Four New Bioactive Pyrrole-Derived Alkaloids from the Marine Sponge *Axinella brevistyla*

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Four new alkaloids (**1**−**4**) were isolated from the marine sponge *Axinella brevistyla*, and their structures were determined on the basis of spectroscopic analysis. The alkaloids **1**−**4** were antifungal against the yeast *Saccharomyces cerevisiae* at <1.0, <1.0, 30, and 100 μg/disk, respectively. Compounds **1**−**3** also exhibited cytotoxicity against L1210 cells with IC_{50} values of 1.1, 0.66, and 2.5 μg/mL, respectively.

Bromopyrrole derivatives often found in marine sponges of the genera *Axinella*,[1] *Agelas*,[2] *Hymeniacidon*,[3] *Stylotella*,[4] and *Pseudoceratina*[5] show a variety of biological activities including antimicrobial,[1,2] tyrosine kinase inhibitory,[3] and antifouling.[4,5] During our search for new antifungal metabolites from marine invertebrates using an *erg6* mutant of the budding yeast *Saccharomyces cerevisiae*,[6] we found significant activity in the MeOH extract of the marine sponge *Axinella brevistyla* (Hoshino, 1981) (family Axinellidae) collected in western Japan. Bioassay-monitored isolation afforded five new alkaloids, whose structures were elucidated to be bromopyrrole derivatives by spectroscopic methods. This paper describes the isolation, structure elucidation, and biological activities of these compounds.

The frozen sponge (1 kg, wet weight) was extracted with MeOH. The concentrated aqueous residue was successively extracted with Et_2O and *n*-BuOH. The active organic extracts were combined and fractionated by a combination of Si gel and ODS chromatographies, gel-filtration on Sephadex LH-20, and HPLC to afford four new alkaloids, 3-bromomaleimide (**1**, 5.0 mg), 3,4-dibromomaleimide (**2**, 12.0 mg), 12-chloro-11-hydroxydibromoisophakellin (**3**, 26.8 mg), and *N*-methylmanzacidin C (**4**, 10.4 mg) along with the known dibromoisophakellin (**5**, 26.8 mg),[8] tauroacidin A (**6**, 7.5 mg),[3] taurodispacamide A (**7**, 4.0 mg),[9] girolline (**8**, 20.0 mg),[10] and 4,5-dibromopyrrole-2-carboxylic acid (**9**, 44.0 mg) (Chart 1).[11]

Compound **1** showed 1:1 doublet ion peaks at *m*/*z* 175 and 177, indicating the presence of a bromine atom; a formula of $C_4H_2BrNO_2$ was established by HREIMS. The ^{1}H NMR spectrum measured in DMSO-d_6 revealed an olefinic proton [δ 7.29 (1H, s, H-4)] and a D_2O-exchangeable hydrogen [δ 11.27 (1H, br s, NH)], while the ^{13}C NMR spectrum displayed signals for two olefinic carbons [δ 131.1 (s) and 133.5 (d)] and two carbonyl carbons [δ 167.0 and 170.4]. These NMR data together with IR bands at 1780, 1760, and 1720 cm^{-1} confirmed structure **1** as 3-bromomaleimide.

The EI mass spectrum of compound **2** exhibited a 1:2:1 ion cluster at *m*/*z* 253/255/257, which matched a formula of $C_4HBr_2NO_2$. The ^{1}H and ^{13}C NMR spectra measured in DMSO-d_6 exhibited signals at δ_H 11.66 (1H, br s, NH) and δ_C 130.0 (s) and 165.4 (s), thus suggesting the symmetrical nature of **2**. Therefore, **2** is 3,4-dibromomaleimide.

