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Anti-mycobacterial alkaloids, cyclic 3-alkyl pyridinium dimers, from the Indonesian marine sponge *Haliclona* sp.Wilmar Maarisit^{a,b}, Delfly B. Abdjul^{a,b,e}, Hiroyuki Yamazaki^{a,*}, Hajime Kato^a, Henki Rotinsulu^c, Defny S. Wewengkang^c, Deiske A. Sumilat^b, Magie M. Kapojos^d, Kazuyo Ukai^a, Michio Namikoshi^a^a Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, Sendai 981-8558, Japan^b Faculty of Fisheries and Marine Science, Sam Ratulangi University, Kampus Bahu, Manado 95115, Indonesia^c Faculty of Mathematic and Natural Sciences, Sam Ratulangi University, Kampus Bahu, Manado 95115, Indonesia^d Faculty of Nursing, University of Pembangunan Indonesia, Bahu, Manado 95115, Indonesia^e North Sulawesi Research and Development Agency, 17 Agustus Street, Manado 95117, Indonesia9
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Three new dimeric 3-alkyl pyridinium alkaloids, named halicyclclamines A–C (1–3), were²⁴ isolated together with five known congeners, cyclostelletamines A (4), B (5), C (6), E (7), and F (8), from the Indonesian marine sponge *Haliclona* sp. The structures of 1–3 were assigned based on their spectroscopic data (1D and 2D NMR, HRFABMS, ESIMS/MS, UV, and IR). Compounds 1–8 exhibited antimicrobial activities against *Mycobacterium smegmatis* with inhibition zones of 17, 10, 13, 14, 8, 8, 12 and 12 mm, respectively, at 10 µg/disc. Compounds 3 and 8 also modestly inhibited the activity of vaccinia H-1-related phosphatase (VHR), a dual-specificity phosphatase, at 17–18 µM.

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Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is one of the most serious infectious diseases that include human immunodeficiency virus (HIV) and malaria.¹ Approximately 10.4 million clinical cases of TB, 1.4 million deaths from TB, and 0.4 million deaths from HIV-co-infected with TB were estimated to have occurred in 2015.² The prevalence of multidrug-resistant TB (MDR-TB), which is defined as TB that is resistant to first-line drugs (rifampicin and isoniazid),^{1,3} is also a serious health issue because there are currently only a limited number of anti-TB agents for clinical use and treatments for TB are required for a minimum of 6 months. Additionally, approximately 9.6% of MDR-TB acquires resistance to some second-line drugs (e.g. fluoroquinolones, amikacin, kanamycin, and capreomycin), and is known as extensive drug-resistant TB (XDR-TB).^{1,3} However, delamanid (Deltyba[®]), a new drug that inhibits cell wall biosynthesis by mycobacteria, was approved for the treatment of MDR-TB in the EU and Japan in 2014.⁴ The discovery of new anti-TB drugs with novel structural features and mechanisms of action is still an urgent global demand.

In the course of our research on antimycobacterial substances from marine resources such as ascidians, sponges, and microorganisms, we have reported new streptocytosine,^{5a} agelasine,^{5b} and

haliclona-diamine derivatives.^{5c} Furthermore, our investigations on the extracts of marine organisms revealed that the EtOH extract of the marine sponge *Haliclona* sp., collected at Manado in Indonesia, inhibited the growth of *M. smegmatis*, which is widely used in research on anti-mycobacterial substances.⁶ The bioassay-guided separation of this extract led to the isolation of three new cyclic 3-alkyl pyridinium dimers, named halicyclclamines A–C (1–3), together with five known analogues,⁷ cyclostelletamines A (4), B (5), C (6), E (7), and F (8) (Fig. 1). We herein describe the isolation, structural elucidation, and biological properties of compounds 1–8.

The EtOH extract (6.1 g) of the marine sponge⁸ (619.7 g, wet weight) showed an inhibition zone of 15 mm at 50 µg/disc and was separated by an ODS flash column (100 g) into seven fractions. The bioactive fractions were purified by repeated HPLC (ODS) to yield compounds 1 (1.4 mg), 2 (1.5 mg), 3 (0.9 mg), 4 (10 mg), 5 (5.4 mg), 6 (14 mg), 7 (5.0 mg), and 8 (5.0 mg).⁹ Compounds 4–8 were identified as the known dimeric 3-alkyl pyridinium alkaloids, cyclostelletamines A, B, C, E, and F, respectively, by comparing the spectroscopic data with those of the reported values.⁷

The ¹H and ¹³C NMR data (Table 1) and UV spectra (267 nm) of compounds 1–3 were very similar to those of 4–8.⁷ Therefore, compounds 1–3 were presumed to have a cyclic bis-1,3-dialkylpyridinium skeleton, and their structures were confirmed by analyses of the ¹H–¹H COSY and HMBC spectra of 1–3 (Fig. 2).

