Studies in Bioactive Marine Alkaloids

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Abstract: In our continuing search for bioactive substances from marine organisms, novel alkaloids were isolated. The structures and functions of these marine alkaloids are described here.

Pinnatoxins and pteriattoxins, potent shellfish poisons, were purified from the Okinawan bivalve *Pinna muricata*. Pinnatoxins activate Ca^{2+} channels. Halichlorine was isolated from the marine sponge *Halichondria okadai*. This compound inhibits the induction of VCAM-1. Drugs that block VCAM-1 may be useful for treating coronary artery diseases, angina, and noncardiovascular inflammatory diseases. Pinnaic acids, which are cPLA₂ inhibitors, were also obtained from *P. muricata*. Interestingly, the structures of pinnaic acids are closely related to that of halichlorine. Norzoanthamine hydrochloride, isolated from the colonial zoanthid *Zoanthus* sp., suppresses decreases in bone weight and strength in ovariectomized mice, and could be a good candidate for an osteoporotic drug. Aburatubolactams, inhibitors of superoxide anion generation, were isolated from *Streptomyces* sp.

1. Introduction

Alkaloids have been defined as nitrogen-containing, basic compounds isolated from plants. Although it is not clear why alkaloids show significant biological activity, they are often useful as drugs or biological probes for physiological studies. As new and more complicated, unexpected diseases appear worldwide, the importance of bioactive alkaloids has increased due to their potential application as chemotherapy. As the application of alkaloids has expanded, the definition of alkaloids has become less restricted. Alkaloids are now widely accepted as nitrogen-containing compounds that occur naturally not only in plants but also in microorganisms, marine organisms, and animals.

2. Pinnatoxins: Ca^{2+} Channel-Activating Shellfish Poisons

Shellfish of the genus *Pinna* live mainly in shallow waters 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 a toxic extract from *P. attenuata*, referred to as pinnatoxin, is a Ca^{2+} channel activator.³ We isolated the following novel toxins from *P. muricata*: pinnatoxins A(1), B(2),

C(3), and D(4).⁴(Fig. 1)

2.1 Isolation and Structure of Pinnatoxins

The 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, Sephadex LH-20, DEAE Sephadex A-25, and ODS-AQ, using a bioassay-guided (intraperitoneal mouse lethality) fractionation. Final purification was achieved by reverse-phase HPLC to give pinnatoxin A (3.5 mg), a mixture of B and C (1.2 mg), and D (2.0 mg).

The structures of pinnatoxins A and D were clarified by 2D NMR experiments. Although pinnatoxins B and C were obtained as a detachable mixture, the gross structures were determined by the analysis of 2D NMR and positive ion ESI MS/MS spectra.⁵ The relative stereochemistry of pinnatoxins was established by NOE experiments.

Pinnatoxins consist of a 20-membered ring, i.e., the 5,6-bicyclo, 6,7-azaspiro, and 6,5,6-triketal moiety in their structure. In particular, pinnaic acids comprise a carboxylate anion and an iminium cation or an ammonium cation.

2.2 Biological Activity of Pinnatoxins

Pinnatoxin A(1) showed potent acute toxicity against mice

Figure 1. Structures of pinnatoxins.

(LD₅₀ 2.7 µg/MU (i.p.)). It was noted that the toxicity of **2**, **3** (LD₅₀ 0.99 µg/MU) was as potent as that of tetrodotoxin. Although the acute toxicity of **4** (LD₅₀ > 10 µg/MU) was weaker than that of the other pinnatoxins, **4** showed the strongest cytotoxicity against a murine leukemia cell line P388 (IC₅₀ 2.5 µg/ml).

