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New Alkaloids from the Indopacific Sponge *Stylissa carteri*

Claudia Eder,[†] Peter Proksch,*[,†] Victor Wray,[‡] Klaus Steube,[§] Gerhard Bringmann,[⊥] Rob W. M. van Soest,[∥] Sudarsono,[⊗] Elisabeth Ferdinandus,[▽] Limy Agan Pattisina,[▽] Sumali Wiryowidagdo,[○] and Willem Moka[○]

Julius-von-Sachs-Institut für Biowissenschaften, Lehrstuhl für Pharmazeutische Biologie, Julius-von-Sachs Platz 2, D-97082 Würzburg, Germany, Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany, Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany, Institut für Organische Chemie, Am Hubland, 97074 Würzburg, Germany, Instituut voor Systematiek en Populatie Biologie, Zoölogisch Museum, P.O. Box 94766, 1090 GT Amsterdam, Netherlands, Marine Science Center, Fisheries Faculty, Pattimura University, Ambon, Indonesia, and Department of Biology, Faculty of Mathematics and Science, Hasanuddin University, Ujung Pandang, Indonesia

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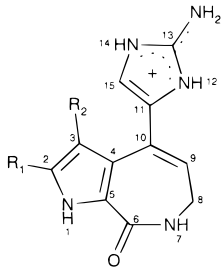
Two samples of the marine sponge *Stylissa carteri* collected in Indonesia yielded two new bromopyrrole alkaloids: debromostevensine (**1**) and debromohymenin (**2**), as well as nine other known congeners (**3**−**11**). The structures of the new compounds were unambiguously established on the basis of their NMR and mass spectra.

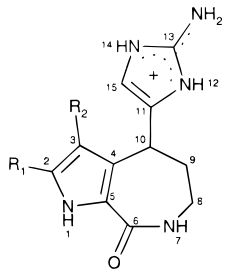
Bromopyrrole alkaloids are typical secondary metabolites of sponges from the families Agelasidae, Axinellidae, and Hymeniacidonidae.[1] Several of these compounds show promising biological activities; they are, for example, cytotoxic,[2] showing α-adrenoceptor blocking activity[3] and protein kinase inhibitory[4] properties. Here we report the results of an investigation of two samples of the sponge *Stylissa carteri* (syn. *Axinella carteri*) collected in 1997 at Ambon and Sulawesi, Indonesia. The samples of *S. carteri* were extracted, and 11 bromopyrrole-type alkaloids were isolated, two of which are novel compounds (**1** and **2**).

Chromatographic separation of the *n*-BuOH-soluble portion of the MeOH extract of *S. carteri* yielded nine bromopyrrole alkaloids as well as two nonbrominated congeners. In addition to the known compounds stevensine (**3**), hymenin (**4**), (*Z*)-debromohymenialdisine (**5**), (*Z*)-hymenialdisine (**6**), (*Z*)-3-bromohymenialdisine (**7**), (*E*)-debromohymenialdisine (**8**), (*E*)-hymenialdisine (**9**), spongiacidin A [= (*E*)-3-bromohymenialdisine] (**10**), and oroidin (**11**), two new alkaloids (**1** and **2**) were isolated and identified from their spectroscopic data and by comparison with published data.[3,5]

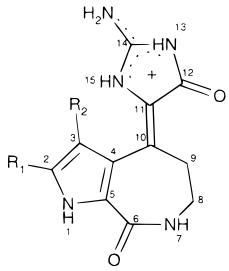
2-Debromostevensine (**1**) was obtained as orange amorphous solid. It showed a $[M + H]^+$ quasimolecular ion at *m*/*z* 308 and 310 in its ESIMS, indicating a bromine substituent. The molecular formula $C_{11}H_{11}N_5OBr$ was established by HRESIMS. UV absorption and ^1H and ^{13}C NMR data were similar to those of stevensine (**3**). The ^{13}C NMR spectrum (Table 1) of **1** contained signals corresponding to seven sp^2 quaternary carbons, three sp^2 methine carbons, and one sp^3 methylene group. The ^1H NMR spectrum of **1** (Table 1) showed three methine and one methylene proton resonances. The ^1H and ^{13}C NMR data are similar to those of stevensine (**3**)[8] but differ from those of the latter by the presence of three instead of two sp^2 methine signals, indicating that **1** is the debromo derivative of stevensine (**3**). The position of the bromine atom at C-3 was evident from $^1J_{C-H}$ (192 Hz)[4,10] for C-2 and the ^{13}C



