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ISOLATION OF AAPTIC ACID FROM THE MARINE SPONGE *AAPTOS LOBATA* AND INHIBITORY EFFECT OF AAPTAMINES ON RANKL-INDUCED FORMATION OF MULTINUCLEAR OSTEOCLASTS

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Abstract – A new aaptamine congener, aaptic acid (**1**), was isolated from the marine sponge *Aaptos lobata* collected in Indonesia, together with aaptamine (**2**), demethyl(oxy)aaptamine (**3**), and isoaaptamine (**4**). The chemical structure of **1** was assigned on the basis of NMR data. The isolated compounds were tested for inhibitory activities against osteoclastogenesis in RAW264 cells. At 5 μM concentration, **2**-**4** inhibited RANKL-induced multinuclear osteoclast formation by 25%, 54%, and 17%, respectively. In contrast, **1** did not inhibit osteoclastogenesis even at 50 μM.

Aaptamine (**2**) is a marine alkaloid bearing a benzo[*de*][1,6]-naphthyridine scaffold and was isolated from the sponge *Aaptos aaptos* collected at Manza beach in Okinawa in 1982.[1] Since its isolation, more than thirty congeners have been isolated from sponges of the genera *Aaptos*,[2] *Xestospongia*,[3] *Suberites*,[4] *Hymeniacidon*,[5] and *Luffariella*.[6,7] Interestingly, the congeners have been reported to show broad spectra of biological activities, such as cytotoxic,[2,3,8] antimicrobial,[2,3] antiviral,[9] antifouling,[10] antioxidant,[11] and enzymatic inhibitory activities.[12] The cytotoxic effect of **2** was well investigated and found to activate the cyclin dependent kinase inhibitor p21, which leads to inhibition of cell cycle progression and induction of apoptosis.[13] Further, proteome analysis indicated that **2** exhibited cytotoxic activity by targeting *myc* and p53 and promoted hypusination of eukaryotic initiation factor 5A-1 (eIF5A).[14] In our continuing search

for new bioactive compounds from marine invertebrates, aaptic acid (**1**), a new aaptamine congener, was isolated from the sponge *A. lobata* collected in Indonesia, together with **2**,[1] demethyl(oxy)aaptamine (**3**),[2] and isoaaptamine (**4**).[4] In our in-house screening, **2**−**4** inhibited RANKL-induced multinuclear osteoclast formation but **1** showed no inhibition.

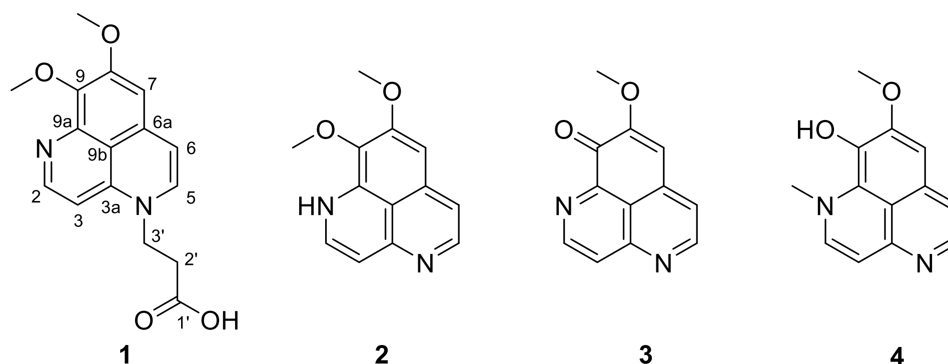

Figure 1. Chemical structures of **1-4**

The sponge was collected in Indonesia and extracted with EtOH and then MeOH. The extract was partitioned between EtOAc and water, and the aqueous layer was extracted with *n*-BuOH. Purification of *n*-BuOH fraction afforded **1**−**4**.

