Isolation and Characterization of Polybrominated Diphenyl Ethers as Inhibitors of Microtubule Assembly from the Marine Sponge *Phyllospongia dendyi* Collected at Palau

Hongwei Liu,[†,‡] Michio Namikoshi,*[,†] Shiori Meguro,[†] Hiroshi Nagai,[†] Hisayoshi Kobayashi,[§] and Xinsheng Yao[‡]

Department of Ocean Sciences, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo 108-8477, Japan, Department of Natural Products, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, People's Republic of China, and Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan

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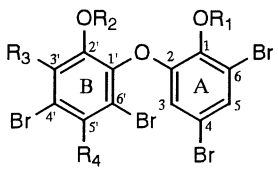
Two new polybrominated diphenyl ethers (**1** and **2**) were isolated by bioassay-guided separations together with nine known compounds (**3**−**11**) from the marine sponge *Phyllospongia dendyi* collected from Palau. The structures were assigned on the basis of their spectral data. Compounds **3**, **7**, and **10** showed inhibitory activities to the assembly of microtubule proteins (IC_{50}: 29.6, 33.5, and 20.9 μM, respectively) and to the meiotic maturation of starfish oocytes (IC_{50}: 3.6, 4.2, and 4.2 μM, respectively), while **1**, **2**, **4**−**6**, **8**, **9**, and **11** were not active at 100 μM. Two phenolic hydroxyls are required for their bioactivities.

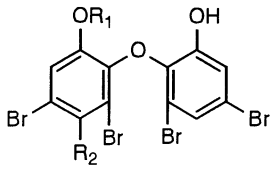
The microtubule system of eukaryotic cells is an important target for the development of anticancer and antifungal agents.[1,2] A variety of compounds, such as vinca alkaloids, colchicine, podophyllotoxin, paclitaxel, and epothilone, act on microtubules to disrupt the cellular microtubule structure and mitotic cell division.[3,4] We have recently reported a screening method for antimitotic substances, which detects the inhibition and stimulation of the assembly and inhibition of the disassembly of microtubules in the sequence utilizing purified porcine brain microtubule proteins.[5]

During our continuous study on biologically active metabolites from the sea, we reported the isolation of seven new meroditerpenoids from the marine sponge *Strongylophora strongylata* by a screening bioassay using starfish oocytes.[6] This screening method detects the inhibitory activity on maturation of oocytes of *Asterina pectinifera* stimulated by 1-methyladenine (1-MeAde). Immature oocytes are arrested at G2/M phase, and 1-MeAde, the maturation-inducing hormone, induces the germinal vesicle breakdown in activating the maturation-promoting factor (MPF). We found that the ethanol extract of the marine sponge *Phyllospongia dendyi* inhibited the maturation of oocytes in this bioassay. The extract also showed inhibitory activity to the assembly of microtubule proteins. Two bioassays were conducted to find cell-cycle inhibitors. Bioassay-guided separation yielded two new (**1** and **2**) and nine known (**3**−**11**) polybrominated diphenyl ethers. Compound **9** and four other polybrominated diphenyl ethers have been reported from the same sponge species.[7] We describe here the isolation, structure characterization, and bioactivities of these compounds.

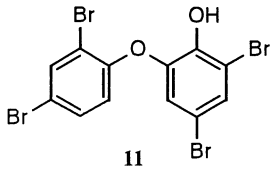
P. dendyi was extracted with EtOH, and the extract was partitioned between water and EtOAc. The EtOAc extract was subjected to bioassay-guided separation using starfish oocytes to give 11 polybrominated diphenyl ethers. The structures of known compounds **3**−**11** were determined on the basis of their spectral data, which were also compared with those of the reported values.[7−10]