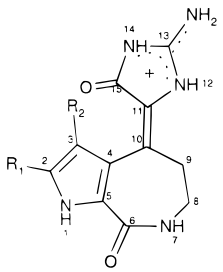
$R_1 = H, R_2 = Br$ **1**
$R_1 = R_2 = Br$ **3**



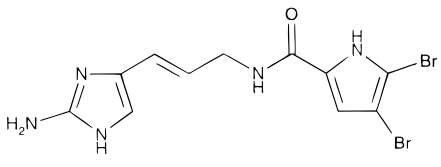
$R_1 = H, R_2 = Br$ **2**
$R_1 = R_2 = Br$ **4**



$R_1 = R_2 = H$ **5**
$R_1 = Br, R_2 = H$ **6**
$R_1 = R_2 = Br$ **7**



$R_1 = R_2 = H$ **8**
$R_1 = Br, R_2 = H$ **9**
$R_1 = R_2 = Br$ **10**



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* To whom correspondence should be addressed. Phone: 0049/931-8886174. Fax: 0049/931-8886182. E-mail: Proksch@botanik.uni-wuerzburg.de.
[†] Julius-von-Sachs-Institut für Biowissenschaften.
[‡] Gesellschaft für Biotechnologische Forschung mbH.
[§] Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH.
[⊥] Institut für Pharmazie und Lebensmittelchemie.
[⊗] Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia.
[∥] Instituut voor Systematiek en Populatie Biologie.
[▽] Pattimura University, Ambon, Indonesia.
[○] Hasanuddin University, Ujung Pandang, Indonesia.

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Table 1. ^{13}C and ^{1}H NMR Data of 2-Debromostevensine (**1**) and 2-Debromohymenin (**2**) in DMSO-d_6

	1		**2**	
	δC^a	δH	δC^a	δH
1		12.40, brs, 1 H		11.74, brs, 1 H
2	123.0, d	7.20, d, $J = 3.0$ Hz, 1 H	122.1, d	7.08, d, $J = 3.1$ Hz, 1 H
3	94.9, s		97.7, s	
4	119.5, s		121.7, s	
5	127.5, s		123.6, s	
6	162.4, s		161.7, s	
7		8.04, t, $J = 5.2$ Hz, 1 H		7.86, m, 1H
8	37.4, t	3.43, m, 2 H	36.5, t	3.11, m, 2 H
9	125.2, d	6.19, t, $J = 7.1$ Hz, 1 H	31.7, t	1.98 and 2.17 (AB), m, 2H
10	126.4, s		33, 4, d	4.08, t, $J = 3.9$ Hz, 1 H
11	125.1, s		129.4, s	
12		12.48, br, 1 H^b		11.74, br, 1 H^b
13	147.3, s		146.9, s	
14		12.23, br, 1 H^b		12.17, br, 1 H^b
15	111.6, d	6.83, s, 1 H	111.0, d	6.12, s, 1 H
13-NH_2		7.48, brs, 2 H		7.35, brs, 2 H

^{a} The signals in these compounds were assigned from the long-range correlations observed in the HMBC spectra. ^{b} Signals of H-12 and H-14 are interchangeable.

NMR chemical shifts of C-2 (123.0, d) and C-3 (94.9, s). Hence, compound **1** was 2-debromostevensine.

Compound **2** was obtained as brown amorphous solid and showed pseudomolecular ion peaks at m/z 310 and 312 in the ESIMS, also indicating one bromine substituent. The molecular formula of $C_{11}H_{13}N_5OBr$ was deduced from HRESIMS. The ^1H and ^{13}C NMR data of **2** partly resembled those of hymenin (**4**), except for the presence of one additional sp^2 methine resonance [δ_H 7.80 (d), δ_C 122.1 (d)], indicating that **2** is the debromo derivative of hymenin (**4**). The position of the bromine atom at C-3 was evident from $^1J_{C-H}$ (192 Hz)[4,10] for C-2 and the ^{13}C NMR chemical shifts of C-2 (122.1, d) and C-3 (97.7, s).

