1,3-Dimethylisoguaninium, an Antiangiogenic Purine Analog from the Sponge *Amphimedon paraviridis*

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Received January 10, 2003; accepted February 21, 2003

An antiangiogenic purine analog, 1,3-dimethylisoguaninium (1), was isolated from the ethanol (EtOH) extract of the Okinawan sponge *Amphimedon paraviridis*. The structure was elucidated on the basis of its spectral properties and X-ray crystallographic analysis. Compound 1 exhibited specific inhibition of the basic fibroblast growth factor (bFGF)-induced proliferation of bovine aorta endothelial cells (BAECs). Moreover, compound 1 reduced the tube formation of BAECs in a time-dependent manner.

Key words antiangiogenesis; purine analog; 1,3-dimethylisoguaninium; marine sponge; *Amphimedon paraviridis*

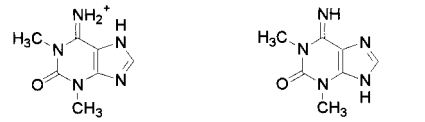
In our continuing efforts to discover angiogenesis inhibitors for the development of new anti-cancer agents from natural resources,[1,2] we have investigated the antiangiogenic activity of Okinawan marine invertebrates. Among these, the EtOH extract of the marine sponge *A. paraviridis* exhibited inhibitory activity against the proliferation of bovine aorta endothelial cells (BAECs). Further investigation has led to the isolation of a purine analog **1** as an active principle. In this paper, we describe the structural determination and antiangiogenic activity of **1**.

The EtOH extract of the sponge was extracted with diethyl ether (Et_2O) and normal butanol (*n*-BuOH), successively. However, both Et_2O and *n*-BuOH fractions exhibited less inhibitory activity than the EtOH fraction on the proliferation of BAECs. For that reason, the active principle was believed to be in the aqueous layer. Thus, the alkaloid extraction was applied to the aqueous fraction, then the active compound **1** was isolated as crystalline needles, followed by chromatographic analysis.

The electron impact mass spectra (EI-MS) of **1** gave a molecular ion peak *m/z* 180 $(M)^+$, corresponding to a molecular formula of $C_7H_{10}N_5O$, which was confirmed by high resolution (HR)-FAB-MS (*m/z*: 180.0882, Calcd for 180.0885). The ^{1}H-NMR spectrum (in CD_3OD) showed an olefinic proton at δ 7.59 (1H, s), and two *N*-methyl protons at δ 3.59 (3H, s), and 3.55 (3H, s). The ^{13}C-NMR spectrum (in DMSO-d_6) exhibited five sp^2 carbon signals at δ 110.3 (s), 148.7 (s), 149.2 (d), 149.9 (s), and 151.0 (s), and two *N*-methyl signals at δ 30.4 and 30.5. The UV spectrum of **1** showed two absorption bands [λ_{max} nm (log ε): 213 (3.82), 296 (3.82)]. From the above data, compound **1** was indicated to be a purine derivative, although further structural determination of **1** using two dimensional (2D)-NMR was not completed. The structure of **1** was established by single-crystal X-ray diffraction. Compound **1** was successfully crystallized in EtOH. The complete structure of **1** was determined to be 1,3-dimethylisoguaninium from a perspective ORTEP drawing, as shown in Fig. 2.

In recent papers, two groups have reported a purine analog, 1,3-dimethylisoguanine (**2**), from the sponge *Amphimedon viridis*.[3,4] The ^{13}C-NMR data of **1**, measured in CD_3OD, agreed very closely with those reported by Ireland's group, but not Berlinck's group. However, the EI-MS data of **1** agreed with those reported by Berlinck's group, not Ireland's group, as shown in Tables 1 and 2. These spectral differences are presumed to result from the isolation procedures. Berlinck's group used trifluoroacetic acid (TFA) containing a solvent with reversed-phase HPLC in the final separation process, because compound **2**, which was reported by Berlinck's group, might be produced as a TFA salt. On the other hand, compound **1** and compound **2**, which was reported by Ireland's group, might be extracted as a salt. Finally, compounds **1** and **2** might be produced in an ionic form and as a hydrochloric salt, respectively, since a pair ion of Cl^- was not detected in the crystal molecular structure of **1**.