1: R_1 = H, R_2 = Me, R_3 = R_4 = Br
2: R_1 = H, R_2 = Me, R_3 = H, R_4 = Br
3: R_1 = R_2 = H, R_3 = R_4 = Br
4: R_1 = R_2 = Me, R_3 = H, R_4 = Br
5: R_1 = H, R_2 = Me, R_3 = R_4 = H
6: R_1 = R_2 = Me, R_3 = R_4 = H
7: R_1 = R_2 = H, R_3 = Br, R_4 = H
8: R_1 = H, R_2 = Me, R_3 = Br, R_4 = H
12: R_1 = R_2 = Me, R_3 = R_4 = Br



9: R_1 = Me, R_2 = H
10: R_1 = H, R_2 = Br



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Compound **1** was obtained as a viscous oil. ESIMS of **1** showed the presence of six Br atoms, and the molecular formula $C_{13}H_6Br_6O_3$ was determined from the FABMS and NMR spectral data. The ^{1}H NMR spectrum of **1** showed signals due to meta-coupled aromatic protons at δ 6.54 and 7.37, a methoxy methyl at δ 3.80, and a hydroxyl proton at δ 5.91. ^{1}H and ^{13}C NMR data for **1** (Table 1) resembled those for **3**, except a methoxy methyl signal of **1**. Compounds **1** and **3** gave the same dimethoxy compound **12**. Therefore, **1** was assigned as a monomethyl ether derivative of **3**. The ^{13}C signals due to C-1−C-6 were assigned by HMQC and HMBC experiments. A correlation was detected from OMe (δ_H 3.80) to the ^{13}C signal at δ_C 151.0 in the HMBC spectrum of **1**, which was ascribed to the C-2′ position by comparing the chemical shift with those of **3** and **12**. Consequently, the structure of **1** was assigned as 2-(3′,4′,5′,6′-tetrabromo-2′-methoxyphenoxy)-4,6-dibromophenol.

Compound **2** was obtained as a viscous oil. The presence of five Br atoms was suggested by the ESIMS of **2**. The molecular formula $C_{13}H_7Br_5O_3$ was deduced from the FABMS and NMR spectral data. The ^{1}H NMR spectrum of **2** showed an aromatic proton singlet at δ 7.28, two meta-coupled aromatic proton doublets at δ 6.51 and 7.34, a methoxy methyl singlet at δ 3.78, and a hydroxyl proton singnal at δ 6.06. ^{1}H and ^{13}C NMR data for **2** (Table 1)

* To whom correspondence should be addressed. Tel: +81-3-5463-0451. Fax: +81-3-5463-0398. E-mail: namikosh@s.kaiyodai.ac.jp.
† Tokyo University of Marine Science and Technology.
‡ Shenyang Pharmaceutical University.
§ The University of Tokyo.

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Table 1. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR Data for Compounds **1** and **2** in $CDCl_3$[a]

	compound **1**			compound **2**		
C#	^{13}C	^{1}H	HMBC	^{13}C	^{1}H	HMBC
1	142.8			142.7		
2	144.5			144.7		
3	116.8	6.54 1H, d, J = 2.1 Hz	1, 2, 4, 5	116.3	6.51 1H, d, J = 2.1 Hz	1, 2, 4, 5
4	111.9			111.5		
5	129.5	7.37 1H, d, J = 2.1 Hz	1, 3, 4, 6	129.3	7.34 1H, d, J = 2.1 Hz	1, 3, 4, 6
6	110.7			110.1		
1′	145.4			140.8		
2′	151.0			152.0		
3′	121.3			116.6	7.28 1H, s	1′, 2′, 4′, 5′
4′	122.3			119.1		
5′	124.5			122.8		
6′	126.8			122.4		
1-OH		5.91 1H, s			6.06 1H, s	
2′-OMe	61.6	3.80 3H, s	2′	56.7	3.78 3H, s	2′

[a] Signals were assigned by $^{1}H-^{1}H$ COSY, HMQC, and HMBC experiments.

were very similar to those for **4**. All ^{13}C signals, except C-4′ and C-5′, were definitely assigned by HMQC and HMBC experiments. A correlation from OMe (δ_H 3.78) to C-2′ (δ_C 152.0) was observed in the HMBC spectrum of **2**. Moreover, an NOE was detected between OMe and H-3′ (δ 7.28) in the NOESY spectrum of **2**. Thus, the structure of **2** was deduced as 2-(4′,5′,6′-tribromo-2′-methoxyphenoxy)-4,6-dibromophenol.