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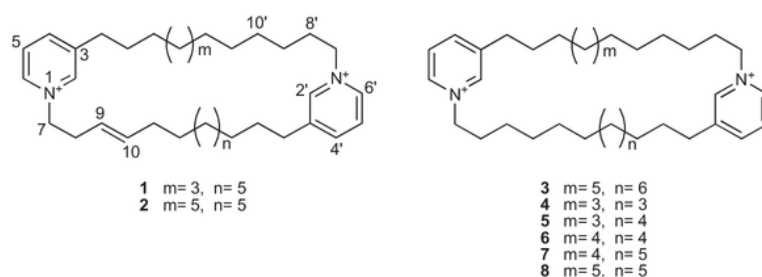
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27 Fig. 1. Structures of compounds **1–8** obtained from the Indonesian marine sponge *Haliclona* sp.

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Table 1

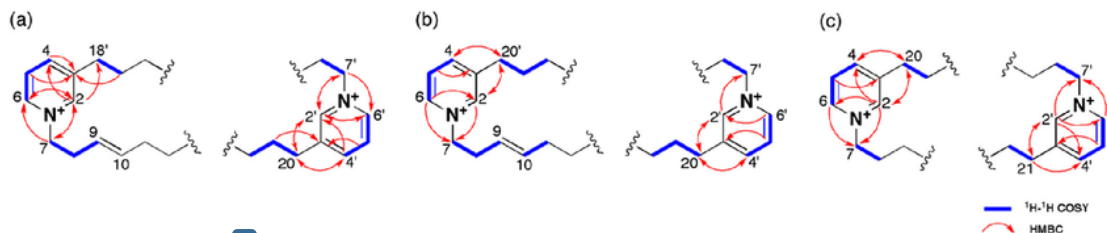
^{13}C (100 MHz) and ^1H (400 MHz) NMR data for **1–3** in CD_3OD .

No.	1		2		3	
	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)
2/2'	145.2	8.90, brs	145.2	8.90, brs	145.2	8.90, brs
3/3'	145.6		145.7		145.6	
4/4'	146.8	8.45, d (7.7)	146.9	8.45, d (8.2)	146.9	8.45, d (7.8)
5/5'	129.1	8.01, dd (7.7, 6.3)	129.2	8.02, dd (8.2, 5.8)	129.2	8.01, dd (7.8, 5.4)
6/6'	143.4	8.82, d (6.3)	143.4	8.82, d (5.8)	143.4	8.82, d (5.4)
7/7'	62.9	4.61, t (6.8)	62.9	4.62, t (6.8)	62.8	4.61, t
8/8'	32.1	1.99, m	32.2	2.00, m	32.0	2.00, m
9	130.5	5.31, m	130.9	5.32, m	26.7	1.24–1.28, m
9'	26.7	1.25–1.32, m	26.7	1.24–1.28, m	26.7	1.24–1.28, m
10	130.5	5.31, m	130.9	5.32, m	29–31 br	1.24–1.28, m
10'	29–31 br	1.25–1.32, m	29–31 br	1.24–1.28, m	29–31 br	1.24–1.28, m
11	30.3	1.99, m	31.3	2.00, m	29–31 br	1.24–1.28, m
11'	29–31 br	1.25–1.32, m	29–31 br	1.24–1.28, m	29–31 br	1.24–1.28, m
12	26.7	1.25–1.32, m	26.8	1.24–1.28, m	29–31 br	1.24–1.28, m
12'	29–31 br	1.25–1.32, m	29–31 br	1.24–1.28, m	29–31 br	1.24–1.28, m
13/13'	29–31 br	1.25–1.32, m	29–31 br	1.24–1.28, m	29–31 br	1.24–1.28, m
14/14'	29–31 br	1.25–1.32, m	29–31 br	1.24–1.28, m	29–31 br	1.24–1.28, m
15/15'	29–31 br	1.25–1.32, m	29–31 br	1.24–1.28, m	29–31 br	1.24–1.28, m
16/16'	29–31 br	1.25–1.32, m	29–31 br	1.24–1.28, m	29–31 br	1.24–1.28, m
17	29–31 br	1.25–1.32, m	29–31 br	1.24–1.28, m	29–31 br	1.24–1.28, m
17'	29–31 br	1.72, m	29–31 br	1.24–1.28, m	29–31 br	1.24–1.28, m
18	29–31 br	1.25–1.32, m	29–31 br	1.33, m	29–31 br	1.24–1.28, m
18'	33.3	2.88, m	29–31 br	1.33, m	29–31 br	1.24–1.28, m
19	29–31 br	1.72, m	29–31 br	1.73, m	29–31 br	1.24–1.28, m
19'			29–31 br	1.73, m	29–31 br	1.73, m
20	33.3	2.88, m	33.3	2.89, m	33.3	1.73, m
20'			33.3	2.89, m	29–31 br	2.89, m
21					33.3	2.89, m

