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ORIGINAL PAPER

Aaptamines, marine spongean alkaloids, as anti-dormant mycobacterial substances

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Abstract A new aaptamine class alkaloid, designated 2-methoxy-3-oxoaaptamine (**1**), together with seven known aaptamines (**2**–**8**) were isolated from a marine sponge of *Aaptos* sp. as anti-mycobacterial substances against active and dormant bacilli. The chemical structure of **1** was determined on the basis of spectroscopic analysis. Compound **1** was anti-mycobacterial against *Mycobacterium smegmatis* in both active growing and dormancy-inducing hypoxic conditions with a minimum inhibitory concentration (MIC) of 6.25 μg/ml, and compounds **2**, **5**, **6**, and **7** showed anti-mycobacterial activities under hypoxic condition selectively, with MIC values of 1.5–6.25 μg/ml.

Keywords Aaptamine · Marine sponge · Anti-mycobacterial activity · Dormant · Tuberculosis

Introduction

Tuberculosis (TB) is one of the most common causes of morbidity and mortality in HIV-positive adults living in poverty [1]. In 2011, there were an estimated 8.7 million new TB cases and 1.4 million deaths by TB [2]. It is now generally accepted that a minimum of 6 months of TB treatment is required owing to the difficulty of eradicating non-replicating persistent *Mycobacterium tuberculosis*. Therefore, new lead compounds which exhibit anti-bacterial activity against *M. tuberculosis* in both its active and dormant states are urgently needed. Although the physiology of latent *M. tuberculosis* infection is still unclear, hypoxic conditions have been found to induce the dormant state of *Mycobacterium* sp., which has a drug-susceptibility profile resembling that of latent *M. tuberculosis* infection [3–5]. Based on this background, we established a screening system to search for substances that have anti-bacterial activity against dormant mycobacteria, and isolated halicyclamines [6, 7] (macrocyclic alkaloids) from a marine sponge of *Haliclona* sp., trichoderins [8] (new aminolipopeptides) from a culture of marine sponge-derived fungus of *Trichoderma* sp., and neamphamide B [9] (a new cyclic depsipeptide) from a marine sponge of *Neamphius* sp. on the basis of bioassay-guided separation. In the continuing screening of marine organisms, a new aaptamine class alkaloid, named 2-methoxy-3-oxoaaptamine (**1**), together with seven known aaptamines (**2**–**8**) were isolated from an Indonesian marine sponge of *Aaptos* sp. In this paper, we present the structure elucidation of compound **1** and the anti-microbial activity against *M. smegmatis* of the compounds isolated.

Materials and methods

General experimental procedures

NMR (600 MHz for ^{1}H-NMR, 150 MHz for ^{13}C-NMR, referenced to TMS) spectra were measured on a Varian Unity Inova 600 (Varian). Electrospray ionization time-of-flight mass spectrometry (ESI-TOF–MS) was recorded on a

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Q-Tof Ultima (Waters Co.). IR spectra were obtained with a Jasco FT/IR-5300 (KBr pellets). Column chromatography was performed on silica gel 60N (63–210 μm, Kanto Chemical Co., Inc., Tokyo, Japan). TLC analysis was carried out by silica gel $60F_{254}$ (Merck Chemical, Darmstadt, Germany). HPLC was performed by a Hitachi High Sensitivity Series system (UV detector: L-4000H) with Cosmosil 5C18-AR-II column (5 μm, 20 mm i.d. × 250 mm, Nacalai tesque, Kyoto, Japan) and Capcellpak MGII S5 (5 μm, 10 mm i.d. × 250 mm, Shiseido Co., Ltd., Tokyo, Japan). Other chemicals were purchased from Sigma (St. Louis, MO, USA) or Nacalai tesque (Kyoto, Japan).

Extraction and isolation of active compounds

The dried marine sponge (400 g), which was collected in 2009 at Kupang, Indonesia, was extracted with MeOH. The MeOH extract [32 g, minimum inhibitory concentration (MIC) against *M. smegmatis* = 200 μg/ml (aerobic condition), 100 μg/ml (hypoxic condition)] was then partitioned by the alkaloid extraction procedure [10]. Using bioassay guidance, the alkaloid fraction [2 g, MIC = 6.25 μg/ml (aerobic condition), 6.25 μg/ml (hypoxic condition)] was subjected to silica gel column chromatography [eluted with $CHCl_3$:MeOH:H_2O (lower phase) containing 0.1 % triethylamine] to obtain nine fractions (Fr. A1–Fr. A9). Of the nine fractions, Fr. A2 and A3 [eluted with $CHCl_3$:MeOH:H_2O = 65:3:1 (lower phase) containing 0.1 % triethylamine], and A6 [eluted with $CHCl_3$:MeOH:H_2O = 15:3:1 (lower phase) containing 0.1 % triethylamine] showed potent anti-microbial activity against *M. smegmatis* under both aerobic and hypoxic conditions.