Compounds **1**−**11** were tested for their inhibitory activities to the assembly of purified porcine brain microtubule proteins[5] and to the meiotic maturation of starfish oocytes stimulated by 1-MeAde.[6] Compounds **3**, **7**, and **10** inhibited the assembly of microtubule proteins at 29.6, 33.5, and 20.9 μM (IC_{50}), respectively (colchicine = 10.0 μM). These compounds also inhibited the maturation of oocytes at 3.6, 4.2, and 4.2 μM (IC_{50}), respectively. Compounds **1**, **2**, **4**−**6**, **8**, **9**, and **11** did not show inhibitory activity in either bioassay at 100 μM. Two phenolic hydroxyls are, therefore, necessary for their bioactivities. Substitution patterns of Br may less affect the activity.

A few bioactivities have been reported for polybrominated diphenyl ethers, such as antibacterial and antifungal activities,[11−13] antimicroalgal activity against *Prorocentrum micans* and *Brachiomonas submaria*,[7] toxicity to brine shrimp,[14] antiinflammatory activity,[15] and inhibitory activity to several enzymes including inosine monophosphate dehydrogenase, guanosine monophosphate synthetase, and 15-lipoxygenase.[10] This is, therefore, the first to report inhibitory activities of polybrominated diphenyl ethers to the assembly of microtubule proteins and to the maturation of starfish oocytes.

Experimental Section

General Experimental Procedures. UV and IR spectra were recorded on a Hitachi U-3000 and on a JASCO A-102, respectively. NMR spectra were measured on either a JEOL JNM A-500 NMR spectrometer or a Varian Unity Inova-500 spectrometer. Mass spectra were obtained by either a JEOL HX-110 mass spectrometer (FAB mode, *m*-nitrobenzyl alcohol as matrix) or a Finnigan TSQ 700 triple quadrupole mass spectrometer (ESI mode).

Marine Sponge. *Phyllospongia dendyi* was collected by scuba diving in July 2000 at Palau and kept in a freezer at −30 °C until extraction. The voucher specimen is deposited at the Department of Ocean Sciences, Tokyo University of Marine Science and Technology, as TUF number 00-07-11=2-1.[16] The sponge was identified by Professor P. Bergquist (University of Auckland, New Zealand).[16]

Isolation of Compounds 1−11. The sponge (284 g) was thawed, cut into small pieces, and extracted with ethanol (2 × 1000 mL). The ethanol extract was evaporated, and the residue was partitioned between water and EtOAc. The organic extract (5.0 g) was chromatographed on SiO_2 with $CHCl_3$−MeOH (gradient elution), and the bioactivity was detected in $CHCl_3$ (300 mg) and 1% MeOH/$CHCl_3$ (1.0 g) fractions. The $CHCl_3$ fraction (300 mg) was further separated by an ODS column with MeOH to afford 200 mg of bioactive fraction, which was subjected to HPLC (SiO_2, *n*-hexane−ethyl acetate) to afford **1** (1.6 mg), **2** (5.7 mg), **4** (18.0 mg), **5** (3.2 mg), **6** (1.0 mg), **8** (13.0 mg), **9** (102.0 mg), **11** (5.6 mg), and a mixture of **3** and **7**. The 1% MeOH/$CHCl_3$ fraction (0.5 g) was separated by HPLC (ODS) with 87% MeOH−H_2O (0.1% TFA) to yield **3** (118.7 mg), **7** (33.3 mg), and **10** (1.7 mg).