The CD spectrum of **2** was similar to that of hymenin (**4**) and showed a positive Cotton effect with λ MeOH 240 nm (Δ +2.38). Thus, both molecules have the same stereochemistry but their absolute configuration remains to be assigned.

To our knowledge, this is the first time that all three compounds with the hymenialdisine moiety have been isolated in both geometrical configurations (**4**−**10**) from one sponge species.

Upon standing in DMSO-d_6, compounds **8**−**10** converted into their respective isomers (**5**−**7**), which have the Z configuration of the 10,11-double bond.[5,6]

The smooth conversion of (*E*)-debromohymenialdisine (**8**) into the respective Z isomer (**5**) by E/Z isomerization of a C−C double bond is easily explained by the push−pull character of the two substituents at this double bond: the entire imidazolone heterocycle on the one side and electron-rich pyrrole substituent on the other. This is best expressed by zwitterionic mesomeric structure **8a** (Figure 1), which, besides establishing the positive charge in the form of a relatively stable iminium salt, also allows an additional formal aromatization of the imidazolone ring. The expected high stability of the mesomeric structure **8a** and thus its presumably great contribution to the true structure of (*E*)-debromohymenialdisine (**8**) explains the drastically reduced double-bond character of the C-10−C-11 central bond and, hence, the configurative instability of this double bond. The unidirectional reaction course from **8** to **5** is understandable from the release of steric strain as exerted between the proton at C-3 and the oxygen at C-15 in **8**, replacing the sterically demanding carbonyl group by the distinctly smaller NH array. The facile and irreversible isomerization of **8** to give **5**, apparently without the necessity of a photochemical activation, makes it somewhat understandable why **8** has only been detected in one previous isolation work although it seems to be a constitutive metabolite.[8] The same mechanism can be deduced for the other two pairs of geometric isomers.

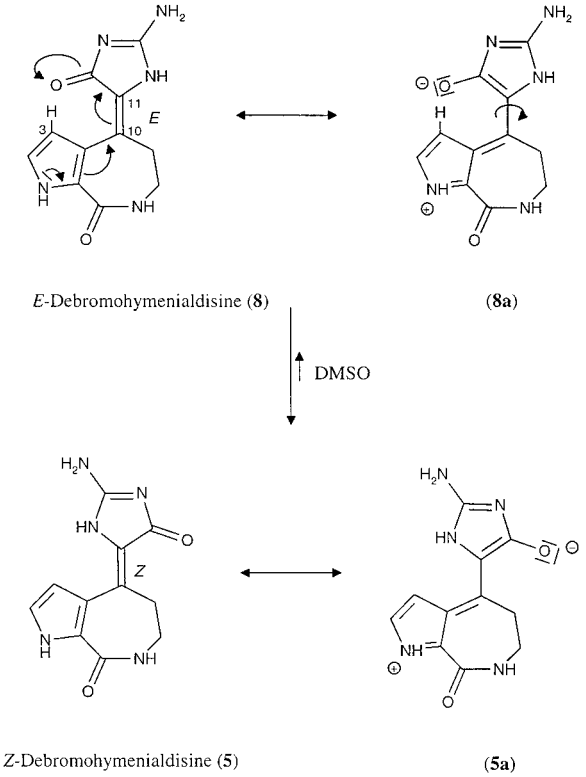

Figure 1. Proposed mechanism of interconversion of (*E*)-debromohymenialdisine into the respective Z isomer.

Cytotoxicity assays were carried out for (*Z*)-debromohymenialdisine (**5**), (*Z*)-hymenialdisine (**6**), (*Z*)-3-bromohymenialdisine (**7**), debromostevensin (**1**), stevensin (**3**), debromohymenin (**2**), and hymenin (**4**) using human monocytic leukemia cells (MONO-MAC 6). Due to the instability of the E isomers (**8**−**10**) under the assay conditions they were not screened for cytotoxicity. From the compounds investigated, only (*Z*)-debromohymenialdisine (**5**) and (*Z*)-hymenialdisine (**6**) proved to be active against MONO-MAC-6 cells. The IC_{50} values of the latter two compounds were 2.4 (**5**) and 0.2 μg/mL (**6**), respectively.