The active Fr. A2 [15.8 mg, MIC = 6.25 μg/ml (aerobic condition), 3.13 μg/ml (hypoxic condition)] was purified by ODS-HPLC [Capcellpak MGII S5, 10 mm i.d. × 250 mm, a linear gradient from 40 % MeOHaq. to 100 % MeOH over 30 min] to provide 2-methoxy-3-oxoaaptamine (**1**, 0.7 mg) and demethyl(oxy)aaptamine [11] (**5**, 1.0 mg). The active Fr. A3 [67.9 mg, MIC = 6.25 μg/ml (aerobic condition), 3.13 μg/ml (hypoxic condition)] was further separated by ODS-HPLC [Cosmosil 5C18-AR-II, 20 mm i.d. × 250 mm, a linear gradient from 40 % MeOHaq. to 100 % MeOH over 30 min] to give 2,3-dihydro-2,3-dioxoaaptamine [12] (**2**, 1.4 mg), compound **4** [13] (2.7 mg), 3-(methylamino)demethyl(oxy)aaptamine [12] (**7**, 2.2 mg), and compound **8** [13] (1.2 mg). Moreover, the active Fr. A6 [146.9 mg, MIC = 1.75 μg/ml (aerobic condition), 12.5 μg/ml (hypoxic condition)] was then purified by ODS-HPLC [Capcellpak MGII S5, 10 mm i.d. × 250 mm, a linear gradient from 20 % MeOHaq. to 100 % MeOH over 30 min] to afford aaptamine [14] (**3**, 5.3 mg) and 3-aminodemethyl(oxy)aaptamine [15] (**6**, 2.4 mg) (Fig. 1). Each known compound was identified by ESI-TOF–MS and 2D-NMR analysis and comparison with authentic spectral data [11–15].

2-Methoxy-3-oxoaaptamine (1): Yellow amorphous solid. IR ν_{max} (KBr) cm^{-1}: 2,926, 1,870, 1,487, 1,282, 1,086. UV λ_{max} (MeOH) nm (ε): 244 (3,500), 258 (2,200), 271 (2,300), 312 (5,850). ESI-TOF–MS: *m/z* 295 $[M + Na]^+$. High resolution (HR)-ESI-TOF–MS: Calcd for $C_{14}H_{12}N_2O_4Na$: *m/z* 295.0695. Found 295.0677. ^{1}H-NMR (600 MHz, DMSO-d_6, δ_H), ^{13}C-NMR (150 MHz, DMSO-d_6, δ_C) spectra: as shown in Table 1.

Bacterial culture

Mycobacterium smegmatis mc^2155 was grown in Middlebrook 7H9 broth (BD, Franklin, NJ, USA) containing 10 % OADC (BD), 0.5 % glycerol and 0.05 % Tween 80 or on Middlebrook 7H10 agar (BD) containing 10 % OADC and 0.5 % glycerol.

Determination of MIC values under aerobic and hypoxic conditions

Determination of MIC values against *M. smegmatis* was performed by the established MTT method [16]. Mid-log phase of *M. smegmatis* (1×10^4 CFU/0.1 ml) was inoculated in 96-well plates, and then serial diluted samples were