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The molecular formula of halicyclamine A (**1**) was deduced as $\text{C}_{36}\text{H}_{58}\text{N}_2$ from the $[\text{M}-\text{H}]^+$ ion peak at m/z 517.4536 ($\Delta +1.4$ mmu) generated by Hofmann-type fragmentation in the HRFABMS of **1**.¹⁰ The ESIMS/MS analysis of **1** revealed the presence of a pyridinyl saturated C_{12} alkyl chain (m/z 246) and pyridinyl C_{14} mono-alkenyl chain (m/z 272) (Fig. 3a). The location of the double bond was assigned by $^1\text{H}-^1\text{H}$ COSY and HMBC data of **1** (Fig. 2a), and the *E*-configuration was defined from the ^{13}C chemical shifts of C-8 (δ_{C} 32.1) and C-11 (30.3).¹¹ Consequently, the structure of **1** was assigned as shown in Fig. 1.

The molecular formula of halicyclamine B (**2**), $\text{C}_{38}\text{H}_{62}\text{N}_2$, established from HRFABMS data (m/z 545.4827 $[\text{M}-\text{H}]^+$, $\Delta -0.7$ mmu), was two methylene units larger than that of **1**. Two fragment ion peaks at m/z 274 and 272 in the ESIMS/MS of **2** showed the presence of a pyridinyl saturated C_{14} alkyl chain and pyridinyl mono-alkenyl chain, respectively (Fig. 3b). The location and configuration of the double bond in **2** were considered to be the same as those in **1** from 1D and 2D NMR data for **2** (Fig. 2b and Table 1). Thus, the structure of **2** was assigned as shown in Fig. 1.



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Fig. 2. $^1\text{H}-^1\text{H}$ COSY and key HMBC correlations for halicyclamines A (a), B (b), and C (c).

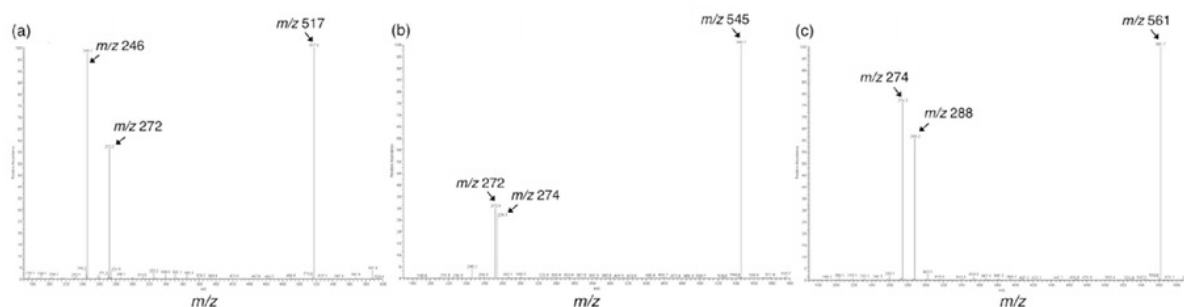


Fig. 3. Positive ESIMS/MS of halicocyclamines A (a), B (b), and C (c).

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Table 2

Antibacterial activities of compounds 1–8 against *Mycobacterium smegmatis*.