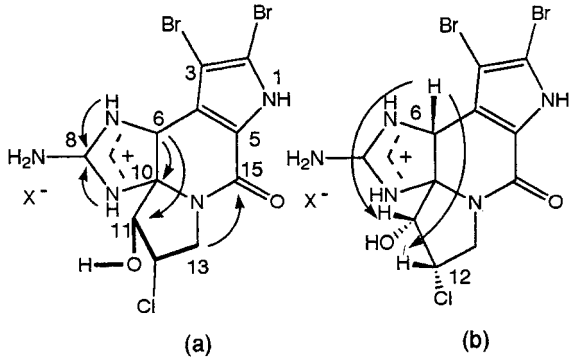

Figure 1. (a) COSY (bold lines) and HMBC (arrows) connectivities for **3**. (b) Selected NOE correlations observed for **3**.

The positive FABMS of compound **3** displayed a 1:2:1.5:0.3 ion cluster at *m*/*z* 438/440/442/444, indicating the presence of a chlorine and two bromine atoms. A molecular formula of $C_{11}H_{11}Br_2ClN_5O_2$ was obtained by HRFABMS. The ^{1}H NMR spectrum in DMSO-d_6 revealed five deshielded sp^3 proton signals at δ 3.60, 4.26, 4.30, 4.38, and 5.36 and six D_2O-exchangeable signals at δ 6.83, 8.17 (2H), 8.72, 9.07, and 12.97. Analysis of the HMQC spectrum revealed a nitrogenous methylene [δ 3.60 and 4.26/51.8 (C-13)] and three heteroatom-bearing methines [δ 4.38/56.7 (C-12), δ 4.30/82.6 (C-11), and δ 5.36/50.3 (C-6)], of which the signals at δ 4.38/56.7 (C-12) were consistent with a chlorinated methine.[10] A COSY cross-peak 11-OH/H-11 placed the hydroxyl group at C-11, thereby resulting in a partial structure, −C(11)HOH−CHCl−C(13)H_2− (Figure 1a). The signals at δ_H 8.17 (2H, br s, 8-NH_2), 8.72 (1H, br s, H-9), 9.07 (1H, br s, H-7) and δ_C 157.0 (s, C-8) were reminiscent of a guanidinium group, which was supported by HMBC cross-peaks δ_H 8.72, 9.07/δ_C 157.0. HMBC cross-peaks H-6/C-8, C-10, and C-11 accommodated the guanidinium group in a five-membered ring. Connectivities of C-6−C-10−C-11 were inferred from HMBC correlations, H-6/C-10 and H-6/C-11 (Figure 1a). The exchangeable proton signal resonating at δ 12.97 (1H, br s, H-1) and sp^2 carbon signals at δ 95.8 (C-5), 105.1 (C-2), 110.9 (C-3), and 133.1 (C-4) were consistent with a disubstituted 2,3-dibromopyrrole.

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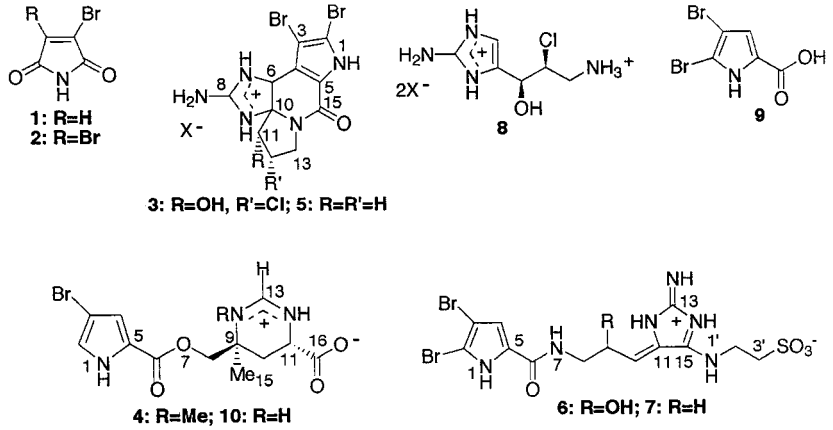
Chart 1



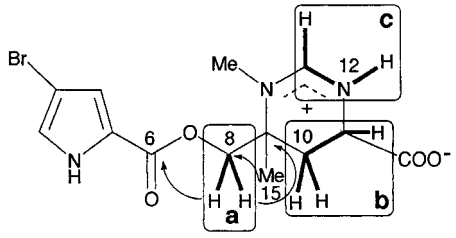


Figure 2. Partial structures **a**−**c** of **4** and COSY (bold lines) and HMBC (arrows) correlations.