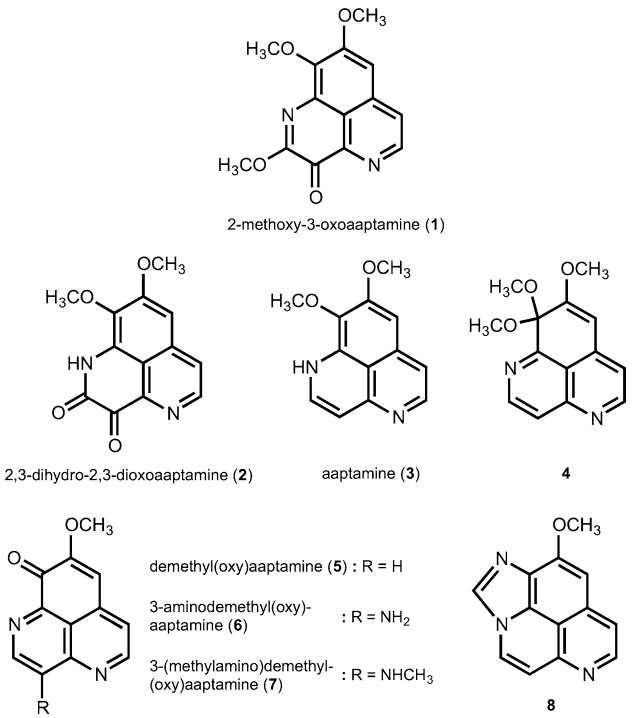

Fig. 1 Chemical structures of the isolated compounds **1**–**8**

Table 1 ^{1}H- and ^{13}C-NMR data for **1**

Position	δ_C^a	δ_H^b
2	156.3	
3-CO	172.0	
3a	144.3	
5	146.4	8.85 (d, $J = 4.8$)
6	125.0	8.07 (d, $J = 4.8$)
6a	134.2	
7	105.1	7.45 (s)
8	156.6	
9	149.0	
9a	125.3	
9b	119.4	
2-OCH_3	54.7	4.01 (s)
8-OCH_3	56.8	3.97 (s)
9-OCH_3	63.6	4.38 (s)

[a] ^{13}C-NMR: δ_C (ppm), (150 MHz, DMSO-d_6)

[b] ^{1}H-NMR: δ_H (ppm, J in Hz), (600 MHz, DMSO-d_6)

added to the 96-well plate. For aerobic conditions, the bacteria were incubated at 37 °C for 24 h, whereas the hypoxic model was performed based on the description by Rustad et al. [17] with minor modification. The mycobacterial bacilli were grown in Middlebrook 7H9 broth at 37 °C under nitrogen atmosphere containing 0.2 % oxygen until the optical density reached 0.8 at 600 nm. The bacilli were then inoculated to the 96-well plate at the same density as the aerobic condition and incubated at 37 °C under nitrogen atmosphere containing 0.2 % oxygen for 96 h. After incubation, 50 µl of MTT solution (0.5 mg/ml) was added into each well and incubated at 37 °C for an additional 12 h under aerobic or hypoxic conditions. The optical density at 560 nm was measured to determine the MIC value.

Results and discussion

The MeOH extract (32 g) of the dried marine sponge of *Aaptos* sp. showed anti-microbial activity against *M. smegmatis* in both active growing aerobic condition and dormancy-inducing hypoxic condition. From a preliminary result obtained by bioautography [18, 19] suggesting that the active constituents might be alkaloidal compounds, the MeOH extract was fractionated by the alkaloid extraction procedure [10]. Using bioassay guidance, the alkaloid fraction (2 g) was further fractionated by silica gel column chromatography and ODS-HPLC to afford compound **1** (0.7 mg, 0.035 % yield from the alkaloid fraction) together with 2,3-dihydro-2,3-dioxoaaptamine (**2**, 1.4 mg, 0.07 %) [12], aaptamine (**3**, 5.3 mg, 0.27 %) [14], dimethyl ketal

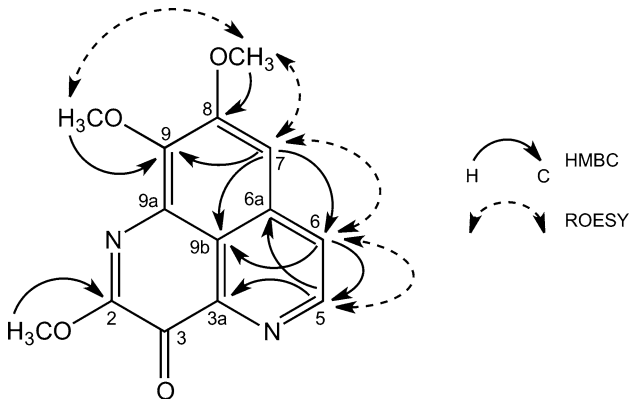

Fig. 2 HMBC and ROESY correlations for **1**

derivative of demethyl(oxy)aaptamine (**4**, 2.7 mg, 0.14 %) [13], demethyl(oxy)aaptamine (**5**, 1.0 mg, 0.05 %) [11], 3-aminodemethyl(oxy)aaptamine (**6**, 2.4 mg, 0.12 %) [15], 3-(methylamino)demethyl(oxy)aaptamine (**7**, 2.2 mg, 0.11 %) [12], and compound **8** (1.2 mg, 0.06 %) [13] (Fig. 1). Of them, compound **4** having the dimethyl ketal moiety is assumed to be an artifact product from compound **5** in the isolation process [20].