1,3-Dimethylisoguaninium (**1**) was evaluated for antiangiogenic activity using methods described previously.[1] 1,3-Dimethylisoguaninium (**1**) inhibited the proliferation of BAECs in medium containing 10% fetal bovine serum (FBS) with an IC_{50} value of 5.2 μM (Fig. 3A).



1,3-dimethylisoguaninium (**1**) 1,3-dimethylisoguanine (**2**)

Fig. 1. Structures of 1,3-Dimethylisoguaninium (**1**) and 1,3-Dimethylisoguanine (**2**)

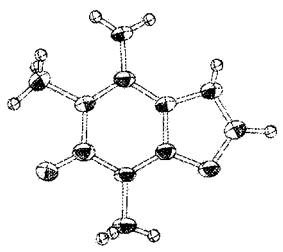


Fig. 2. Perspective ORTEP Drawing of **1**

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On the other hand, 1,3-dimethylisoguaninium (**1**) did not show significant inhibitory activity against the proliferation of KB cells (Fig. 3B).

To determine whether **1** inhibits angiogenic factor-induced BAECs proliferation, we tested **1** for its ability to inhibit bFGF-induced BAECs proliferation. 1,3-Dimethylisoguaninium (**1**) inhibited bFGF-induced proliferation of BAECs in a dose-dependent manner, and exhibited significant inhibition of bFGF-induced proliferation of BAECs at the concentration of 1 μM (Fig. 3C).

Additionally, we tested for the ability of **1** to inhibit tube formation of BAECs. As shown in Fig. 4, treatment with **1** at 100 nM reduced the tube formation of BAECs in a time-dependent manner.

bFGF and vascular endothelial growth factor (VEGF) are the most potent angiogenic factors, regulating endothelial cell proliferation and vascular permeability. Receptor tyrosine kinase inhibitors against bFGF and VEGF have been developed for the treatment of human cancer. 1,3-Dimethyl-

Table 1. ^{13}C-NMR Data of **1** and **2** in CD_3OD

C	**1**[a]	**2**[b]	**2**[c]
N1–CH_3	31.5	30.4	32.1
N3–CH_3	31.1	30.6	32.1
C2	150.9	150.8	150.3
C4	152.3	152.7	131.6
C5	111.4	111.2	116.7
C6	151.5	152.3	151.7
C8	151.5	—	144.5

a) 150 MHz, spectrum was referenced to solvent signal. *b*) 125 MHz, spectrum was referenced to solvent signal and the data were cited of ref. 3. *c*) 100 MHz, spectrum was referenced to TMS as internal reference and the data were cited of ref. 4.

Table 2. EI-MS Data of **1** and **2**

	Relative intensities (%)		
m/z	**1**	**2**[a]	**2**[b]
180	10	—	—
179	100	34	100
150	96	—	57
122	8	100	—
121	40	78	27
94	44	65	17
57	20	9	—

a) 70 eV, data were cited of ref. 3. *b*) 70 eV, data were cited of ref. 4.

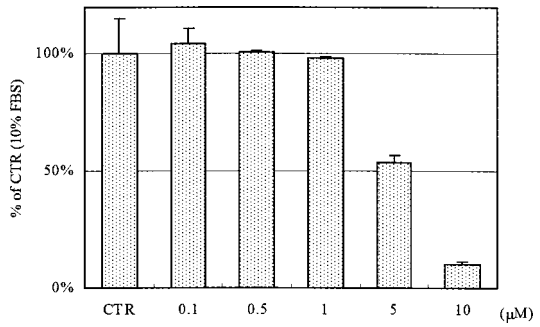

Fig. 3A. Inhibitory Effect of 1,3-Dimethylisoguaninium (**1**) on Proliferation of BAECs

Data shown are mean values with bars indicating the S.D. of the mean (n=3).

