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ORIGINAL PAPER

An anti-mycobacterial bisfunctionalized sphingolipid and new bromopyrrole alkaloid from the Indonesian marine sponge *Agelas* sp.

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Abstract In the course of our studies on anti-mycobacterial substances from marine organisms, the known dimeric sphingolipid, leucettamol A (**1**), was isolated as an active component, together with the new bromopyrrole alkaloid, 5-bromophakelline (**2**), and twelve known congeners from the Indonesian marine sponge *Agelas* sp. The structure of **2** was elucidated based on its spectroscopic data. Compound **1** and its bis TFA salt showed inhibition zones of 12 and 7 mm against *Mycobacterium smegmatis* at 50 µg/disk, respectively, while the *N,N'*-diacetyl derivative (**1a**) was not active at 50 µg/disk. Therefore, free amino groups are important for anti-mycobacterial activity. This is the first study to show the anti-mycobacterial activity of a bisfunctionalized sphingolipid. Compound **13** exhibited weak PTP1B inhibitory activity (29% inhibition at 35 µM).

Keywords Leucettamol A · Bromopyrrole alkaloid · Marine sponge *Agelas* sp. · *Mycobacterium smegmatis* · Protein tyrosine phosphatase 1B

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Introduction

Marine sponges are an attractive resource for drug candidates, with unique structural characteristics [1–4]. To date, a number of metabolites have been isolated and found to exhibit various biological activities, including antibacterial, antifungal, anti-HIV, cytotoxic, anti-inflammatory, and several enzyme inhibitory activities [4]. Some sponge-derived natural and synthetic compounds have been approved for clinical use [5], such as Eribulin (an anti-cancer agent).

During our studies on new anti-mycobacterial substances from marine invertebrates and microorganisms collected in tropical and subtropical regions, we have reported new streptcytosine, agelasine, and halichondramine derivatives [6–8] and found that the EtOH extract of the Indonesian marine sponge *Agelas* sp. inhibited the growth of *Mycobacterium smegmatis*. *Mycobacterium smegmatis* is used as an alternative microorganism to detect antibacterial activity against tuberculous bacteria. Bioassay-guided separation of the extract led to the isolation of the known bisfunctionalized sphingolipid, leucettamol A (**1**) [9, 10], as an active component against *M. smegmatis*, and thirteen bromopyrrole alkaloids **2**–**14**, including the new compound **2**, 5-bromophakelline (Fig. 1). We herein describe the isolation, structural elucidation, and biological activities of compounds **1**–**14**.

The EtOH extract of the marine sponge *Agelas* sp., collected in the coral reefs of North Sulawesi, Indonesia in 2013, displayed an inhibition zone of 10 mm at 50 µg/disk against *M. smegmatis* in the screening bioassay. The extract was purified using an ODS column followed by preparative HPLC (ODS) to give compounds **1** (81 mg), **2** (4.5 mg), **3** (3.0 mg), **4** (3.5 mg), **5** (6.5 mg), **6** (45 mg), **7**

