Bryoanthrathiophene, a New Antiangiogenic Constituent from the Bryozoan *Watersipora subtorquata* (d'Orbigny, 1852)

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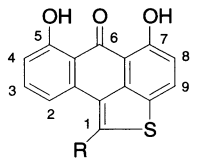
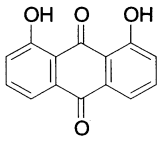
A new antiangiogenic compound, bryoanthrathiophene (**1**), together with two known compounds, 5,7-dihydroxy-1-methoxycarbonyl-6-oxo-6*H*-anthra[1,9-*bc*]thiophene (**2**) and 1,8-dihydroxyanthraquinone (**3**), was isolated from the bryozoan *Watersipora subtorquata* (d'Orbigny, 1852) using bioassay-guided fractionation methods. Among them, bryoanthrathiophene (**1**) exhibited the most potent antiangiogenic activity on BAEC (bovine aorta endothelial cell) proliferation.

Angiogenesis, the growth and formation of new blood vessels from pre-existing ones, is critical for the development of tumor growth and metastasis. Agents that selectively inhibit the proliferation of endothelial cells may be used potentially as treatments for cancer and angiogenesis-dependent diseases such as diabetic retinopathy and arthritis.[1] In our continuing efforts to discover angiogenesis inhibitors for development of new anticancer agents from natural sources, we have investigated the antiangiogenic activity of 120 marine invertebrates. Among them, the acetone extract of the bryozoan *Watersipora subtorquata* (d'Orbigny, 1852)[2] showed an inhibitory effect on bovine aortic endothelial cell (BAEC) proliferation. Herein, we describe the structure determination for the new compound **1** and the antiangiogenic effect of compounds **1**−**3**.

Specimens were collected in Tsutsumi Island, Fukuoka Prefecture, Japan, at depths of 5−10 m, and stored in a freezer until extraction. The acetone extract of *W. subtorquata* exhibited significant inhibition of BAEC proliferation. Bioassay-guided fractionation resulted in the isolation of three antiangiogenic compounds, bryoanthrathiophene (**1**), 5,7-dihydroxy-1-methoxycarbonyl-6-oxo-6*H*-anthra[1,9-*bc*]thiophene (**2**), and 1,8-dihydroxyanthraquinone (**3**).



1 R = CH_3
2 R = $COOCH_3$



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Bryoanthrathiophene (**1**) was obtained as a yellow powder. The EIMS of **1** displayed molecular ion peaks at *m*/*z* 284 [$M^+ + 2$, 7%] and 282 [M^+, 100%] due to isotopic cluster of sulfur, and the molecular formula was determined as $C_{16}H_{10}O_3S$ from the positive HRFABMS. The ^1H NMR spectrum of **1** showed signals for an ABC-type system [δ 7.62 (1H, t, J = 7.9, H-3), 7.59 (1H, d, J = 7.9, H-2), and 6.98 (1H, d, J = 7.9, H-4)], an AB-type system [δ 7.90 (1H, d, J = 8.8, H-9) and 7.05 (1H, d, J = 8.8, H-8), and two hydroxyl groups [δ 13.23 (1H, brs, OH-5) and 12.04 (1H, brs, OH-7)]. These results showed **1** to have a 6*H*-anthra[1,9-*bc*]thiophene skeleton as exhibited by the known compound **2** isolated from this bryozoan previously.[3] In addition, the ^1H NMR signal at δ 2.97 (3H, s, 1-CH_3) and the ^{13}C NMR signal at δ 18.5 indicated that **1** has an olefinic methyl moiety replacing the carboxymethyl ester moiety of **2**. Interpretation of the HSQC and HMBC spectra (see Table 1) enabled its structure (**1**) to be established as 5,7-dihydroxy-1-methyl-6-oxo-6*H*-anthra[1,9-*bc*]thiophene. The known compounds **2** and **3** were also characterized by comparison of their spectral data (NMR and MS) with literature values.[3]