Compound 1: UV (EtOH) λ_{max} (ϵ) 232 (22 600), 292 (5000) nm; IR (KBr) ν_{max} 3434, 2924, 2854, 1584, 1477, 1445, 1406, 1390, 1344, 1264, 1206, 997, 963, 880, 835 cm^{-1}; ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data, Table 1; ESIMS *m*/*z* 684, 686, 688, 690, 692, 694, and 696 (1:2:4:8:4:2:1, $[M]^+$); HRFABMS *m*/*z* 685.5380 (calcd for $C_{13}H_6{}^{79}Br_5{}^{81}BrO_3$, 685.5397), 687.5327 (calcd for $C_{13}H_6{}^{79}Br_4{}^{81}Br_2O_3$, 687.5377), 689.5277 (calcd for $C_{13}H_6{}^{79}Br_3{}^{81}Br_3O_3$, 689.5357), 691.5228 (calcd for $C_{13}H_6{}^{79}Br_2{}^{81}Br_4O_3$, 691.5337), 693.5294 (calcd for $C_{13}H_6{}^{79}Br{}^{81}Br_5O_3$, 693.5318).

Compound 2: UV (EtOH) λ_{max} (ϵ) 232 (25 000), 294 (6100) nm; IR (KBr) ν_{max} 3503, 3085, 2924, 1596, 1573, 1476, 1429, 1405, 1350, 1287, 1263, 1246, 1033, 942, 890, 835 cm^{-1}; ^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$) NMR data, Table 1; ESIMS *m*/*z* 606, 608, 610, 612, 614, and 616 (1:4:8:8:4:1, $[M]^+$); HRFABMS *m*/*z* 607.6285 (calcd for $C_{13}H_7{}^{79}Br_4{}^{81}BrO_3$, 607.6292), 609.6259 (calcd for $C_{13}H_7{}^{79}Br_3{}^{81}Br_2O_3$, 609.6272), 611.6224 (calcd for $C_{13}H_7{}^{79}Br_2{}^{81}Br_3O_3$, 611.6252), 613.6219 (calcd for $C_{13}H_7{}^{79}Br{}^{81}Br_4O_3$, 613.6233).

Bioassay. The microtubule assembly assay was performed as reported.[5,17] In brief, fresh porcine brains were homogenized at 0 °C in a buffer solution (100 mM 4-morphorineethansulfonic acid, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM GTP, and 1 mM 2-mercaptoethanol, pH 6.5) and centrifuged at 50000*g* at 4 °C. A glycerol buffer (8 M glycerol in the above buffer solution, pH 6.5) was added to the supernatant, and the mixture was incubated at 37 °C for 30 min and centrifuged at 100000*g* to afford the precipitate (microtubule). The depolymerization and polymerization procedure was further performed twice to purify the microtubule proteins. The concentration of proteins was quantified using the Coomassie Protein Assay Kit (Pierce). Polymerization was observed by the turbidity at 400 nm in a glass UV cell at 37 °C with a Shimadzu model U-3000 spectrophotometer equipped with an electronic temperature controller.[5] Each sample was dissolved in DMSO and added to the suspension of microtubule proteins in the buffer solution (1.3 mg in 1 mL). The final concentration of DMSO was less than 2%.

Effects of compounds on meiotic maturation of starfish oocytes were measured according to the previous paper[6] and earlier report.[18] Starfish, *Asterina pectinifera*, were collected from the coastal waters off Japan during the breeding season. Immature oocytes were obtained from the starfish ovaries and adjusted to 100 cells/mL. This oocyte suspension (1 mL) and 10 μL of each test sample were added into a 24-well plate and placed on ice for 10 min. After addition of 10 μL of 1-MeAde (1.25 μM), the plate was incubated at 20 °C for 60 min, and the states of germinal vesicles were observed under an inverted microscope. DMSO was used to dissolve the samples. The final concentration of DMSO was adjusted to less than 1%.

Preparation of Methyl Ethers. A solution of compound (0.01 mmol), MeI (1 mL), and anhydrous K_2CO_3 (500 mg) in dry acetone (10 mL) was refluxed for 12 h. The residue, after evaporation, was partitioned between water (10 mL) and chloroform (3 × 10 mL), and the organic extract was dried (Na_2SO_4) and evaporated to give the corresponding methyl ether.

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