Haliclonacyclamines, Tetracyclic Alkylpiperidine Alkaloids, as Anti-dormant Mycobacterial Substances from a Marine Sponge of *Haliclona* sp.

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A new tetracyclic alkylpiperidine alkaloid, 22-hydroxyhaliclonacyclamine B (1), together with two known alkaloids, haliclonacyclamine A (2) and B (3), were isolated from a marine sponge of *Haliclona* sp. as anti-dormant mycobacterial substances. The chemical structure of 22-hydroxyhaliclonacyclamine B (1) was determined on the basis of spectroscopic study. The compounds 2 and 3 showed strong anti-mycobacterial activity against *Mycobacterium smegmatis* and *M. bovis* Bacille de Calmette et Guérin (BCG) under both aerobic condition and hypoxic condition inducing dormant state with minimum inhibitory concentrations (MICs) in the ranges of 1.0—2.5 μg/ml. In addition, the anti-microbial activity of compound 3 was bactericidal against *M. bovis* BCG under both aerobic and hypoxic conditions. The 22-hydroxy group in 1 was found to reduce anti-mycobacterial activity, because 22-hydroxyhaliclonacyclamine B (1) exhibited weaker anti-microbial activities against *Mycobacterium* bacilli with MICs in the ranges of 12.5—50 μg/ml.

Key words 22-hydroxyhaliclonacyclamine B; marine sponge; anti-mycobacterial activity; dormant; tuberculosis

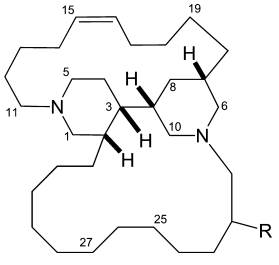
Tuberculosis, caused by *Mycobacterium tuberculosis* infection, is an infectious disease that is responsible for the deaths of around two million people a year.[1)] Aggravation of tuberculosis is evaded by host immune systems, even if infection is concluded completely. However, a small population of bacilli changes their phenotype into dormant state in the granuloma formed by immune cells. Then, the bacilli keep their ability to resume growth and aggravate disease as a result of deterioration of immune systems. This unique property also relates to resistance against conventional anti-tuberculosis drugs such as isoniazid.[2)] Therefore, the lead compounds, which are effective to *M. tuberculosis* in both active state and dormant state, are urgently needed.

Although physiology of the latent *M. tuberculosis* infection is still unclear, hypoxic condition was found to induce dormant state of *Mycobacterium* sp., which has a drug susceptibility profile resembling that of the latent *M. tuberculosis*.[3—5)] Recently, we have established a screening system for anti-dormant mycobacterial substances, and then, re-discovered halicyclamine A from a marine sponge of *Haliclona* sp. 05A08.[6)] In the continuous screening from marine organisms, we isolated a new compound named 22-hydroxyhaliclonacyclamine B (**1**) together with two known alkaloids, haliclonacyclamine A (**2**) and B (**3**), were isolated from an Indonesian marine sponge of *Haliclona* sp. as anti-dormant mycobacterial compounds. In this paper, the structure elucidation of 22-hydroxyhaliclonacyclamine B (**1**) and the anti-mycobacterial activity of haliclonacyclamines are presented.

Results and Discussion

The MeOH extract of the dried marine sponge (1 kg) of *Haliclona* sp. 01D53, which was collected in 2001 at Indonesia, showed anti-microbial activity against *Mycobacterium smegmatis* under both aerobic condition and hypoxic condition inducing dormant state. On the guidance of bioassay, the MeOH extract was partitioned into a water–EtOAc mixture. The aqueous phase was further partitioned with *n*-BuOH. The *n*-BuOH soluble portion (5.5 g) was fractionated by Diaion HP-20 column chromatography, Sephadex LH-20 column chromatography, ODS column chromatography, MCI gel column chromatography, and HPLC using COSMOSIL Sugar-D column to afford 22-hydroxyhaliclonacyclamine B (**1**, 3.5 mg, 0.06% yield from the *n*-BuOH soluble portion), haliclonacyclamine A (**2**, 5 mg, 0.09%), and haliclonacyclamine B (**3**, 10 mg, 0.17%) (Fig. 1). Compounds **2** and **3** were identified by physico-chemical properties, ESI-TOF-MS and 2D-NMR analysis and comparison with authentic spectral data.[7)]

22-Hydroxyhaliclonacyclamine B (**1**) was obtained as colorless solid. The ESI-TOF-MS of **1** showed a quasi-molecular ion peak $[M+H]^+$ at *m*/*z* 485, which was larger than that of **3** by 16 amu, and the molecular formula was determined as $C_{32}H_{56}N_2O$ by high-resolution (HR-) ESI-TOF-MS and NMR analysis. The IR absorption at 3412 cm^{-1} suggested the presence of hydroxyl group. The ^1H- and ^{13}C-NMR spectra of **1** showed the signals ascribable to four olefinic protons (δ_H 5.31, 5.32, 5.41, 5.41), four olefinic carbons (δ_C 130.8, 132.6, 131.0, 130.7), six aminomethylene carbons (δ_C 52.6,