Compound	Inhibition zone (mm)		
	1 µg/disc	5 µg/disc	10 µg/disc
1	– ^a	10	17
2	–	7	10
3	–	9	13
4	–	9	14
5	–	–	8
6	–	–	8
7	–	7	12
8	–	8	12
Streptomycin sulfate ^b	20	35	

^a Not active.

^b Positive control.

Halicocyclamine C (**3**) had the molecular formula $C_{39}H_{66}N_2$, established from HRFABMS data (m/z 561.5132 $[M-H]^+$, Δ –1.5 mmu). The lengths of two pyridinyl alkyl chains were C_{14} and C_{15} from the two product ion peaks at m/z 274 and 288, respectively, in the ESIMS/MS of **3** (Fig. 3c). Therefore, the structure of **3** was elucidated as shown in Fig. 1.

The growth inhibitory activities of **1–8** against *M. smegmatis* were examined using the paper disc method.¹² Compounds **1–8** showed inhibition zones against *M. smegmatis* in a dose-dependent manner, and, among them, compound **1** exhibited the most potent activity with an inhibition zone of 10 mm at 5 µg/disc (Table 2). Streptomycin sulfate was used as a positive control and showed an inhibition zone of 35 mm at 5 µg/disc (Table 2). Compounds **4–8** were previously reported to exhibit antimicrobial activities against *M. tuberculosis*,¹³ and, thus, compounds **1–3** will also likely be active against *M. tuberculosis*. Interestingly, although cyclostelletamines B and C (**5** and **6**) showed the weakest inhibition zone against *M. smegmatis* in this study (Table 2), compounds **5** and **6** have been reported to indicate the most potent MIC values against *M. tuberculosis*.¹³ Anti-mycobacterial activities against pathogenic mycobacteria are now being examined, and the results obtained will be described elsewhere.

The inhibitory activities of **1–8** against four protein tyrosine phosphatases (PTPs), PTP1B, T-cell PTP (TCPTP), CD45 tyrosine phosphatase (CD45), and vaccinia H-1-related phosphatase (VHR), were also evaluated in an enzyme assay.¹⁴ Compounds **3** and **8** at 17 and 18 µM, respectively, inhibited VHR activity by 28 and 38%, respectively. VHR is known as a dual-specificity phosphatase and catalyzes the dephosphorylation of p-Tyr and p-Ser/p-Thr residues. VHR²⁹ inhibitors were recently suggested to be novel drug candidates for the treatment of cancer and useful chemical probes for investigating the role of VHR in several cell functions.¹⁵

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Acknowledgments

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- The marine sponge was collected by scuba diving in the coral reef at Manado, North Sulawesi, Indonesia, in December 2013 and identified as *Haliclona* sp. A voucher specimen was deposited at the Faculty of Mathematic and Natural Sciences, Sam Ratulangi University as 13-12-10 = 2-147.
- The marine sponge (619.7 g, wet weight) was cut into small pieces and soaked in EtOH (1.5 L) on the boat immediately after its collection. The extract (6.1 g) after the evaporation of EtOH, was dissolved in $CH_3OH:H_2O$ (200 mL, 9:1, v/v) and extracted with EtOAc (200 mL) to give a crude material (2.6 g). The EtOAc extract was separated using an ODS flash column (100 g) with the stepwise elution of CH_3OH in H_2O into seven fractions (Fr. 1–7). Compounds **1** (1.4 mg), **2** (1.5 mg), **4** (10 mg), **5** (5.4 mg), and **6** (14 mg) were isolated from Fr. 2 (400 mg) by preparative HPLC [column, Inertsil ODS-P (GL Science, Inc., Tokyo, Japan), i.d. 10 mm × 250 mm; solvent, 60% CH_3OH in H_2O containing 0.05% TFA; flow rate, 2.0 mL/min; detection, UV 210 nm]. Compound **3** (0.9 mg) was purified from Fr. 6 (202 mg) by preparative HPLC [column, Inertsil ODS-P, i.d. 10 mm × 250 mm; solvent, 65% CH_3OH in H_2O containing 0.05% TFA; flow rate, 2.0 mL/min; detection, UV 210 nm]. Fr. 5 (219 mg) was separated by repeated HPLC [column, PEGASIL ODS (Senshu Sci. Co., Ltd., Tokyo, Japan), i.d. 10 mm × 250 mm; solvent, 65% CH_3OH in H_2O containing 0.05% TFA; flow rate, 2.0 mL/min; detection, UV 210 nm] to give compounds **7** (5.0 mg) and **8** (5.0 mg). *Halicocyclamine A* (**1**): colorless solids; IR (KBr) ν_{max} 2931, 1683, 1465 cm^{-1} ; UV (CH_3OH) λ_{max} (log ϵ) 267 (3.9) nm; FABMS m/z 517 $[M-H]^+$; HRFABMS m/z (517.4536 $[M-H]^+$, calcd for $C_{36}H_{57}N_2$, 517.4521); 1H and ^{13}C NMR (CD_3OD), see Table 1. *Halicocyclamine B* (**2**): colorless solids; IR (KBr) ν_{max} 2927, 1686, 1466 cm^{-1} ; UV (CH_3OH) λ_{max} (log ϵ) 267 (4.0) nm; FABMS m/z 545 $[M-H]^+$; HRFABMS m/z (545.4827 $[M-H]^+$, calcd for $C_{38}H_{61}N_2$, 545.4834); 1H and ^{13}C NMR (CD_3OD), see Table 1. *Halicocyclamine C* (**3**): colorless solids; IR (KBr) ν_{max} 2931, 1682, 1466 cm^{-1} ; UV (CH_3OH) λ_{max} (log ϵ) 267 (3.9) nm; FABMS m/z 561 $[M-H]^+$; HRFABMS m/z 561.5132 $[M-H]^+$, calcd for $C_{39}H_{65}N_2$, 561.5147); 1H and ^{13}C NMR (CD_3OD), see Table 1.
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- The antibacterial assay was performed using *M. smegmatis* NBRC 3207 with the paper disc method.^{5,16} Strain NBRC 3207 was obtained from the Biological