Aaptic acid (**1**) was obtained as dark brown amorphous solid. The molecular formula of $C_{16}H_{16}N_2O_4$ was determined by HRESIMS. ^{1}H NMR spectrum of **1** showed two sets of mutually coupled protons [δ_H 8.09 (d, J = 5.6 Hz, H-2)/δ_H 6.23 (d, J = 5.6 Hz, H-3) and δ_H 7.03 (d, J = 7.2 Hz, H-5)/δ_H 6.36 (d, J = 7.2 Hz, H-6)], a singlet proton [δ_H 6.75 (H-7)], mutually coupled methylene protons [δ_H 2.56 (t, J = 6.2 Hz, H-2')/δ_H 3.95 (t, J = 6.2 Hz, H-3')], and two singlet methyls [δ_H 3.85 (8-OMe) and 3.73 (9-OMe)] (Table 1). ^{13}C NMR spectrum and HSQC spectrum of **1** revealed the presence of one carbonyl carbon [δ_C 172.3 (C-1')], five methine carbons [δ_C 149.2 (C-2), 95.5 (C-3), 134.3 (C-5), 109.3 (C-6), and 100.1 (C-7)], six quaternary carbons [δ_C 147.0 (C-3a), 130.1 (C-6a), 154.1 (C-8), 135.5 (C-9), 141.8 (C-9a), and 117.8 (C-9b)], two methylene carbons [δ_C 31.9 (C-2') and 47.6 (C-3')], and two methoxy carbons [δ_C 56.0 (8-OMe) and 59.5 (9-OMe)] (Table 1).

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for **1** (DMSO-d_6)

No.	δ_C	δ_H, mult (J in Hz)	HMBC
2	149.2, CH	8.09, d (5.6)	3, 3a, 9a
3	95.5, CH	6.23, d (5.6)	9b
3a	147.0, C		
5	134.3, CH	7.03, d (7.2)	6, 3a, 6a
6	109.3, CH	6.36, d (7.2)	5, 7, 9b
6a	130.1, C		
7	100.1, CH	6.75, s	6, 8, 9, 9b
8	154.1, C		
9	135.5, C		
9a	141.8, C		
9b	117.8, C		
8-OMe	56.0, CH_3	3.85, s	8
9-OMe	59.5, CH_3	3.73, s	9
1’	172.3, C		
2’	31.9, CH_2	2.56, t (6.2)	1’, 3’
3’	47.6, CH_2	3.95, t (6.2)	3a, 5, 1’, 2’

Key HMBC correlations, H-2/C-3a and C-9a, H-3/C-9b, H-5/C-3a and C-6a, H-6/C-7 and C-9b, H-7/C-8, C-9, and C-9b, 8-OMe/C-8, and 9-OMe/C-9, established the aaptamine nucleus. Additional HMBC correlations from H-2’ and H-3’ to C-1’ and from H-3’ to C-3a and C-5 assigned the substitution of propionic acid at the N-4 position, and therefore the chemical structure of **1** was determined (Figure 2).

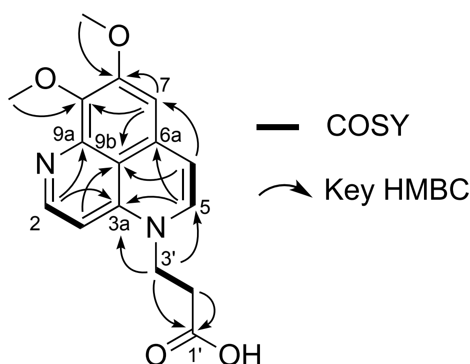

Figure 2. COSY and key HMBC correlations of **1**

Compounds **1**−**4** were evaluated for inhibitory effects on RANKL-induced osteoclastogenesis in RAW264 cells. RANKL induces the osteoclastogenesis of RAW264 cells to form TRAP-positive cells. Subsequently, cell-cell fusion is initiated and then giant multinuclear osteoclasts are formed. In our *in vitro* bioassay, four days after the treatment with RANKL (50 ng/mL) and each compound (5 μM), cells were fixed and stained with TRAP staining solution (Figure 3a), and the numbers of multinuclear osteoclasts were counted (Figure 3b).[15,16] Compared with the control containing RANKL, **3** inhibited the formation of multinuclear osteoclasts by 54% without cytotoxicity, and **2** and **4** moderately inhibited it by 25% and 17%, respectively (Figure 3b). In contrast, **1** did not inhibit the osteoclastogenesis even at a concentration of 50 μM.