22-hydroxyhaliclonacyclamine B (**1**) : R = OH, Δ^{27}
haliclonacyclamine A (**2**) : R = H, Δ^{25}
haliclonacyclamine B (**3**) : R = H, Δ^{27}

Fig. 1. Structure of Compounds **1**—**3**

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Table 1. ^{1}H- and ^{13}C-NMR Data for **1** (600 MHz and 150 MHz in CD_3OD)

No.	22-Hydroxyhaliclonacyclamine B (**1**)	
	δ_C	δ_H (mult., J in Hz)
1	52.6	3.22 (m), 2.98 (m)
2	40.3	1.97 (m)
3	33.8	2.30 (m)
4	34.7	2.33 (2H, m)
5	49.8	3.34 (2H, m)
6	68.7	3.23 (m), 3.01 (m)
7	47.4	3.09 (m)
8	35.9	2.15 (m), 1.26 (m)
9	38.6	2.70 (m)
10	69.0	2.98 (m), 2.92 (m)
11	57.0	3.09 (2H, m)
12	21.7	1.72 (m), 1.60 (m)
13	27.5	1.41 (m), 1.22 (m)
14	28.4	2.25 (m), 2.09 (m)
15	130.8	5.31 (m)
16	132.6	5.32 (m)
17	27.8	2.28 (m), 2.20 (m)
18	30.6	1.59 (m), 1.42 (m)
19	25.7	1.47 (m), 1.36 (m)
20	33.4	1.51 (m), 1.02 (m)
21	74.0	3.38 (m), 3.22 (m)
22	71.5	3.63 (m)
23	33.2	1.89 (m), 1.26 (m)
24	29.1	1.46 (m), 1.35 (m)
25	28.8	2.28 (m), 2.26 (m)
26	26.7	2.31 (m), 1.77 (m)
27	131.0	5.41 (m)
28	130.7	5.41 (m)
29	28.4	1.52 (m), 1.46 (m)
30	32.1	1.52 (m), 1.18 (m)
31	27.3	1.52 (m), 1.43 (m)
32	32.4	1.54 (m), 1.41 (m)

49.8, 68.7, 69.0, 57.0, 74.0), and an oxymethine carbon (δ_C 71.5) were closely similar to those of **3**, except for the signals adjacent to C-22. The two partial structures (A, B) in **1** were revealed by COSY and HSQC analysis (Fig. 2). And, the connectivity of these partial structures was figured out on the basis of the HMBC correlations as shown in Fig. 2. All the proton- and carbon-signals were assigned as shown in Table 1, and the planar structure of **1** was elucidated as shown in Fig. 2.

The relative stereostructure of **1** was revealed by the ROESY correlations between H-5a, H-3 and H-1a; H-3, H-2, H-7 and H-9; H-7 and H-6b; H-10a and H-6a; as shown in Fig. 3. For the scarcity of the isolated amount, the stereochemistry of the 22-hydroxyl group in **1** was not investigated.

The dormant *M. tuberculosis* was highly resistant against isoniazid, which is conventional drug and inhibits inhA of type II fatty acid biosynthetic enzyme.[3—5,8] The MIC values of isoniazid against *M. smegmatis* and *M. bovis* Bacille de Calmette et Guérin (BCG) were 2.5 and 0.03 μg/ml under aerobic condition, respectively. While, the MIC values of isoniazid against these strains were more than 25 μg/ml under nitrogen atmosphere containing 0.2% oxygen. On the other hand, the MIC values of haliclonacyclamines A (**2**) and B (**3**) were 2.5 and 1.0 μg/ml against *M. smegmatis* and *M. bovis* BCG under both aerobic and hypoxic conditions, respectively (Table 2). These results indicated that compounds **2** and **3** were effective against *Mycobacterium* sp. in both actively growing state and dormant state. On the other hand, 22-hydroxyhaliclonacyclamine B (**1**) showed weaker anti-mycobacterial activity with MICs in the range of 12.5—50 μg/ml. Therefore, the 22-hydroxy group in **1** reduced anti-mycobacterial activity. Moreover, to examine whether the anti-mycobacterial activity of haliclonacyclamines is bactericidal or bacteriostatic, colony forming unit (CFU) assay was executed using compound **3** in both aerobic and hypoxic conditions. As shown in Fig. 4, the CFU of *M. bovis*