Extracts from the digestive glands of several *Pinna* sp., including *P. muricata*, *P. attenuata*, *P. atropurpurea*, and the commonly eaten shellfish *Atrina pectinata*, all 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.⁶

2.3 Biogenesis of the Pinnatoxin Family

Pteriatoxins A(**5**), B, and C(**6**, **7**: a 1:1 mixture) were also isolated from the Okinawan bivalve *Petria penguin*.⁶ Pteriatoxins (**5**, **6**, and **7**) showed significant acute toxicity against mice (LD₉₉ 100; 8 mg/kg). Based on an analysis of positive ESI MS/MS spectra,⁵ pteriatoxins were determined to be pinnatoxin analogs containing a cysteine moiety. (Fig. 2)

Figure 2. Structures of pteriatoxins.

Interestingly, the backbone of pinnatoxins and their analogues could be configured from C1 to C34 in a single carbon chain. Marine organisms usually produce super carbon chain molecules with a terminal amino group, e.g., palytoxin. In this study, we have proposed a polyketide biogenetic pathway for pinnatoxins, shown in Fig. 3.

Figure 3. Proposed biogenesis of pinnatoxin A.

3. Halichlorine: an Inhibitor of VCAM-1 Induction

Adhesion molecules are involved in the processes of adhesion between cells and adhesion between cells and the extracellular matrix in the formation of multicellular bodies. In addition, it is known that the activity of adhesive molecules is very important for the maintenance of function and performance. A recent study indicated that the clinical application of adhesion molecules as anti-inflammatory agents and immunosuppressive agents may some day be possible, provided that the function of the adhesive molecules is controlled.⁷

A simple model of multistage adhesion between leukocyte and vascular cells is shown in Fig. 4. This process can be classified into four stages, i.e., 1) Rolling, 2) Triggering, 3) Strong Adhesion, and 4) Transmigration. VCAM-1⁸ (vascular cell adhesion molecule-1) is affected during the phase of Strong Adhesion. Drugs that block the induced expression of VCAM-1 may be useful for treating atherosclerosis, coronary artery diseases, angina, and noncardiovascular inflammatory diseases.⁷

Figure 4. A model of adhesion between leukocyte and vascular cells.

3.1 Isolation and Structure of Halichlorine

The marine sponge *H. okadai* Kadota (200 kg) was immersed in ethanol at room temperature. The ethanolic extract was filtered, concentrated under reduced pressure, and extracted with ethyl acetate. The ethyl acetate extract was partitioned between 70% aqueous methanol and hexane. The aqueous methanol layer was separated by column chromatography on TSK G3000S, then on ODS, and then on SiO₂. Finally, the active fraction was purified by preparative TLC on SiO₂ to give crystalline halichlorine (**7**) (70.8 mg).⁹

The structure of **7** was elucidated by an analysis of the MS and the extensive 2D NMR spectra, as shown in Fig. 5. Halichlorine consists of a sterically hindered 15-membered lactone, an azabicyclo [4.4.0] ring, and a [5.6]-spiro ring moiety. The relative stereochemistry of **7** was clarified mainly by the coupling constants and NOESY spectral data. Furthermore, the Bohlmann band¹⁰ in the IR spectrum suggested the

Figure 5. Structure of halichlorine.

conformation around the tertiary amine functionality.

The absolute stereochemistry of **7** was clarified by compositional techniques. Oxidative degradation of **7**, as well as the asymmetric synthesis of the degradation product, allowed us to determine the absolute stereochemistry of halichlorine.¹¹ The first total synthesis by Danishefsky *et al.*¹² also supported our conclusions for the structure of **7**.

3.2 Biological Activity of Halichlorine

Halichlorine inhibits the induction of VCAM-1 at IC₅₀ 7 µg/ml. Although VCAM-1 and ICAM belong to the same immunoglobulin superfamily, halichlorine does not affect ICAM (IC₅₀ > 100 µg/ml).^{6,13} It remains largely unknown why halichlorine affects only VCAM-1. Thus, additional research will be needed to clarify the functions and mechanisms of action of VCAM-1.

