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Isolation of Araguspongine M, a New Stereoisomer of an Araguspongine/Xestospongin alkaloid, and Dopamine from the Marine Sponge *Neopetrosia exigua* Collected in Palau

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Abstract: A new stereoisomer of an araguspongine/xestospongin alkaloid, named araguspongine M (**1**), has been isolated together with 12 known compounds, araguspongines B (**2**) and D (**3**), dopamine, three galactosyl diacylglycerols, 24-methyl cholesterol, 5,6-dihydrocholesterol, β-sitosterol, and three 5α,8α-epidioxy sterols (**11**–**13**), from the marine sponge *Neopetrosia exigua* (formerly *Xestospongia exigua*) collected in Palau. The structure of **1** was assigned on the basis of its spectral data analysis. This is the first report on the isolation of dopamine from a marine sponge. This compound may be produced by an endosymbiotic *Synechococcus*-like cyanobacterium. Compounds **1**–**3** and **11**–**13** showed cytotoxicity against HL-60 at IC_{50}'s of 5.5, 5.5, 5.9, 22.4, 9.5, and 9.6 μM, respectively. The possible biosynthesis origin of the isolated metabolites is discussed.

Keyword: Marine sponge**,** *Neopetrosia exigua,* araguspongine/xestospongin alkaloid, cytotoxicity, HL-60.

Introduction

Marine sponges (Porifera) are rich sources of bioactive compounds. It has also been well known that they harbor bacteria in their tissues [1]. In some cases, the associated microorganisms constitute about a half of the biomass [2, 3]. Moreover, numbers of marine natural products obtained from sponges have been found to show structural similarities to the metabolites of marine and terrestrial microorganisms. These evidences presented the question whether the bioactive compounds are produced by the sponges or by the associated microorganisms [4]. A few studies have revealed the localization of compounds previously obtained from sponges in the symbiotic microorganisms and the involvement of bacteria in the biosynthesis of the compounds [5].

We have started the chemical ecology study on the sponge *Neopetrosia exigua,* previously named as *Xestospongia exigua*, and describe here the constituents of the Palauan species. A new stereoisomer of an araguspongine/xestospongin alkaloid, named araguspongine M (**1**), and 12 known compounds (**2-13**) were obtained from the EtOH extract. It should be noted that this is the first report on the isolation of dopamine from a marine sponge.

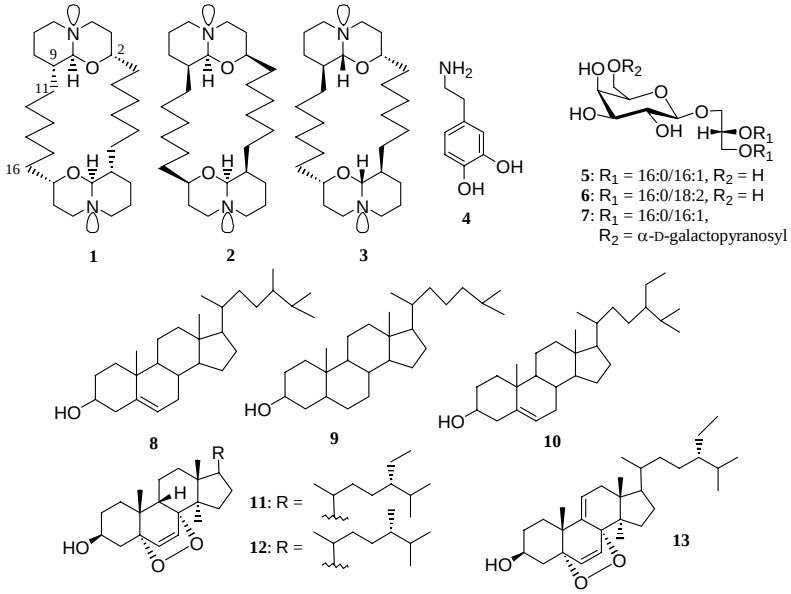

Figure 1. Structures of compounds isolated from marine sponge *Neopetrosia exigua*

Results and discussion

N. exigua is a common and widely distributed sponge in the shallow lagoon of Palau. A *Synechococcus*-like cyanobacterium and several types of bacteria were detected in the tissue by visible and fluorescent microscopic observations [6]. The EtOH extract of the sponge was

partitioned between water and 1-butanol. The butanol extract was subjected to repeated chromatographic separations to give the new compound, araguspongine M (**1**), araguspongines B (**2**) and D (**3**) [7, 8], dopamine (**4**), three galactosyl diacylglycerols (**5**-**7**) [9-13], 24-methyl cholesterol (**8**), 5, 6-dihydrocholesterol (**9**), β-sitosterol (**10**) [14-16], and three 5α,8α-epidioxy sterols (**11**–**13**) [17, 18]. The structures of known compounds were determined on the basis of their spectral data and comparison with the previously reported values.