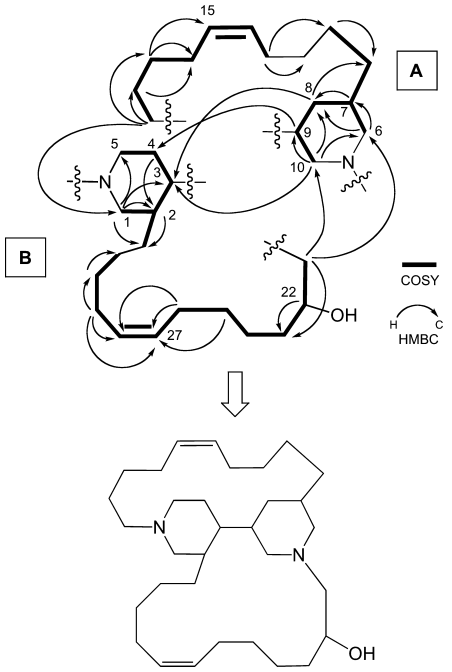

Fig. 2. COSY and HMBC Correlations for **1**

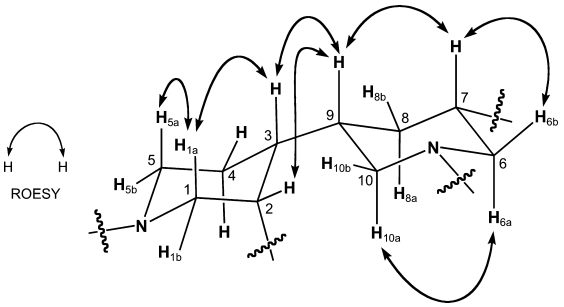

Fig. 3. ROESY Correlations for **1**

Table 2. MIC of Haliclonacyclamines and Isoniazid against *M. smegmatis* and *M. bovis* BCG under Aerobic or Hypoxic Conditions

Compounds	MIC (μg/ml)			
	M. smegmatis		*M. bovis* BCG	
	Aerobic	Hypoxic	Aerobic	Hypoxic
1	12.5	25	25	50
2	2.5	2.5	1.0	1.0
3	2.5	2.5	1.0	1.0
Isoniazid	2.5	25	0.03	>100

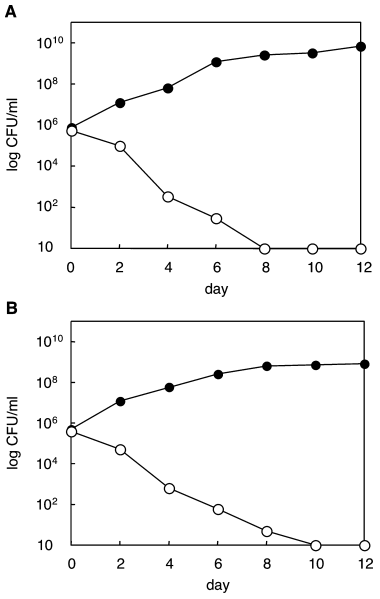

Fig. 4. Bactericidal Effect of Haliclonacyclamine B (**3**) against *M. bovis* BCG

The culture of *M. bovis* BCG (1×10^6 CFU/ml) was incubated in the presence (open circle) or absence (closed circle) of haliclonacyclamine B (**3**) under both aerobic (A) and hypoxic (B) conditions for indicated time. The 100 μl of portion was collected at each time point, and serial diluted culture was plated on the Middlebrook 7H10 agar. Number of colony was counted after 4 weeks incubation.

BCG decreased in time-dependent fashion in the presence of 5.0 μg/ml of compound **3**, and the colony was not detected after 8 d incubation under aerobic condition or 10 d incubation under hypoxic condition. This data indicated that haliclonacyclamine B (**3**) exhibits bactericidal effect against *M. bovis* BCG in both aerobic and hypoxic conditions. The further analysis of molecular target of haliclonacyclamines is currently under way.

Experimental

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 Q-Tof Ultima (Waters Co.). IR spectra were obtained with a JASCO FT/IR-5300 (KBr pellets). Column chromatography was performed on COSMOSIL ODS (75C18-OPN, Nacalai Tesque, Kyoto, Japan), Sephadex LH-20 (GE Healthcare Bio-Sciences, Buckinghamshire, U.K.), Diaion HP-20 (Mitsubishi Chemical Corp., Tokyo, Japan), and MCI GEL (75—150 μm, Mitsubishi Chemical Corp., Tokyo, Japan). TLC analysis was carried out by silica gel $60F_{254}$ (Merck Chemical, Darmstadt, Germany). HPLC was performed by HITACHI High Sensitivity Series system (UV-detector: L-4000H) with COSMOSIL Sugar-D column (5 μm, 10×250 mm, Nacalai Tesque, Kyoto, Japan).