4. Pinnaic acids: cPLA₂ Inhibitors

Specific inhibitors of phospholipase A₂ (PLA₂) have been considered as potential drugs for the treatment of inflammation and other disease states, since PLA₂ is linked to the initial step in the cascade of enzymatic reactions which lead to the generation of inflammatory mediators.¹⁴ Marine natural products such as manoalide¹⁵ and luffariellolide¹⁶ have been reported to be potent PLA₂ inhibitors.¹⁷ A cytosolic 85-kDa phospholipase (cPLA₂)¹⁸ exhibits specificity for the release of arachidonic acid from membrane phospholipids.¹⁹ Therefore, compounds that inhibit cPLA₂ activity have been targeted as anti-inflammatory agents.

4.1 Isolation and Structure of Pinnaic Acids

The 80% EtOH extract of the viscera (10 kg) of *P. muricata* (3,000 individuals) was partitioned between EtOAc and water. The water layer was fractionated by column chromatography on TSK G-3000S polystyrene gel, Sephadex LH-20, DEAE Sephadex A-25, ODS-AQ, and silica gel to obtain two novel fatty acids, i.e., pinnaic acid (**8**) (1 mg) and taupinnaic acid (**9**) (4 mg).²⁰ (Fig. 6)

Figure 6. Structures of pinnaic acids.

The structure of **9** was determined by the analysis of NMR spectral data. Taupinnaic acid (**9**) has a 6-azaspiro[4.5]decane unit and a taurine moiety. The relative stereochem-

istry of **9** was deduced from phase-sensitive NOE correlations. Furthermore, the gross structure of **8** was elucidated by a detailed comparison of the EI-MS fragment peaks with the corresponding peaks of **9**. Interestingly, the structure of pinnaic acid has been shown to be quite similar to that of halichlorine (**7**). Although the absolute stereochemistry of **7** has been fully established, the scarcity of **8** among natural sources has allowed only the assignment of the relative stereochemical relationships between C5, C9, and C13. We tentatively reported the C14 configuration as epimeric to the structure of **8**.²⁰ However, from a biogenetic point of view, this conclusion is open to question, in particular when the configuration at C14 is compared to that of halichlorine. Synthetic studies of pinnaic acid (*vide infra*) unambiguously establish that the relative stereochemistry of pinnaic acid is similar to that of **7**.^{21,22}

4.2 Biological Activities of Pinnaic Acids

Pinnaic acid (**8**) and taupinnaic acid (**9**) inhibited cPLA₂ activity *in vitro* with IC₅₀ values of 0.2 mM and 0.09 mM, respectively. Although the activity of pinnaic acids was moderate, inhibitors of cPLA₂ have not yet been reported to date. Therefore, it is necessary to clarify the mechanism of action of these inhibitors.

4.3 Biogenesis of Pinnaic Acids

As described above, pinnaic acids are closely related to halichlorine. Therefore, each carbon atom has been tentatively numbered according to biogenetic consideration of the formation of the N-C23 bond. (Fig. 7) It is possible that these bioactive compounds from marine organisms are produced by symbiotic microorganisms. Further studies of the detailed chemistry of pinnaic acids, including biogenetic pathways and structure-activity relationships, are currently underway in our laboratory.

Figure 7. Biogenesis of pinnaic acids and halichlorine.

4.4 Total Synthesis of Pinnaic Acid

These architecturally novel alkaloids have attracted the attention of synthetic chemists; to date, 14 research groups have published synthetic studies of these alkaloids. The Danishefsky group has achieved the total synthesis of halichlorine²⁰ and pinnaic acid²¹ in an asymmetric manner.

In their synthetic process, it was assumed that pinnaic acid had the same absolute configuration around the aza-spirocyclic moiety (C5, C9, C13), and that all four possible diastereomers at C14 and C17 were synthesized. Comparison of the NMR spectra of these synthetic samples with the data reported for the natural products made it possible to revise the relative structure for pinnaic acid as **8**.