An HMBC cross-peak H_2-13/C-15 could incorporate the remaining element of CNO in a 2-piperidone ring (Figure 1a). Hence, **3** was 12-chloro-11-hydroxyldibromoisophakellin.[8] NOESY cross-peaks from H-6 to H-11 and H-12 suggested the relative stereochemistry of **3** as shown in Figure 1b.

Compound **4** possessed a molecular formula of $C_{13}H_{16}BrN_6O_4S$ as established by HRFABMS. The presence of a 2-carboxy-3-bromopyrrole moiety was readily inferred from NMR data. Interpretation of COSY and HMQC data resulted in three partial structures **a**, **b**, and **c** (Figure 2). There were three functional groups to be located, e.g., an *N*-methyl, a carboxyl group, and a tertiary methyl group. HMBC cross-peaks, H_2-10/C-15, C-16, H-11/C-16, H-13/C-9, C-11, NMe, H_3-15/C-9, C-10, and Me-14/C-9, C-13, constructed an *N*-methyltetrahydropyrimidinium ring, to which the carboxyl and tertiary methyl groups were attached at C11 and C9 positions, respectively.[10] The 2-carboxy-4-bromopyrrole and *N*-methyltetrahydropyrimidinium units could be connected through the C-8 methylene by the HMBC cross-peaks, H_2-8/C6, C9 and H_3-15/C8 (Figure 2). Thus, **4** possessed the pyrimidine ring identical with that of manzacidin D[12] except for stereochemistry. Since an NOE between H_3-15 and H-11 was not observed, **4** had the same relative stereostructure as manzacidin C (**10**).[13] The optical rotation of **4** ($[\alpha]_D^{23}$ +36.4°) comparable to that of **10** ($[\alpha]_D^{22}$ +37°) indicated their identical absolute stereochemistry. Therefore, **4** was established as *N*-methylmanzacidin C. A recent synthesis of **10** disclosed the 9*S*,-11*S*-stereochemistry.[14]

Compounds **1**−**4** inhibited the growth of the *erg6* mutant[1] of the yeast *S. cerevisiae* at <1.0, <1.0, 30, and 100 μg/disk, respectively, while **1**−**3** were also cytotoxic against L1210 cells with IC_{50} values of 1.1, 0.66, and 2.5 μg/mL, respectively. Although compounds **1** and **2** have been synthesized,[15] this is the first report of their isolation from nature. Interestingly, **3** is biogenetically derived from a potent antitumor agent, girolline (**8**),[10] and 4,5-dibromopyrrole-2-carboxylic acid (**9**),[11] both of which were also present in the sponge.

Experimental Section

General Experimental Conditions. UV spectra were measured on a SHIMADZU UV-1600 UV−visible spectrophotometer. IR spectra were recorded on a SHIMADZU IR-460 infrared spectrophotometer. Optical rotations were determined with a JASCO DIP-1000 digital polarimeter or a HORIBA SEPA-300 high-sensitive polarimeter. NMR spectra were recorded on a JEOL A600 NMR or JEOL GSX500 NMR spectrometer in DMSO-d_6. All chemical shifts were reported with respect to DMSO-d_6 (δ_H 2.49, δ_C 39.5). Mass spectra were measured on a JEOL SX-102 mass spectrometer.

Antifungal Assay Against the Yeast *Saccharomyces cerevisiae*. The strain of *S. cerevisiae* used in this study was the *erg6* strain YAT2285 (*MAT*α *leu2 his3 trp1 ura3 ade2 can1 erg6*::*URA3*) generously supplied by Prof. A. Toh-e of the University of Tokyo. Growth inhibitory activity was determined by the paper disk method. Paper disks (6 mm ϕ) loaded with samples were incubated on agar plates at 27 °C.