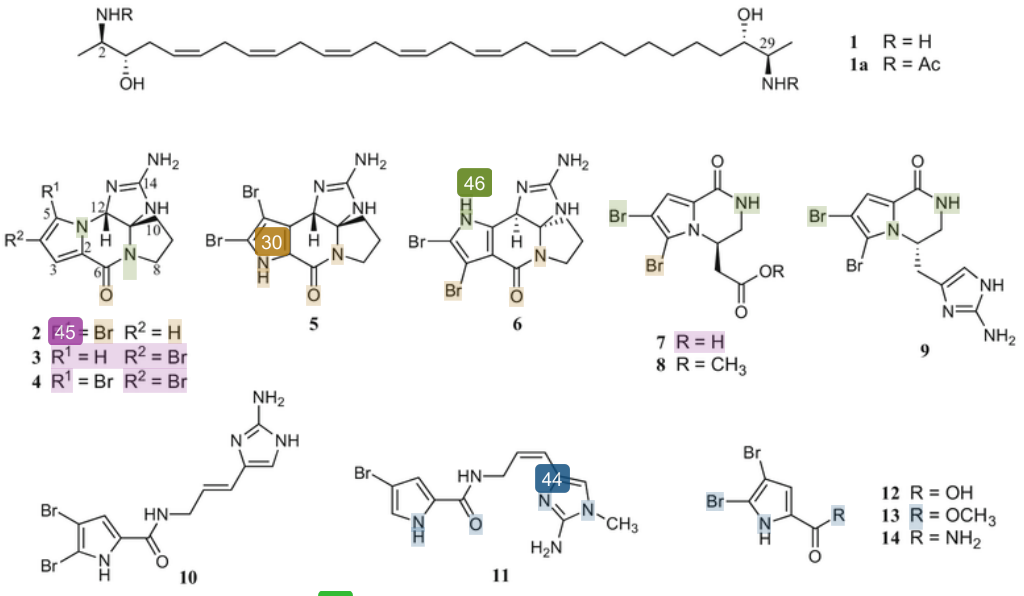

Fig. 1 Structures of compounds **1**–**14** obtained from the Indonesian marine sponge *Agelas* sp.

(1.1 mg), **8** (1.2 mg), **9** (61 mg), **10** (68 mg), **11** (1.7 mg), **12** (24 mg), **13** (2.0 mg), and **14** (7.0 mg).

Compounds **1** and **3**–**14** were identified by comparing their spectroscopic data with reported values for leucettamol A [9, 10], monobromophakelline [11, 12], dibromophakelline [11], dibromoisophakelline [13, 14], cylindradine A [15], (−)-longamide B [16], (−)-longamide B methyl ester [17], cyclooroidin [18], oroidin [19, 20], keramadine [21], 4,5-dibromopyrrole-2-carboxylic acid [19, 20], 4,5-dibromopyrrole-2-methylcarboxylate [19, 22], and 4,5-dibromopyrrole-2-carbamide [19, 22], respectively.

Compound **2** showed two protonated molecule ion peaks at *m/z* 310 $[M + H]^+$ and 312 $[M + 2 + H]^+$, with a 1:1 ratio in the FAB mass spectrum. The molecular formula of **2** was found to be $C_{11}H_{12}BrN_5O$ from HRFABMS (*m/z* 310.0300 $[M + H]^+$, Δ −0.4 mmu) and NMR data (Table 1), which was the same as that of **3**. The ^1H and ^{13}C NMR spectra of **2** were very similar to those of **3**, except for the chemical shifts and coupling constants of two aromatic protons. These differences suggested that compound **2** was a regioisomer of **3**, and an analysis of COSY and HMBC data for **2** revealed the planar structure of **2** as 5-bromophakelline (Fig. 2).

The relative configuration of **2** based on the NOESY correlation between H-10b (δ 2.41) and H-12 (δ 6.30) was found to be the same as those of **3** and **4**. The absolute configurations of **3** and **4** were established by an X-ray crystallographic analysis [11] and synthesis [12]. Since compound **2** was obtained together with **3** and **4** from the same marine sponge in this study, compounds **2**–**4** appear to be biosynthesized through the same pathway. Moreover, the specific rotation of **2** ($[\alpha]_D^{23}$ −113.0, *c* 0.25, CH_3OH) showed the same sign as that of **3** ($[\alpha]_D^{23}$ −114.3, *c* 0.03, CH_3OH; lit. $[\alpha]_D^{23}$ −123, *c* 3.015, CH_3OH) [11]. Thus, the absolute configuration of **2** was assigned as shown in Fig. 1.

Table 1 ^1H and ^{13}C NMR data for compound **2** in DMSO-d_6

No.	δ_C	δ_H mult. (*J* in Hz)	COSY	HMBC
1				
2	124.6			
3	114.0	6.85 d (4.4)	3	2, 3, 5
4	114.4	6.52 d (4.4)	4	2, 4, 5, 6
5	104.1			
6	154.5			
7				
8	44.5	3.48 m	9	
		3.66 m		
9	19.1	2.06 m	8a, 10b	
10	38.5	2.28 m		
		2.41 m	9	
11	82.5			
12	67.6	6.30 s		2, 5, 10, 11, 14
13				
14	156.3			
15 (NH)		10.2 brs		
16 (NH_2)		8.35 brs		

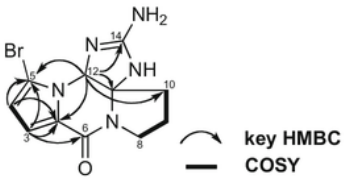

Fig. 2 ^{1}H-^{1}H COSY and key HMBC correlations of **2**

The antimicrobial activities of **1**–**14** against *M. smegmatis* NBRC 3207 were examined using the paper disk method [6, 23]. Compound **1** exhibited moderate anti-mycobacterial activity, with inhibition zones of 7–12 mm at 10–50 μg/disk (Table 2), while the other compounds were not active at 50 μg/disk. The bis TFA salt of **1**, obtained from HPLC purification with TFA, showed a reduced inhibition zone of 7 mm at 50 μg/disk, and the *N,N'*-diacetyl derivative (**1a**), prepared from **1**, was inactive up to 50 μg/disk. Therefore, the free amino groups in **1** are important for anti-mycobacterial properties.