As pinnaic acid is a zwitterionic molecule, the NMR spectrum is quite sensitive to the measurement conditions. We recently reported a racemic total synthesis of **8** (Scheme 1),²²

Scheme 1. Synthesis of (±)-pinnaic acid²².

and our detailed comparison of the ¹H-NMR spectra of both synthetic and natural samples supported Danishefsky's revision of the configuration at C14.

5. Norzoanthamine: a Significant Inhibitor of Osteoporosis

Osteoporosis is caused by an imbalance between bone resorption and bone formation, which results in bone loss and fractures after mineral flux occurs. The frequency of fracture is significantly increased in patients with osteoporosis, and hip fracture in elderly patients with osteoporosis is a very serious problem because it often limits their quality of life. Therefore, in addition to preventing the loss of bone mass, the maintenance of the mechanical strength of bone tissue is a very important point to consider in the development of novel anti-osteoporotic drugs.²³

Norzoanthamine (**10**)²⁴ and its homologues (**11–18**)^{24,25} were isolated from the genus *Zoanthus* sp. (Fig. 8) Norzoanthamine inhibits IL-6 production.²⁶ Furthermore, norzoanthamine and norzoanthamine hydrochloride, both of which counteract decreases in bone weight and strength in ovariectomized mice, could be a good candidate for osteoporotic drug.^{23,27} The relative stereochemistry of norzoanthamines was determined by X-ray analysis.²⁴ Furthermore, the absolute stereochemistry of norzoanthamine was determined by

an advanced version of Mosher's method, as shown in Fig. 8.^{26a}

5.1 Biological Activity of Norzoanthamine

The effect of norzoanthamine hydrochloride on bone weight and strength was tested in ovariectomized mice, a postmenopausal osteoporosis model.^{26,28} Norzoanthamine hydrochloride (0.08 mg/kg/day, p.o.) significantly suppressed the decrease in femoral weight caused by ovariectomy without an increase in uterine weight. (Fig. 9) Such data suggested that the mode of action of norzoanthamine hydrochloride differs from that of estrogen.²⁹ Furthermore, failure load and yield energy of the femur were maintained by the administration of norzoanthamine hydrochloride at a dose of 0.4 mg/kg/day (p.o.), respectively. (Fig. 10) Finally, the thickness of the cortical bone was measured by a photograph of the ground bone. Ovariectomy caused a decrease in the humeralis trabeculae. (Fig. 11) Norzoanthamine hydrochloride significantly suppressed this decrease in a dose-dependent manner. (Fig. 11C, D, E) In ovariectomized mice treated with norzoanthamine hydrochloride, the primary spongiosa had not significantly increased, and the morphology of the metaphysis remained nearly normal.

IL-6 is known as a stimulator of osteoclast formation, and suppression of IL-6 secretion can be effective in the preven-

Figure 8. Structures of zoanthamine-type alkaloids.

Figure 9. Effects of norzoanthamine hydrochloride on uterine and femoral weight.

Figure 10. Effects of norzoanthamine hydrochloride on failure load and yield energy.

Figure 11. Effect of norzoanthamine hydrochloride on humeralis morphology in ovariectomized mice.

tion of osteoporosis. Norzoanthamine and norzoanthamine hydrochloride inhibit IL-6 induction at values of 13 and 4.7 $\mu\text{g/ml}$, respectively.

5.2 Biogenesis of Zoanthamines

Based on their molecular formulas, zoanthamines have been regarded as terpenoids; however, the biogenetic pathway of zoanthamines remains unclear. As described above, marine organisms usually produce super carbon chain molecules with a terminal amino group. Here, we propose a polyketide biogenetic pathway for zoanthamines, shown in Fig. 12.

Interestingly, Nakamura's group isolated zoanthellamine

Figure 12. Proposed biogenesis of zoanthamines.

