM-A600 (600 MHz for ^{1}H, 150 MHz for ^{13}C), Unity INOVA600 (600 MHz for ^{1}H, 150 MHz for ^{13}C), or JEOL JNM-ECP800 (800 MHz for ^{1}H, 201 MHz for ^{13}C) spectrometers. FABMS were recorded on a JEOL JMS-700 spectrometer. The matrix used in FABMS analysis was *m*-nitrobenzyl alcohol. ESITOFMS was recorded on a Bruker Daltonics micrOTOF-focus spectrometer. DMAP, MNBA, CH_2Cl_2 (anhydrous), and all other solvents for the workup and isolation procedure were used as received from commercial suppliers. Column chromatography was performed with silica gel FL-60D (Fuji Silysia Chem. Ltd.), TSK G3000S polystyrene gel (Tosoh Co.), or Develosil NH_2 silica gel (Nomura Chemical Co.). Analytic TLC was performed with glass TLC plates (Merck 0.25 mm coated silica gel $60F_{254}$ plates or NH_2 F_{254S} plates). Preparative TLC was performed with glass TLC plates (Merck 0.25 mm coated silica gel NH_2 F_{254S} plates).

Biological Material. The black sponge *Halichondria okadai* Kadota was collected around anorisaki and daiozaki (shima) and toshijima (toba) in Mie prefecture, Japan (about 300 km southwest of Kanagawa prefecture), in 2006. An 80 kg (wet weight) amount of the sponge, which is common in this area, was collected and did not result in any environmental damage. A voucher specimen (YH-0609) has been stored at Keio University. In addition to pinnarine and halichlorine, other known and new marine natural products were also isolated from the extract, which will be disclosed in a future report.

Extraction and Isolation. The black sponge *H. okadai* Kadota was crushed by a mixer and then extracted with MeOH (80 L) at room temperature for one week. After filtration and evaporation under reduced pressure, the MeOH extract was partitioned with EtOAc

(3 × 3 L). The EtOAc layer was evaporated under reduced pressure and then fractionated by column chromatography on TSK G3000S polystyrene gel (Φ 60 × 200 mm) using a gradient elution of EtOH−H_2O (25:75 → 40:60 → 50:50 → 55:45 → 60:40 → EtOH). The fraction (4.40 g) eluted with 40% aqueous EtOH was subjected to Develosil NH_2 silica gel column chromatography (Φ 23 × 75 mm, hexane−EtOAc (1:2) → $CHCl_3$ → $CHCl_3$−MeOH (9:1) → MeOH) to afford four fractions (A1−A4). Fraction A1 (33.0 mg) was developed on silica gel NH_2 F_{254S} preparative TLC (10 × 10 cm, 2 pieces, $CHCl_3$) to afford six fractions. Fraction A1-3 (3.9 mg) was further developed on silica gel NH_2 F_{254S} preparative TLC (10 × 10 cm, hexane−EtOAc, 2:1) to afford five fractions, among which fraction A1-3-3 was assigned to be pinnarine (0.5 mg, 6 × 10^{-7}% isolation yield). From fraction A3, the known halichlorine (3.8 mg) was obtained.

Natural Pinnarine (1): white foam; R_f 0.38 (hexane−EtOAc, 2:1, silica gel NH_2 F_{254S}), 0.62 (MeOH−CH_2Cl_2, 10:1, silica gel 60F_{254}); CD (c 0.6 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 240 (7.27) and 221 (−5.31); ^{1}H NMR (CD_3OD, 800 MHz, 3.31 (CHD_2OD) as the reference) δ 6.83 (1 H, dd, J = 7.1, 7.1 Hz), 5.78 (1 H, m), 5.52 (1 H, d, J = 8.3 Hz), 5.22 (1 H, br d, J = 16 Hz), 5.02 (1 H, br d, J = 8.2 Hz), 4.62 (1 H, br dd, J = 12.0, 12.0 Hz), 4.07 (1 H, br d, J = 11.0 Hz), 2.94 (1 H, br d, J = 10.0 Hz), 2.80 (1 H, m), 2.58 (1 H, m), 2.50 (1 H, br d, J = 16.0 Hz), 2.34 (1 H, m), 2.21 (1 H, m), 1.86 (3 H, s), 1.74 (1 H, m), 1.73 (1 H, m), 1.70 (1 H, m), 1.68 (1 H, m), 1.66 (1 H, m), 1.62 (1 H, m), 1.60 (1 H, m), 1.53 (1 H, m), 1.45 (1 H, m), 1.40 (1 H, m), 1.36 (1 H, m), 1.25 (1 H, m), 1.13 (1 H, m), 0.98 (3 H, d, J = 8.2 Hz); ^{13}C NMR (CD_3OD, 150 MHz, 49.0 (CD_3OD) as the reference) δ 138.5, 137.6, 132.2, 132.0, 131.4, 129.0, 69.5, 64.6, 61.3, 58.0, 52.0, 40.9, 39.0, 38.8, 38.4, 35.6, 32.3, 32.0, 24.21, 24.20, 23.4, 13.0 (the lactone carbon could not be observed due to the limited amount of sample); HRESIMS m/z 408.2305 $[M + H]^+$ (calcd for $C_{23}H_{35}{}^{35}ClNO_3$, 408.2305).