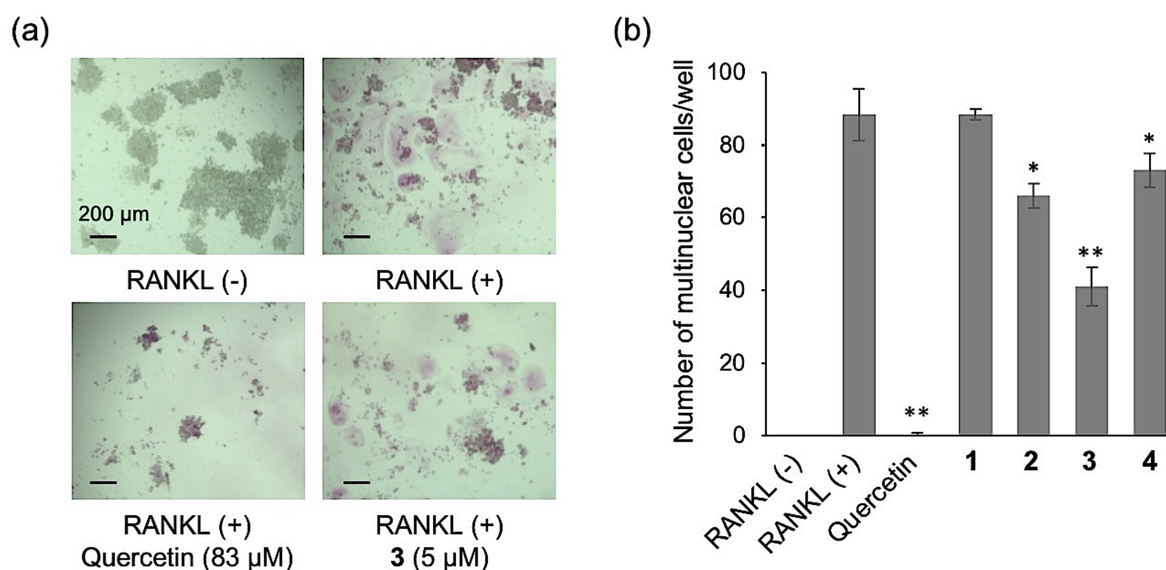

Figure 3. Inhibitory effects of **1**−**4** on RANKL-induced multinuclear osteoclast formation in RAW264 cells. Cells were incubated with RANKL (50 ng/mL) in the presence or absence of **1**−**4** (5 μM) for four days. (a) The cells were stained with TRAP-staining solution. (b) Numbers of TRAP-positive multinuclear osteoclasts (nuclei ≥ 3) stained in red were counted. Quercetin (25 μg/mL, 83μM) was used as a positive control. The bars represent means ± S.D. *P < 0.05 and **P < 0.001.

Aaptic acid (**1**), substituted with a propionic acid at N-4, and three known aaptamines (**2**−**4**) were isolated from the marine aponge *A. lobata* collected in Indonesia. Although more than thirty aaptamine congeners have been isolated from marine sponges so far, **1** is the first compound substituted with a carboxylic acid at the aaptamine nucleus. Aaptamines **2**−**4** inhibited the formation of RANKL-induced multinuclear

osteoclasts at 5 μM. However, **1** did not exhibit the inhibition even at 50 μM. Introduction of an acyl group and methylation at the N-4 position have been reported to decrease the cytotoxicity, which clearly indicates that the nitrogen atom at N-4 of aaptamines may play a key role in their biological activities.[8,17,18] The mechanism of the inhibitory effect of aaptamines on osteoclastogenesis is currently under investigation in our laboratory.