Leucettamol A (**1**) was initially isolated from the marine sponge *Leucetta microraphis* as an antimicrobial compound [9], and its inhibitory effects on the Ubc13–Uev1A interaction and modulatory effects on TRPA1 and TRPM8 channels were recently reported [24, 25]. This is the first study to demonstrate that leucettamol A (**1**) exhibits anti-mycobacterial activity.

The inhibitory activities of isolated compounds **1**–**14** were also tested against protein tyrosine phosphatase 1B (PTP1B) using the enzyme assay method [26], and compound **13** exhibited weak activity, with 29% inhibition at 35 μM. Oleanolic acid, a positive control [27], inhibited the PTP1B activity with an IC_{50} value of 1.3 μM. PTP1B is an attractive target for the treatment of type 2 diabetes and obesity because it plays an important role as a negative regulator in the insulin and leptin signaling pathways [28–30]. On the other hand, compound **13** was not cytotoxic up to 50 μM against two human cancer cell lines, A549 (lung carcinoma) and Huh-7 (hepatoma) [31]. In the same experiment, doxorubicin, an anticancer agent, showed the IC_{50} values of 0.36 and 0.043 μM against A549 and Huh-7, respectively. Huh-7 cells have been used in cell-based experiments to investigate the insulin signaling pathway [32]. Therefore, compound **13** will be an interesting candidate in the study of new PTP1B inhibitors to develop agents for the treatment of type 2 diabetes and obesity.

Table 2 Antimicrobial activities of **1** and **1a** against *Mycobacterium smegmatis*

Compound	*M. smegmatis* (inhibition zone, mm)		
	10 μg/disk	20 μg/disk	50 μg/disk
1	7	9	12
1-bis TFA salt	–[a]	–	7
1a	–	–	–
Streptomycin sulfate[b]	32		

Compounds **2**–**14** were not active at 50 μg/disk

[a] Not active

[b] Positive control (5 μg/disk)

Materials and methods

General experimental procedures

Specific rotations were assessed with a JASCO P-2000 digital polarimeter (JASCO, Ltd., Tokyo, Japan). UV spectra were measured on a U-3310 UV–Visible spectrophotometer (Hitachi, Ltd., Tokyo, Japan) and IR spectra on a PerkinElmer Spectrum One Fourier transform infrared spectrometer (PerkinElmer, Waltham, MA, USA). NMR spectra were recorded on a JEOL JNM-AL-400 NMR spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) in CD_3OD (δ_H 3.30, δ_C 49.0) or DMSO-d_6 (δ_H 2.49, δ_C 39.7). EIMS and FABMS were performed using a JMS-MS 700 mass spectrometer (JEOL, Tokyo, Japan). Preparative HPLC was performed with the Hitachi L-6200 system (Hitachi, Ltd.).

Materials

Middlebook 7H9 broth, polysorbate 80, and Middlebook OADC were purchased from BD (Franklin Lakes, NJ, USA). PTP1B was purchased from Enzo Life Sciences (Farmingdale, NY, USA). *p*-Nitrophenyl phosphate (*p*NPP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Oleanolic acid was purchased from Tokyo Chemical Industry (Tokyo, Japan). Plastic plates (96-well) were purchased from Corning Inc. (Corning, NY, USA). All other chemicals, including organic solvents, were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Marine sponge and isolation of compounds 1–14

The marine sponge was collected by scuba diving at Manado, North Sulawesi, Indonesia, in December 2013 and identified as *Agelas* sp. by Dr. Kazunari Ogawa (Nakai Laboratory). A voucher specimen is deposited at the Faculty of Mathematic and Natural Sciences, Sam Ratulangi University, as 13–12–10 = 1–146.