Table 1. ^1H, ^{13}C, and HMBC Spectral Data of Compound **1**[a]

position	δ_C	δ_H (mult., J in Hz)	HMBC
1	122.4[b]		
1a	147.5[b]		
1b	135.0		
2	115.8	7.59 (d, J = 7.9)	C-1a, C-4
3	136.0	7.62 (t, J = 7.9)	C-1b, C-5
4	115.9	6.98 (d, J = 7.9)	C-2
5	164.2[b]		
5a	115.1		
6	n.r.[c]		
6a	110.9[b]		
7	162.1[b]		
8	115.0	7.05 (d, J = 8.8)	C-6a, C-9a
9	130.5	7.90 (d, J = 8.8)	C-7, C-9b
9a	125.2[b]		
9b	138.2[b]		
CH_3-1	18.5	2.97 (s)	C-1, C-1a
OH-5		13.23 (brs)	C-5a, C-5
OH-7		12.04 (brs)	C-6a, C-7, C-8

[a] Data recorded in $CDCl_3$ at 600 MHz (^1H) and 150 MHz (^{13}C). [b] Assignments are based on the HMBC experiment. [c] Signal was not recorded.

These three compounds were tested for antiangiogenic activities. The IC_{50} values of **1**−**3**, when evaluated against BAEC proliferation, are shown in Table 2. Bryoanthrathiophene (**1**) is the most active compound of the series and was 90−120 times more potent than compounds **2** and **3** for BAEC proliferation in medium containing 10% fetal bovine serum (FBS). To determine whether **1**−**3** selectively inhibit angiogenic factor-induced proliferation of endothelial cells, the ability of **1**−**3** to modulate basic fibroblast growth factor (bFGF)-induced proliferation of BAEC was examined. Compounds **1**−**3** inhibited bFGF-induced proliferation of BAEC (Table 2). Among them, bryoanth-

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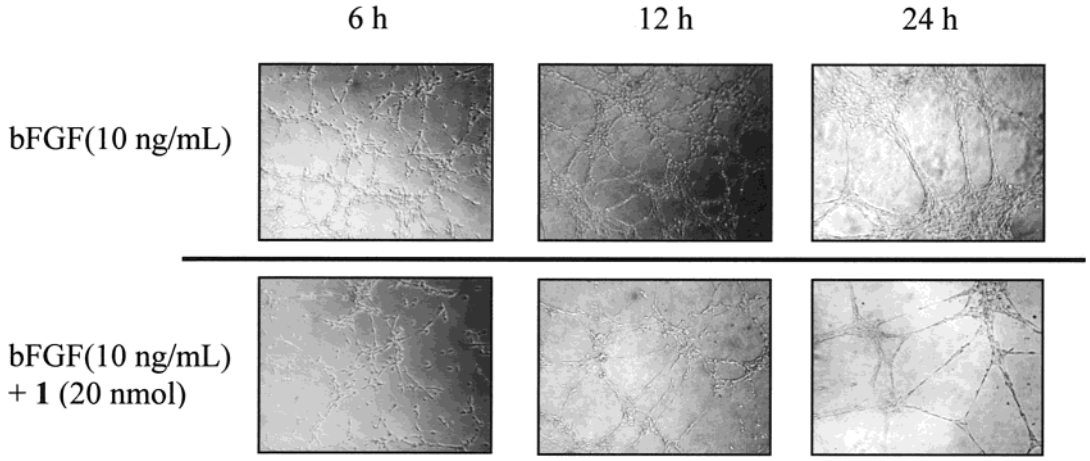

Figure 1. Effect of compound **1** on BAEC tube formation. BAEC was incubated with or without compound **1** (20 nmol) on polymerized Matrigel. After 6, 12, or 24 h, morphological changes of BAEC were observed and photographed with a phase-contrast microscope (×100).