Resource Center (NBRC), NITE (Chiba, Japan) and maintained in 20% glycerol at -80°C . The test microorganism was cultured in Middlebrook 7H9 broth containing 0.05% polysorbate 80, 0.5% glycerol, and 10% Middlebrook OADC at 37°C for 2 days and adjusted to 1.0×10^8 CFU/mL. The inoculum was spread on the above medium containing 1.5% agar. Each sample in CH_3OH was adsorbed on a sterile filter disc (6 mm), and, after the evaporation of CH_3OH , the disc was placed on an agar plate and incubated at 37°C for 2 days. Streptomycin sulfate (5 $\mu\text{g}/\text{disc}$) and CH_3OH were used as positive and negative controls, respectively.

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14. The effects of compounds 1–8 on PTPs were examined by measuring the rate of hydrolysis of the substrate, pNPP, according to the previously described method with slight modifications.¹⁷ PTP1B (100 μL of a 0.5 $\mu\text{g}/\text{mL}$ stock solution), TCPTP (100 μL of a 0.5 $\mu\text{g}/\text{mL}$ stock solution), CD45 (100 μL of a 0.5 $\mu\text{g}/\text{mL}$ stock solution), or VHR (100 μL of a 1.0 $\mu\text{g}/\text{mL}$ stock solution) in 50 mM citrate buffer (pH 6.0) containing 0.1 M NaCl, 1 mM dithiothreitol (DTT), and 1 mM EDTA was added to each well of a 96-well plastic plate. A sample (2.0 μL in CH_3OH) was added to each well to make the final

concentration, and the plate was incubated at 37°C for 10 min. The reaction was initiated by the addition of pNPP in citrate buffer (100 μL of a 4.0 mM stock solution), incubated at 37°C for 30 min, and then terminated using 10 μL of a stop solution (10 M NaOH). The optical density of each well was measured at 405 nm using a MTP-500 microplate reader (Corona Electric Co.). PTP1B inhibitory activity (%) was defined as $[1 - (\text{ABS}_{\text{sample}} - \text{ABS}_{\text{blank}}) / (\text{ABS}_{\text{control}} - \text{ABS}_{\text{blank}})] \times 100$. $\text{ABS}_{\text{blank}}$ is the absorbance of wells containing only the buffer and pNPP. $\text{ABS}_{\text{control}}$ is the absorbance of p-nitrophenol liberated by the enzyme in the assay system without a test sample, whereas $\text{ABS}_{\text{sample}}$ is that with a test sample. Assays were performed in three duplicate experiments for all test samples. Oleanolic acid, a known phosphatase inhibitor,¹⁸ was used as a positive control.

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