Animal Material. The marine sponge was collected by scuba at a depth of 25 m in Kamagi Bay on the Sada Peninsula, 600 km west of Tokyo, frozen immediately, and kept frozen until processed. The sponge was identified as *Axinella brevistyla (*Hoshino, 1981) (family Axinellidae). It was originally described as *Homaxinella brevistyla* by Hoshino, 1981. The genus *Homaxinella* is now considered to belong to the Suberitidae (order Hadromerida), but Hoshino's species is a clear Axinellidae (order Halichondrida). Our investigations show that some *Axinella*s, such as *A. brevistyla*, have few if any oxeas in addition to the styles, but that is not sufficient for it to belong to a separate genus. The structure of the sponge is quite similar to typical *Axinella*s with both styles and oxeas. A voucher specimen (ZMA POR. 15732) was deposited at the Institute for Systematics and Ecology, University of Amsterdam, The Netherlands.

Extraction and Isolation. The frozen sponge (1.0 kg, wet wt) was extracted with MeOH. The combined extracts were concentrated under reduced pressure and extracted with ether and then with *n*-BuOH. A portion (4.4 g) of the *n*-BuOH layer (8.3 g) was subjected to ODS chromatography with MeOH/H_2O. The fraction eluted with 60% MeOH/H_2O was purified by gel-filtration on Sephadex LH-20 with MeOH, followed by Si gel chromatography with $CHCl_3$/*n*-BuOH/AcOH/H_2O and reversed-phase HPLC with CH_3CN/H_2O/TFA to afford taurodispacamide A (**7**, 4.0 mg) and *N*-methylmanzacidin C (**4**, 10.4 mg) together with known tauroacidin A (**6**, 7.5 mg) and dibromoisophakellin (**5**, 26.8 mg). A portion (4.0 g) of the ether layer (11.0 g) and another portion (2.0 g) of the *n*-BuOH layer were combined and subjected to ODS chromatography with MeOH/H_2O. The 2-PrOH-soluble portion of the 60% MeOH/H_2O eluate was purified by gel filtration on TOYOPEARL HW-40 with 2-PrOH, followed by ODS chromatography with

CH_3CN/H_2O to afford 3-bromomaleimide (**1**, 5.0 mg) and 3,4-dibromomaleimide (**2**, 12.0 mg) along with known 4,5-dibromopyrrole-2-carbamide (**9**, 44.0 mg). The remaining ether (7.0 g) and *n*-BuOH layers (1.0 g) were combined and subjected to ODS chromatography with $MeOH/H_2O$. The MeOH-soluble portion of the 30% $MeOH/H_2O$ eluate was purified by ODS chromatography with CH_3CN/H_2O, Si gel chromatography with EtOAc, and normal phase HPLC with EtOAc to afford 12-chloro-11-hydroxydibromoisophakellin (**3**, 26.8 m). The aqueous layer was fractionated by ODS chromatography with H_2O and gel-filtration on Sephadex LH-20 with H_2O, followed by reversed-phase HPLC with H_2O/TFA to afford girolline (**8**, 20.0 mg).

3-Bromomaleimide (1): UV (MeOH) λ_{max} (log ϵ) 201.5 (3.8), 229.5 (3.7), 245.5 nm (3.5, sh); IR (KBr) ν_{max} 1780, 1760, 1720 cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.29 (1H, s, H-4) and 11.27 (1H, br s, NH); ^{13}C NMR (DMSO-d_6) δ 131.1 (C-3), 133.5 (C-4), 167.0 (C-5 or C-2), and 170.4 (C-2 or C-5); EIMS *m/z* 175 $[M]^+$, 177 $[M + 2]^+$ (1:1); HREIMS *m/z* 174.9280 ($C_4H_279BrNO_2$, Δ +1.1 mmu).