(19, Fig. 8) from a symbiotic dinoflagellate *Symbiodinium* sp.³⁰ The absolute stereochemistry of 19 was the same as that of norzoanthamine. The structural similarity of 19 to that of the zoanthid alkaloids suggests an algal origin for these zoanthamines. Furthermore, a feeding experiment with a labeled compound suggested the biosynthetic pathway of 19. This pathway was similar to that suggested in our previous proposal.

6. Aburatubolactams: Inhibitors of Superoxide Anion Generation from Marine Microorganisms

As described above, extremely bioactive, structurally novel compounds have been found in marine organisms. However, their practical use in drugs is considerably limited because of the extraordinarily low amount of physiologically active compounds obtainable from these marine organisms. Therefore, research has necessarily focused on the metabolites of such marine organisms, which most likely are the true producers of the active compounds. Aburatubolactams were isolated from the cultured broth of a *Streptomyces* sp., SCRC-A20, in a study monitoring the inhibition of superoxide anion generation.³¹

6.1 Isolation and Structure of Aburatubolactams

The cultured broth (100 L) of SCRC-A20 was separated into supernatants and mycelium by filtration. The supernatant was AcOEt, and the mycelium was then extracted with MeOH. The combined extract was fractionated by a column chromatography on SiO_2 , LH-20, and ODS, while bioactivity was monitored. Finally, aburatubolactam A (20, 1 mg), B (21, 16 mg), and C (22, 140 mg) were purified by preparative TLC.³²

Figure 13. Structures of aburatubolactams.

The structures of these compounds were mainly determined by NMR analysis. Fortunately, a single crystal of aburatubolactam A was obtained from MeOH. The structure of the 20-membered macrocyclic structure is thought to be with the diene amide and dienone functionalities. The coplanar structure of the diene amide and the triketone moiety is very remarkable in this molecule. The structures of other aburatubolactams were deduced by detailed comparison of the NMR spectra with that of aburatubolactam A (Fig. 13)

Aburatubolactams, which possess an acyl tetramine structure, are biogenetically related to the following: ikarugamycin³³ from a terrestrial actinomycete, alteramide A³⁴ from a marine bacterium, and cyrindramine³⁵ from a marine sponge. Such results have suggested that microorganisms may be the true producers of most marine metabolites.

6.2 Biological Activity of Aburatubolactams

Superoxide anions are thought to be closely associated with inflammation, cancer, and aging.³⁶ Superoxide anion generation was assayed using a modification of cytochrome C reduction methods. Aburatubolactams (**20**, **21**, **22**) inhibited TPA-induced superoxide anion generation by human neutrophils (IC₅₀ 26, 6.3, 2.7 µg/ml, respectively). The mechanism of action and the in vivo behavior of aburatubolactams are currently under investigation.

7. Conclusion

As described above, bioactive alkaloids were isolated from marine organisms in the present study. These structures were clarified by spectroscopic analysis and synthetic methods. The biogenesis of these compounds was proposed based on comparisons with their analogs. Additional biological activities of these compounds *in vivo* are currently under investigation in our laboratories.

Thanks to the development of new analytical instruments and techniques, numerous compounds have been isolated and elucidated from natural resources during the past 30 years. Continued study of natural resources is a promising field for the discovery of novel bioactive compounds.

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PROFILE

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Hirokazu Arimoto is an Associate Professor of Nagoya University. He was born in Yokohama in 1966. He received his Ph.D. in 1995 from Keio University. After spending 7 years (Assistant Professor 1994-1999, Associate Professor 1999-2002) at Shizuoka University, he moved to Nagoya University as an Associate Professor of Chemistry in 2001. He is also a researcher at the Institute for Advanced Research at Nagoya University (2003-2005), and in the Precursory Research for Embryonic Science and Technology Program of the JST (2001-2004). He was a recipient of the 2001 Incentive Award in Synthetic Organic Chemistry, Japan. His current research interests are in the total synthesis of natural products and polyvalent biologically active polymers.

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