The sponge (231.1 g, wet weight) was cut into small pieces and soaked in EtOH (1.5 L) on a boat immediately after collection. The EtOH extract (3.6 g) was separated

into seven fractions (Frs. 1–7) using an ODS column (100 g) by a stepwise elution with CH_3OH in H_2O. Fr. 2 (377 mg, 30% CH_3OH eluate) was subjected to HPLC separation [column; PEGASIL ODS (Senshu Scientific Co., Ltd., Tokyo, Japan), 10 × 250 mm; mobile phase, 28% CH_3OH in H_2O containing 0.05% TFA; detection, UV at 210 nm; flow rate, 2.0 mL/min] to give **6** (45 mg, t_R = 32 min), **9** (61 mg, t_R = 46 min), and two subfractions (Frs. 2-1 and 2-2). The purification of Fr. 2-1 (156 mg) by preparative HPLC (column; PEGASIL ODS, 10 × 250 mm; mobile phase, 15% CH_3OH in H_2O containing 0.05% TFA; detection, UV at 210 nm; flow rate, 2.0 mL/min) gave 4.5 mg of **2** (t_R = 47 min) and 3.0 mg of **3** (t_R = 62 min). Compounds **4** (3.5 mg, t_R = 47 min), **5** (6.5 mg, t_R = 43 min), and **11** (1.7 mg, t_R = 55 min) were isolated from Fr. 2-2 (54 mg) by preparative HPLC (column; PEGASIL ODS, 10 × 250 mm; mobile phase, 25% CH_3OH in H_2O containing 0.05% TFA; detection, UV at 210 nm; flow rate, 2.0 ml/min). Fr. 3 (230 mg, 50% CH_3OH eluate) was separated by preparative HPLC (column; PEGASIL ODS, 10 × 250 mm; mobile phase, 46% CH_3OH in H_2O containing 0.05% TFA; detection, UV at 210 nm; flow rate, 2.0 ml/min) to give compounds **7** (1.4 mg, t_R = 39 min), **10** (68 mg, t_R = 25 min), and **14** (7.0 mg, t_R = 29 min). Compounds **8** (1.2 mg, t_R = 28 min), **12** (24 mg, t_R = 35 min), and **13** (2.0 mg, t_R = 49 min) were obtained from Fr. 4 (171 mg, 70% CH_3OH eluate) by preparative HPLC (column; PEGASIL ODS, 10 × 250 mm; mobile phase, 50% CH_3OH in H_2O containing 0.05% TFA; detection, UV at 210 nm; flow rate, 2.0 ml/min). Fr. 5 (81 mg, 85% CH_3OH eluate) was obtained as pure compound **1**, and further HPLC purification (column; PEGASIL ODS, 10 × 250 mm; mobile phase, 70% CH_3OH in H_2O containing 0.05% TFA; detection, UV at 210 nm; flow rate, 2.0 ml/min) of Fr. 5 gave the TFA salt of **1** (t_R = 36 min).

Leucettamol A (**1**): a yellow oil; $[\alpha]_D^{23}$ −3.5 (*c* 1.0, CH_3OH); lit. $[\alpha]_D^{24}$ −3.8 (*c* 4.4, CH_3OH) [9]; UV λ_{max} (CH_3OH) nm (log ε) 202 (4.2); EIMS 472 $[M]^+$; ^1H NMR (CD_3OD) δ 5.48 (1H, m), 5.46 (1H, m), 5.37 (9H, m), 5.33 (1H, m), 3.77 (1H, m), 3.69 (1H, m), 3.29 (1H, m), 3.27 (1H, m), 2.84 (8H, m), 2.81 (2H, m), 2.27 (2H, m), 2.07 (2H, m), 1.35–1.51 (10H, m), 1.23 (3H, *d*, *J* = 8.0 Hz), 1.20 (3H, *J* = 8.0 Hz).

5-Bromophakelline (**2**): a yellow oil; $[\alpha]_D^{23}$ −113.0 (*c* 0.25, CH_3OH); UV λ_{max} (CH_3OH) nm (log ε) 202 (4.2), 281 (3.8); IR (KBr) ν_{max} 3382, 2924, 1679, 1562, 1437, 1385, 1204, 1136, 1029, 840, 802 cm^{-1}; FABMS *m/z* 310 $[M + H]^+$ and 312 $[M + 2 + H]^+$ (1:1); HRFABMS *m/z* 310.0300 ($[M + H]^+$, −0.4 mmu; calcd. for $C_{11}H_{13}BrN_5O$, 310.0304); ^1H and ^{13}C NMR (DMSO-d_6), see Table 1.