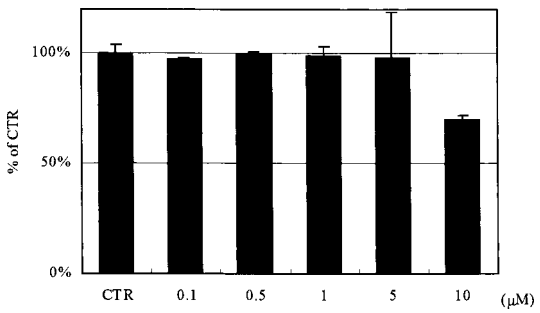

Fig. 3B. Inhibitory Effect of 1,3-Dimethylisoguaninium (**1**) on Proliferation of KB Cells

Data shown are mean values with bars indicating the S.D. of the mean (n=3).

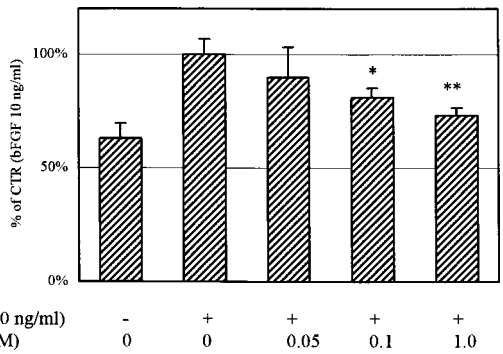

Fig. 3C. Inhibitory Effect of 1,3-Dimethylisoguaninium (**1**) on bFGF-Induced Proliferation of BAECs

Data shown are mean values with bars indicating the S.D. of the mean (n=3). $*p<0.05$, $**p<0.01$ compared with bFGF alone.

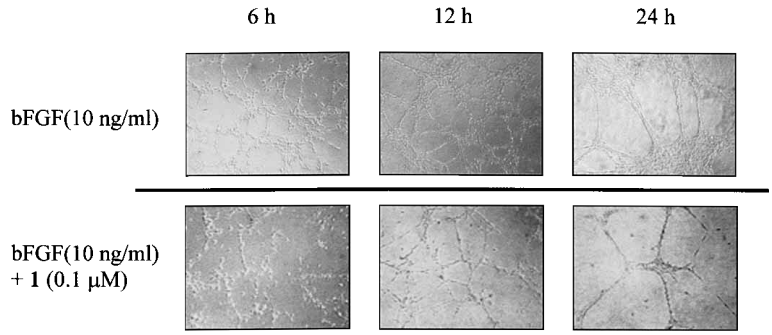

Fig. 4. Effect of Compound **1** on BAECs Tube Formation

BAECs were incubated with or without compound **1** (100 nM) on polymerized Matrigel. After 6, 12 or 24 h, morphological changes of BAECs were observed and photographed with a phase-contrast microscope (×100).

isoguaninium (**1**) may be useful for the development of novel antiangiogenic agents.

Experimental

General Experimental Procedures UV spectra were recorded on a JASCO U-best30 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on JEOL GX-270 (270 MHz) and Varian Unity 600 (600 MHz) spectrometers. The chemical shifts are reported in parts per million, relative to the residual solvent. EI-MS was recorded on a Shimadzu GCMS-QP5050A. FAB-MS and HR-FAB-MS were recorded on a JEOL SX-102 spectrometer. X-Ray crystallographic measurement was made on a Rigaku AFC5R automatic single crystal structure analysis system.

Animal Material The sponge *A. paraviridis* was collected at depths of 1.0—1.5 m off Sesoko Island, Okinawa Prefecture, Japan, in July 2000. A voucher specimen (ZMA POR. 16723) is deposited at the Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Netherlands.

Extraction and Isolation Wet specimens (1.4 kg) were macerated in a blender and extracted with EtOH (1.5 l×2). The EtOH extract was evaporated *in vacuo*, and the resulting aqueous suspension was diluted with H_2O (0.5 l), then extracted with Et_2O (1 l×2) and *n*-BuOH (1 l×2), successively. The remaining aqueous layer was then acidified with HCl to produce a 3% HCl aqueous layer, and was then extracted with $CHCl_3$. The aqueous layer was then basified with NaOH solution to pH 9, and the mixture was extracted with $CHCl_3$. The crude alkaloid fraction was repeatedly chromatographed on Si gel ($CHCl_3$: MeOH : H_2O, 7 : 3 : 1, lower layer) to give compound **1** (2.5 mg).