Compound **1** was obtained as a yellow amorphous solid. The ESI-TOF–MS of **1** showed a quasi-molecular ion peak $[M + Na]^+$ at *m/z* 295, and the molecular formula was determined as $C_{14}H_{12}N_2O_4$ by high-resolution (HR-) ESI-TOF–MS. In the ^{1}H-NMR spectrum of **1**, the signals observed at δ_H 8.07 and 8.85 (each 1H, d, $J = 4.8$ Hz), along with one isolated singlet at δ_H 7.45 (1H, s), resembled with those of aaptamine alkaloids having benzo[*de*][1,6]naphthyridine skelton. Compound **1** also exhibited three methoxyl signals observed at δ_H 3.97, δ_H 4.01, and δ_H 4.38 (each 3H, s). In addition, the IR absorptions at 1,670 cm^{-1} and the signal at 172.0 ppm in the ^{13}C-NMR spectrum suggested the presence of a carbonyl group. Then, the HMBC correlations between δ_H 3.97 and δ_C 156.6 (C-8); δ_H 4.38 and δ_C 149.0 (C-9) suggested that two of the three methoxyl groups were positioned at C-8 and C-9. The ROESY correlations from H-5 (δ_H 8.85) and H-7 (δ_H 7.45) to H-6 (δ_H 8.07); from the methoxyl proton (δ_H 4.38) and H-7 (δ_H 7.45) to the other methoxyl proton (δ_H 3.97) also supported the positions of these methoxyl groups (Fig. 2). In addition, the ten unsaturations and the HMBC correlation between δ_H 4.01 and δ_C 156.3 (C-2) as shown in Fig. 2 provided the chemical structure (2-methoxy-3-oxoaaptamine) for compound **1**. All the proton and carbon signals were assigned as shown in Table 1.

It is well known that these benzonaphthyridine alkaloids show various biological properties such as α-adrenoreceptor antagonistic, anti-microbial, anti-proliferative, anti-protozoal, and anti-viral activities [21]. Our group have also reported that aaptamine (**3**) activated p21 promoter

Table 2 MIC of aaptamines against *M. smegmatis* under aerobic and hypoxic conditions

Compounds	MIC (μg/ml)	
	Aerobic	Hypoxic
1	6.25	6.25
2	25	6.25
3	100	200
4	200	100
5	25	6.25
6	6.25	1.5
7	6.25	1.5
8	25	12.5
Isoniazid	2.5	25

stably transfected in human osteosarcoma cell MG63 in a p53-independent way [22]. To date, some semi-synthetic derivatives of aaptamine (**3**) and isoaaptamine [23] have been reported to exhibit growth inhibitory activity against *M. tuberculosis* or *M. intracellulare* under active growing condition, while no anti-mycobacterial activity has been reported for aaptamine (**3**) and other natural related compounds [24, 25].

On the other hand, hypoxia is known to be a major factor inducing a nonreplicating persistence of tubercule bacilli. Wayne et al. [3, 26] proved that oxygen depletion triggered the dormancy response, such as isoniazid resistance, in mycobacterial bacilli. Based on these observations, we established a screening system to search for substances that exhibit anti-bacterial activity against dormant mycobacteria. Indeed, the minimum inhibitory concentration (MIC) value of isoniazid against *M. smegmatis* is observed to be 2.5 μg/ml under aerobic conditions, whereas it shifted to 25 μg/ml under nitrogen atmosphere containing 0.2 % oxygen, as shown in Table 2. Compounds **1**–**8** were then examined for their anti-microbial effects against *M. smegmatis* using this assay system (Table 2). Under active growing aerobic condition, compounds **1**, **6**, and **7** showed potent anti-microbial activities with MIC values of 6.25 μg/ml, and compounds **2**, **5**, and **8** exhibited moderate activities with MIC values of 25 μg/ml. On the other hand, the activity of compounds **3** and **4** was very weak. Although further investigation is necessary for structure–activity relationship (SAR) studies, these observations suggest that the existence of the carbonyl group at the C-3 or C-9 positions would provide the positive effect for the anti-microbial activity against *M. smegmatis* under active growing condition. Interestingly, compounds **2**, **5**, **6**, and **7** exhibited anti-microbial activity against dormancy-induced *M. smegmatis* selectively, with MIC values of 6.25, 6.25, 1.5, and 1.5 μg/ml, respectively. Taken together, compounds **6** and **7** showed the most potent and selective anti-microbial activity, with MIC values of 6.25 and 1.5 μg/ml under aerobic condition and hypoxic condition, respectively (Table 2). Detailed evaluation using pathogenic *Mycobacterium* spp. and synthetic study of analog compounds for SAR analysis are in progress.

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