Table 2. Effect of Compounds **1**−**3** on Bovine Aortic Endothelial Cell (BAEC) Proliferation

	IC_{50} values for inhibition of BAEC growth (μmol)	
compound	bFGF	10% FBS
1	0.005	0.22
2	2.2	25.8
3	5.7	20.1

rathiophene (**1**) exhibited the most significant antiangiogenic activity on bFGF-induced proliferation of BAEC. Additionally, we tested for the ability of **1** to inhibit tube formation of BAEC. As shown in Figure 1, treatment with **1** reduced the tube formation of BAEC in a time-dependent manner.

bFGF and vascular endothelial growth factor (VEGF) are the most potent angiogenic factors and regulate endothelial cell proliferation and vascular permeability. Receptor tyrosine kinase inhibitors against bFGF and VEGF have been developed for the treatment of human cancer.[4] Bryoanthrathiophene (**1**) and its analogues may be useful for the development of novel antiangiogenic agents.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a JASCO U-best30 spectrophotometer. IR spectra were recorded on a JASCO FT/IR-410 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 from TMS as an internal standard. EIMS data were obtained using a Shimadzu GCMS-QP5050A. HRFABMS were recorded on a JEOL SX-102 spectrometer.

Animal Material. The bryozoan *W. subtorquata* (Watersiporidae) was collected in Tsutsumi Island, Fukuoka Prefecture, Japan, in June 2000 and July 2001, at depths of 5−10 m, and stored in a freezer until extraction. A voucher specimen (No. ZIHU-02036) is deposited in the Zoological Institute of Hokkaido University.

Extraction and Isolation. Wet specimens (0.64 kg) were macerated in a blender and extracted two times with acetone (1.5 L). The acetone extract was evaporated in vacuo, and the resulting aqueous suspension was diluted with H_2O (0.5 L) and extracted with Et_2O (1 L × 2). The Et_2O-soluble extract (1.22 g) was diluted with MeOH and centrifuged at 10 000 rpm (15 min). The MeOH-soluble extract inhibited BAEC proliferation. The MeOH-soluble extract (0.91 g) was subjected to elution over Sephadex LH-20 with MeOH to give five fractions (Fr. 1−Fr. 5). The active Fr. 3 (84.0 mg) was chromatographed on Sephadex LH-20 with $CHCl_3$/MeOH (1:1−1:2) to give four other fractions. The active subfraction Fr. 3-3 (25 mg) was chromatographed on Si gel 60 with *n*-hexane/acetone (5:2) followed by RP C_{18} with MeOH and purified by HPLC using a Cosmosil 5C_{18}-AR-II column (UV 254 nm; flow rate, 5 mL/min) with the mobile phase MeOH to give compounds **1** (0.6 mg), **2** (3.9 mg), and **3** (5.6 mg).

Bryoanthrathiophene (1): yellow powder; UV (MeOH) λ_{max} (log ϵ) 213 (4.28), 283 (3.81), 324 (3.56), 429 (3.77) nm; IR ($CHCl_3$) ν_{max} 3025, 1604 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m/z* 284 (7), 283 (20), 282 $[M]^+$ (100), 265 (5), 253 (24), 237 (5), 225 (7), 208 (9), 195 (8), 163 (16), 127 (25), 104 (33); HRFABMS *m/z* 283.0451 (calcd for $C_{16}H_{11}O_3S$ $[M + H]^+$, 283.0429).

Cell Culture. BAEC was isolated from the bovine aorta as described previously.[5] These cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM, Asahi Techno Glass Co., Japan) containing 10% heat-inactivated FBS, 60 μg/mL penicillin (Life Technologies, Grand Island, NY), 60 U/mL streptomycin (Life Technologies, Grand Island, NY), and 5 μg/mL Fungizone (Life Technologies, Grand Island, NY) in a 37 °C incubator humidified atomosphere containing 5% CO_2. BAEC was used at passages 4−10.

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). After 3 days, the MTT assay was performed.

BAEC Tube Formation Assay. Matrigel (250 μL, Becton Dickinson Labware, Bedford, MA) 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.

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