4,5-Dibromopyrrole-2-methylcarboxylate (**13**): a yellow oil; UV λ_{max} (CH_3OH) nm (log ε) 202 (4.1), 277 (3.9); EIMS *m/z* 281/283/285 $[M]^+$ (1:2:1); ^1H NMR (DMSO-d_6) δ 12.7 (1H, brs), 6.90 (1H, s), 3.76 (3H, s).

Preparation of *N,N'*-diacetyl-leucettamol A (1a)

Acetic anhydride (120 µL) was added to a solution of **1** (8.0 mg) in CH_3OH (100 µL) at room temperature. The mixture was stirred for 12 h and evaporated. The residue was purified by preparative HPLC (70% CH_3OH containing 0.05% TFA) using an ODS column (PEGASIL ODS) to give 4.6 mg of **1a**.

N,N'-Diacetyl-leucettamol A (**1a**): a yellow oil; EIMS *m/z* 556 $[M]^+$; ^1H NMR (CD_3OD) δ 5.47 (1H, m), 5.45 (1H, m), 5.34–5.37 (10H, m), 3.83 (2H, m), 3.55 (1H, m), 3.48 (1H, m), 2.80–2.86 (8H, m), 2.28 (2H, m), 2.18 (1H, m), 2.07 (3H, m), 1.93 (6H, s) 1.28–1.50 (10H, m), 1.11 (3H, *d*, *J* = 8.0 Hz), 1.08 (3H, *J* = 8.0 Hz).

Anti-mycobacterial assay

The antibacterial assay was performed using *M. smegmatis* NBRC 3207 with the paper disk method [6, 23]. 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 Middlebook 7H9 broth containing 0.05% polysorbate 80, 0.5% glycerol, and 10% Middlebook OADC at 37 °C for 2 days and adjusted to 1.0 × 10^6 CFU/mL. The inoculum was spread on the above medium containing 1.5% agar in square plate. Each sample in CH_3OH was adsorbed to a sterile filter disk (6 mm, Advantec), and, after the evaporation of CH_3OH, the disk was placed on an agar plate and incubated at 37 °C for two days. Streptomycin sulfate (5 µg/disk) was used as a positive control.

PTP1B inhibitory assay

PTP1B inhibitory activity was assessed by measuring the rate of hydrolysis of a substrate, *p*-nitrophenyl phosphate (*p*NPP), according to the previously described method with a slight modification [26, 33]. Briefly, PTP1B (100 µL of 0.5 µg/mL stock solution) in 50 mM citrate buffer (pH 6.0) containing 0.1 M NaCl, 1 mM dithiothreitol (DTT), and 1 mM *N,N,N',N'*-ethylenediaminetetraacetate (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 then incubated at 37 °C for 10 min. The reaction was initiated by the addition of *p*NPP to 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 in each well was measured at 405 nm using an MTP-500 microplate reader (Corona Electric Co., Ltd., Ibaraki, Japan). PTP1B inhibitory activity (%) was defined as $[1 - (ABS_{sample} - ABS_{blank})/(ABS_{control} - ABS_{blank})] \times 100$. ABS_{blank} is the absorbance of wells containing only the buffer and *p*NPP. $ABS_{control}$ is the absorbance of *p*-nitrophenol liberated by the enzyme in the assay system without a test sample, whereas ABS_{sample} is that with a test sample. Assays were performed in three duplicate experiments for all test samples. Oleanolic acid, a known phosphatase inhibitor [27], was used as a positive control. Data are expressed as averages of three independent experiments performed in duplicate.

WST-1 assay

Cytotoxicity was assessed using the WST-1 [sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H tetrazolium inner salt] assay, which detects metabolically competent cells with an intact mitochondrial electron transport chain [31]. Briefly, 1×10^4 cells were seeded on each well of a 96-well plastic plate and cultured overnight. Cells were treated with each test compound and incubated for 48 h, and this was followed by the addition of medium containing WST-1 solution (0.5 mM WST-1 and 0.02 mM 1-methoxy-5-methylphenazinium methyl sulfate; 1-PMS) to each well. The plate was incubated at 37 °C for 60 min, and absorption at 438 nm (reference 620 nm) was measured using an SH-1200 Microplate Reader (Corona Electric). Control cells were treated with 0.1% EtOH. Cell viability was calculated using the formula: absorbance in the treated sample/absorbance in the control × 100 (%). Data are represented as averages of four independent experiments.

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