Araguspongine M (**1**) was obtained as colorless tiny needles. The molecular formula $C_{28}H_{50}N_2O_2$ was deduced from the HRFABMS and NMR spectral data. The ^1H and ^{13}C NMR spectra of **1** resembled those of **2** and **3**. The ^{13}C NMR spectrum of **1** showed 14 signals ascribed to three methines and 11 methylenes (Table 1) as similar to those of **2** and **3**, which suggested that **1** has a C_2-symmetric structure. There are only two compounds (**2** and **3**) thus far isolated from sponges possessing the molecular formula of $C_{28}H_{50}N_2O_2$ and C_2-symmetric structures. Therefore, **1** was revealed to be a new compound of aragustongine/xestospongin alkaloids [7, 8, 19-23].

Table 1. ^{13}C and ^1H NMR data for compound **1** in $CDCl_3$[a]

Atom No.	^{13}C	^1H (*J* in Hz)
2	76.4	3.68 br t (10.8)
3	29.5	1.74 m, 2.26 m
4	52.7	3.01 m, 3.54 br d (10.4)
6	53.3	2.73 m, 3.35 br d (11.2)
7	22.2	1.72 m, 2.33 m
8	24.8	1.30 m, 1.43 m
9	39.1	2.25 m
10	94.9	4.09 d (10.0)
11	30.5	1.36 m, 1.64 m
12	28.7	1.14 m, 1.32 m
13	31.1	1.16 m, 1.32 m
14	27.3	1.78 m, 1.84 m
15	24.3	1.14 m, 1.27 m
16	34.5	1.48 m, 1.70 m

[a] Signals were assigned by ^1H-^1H COSY and HMQC experiments and comparison with the NMR data for **2** and **3**.

Compound **3** showed the characteristic Bohlmann absorptions at 2754 and 2812 cm^{-1} in its IR spectrum. However, these bands were not detected in the IR spectra of **1** and **2**, indicating that **1** have cis-fused 1-oxaquinolizidine rings as similar to the structure of **2** [24]. The ^1H and ^{13}C NMR signals of **1** were assigned by the analysis of ^1H–^1H COSY and HMQC spectral data and comparison with the signals of **2** and **3**. The ^1H–^1H COSY spectrum of **1** revealed the carbon

sequences of 2–3–4 and 6–7–8–9–10 together with the geminal couplings of H_2-3, H_2-4, H_2-6, H_2-7, and H_2-8. The larger coupling constant observed for H-10 (δ 4.09, *J* = 10.0 Hz) indicated that H-9 is axially oriented. H-2 with a larger coupling constant (3.68, J = 10.8 Hz) is also at axial orientation confirmed by the NOE correlations observed between H-2 and H-9 in the NOESY and ROESY spectra of **1**. These data suggested that 1-oxaquinolizidine rings in **1** have the ααα-II type structure reported by Hoye et al. [25]. Consequently, the structure of araguspongine M was assigned as **1**.

Araguspongine/xestospongin alkaloids have been principally detected in the genus *Xestospongia* (*Neopetrosia*), more precisely, *N. exigua* collected at different areas, such as Australia [20, 21], Red Sea [19], Okinawa Japan [7], India [23], Philippines [29], and Palau [21] (this study). While, araguspongine A (xestospongin D) was found in *Niphates* sp. from Singapore [27]. A variety of bioactivities have been reported for araguspongines and xestospongins, such as inhibition of rat brain nitric oxide synthase activity [26], vasodilation activity [7, 21], cytotoxicity [27], antifungal activity [20], somatostatin and vasoactive intestinal peptide inhibition activity [28], antimalarial and antituberculosis activities [19], and inhibition of platelet aggregation [29].