3,4-Dibromomaleimide (2): UV (MeOH) λ_{max} (log ϵ) 236.0 (4.1), 243.0 (4.1), 251.5 (3.9, sh), 303.5 nm (3.1); IR (KBr) ν_{max} 1760, 1720 cm^{-1}; ^1H NMR (DMSO-d_6) δ 11.66 (1H, br s, NH); ^{13}C NMR (DMSO-d_6) δ 130.0 (C-3, C-4), 165.4 (C-2, C-5); EIMS *m/z* 253 $[M]^+$, 255 $[M + 2]^+$, 257 $[M + 4]^+$ (1:2:1); HREIMS *m/z* 252.8385 ($C_4H^{79}Br_2NO_2$, Δ +1.1 mmu).

12-Chloro-11-hydroxydibromoisophakellin (3): $[\alpha]_D^{23}$ +51.0° (*c* 0.408, MeOH); UV (MeOH) λ_{max} (log ϵ) 207.5 (4.4), 242.5 nm (3.8, sh); IR (KBr) ν_{max} 3300, 1670, 1560, 1490, 1200, 1130 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.60 (1H, dd, J = 12.8, 3.9 Hz, H-13), 4.26 (1H, dd, J = 12.8, 6.8 Hz, H-13), 4.30 (1H, d, J = 2.0 Hz, H-11), 4.38 (1H, ddd, J = 6.8, 3.9, 2.0 Hz, H-12), 5.36 (1H, s, H-6), 6.83 (1H, br s, 11-OH), 8.17 (2H, br s, 8-NH_2), 8.72 (1H, br s, H-9), 9.07 (1H, br s, H-7), 12.97 (1H, br s, H-1); ^{13}C NMR (DMSO-d_6) δ 50.3 (C-6), 51.8 (C-13), 56.7 (C-12), 82.6 (C-11), 85.2 (C-10), 95.8 (C-5), 105.1 (C-2), 110.9 (C-3), 133.1 (C-4), 157.0 (C-8), 158.1 (C-15); FABMS (positive, NBA matrix) *m/z* 438 $[M]^+$, 440 $[M + 2]^+$, 442 $[M + 4]^+$, 444 $[M + 6]^+$ (1:2:1.5: 0.3); HRFABMS *m/z* 441.8915 ($C_{11}H_{11}79Br^{81}Br^{37}ClN_5O_2$, Δ −0.3 mmu).

***N*-Methylmanzacidin C (4):** $[\alpha]_D^{23}$ +36.4° (*c* 0.169, MeOH); UV (MeOH) λ_{max} (log ϵ) 202.5 (3.7), 220.5 (3.7), 273.0 nm (3.7); IR (KBr) ν_{max} 3400, 1680, 1620, 1400, 1310, 1200, 1180, 1130 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.38 (3H, s, H_3-15), 1.99 (1H, dd, J = 13.8, 10.8 Hz, H-10), 2.59 (1H, dd, J = 13.8, 5.4 Hz, H-10), 3.17 (3H, s, Me-14), 4.26 (1H, d, J = 12.0 Hz, H-8), 4.42 (1H, dd, J = 10.8, 5.4 Hz, H-11), 4.47 (1H, d, J = 12.0 Hz, H-8), 6.87 (1H, s, H-4), 7.25 (1H, s, H-2), 8.25 (1H, d, J = 0.6 Hz, H-13), 10.38 (1H, d, J = 0.6 Hz, H-12), 12.43 (1H, s, H-1); ^{13}C NMR (DMSO-d_6) δ 20.1 (C-15), 31.6 (C-10), 36.0 (NMe), 48.5 (C-11), 55.9 (C-9), 66.0 (C-8), 96.2 (C-3), 116.7 (C-4), 121.7 (C-5), 124.8 (C-2), 153.3 (C-13), 158.7 (C-6), 170.3 (C-16); FABMS (positive, glycerol matrix) *m/z* 358 $[M + H]^+$, 360 $[M + 2 + H]^+$ (1:1); HRFABMS *m/z* 358.0404 ($C_{13}H_{17}79BrN_3O_4$, Δ +0.2 mmu).

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