Experimental Section

General Experimental Procedures. ^{1}H (1D, 2D COSY) and ^{13}C (1D, DEPT-135, 2D HMBC) NMR spectra were recorded on Bruker AM 300 and ARX 400 NMR spectrometers. Mass spectra (ESIMS) were recorded on a Finnigan MAT TSQ-7000 mass spectrometer with a capillary temperature of 220 °C and a drift voltage of 3.5 kV. HRESIMS were obtained on a Finnigan MAT 900 mass spectrometer. UV and CD spectra were measured in methanol on a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer and ISA Jobin CD6 spectropolarimeter, respectively. Solvents were distilled prior to use, and spectral-grade solvents were used for spectroscopic measurements. TLC was performed on precoated TLC plates with silica gel F_{254} (Merck, Darmstadt, Germany). The compounds were detected by their UV absorbance at 254 and 366 nm. Semipreparative HPLC was performed on a HPLC system (Merck, Darmstadt, Germany) coupled with a UV detector (UV detection at 254 nm). The separation column (8 × 250 mm) was prefilled with Eurospher C_{18} (Knauer, Berlin, Germany). The compounds were eluted with a solvent system of MeOH/H_2O, containing 0.1% CF_3COOH for improved separation, at a flow rate of 5 mL/min.

Sponge Materials. Specimens of *S. carteri* were collected at a depth of 3−23 m near the islands of Sulawesi (Pulau Baranglompo) and Ambon (Seram Tanjung Sial), Indonesia, in August 1997. *S. carteri* (Dendy, 1889) is a red-orange flabellate sponge with a ridged conulose but in between smooth slippery surface with a faint light orange veneer (below the surface the color becomes darker). The surface has no spicule cover (thus is organic). The choanosomal skeleton is irregularly plumoreticulate, with thin tracts of spicules becoming thicker toward the interior. The stem and its ramifications consist of thick spicule masses. Spicules are exclusively styles, mostly rather robust and curved near the blunt end; the length and width are variable, with the typical sizes being 350 × 15 to 500 × 20 μm. The sponge samples were immersed in EtOH immediately after collection. A voucher specimen of each sample is kept in ethanol under the registration numbers ZMA POR. 12662 (specimen from Sulawesi) and 12668 (specimen from Ambon).

Extraction and Isolation. The sponges (each sample ca. 500 g wet weight) were extracted exhaustively with methanol, and the methanolic extracts were combined with the EtOH-soluble material. The resulting crude extracts were first partitioned between H_2O and EtOAc followed by extraction of the aqueous layer with *n*-BuOH.

The *n*-BuOH-soluble material of *S. carteri* from Sulawesi (5.29 g) was subjected to vacuum−liquid chromatography on silica gel, using a step gradient consisting of different portions of CH_2Cl_2−MeOH. Elution started with 100% CH_2Cl_2, and the MeOH concentrations were increased gradually. Twenty fractions were obtained. Fraction 65:35 (CH_2Cl_2−MeOH) yielded compounds **8** (26.5 mg, 0.50%) and **5** (200 mg, 3.8%). Further purification of this fraction was achieved by C_{18} column chromatography (Lobar, RP-18, Merck, size B; MeOH−H_2O−CF_3COOH, 65:35:0.1). Compound **8** was finally obtained by semipreparative RP-18 HPLC with gradient elution starting with a concentration of 10% MeOH and increasing the concentration in a linear manner within 25 min up to 30%. The less polar fraction 70:30 (CH_2Cl_2−MeOH) yielded compounds **9** (25.9 mg, 0.48%) and **6** (90 mg, 1.7%). It was subjected to C_{18} column chromatography (Lobar, RP-18, Merck, size B; MeOH−H_2O−CF_3COOH, 55:45:0.5). Compound **9** was finally purified by semipreparative RP-18 HPLC with gradient elution starting with a concentration of 10% MeOH and increasing the concentration in a linear manner within 25 min up to 40%. Fraction 80:20 (CH_2Cl_2−MeOH) was chromatographed on a C_{18} column (Lobar, RP-18, Merck, size B; MeOH−H_2O−CF_3COOH, 70:30:0.25) to afford compound **11** (40 mg, 0.8%). All compounds were identified from the comparison of their UV, MS, and NMR data with those of the literature.[6−9]