Bacterial Culture *M. smegmatis* mc^{2}155 and *M. bovis* BCG Pasteur were grown in Middlebrook 7H9 broth (BD, Franklin, NJ, U.S.A.) containing 10% OADC (BD, Franklin, NJ), 0.5% glycerol and 0.05% Tween 80 or on Middlebrook 7H10 agar (BD, Franklin, NJ, U.S.A.) containing 10% OADC and 0.5% glycerol.

Extraction and Isolation of Active Compounds The dried marine sponge of *Haliclona* sp. 01D53 (1 kg), which was collected in August, 2001 at Flores Island, Indonesia, was extracted with MeOH. The MeOH extract (100 g), which exhibited anti-mycobacterial activity against *M. smegmatis* under both aerobic and hypoxic conditions with MIC 100 μg/ml, was partitioned into a water–EtOAc mixture (1 : 1). The aqueous phase was further partitioned with *n*-BuOH. The *n*-BuOH soluble portion (5.5 g, MIC 6.0 μg/ml) was fractionated by Diaion HP-20 column chromatography to give an active MeOH containing 0.1% TFA eluate, which was further separated by Sephadex LH-20 column (MeOH) to afford an active fraction (1.5 g, MIC 6.0 μg/ml). The active fraction was then separated by ODS column (MeOH–H_2O) to give an active 50% MeOH eluate (Fr. A6). The Fr. A6 (820 mg, MIC 6.0 μg/ml) was fractionated by MCI-gel column (MeOH–H_2O) to give five fractions (Fr. B1—Fr. B5). The active Fr. B3 (440 mg, MIC 3.0 μg/ml) was purified by HPLC [COSMOSIL Sugar-D column; eluted with CH_3CN–$CHCl_3$=95 : 5] to provide **2** (5 mg, 0.09% yield from the *n*-BuOH soluble portion), **3** (10 mg, 0.17% yield from the *n*-BuOH soluble portion) and an active fraction containing **1** (Fr. C1). The Fr. C1 (7 mg, MIC 25 μg/ml) was further purified by HPLC [COSMOSIL Sugar-D column; eluted with CH_3CN–H_2O=95 : 5] to afford **1** (3.5 mg, 0.06% yield from the *n*-BuOH soluble portion).

22-Hydroxyhaliclonacyclamine B (**1**): Colorless solid. $[\alpha]_D^{20}$ +11.8° (*c*=0.1, MeOH). ESI-MS: *m/z* 485 $[M+H]^+$. High resolution ESI-MS *m/z*: Calcd for $C_{32}H_{57}N_2O$: 485.4479; Found: 485.4471. IR (KBr) cm^{-1}: 3412, 2928, 2858, 1653, 1462. UV λ_{max} (MeOH) nm (ε): 268 (3400). ^{1}H-NMR (600 MHz, CD_3OD, δ_H), ^{13}C-NMR (150 MHz, CD_3OD, δ_C) spectra: as shown in Table 1.

Determination of MIC Values under Aerobic and Hypoxic Conditions Determination of MIC values against *M. smegmatis* and *M. bovis* BCG was performed by the established MTT method.[9] Mid-log phase bacilli (*M. smegmatis* (1×10^4 CFU/0.1 ml) or *M. bovis* BCG (1×10^5 CFU/0.1 ml)) were inoculated in 96-well plate, and then serial diluted sample was added to the 96-well plate. In the case of aerobic condition, the bacteria were incubated at 37 °C for 24 h (for *M. smegmatis*) or for 7 d (for *M. bovis* BCG). On the other hand, the hypoxic model was performed based on the description by Rustad *et al.* with minor modification.[10] 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. Then, the bacilli were inoculated to the 96-well plate in the same density as the aerobic condition and incubated at 37 °C under nitrogen atmosphere containing 0.2% oxygen for 96 h (for *M. smegmatis*) or for 14 d (for *M. bovis* BCG). After incubation, 50 μl of MTT solution (0.5 mg/ml) was added into each well and incubated at 37 °C for additional 12 h under aerobic or hypoxic conditions. The optical density at 560 nm was measured to determine MIC value.

Bactericidal Effect of Haliclonacyclamine B (3) against *M. bovis* BCG under Both Aerobic and Hypoxic Conditions The *M. bovis* BCG were grown in Middlebrook 7H9 broth at 37 °C under aerobic condition or nitrogen atmosphere containing 0.2% oxygen until the optical density reached 0.8 at 600 nm. The culture of *M. bovis* BCG in Middlebrook 7H9 broth was adjusted to 1×10^6 CFU/ml, and then compound **3** (5.0 μg/ml) was added. The 100 μl of portion was collected at each time point, and serial diluted culture was plated on the Middlebrook 7H10 agar to measure CFU. Number of colony was counted after 4 weeks incubation.

Acknowledgements The authors are grateful to Dr. Nicole J. de Voogd, National Museum of Natural History, the Netherlands for identification of the sponge. This study was financially supported by the Hoh-ansha Foundation, the Uehara Memorial Foundation, and Grant-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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