Synthesis of Pinnarine. To a mixture of pinnaic acid sodium salt (2.8 mg, 0.0063 mmol) and anhydrous CH_2Cl_2 (4 mL) under Ar atmosphere was added one portion of a solution of 2-methyl-6-nitrobenzoic anhydride (MNBA, 6.5 mg, 0.019 mmol) and 4-(dimethylamino)pyridine (DMAP, 6.3 mg, 0.052 mmol) in anhydrous CH_2Cl_2 (0.5 mL) at −40 °C. The reaction mixture was stirred at −40 °C for 10 min and then at 25 °C for 16 h. After concentration to approximately 1 mL with a rotary evaporator, the reaction mixture was loaded onto a short column of silica gel, which was eluted with MeOH−CH_2Cl_2 (0:100 to 1:100 to 5:100 to 20:100) to afford pinnarine (1.9 mg, 74%) as a white foam.

Synthetic Pinnarine (1): white foam; $[\alpha]^{12}_D$ +27.7 (c 0.065, MeOH); UV (c 0.6 × 10^{-4} M, MeOH) λ_{max} (log ε) 229 (4.88); CD (c 0.6 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 241 (3.13) and 220 (−12.9); IR (film) ν_{max} 3353 (br), 2924, 2853, 1741, 1714, 1659, 1644, 1536, 1455, 1377, 1275, 1260, 1114, 750 cm^{-1}; R_f 0.62 (MeOH−CH_2Cl_2, 10:1, silica gel 60F_{254}); ^{1}H NMR (CD_3OD, 600 MHz, 3.31 (CHD_2OD) as the reference) δ 6.85 (1 H, dd, J = 7.2, 6.6 Hz, H-3), 5.81 (1 H, ddd, J = 15.6, 9.6, 2.4 Hz, H-15), 5.55 (1 H, d, J = 8.4 Hz, H-18), 5.26 (1 H, dd, J = 15.6, 1.8 Hz, H-16), 5.05 (1 H, ddd, J = 8.4, 2.4, 1.8 Hz, H-17), 4.64 (1 H, ddd, J = 12.6, 11.4, 1.2 Hz, H-21a), 4.09 (1 H, ddd, J = 11.4, 3.6, 2.4 Hz, H-21b), 2.98 (1 H, m, H-5), 2.83 (1 H, ddd, J = 15.0, 12.6, 3.6 Hz, H-20a), 2.60 (1 H, ddd, J = 15.6, 8.4, 3.6 Hz, H-4a), 2.53 (1 H, ddd, J = 15.0, 2.4, 1.2 Hz, H-20b), 2.37 (1 H, ddd, J = 15.6, 8.4, 3.6 Hz, H-4b), 2.25 (1 H, m, H-14), 1.87 (3 H, s, H-23), 1.78−1.60 (7 H, overlapped), 1.56 (1 H, br d, J = 13.2 Hz), 1.46 (1 H, m), 1.45−1.38 (2 H, overlapped), 1.28 (1 H, m), 1.16 (1 H, dddd, J = 12.6, 12.6, 12.6, 4.2 Hz, H-6a), and 1.00 (3 H, d, J = 6.6 Hz, H-22); ^{13}C NMR (CD_3OD, 150 MHz, 49.0 (CD_3OD) as the reference) δ 168.4, 138.2, 137.4, 132.0, 131.8, 131.3, 129.0, 69.3, 64.5, 61.1, 57.9, 51.9, 40.8, 38.9, 38.6, 38.3, 35.4, 32.1, 31.8, 24.09, 24.08, 23.3 and 12.9; HRFABMS m/z 408.2308 $[M + H]^+$ (calcd for $C_{23}H_{35}{}^{35}ClNO_3$, 408.2305).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Copies of the spectra of both natural and synthetic pinnarine. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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(9) In the Danishefsky group's first asymmetric total synthesis, they proved the relative configuration of pinnaic acid; see ref 7c.

(10) The isolated natural pinnaic acid sample was only a minute quantity, and during storage in the freezer it partly decomposed, making repurification and obtaining a sufficient amount for absolute configuration determination very difficult.

(11) For the comparison of NMR spectra of pinnarine and halichlorine, see the Supporting Information.

(12) In our previous report of asymmetric total synthesis of pinnaic acid (see ref 7f), we were not able to measure its optical rotation due to the small amount of the synthetic pinnaic acid sample. In this project, we prepared pinnaic acid following the same route from (*R*)-(+)-pulegone on a larger scale, and we successfully determined the specific rotation of the synthetic pinnaic acid sodium salt to be $[\alpha]^{12}_{D} = -16.4$ (*c* 0.14, MeOH).

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