The *n*-butanol soluble residue of *S. carteri* from Ambon (4.93 g) was subjected to Sephadex LH-20 (MeOH), and eight major fractions were obtained. Fraction 3 afforded compounds **1** (74.4 mg, 1.5%), **2** (18.8 mg, 0.38%), **3** (110 mg, 2.2%), and **4** (32 mg, 0.6%). The pure compounds were obtained by chromatography on a RP-18 silica gel column (Lobar, RP-18, Merck, size B; MeOH−H_2O−CF_3COOH, 60:40:0.2). Compounds **1** and **2** were further purified by semipreparative RP-18 HPLC with gradient elution starting with a concentration of 15% MeOH and increasing the concentration in a linear manner up to 30% MeOH within 20 min. Fraction 4 yielded compounds **5** (70 mg, 1.4%), **6** (50 mg, 1.0%), **7** (13 mg, 0.3%), and **10** (20 mg, 0.4%). After chromatography on a RP-18 silica gel column (Lobar, RP-18, Merck, size B; MeOH−H_2O−CF_3COOH, 50:50:0.25) pure compounds were obtained by semipreparative RP-18 HPLC with gradient elution starting with a concentration of 25% MeOH and increasing the concentration in a linear manner up to 45% MeOH within 20 min. All known compounds were identified from the comparison of their UV, MS, and NMR data with those of the literature.[2−7]

2-Debromostevensine (1): orange amorphous solid; UV (MeOH) λ_{max} (log ϵ) 232 (3.84), 263 (3.62), 288 (3.37) nm; ^{13}C and ^{1}H NMR, see Table 1; ESIMS *m/z* 308 and 310 $[M + H]^+$; HRESIMS *m/z* 308.0206 $[M + H]^+$ (calcd for $C_{11}H_{11}N_5O^{79}Br$, 308.0147).

2-Debromohymenin (2): brown amorphous solid; UV (MeOH) λ_{max} (log ϵ) 220 (3.86), 269 (3.64) nm; CD λ^{MeOH} 240 (Δ +2.38), 273 (Δ −1.38) nm; ^{13}C and ^{1}H NMR, see Table 1; ESIMS *m/z* 310 and 312 $[M + H]^+$; HRESIMS *m/z* 310.0291 $[M + H]^+$ (calcd for $C_{11}H_{13}N_5O^{79}Br$, 310.0304).

Cytotoxicity Studies. The MONO-MAC-6 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The culture was mycoplasma-free and cultivated under standardized conditions.[11] For all experiments, exponentially growing cells were used, with a viability exceeding 90%, as determined by trypan blue staining. The final concentration used in the experiments was 10^5 cells/mL. The experiments were carried out in the presence of 100 IU/mL of penicillin G and 100 mg/mL of streptomycin.

Concentrated stock solutions of the test compounds were prepared in ethylene glycol monomethyl ether and stored at −20 °C. For the cytotoxicity analysis cells were harvested, washed, and resuspended in a final concentration of 10^5 cells/mL. They were seeded in triplicate in 90 μL volumes in 96-well-flat bottom culture plates (Nunc). Test compounds in 10 μL, obtained by diluting the stock solution with a suitable quantity of growth medium, were added to each well. The cultures were incubated for 48 h at 37 °C in a humidified incubator with 5% CO_2.

Cytotoxicity was determined by incorporation of [^{3}H]thymidine.[12] Radioactive incorporation was carried out for the last 3 h of the 48 h incubation period. One μCi of [methyl-^{3}H]-thymidine (Amersham-Buchler, Braunschweig, Germany; specific activity 0.25 mCi/μmol) was added in a 20 μL volume to each well. Cells were harvested on glass fiber filters with a multiple automatic sample harvester, and radioactivity was determined in a liquid scintillation counter (1209 Rackbeta, LKB, Freiburg, Germany). Media with 0.1% ethylene glycol monomethyl ether were included in the experiments as controls.

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