Dopamine (**4**) was obtained in rather higher yield (0.0022% of wet weight). This is the first report of the isolation of dopamine from a marine sponge. Sponges are known as primitive animals without a nerve system, and thus dopamine may not be produced by the sponge as a neurotransmitter. L-Dopa-3-sulfate has been isolated from the brown alga *Ascophyllum nodosum* [30], and recent reports suggested that dopamine act as a grazer deterrent in the green alga *Ulvaria obscura* [31] and be involved in the allellopathic properties of *U. obscura* and *Ulva fenestrate* [32]. TLC analysis of the extracts of 11 different marine sponges collected in Palau showed that Dopamine only contained in *N. exigua*. Therefore, dopamine isolated from *N. exigua* could be produced by a photosynthetic associate to show deterrent and antifouling effects, and correspondingly contribute to the predominance of *N. exigua* in that region. In fact, numerous *Synechococcus*-like cells were observed under a microscope at the outer tissue layer of the sponge [6]. A series of sulfur-containing dopamine derivatives were also discovered in the ascidians of the genera *Lissoclinum*, *Aplydium*, *Eudistoma*, and *Polycitor* [33-36]. These compounds or their precursors may be produced by the symbiotic microorganism.

Galactosyl diacylglycerols are widely distributed in plants as structural components of the thylakoid membrane. These compounds were also isolated from marine sponges, *Phyllospongia foliascens* [37] and *Pseudoceratina* sp. [38], and marine microorganisms, such as cyanobacteria [9-10], dinoflagellates [11, 12], and a lichenized fungus [13]. HPLC analysis of the glycolipids fractions revealed that there were at least seven minor monogalactosyl and digalactosyl diacylglycerols, and several trigalactosyl diacylglycerols in this sponge. Compound **5** was the mostly abundant component among them. These lipids would be produced by the photosynthetic symbiont (most likely a *Synechococcus*-like cyanobacterium) [6].

Sterols possessing the 5α, 8α-epidioxy moiety have been found in eight marine sponges of different genera, *Axinella cannabina*, *Tethya aurantia*, *Raphidostila incise*, *Thalyisias juniperina*,

Haliclona rubens, *Hyrtios* sp., *Axinissa* sp., *Dysidea fragilis*, and *Luffariella* cf. *variabilis* [17]. This report revealed a new source of these sterols. Therefore, the question has been raised whether these sterols are produced by the sponges or by the associated microorganisms.

Separation of microorganisms and sponge cells from *N. exigua*, cultivation of these cells, and analysis of genes for the biosynthesis of the secondary metabolites are now under investigation.

Cytotoxicity against the human leukemia cell line HL-60 and antimicrobial activity of compounds **1**–**13** were examined. The IC_{50} values of compounds **1**, **2**, **3**, **11**, **12**, and **13** against HL-60 were 5.5, 5.5, 5.9, 22.4, 9.5, and 9.6 μM, respectively. Compounds **1**–**13** did not inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Mucor hiemalis*, and marine bacterium *Ruegeria atlantica* even at 100 μg/disc.

Conclusions

A chemical ecology study of the marine sponge *Neopetrosia exigua* resulted in the isolation of a new stereoisomer of an araguspongine/xestospongin alkaloid, named araguspongine M (**1**), together with 12 known compounds. This is also the first report of the isolation of dopamine from a marine sponge. Dopamine may be produced by an endosymbiotic *Synechococcus*-like cyanobacterium to show deterrent and antifouling effects. Compounds **1**–**3** and **11**–**13** showed cytotoxicity against HL-60 cell line, and all isolates were inactive against the five test microorganisms.

Acknowledegments

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Experimental data

Gerenal

Optical rotations were obtained on a JASCO DIP-130 polarimeter. UV and IR spectra were recorded on a Hitachi U-3000 and on a JASCO A-102, respectively. NMR spectra were measured on either a JEOL JNM ECP-600, A-500, or AL-400 NMR spectrometer. Mass spectra were obtained by either a JEOL HX-110 mass spectrometer (FAB mode, *m*-nitrobenzylalcohol as matrix) or a Finnigan TSQ 700 triple quadrupole mass spectrometer (ESI mode).

Marine Sponge

N. exigua was collected by skin diving at Iwayama Bay in Palau during the training vessel Shin'yo Maru was anchored at the Marakal Port in March, 2003. The sponge was kept in a freezer at –50 ˚C on the ship and transported to Japan. The voucher specimen is deposited at the Department of Ocean Sciences, Tokyo University of Marine Science and Technology, as TUF number 03-03-04=2-23. The sponge was identified by Professor P. Bergquist (University of Auckland, New Zealand) [39].