1,3-Dimethylisoguaninium (1) Compound **1** was obtained as colorless needles (EtOH). UV λ_{max} (MeOH) nm (log ε): 213 (3.82), 296 (3.82). ^{1}H-NMR (CD_3OD, 600 MHz) δ: 7.59 (1H, s), 3.59 (3H, s), 3.55 (3H, s), ^{13}C-NMR (CD_3OD, 150 MHz) δ: 31.1, 31.5, 111.4, 150.9, 151.5, 152.3, ^{13}C-NMR (DMSO-d_6, 67.8 MHz) δ: 30.4, 30.5, 110.3, 148.7, 149.2, 149.9, 151.0, EI-MS *m*/*z* (rel. int.): 180 (M^+) (10), 179 (100), 150 (96), 136 (9), 121 (40), 94 (44), 67 (34); FAB-MS *m*/*z*: 180 (M^+) (71), 136 (94), 120 (20), 89 (37), HR-FAB-MS *m*/*z*: 180.0882 (Calcd for $C_7H_{10}N_5O$: 180.0885).

Crystal Data for 1 Crystal Data: $C_7H_{10}N_5O$ (M.W. 180), monoclinic, space group $P2_1/c$, a=8.941(5), b=6.105(4), c=14.682(3) Å, (from 22 orientation reflections, 52.0<2θ<59.1°), V=796.4(7) Å^3, Z=4, D_c=1.494 g/cm^3, F(000)=376, μ(Cu$K\alpha$)=9.07 cm^{-1}. Intensity data were recorded on a Rigaku AFC5R diffractometer {Cu$K\alpha$ radiation, ω–2θ scans, $2\theta_{max}$=120.1°, scanwidth (1.21+0.30 tan θ)°}. The intensities of three standard reflections, re-measured every 150 reflections during data collection to monitor crystal stability, indicated that significant deterioration occurred (overall intensity loss=0.00%). From a total 1264 of measurements, those 826 reflections in which $I>3.00\sigma(I)$ were retained for the analysis. Lorentz-polarization corrections were applied. The crystal structure was solved by a direct method (SHELXS-86).[5] The non-hydrogen atoms were refined anisotropically. The final cycle of full-matrix least-squares refinement was based on 826 observed reflections and 159 variable parameters, and they converged with unweighted and weighted agreement factors of R=0.043, R_w=0.044, GOF=1.32. Crystallographic calculations were performed on indigo2 (Silicon Graphics, Inc.) using teXsan Structure Analysis Software (Molecular Structure Co.). In the least-squares iterations, $\Sigma\omega(|F_O|-|F_C|)^2$, $\omega=[1/\sigma_c^2(F_O)+0.25p^2F_O^2]^{-1}$ was minimized.[6]

BAEC Proliferation Assay BAEC was seeded at 1×10^4 cells/well in 24-well plates and incubated in DMEM supplemented with 10% FBS. After 24 h, the medium was replaced with DMEM, supplemented with 1% FBS, in the presence of the compounds and 10 ng/ml recombinant human bFGF (Biosource Co., Camarillo, CA, U.S.A.). After 3 d, the MTT assay was performed.

BAEC Tube Formation Assay Matrigel (250 μl, Becton Dickinson Labware, Bedford, MA, U.S.A.) was added to 24-well plates, and was then polymerized at 37 °C for 30 min. BAEC (1×10^5) cells/well stimulated by bFGF (10 ng/ml) was grown on the Matrigel with or without compound **1**. After 6, 12, or 24 h, morphological changes of BAEC were photographed at ×100 magnification with a phase-contrast microscope.

Statical Analysis The data were analyzed for statistical significance using Student's *t*-test. p Values less than 0.05 were considered to be significant.

Acknowledgments This work was supported in part by a Grant-in Aid for Scientific Research (C) (No. 12672055) from The Ministry of Education, Culture, Sports, Science and Technology, Japan.

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6) Crystallographic data for compound **1** reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44–(0)1223–336033 or e-mail: deposit@ccdc.cam.ac.uk).