EXPERIMENTAL

General Experimental Procedures

UV spectra were measured on a JASCO V-550 spectrophotometer in MeOH. IR spectra were recorded on a Perkin Elmer Frontier FT-IR spectrophotometer. 1D and 2D NMR spectra were recorded on a Bruker Avance III 600 NMR spectrometer. Chemical shifts were referenced to the residual solvent peaks (δ_H 2.49 and δ_C 39.5 for DMSO-d_6). HRESIMS spectra were measured on a Bruker impact-II mass spectrometer. The preparative HPLC system comprised a Waters 515 HPLC pump, Waters 2489 UV/visible detector. Microscopic images were taken by Keyence BIOREVO BZ-9000.

Animal Material

The marine sponge was collected at a depth of 10 m in North Sulawesi, Indonesia, in December 2010, and immediately soaked in EtOH. The sponge was identified as *Aaptos lobata* by Dr. N. J. de Voogd. A voucher specimen (RMNH POR. 11757) has been deposited in the Naturalis Biodiversity Center, The Netherlands.

Extraction and Isolation

The sponge (562 g wet weight) was extracted with EtOH and then MeOH. After evaporation, the residual aqueous solution was extracted with EtOAc and then *n*-BuOH. The *n*-BuOH fraction (14 g) was subjected to silica gel column chromatography and eluted with a stepwise gradient of CH_2Cl_2/MeOH (4:1 and 3:1), CH_2Cl_2/MeOH/H_2O (6:4:1) and MeOH to yield 8 fractions (Frs. 1−8). Fr. 2 that eluted with CH_2Cl_2/MeOH (4:1) was identified to be **2** (3.2 g). Fr. 1 was subjected to size exclusion HPLC (Asahipak GS-310P, Asahi Chemical Industry Co., Ltd., 21.5 × 500 mm) with CH_2Cl_2/MeOH (4:1) followed by silica gel HPLC (Inertsil Diol, GL Sciences Inc., 20 × 250 mm) with *n*-hexane/CH_2Cl_2/MeOH (40:19:1) to afford **3** (1.0 mg). Fr. 4 that eluted with CH_2Cl_2/MeOH (3:1) was purified by size exclusion HPLC (Asahipak GS-310P, 21.5 × 500 mm) to afford **4** (8.4 mg). Frs. 5 and 6 (1.4 g) were combined and subjected to ODS column chromatography with a stepwise gradient of MeOH/H_2O (2:3 and 4:1) and CH_2Cl_2/MeOH/H_2O (6:4:1) to yield 6 fractions. The second fraction (197.8 mg) that eluted with MeOH/H_2O (2:3) was purified by size exclusion HPLC (Asahipak GS-310P, 21.5 × 500 mm) with MeOH/H_2O (1:4) to yield 13 fractions. The third fraction was further purified by silica gel column

chromatography with a stepwise gradient of CH_2Cl_2/MeOH (17:3, 5:1, 4:1 and 7:3) and CH_2Cl_2/MeOH/H_2O (6:4:1) to afford eight fractions. The eighth fraction that eluted with CH_2Cl_2/MeOH/H_2O (6:4:1) yielded **1** (4.7 mg).

Aaptic Acid (1): dark brown amorphous solid; UV (MeOH) λ_{max} (log ε) 218 (4.21), 240 (4.25), 258 (4.19), 268 (4.15), 278 (4.13) nm; IR (film) ν_{max} 3364, 3083, 2924, 2853, 1651, 1598, 1569, 1459, 1432, 1381, 1350, 1321, 1250, 1196, 1162, 1111, 1093, 1058 cm^{-1}; HRESIMS *m/z* 301.1182 $[M + H]^+$ (calcd for $C_{16}H_{17}N_2O_4$, 301.1183); ^1H and ^{13}C NMR data (DMSO-d_6), see Table 1.

Formation of RANKL-Induced Multinuclear Osteoclasts in RAW264 Cells

This experiment was performed as previously described.[15,16]

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