Extraction and Isolation

The sponge (900g, wet weight) was thawed, cut into small pieces, and extracted with EtOH (3L × 3). The extract was evaporated and partitioned between water and 1-BuOH (saturated with water). The BuOH extract (9.0 g) was separated on an ODS column (eluted with MeOH) to afford four fractions (Fr. B-1–Fr. B-4). Fr. B-1 (1.2 g) was separated by an HW-40 column (MeOH) into two fractions (B-1-1 and B-1-2). Fr. B-1-1 was subjected to ODS column (100% H_2O to100% MeOH) and HPLC (ODS, 0.1% TFA/H_2O) separations to give dopamine (**4**, 20 mg). Araguspongines M (**1**, 6.5 mg), B (**2**, 6.0 mg), and D (**3**, 5.6 mg) were isolated from Fr. B-1-2 by SiO_2 ($CHCl_3$–MeOH–NH_4OH, 30:1:0.05, 20:1:0.2, 10:1:0.5) followed by LH-20 (MeOH) column chromatographies. Fr. B-2 (0.9g) was separated by a SiO_2 column ($CHCl_3$–MeOH–H_2O, 90:10:0.5, 80:20:1, 70:30:5) and then HPLC on ODS (100% MeOH) to give compounds **5** (50 mg), **6** (3.7 mg), and **7** (12.0 mg). Fr. B-4 (2.5g) was subjected to a SiO_2 column ($CHCl_3$–MeOH, 100:0, 100:1, 50:1, 25:1), an LH-20 column (MeOH), and HPLC (ODS, 100% MeOH) separations to afford **8** (2.2 mg), **9** (30 mg), **10** (170 mg), **11** (9.3 mg), **12** (3.2 mg), and **13** (1.4 mg).

Araguspongine M (1): $[\alpha]_D$ –4.6° (*c* 0.5, $CHCl_3$); IR (KBr) λ_{max} 3437, 2934, 2857, 2733, 2072, 1638, 1465, 1420, 1173, 1131, 1092, 1040 cm^{-1}; ^1H and ^{13}C NMR data ($CDCl_3$) see Table 1; HRFABMS *m/z* 447.3932 $[M + H]^+$ (calcd for $C_{28}H_{51}N_2O_2$, 447.3951).

Araguspongine B (2): $[\alpha]_D$ –3.6° (*c* 0.5, $CHCl_3$); IR (KBr) λ_{max} 3421, 2927, 2850, 1454, 1350, 1141, 1095, 1033, 964, 910 cm^{-1}; ESIMS *m/z* 447 ($[M+H]^+$).

Araguspongine D (3): $[\alpha]_D$ –10.3° (*c* 0.5, $CHCl_3$); IR (KBr) λ_{max} 3421, 2931, 2850, 2812, 2754, 2360, 2341, 1465, 1346, 1276, 1157, 1134, 1095, 1037, 948cm^{-1}; ESIMS *m/z* 447 ($[M+H]^+$).

TLC analysis of dopamine in marine sponges

Freeze dried small pieces of 11 species of marine sponges including *N. exigua* were each extracted with 50% EtOH. The extract was evaporated, and the residue was redissolved with 50% EtOH to adjust the concentration at 1 g dry sponge weight/mL. Five microliter of each extract was loaded on a SiO_2 TLC plate together with 2 μL of dopamine HCl (1 mg/mL) as control. TLC plates were developed with 1-BuOH–AcOH–H_2O (4:1:2, R_f = 0.45) and detected with the Ninhydrin reagent.

Cytotoxicity assay

The human leukemia cell line HL-60 was incubated in RPMI 1640 using 24-well assay plates. Samples were dissolved in MeOH, and 10 μL of each sample was poured in a well and the solvent

evaporated in a clean bench. The suspension (1 mL, 4 x 10^4 cells/mL) of HL-60 was added to each well and incubated at 37 ˚C for 72 hours in a CO_2 incubator. The shape of the cells was observed after 72 hours under an inverted microscope. The number of vital cells in the sample wells after 72 hours was compared with those in the control wells using XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (cell proliferation kit II®).

Antimicrobial activity

The growth inhibitory activity of samples was evaluated by a paper disc method against *Escherichia coli* IAM 12119T, *Staphylococcus aureus* IAM 12544T, *Saccharomyces cerevisiae* IAM 1438T, *Mucor hiemalis* IAM 6088, and the marine bacterium *Ruegeria atlantica* TUF-D as described in the previous report [40].

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Sample Availability: Samples are available from the authors.