Bioactive Polybrominated Diphenyl Ethers from the Marine Sponge *Dysidea* sp.

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A new polybrominated diphenyl ether (**9**), together with eight known compounds, were isolated from the crude organic extract of the marine sponge *Dysidea* sp. collected from the Federated States of Micronesia. Their structures were elucidated on the basis of various NMR spectroscopic data. These compounds exhibited inhibitory activities against *Streptomyces* 85E in the hyphae formation inhibition (HFI) assay and displayed antiproliferative activities against the human breast adenocarcinoma cancer cell line MCF-7. Compound **6** was selected for further evaluation in a cell cycle progression study.

The phosphorylation of proteins on serine/threonine and tyrosine residues by protein kinases is one of the major regulatory mechanisms in biological processes including apoptosis, cell proliferation, cell differentiation, and metabolism. Deregulated phosphorylation associated with these pathways can result from genetic alterations acquired early in tumorigenesis and are often the cause of cancer. In this regard, protein kinases have emerged as promising inhibitory targets in cancer treatment.[1,2]

Marine natural products offer a rich source of as yet unexamined potent bioactive molecules that are of potential value in the development of new pharmaceutical agents.[3] As part of our ongoing effort to discover natural product-based protein phosphorylation inhibitors, we have evaluated over 2500 terrestrial, microbial, and marine extracts received from the National Cancer Institute Natural Products Branch. To achieve this aim, a hyphae formation inhibition (HFI) assay in *Streptomyces* 85E[4] was adopted to rapidly screen and identify candidate compounds targeting general serine/threonine and/or tyrosine kinase activities. Aerial hyphae formation in *Streptomyces* species requires protein kinase activity, and a variety of kinase inhibitors that block this process have been reported.[4,5] In the HFI assay, a clear inhibition zone indicates a test agent that inhibits both growth and sporulation, while a zone of "bald" cells indicates the presence of substrate hyphae without aerial hyphae as compared to the surrounding colonies.[4] By recording the presence and extent of clear and bald zones in the HFI assay, test compounds can be rapidly screened for presumptive kinase activity. Furthermore, it has been hypothesized that natural products that inhibit hyphae formation in *Streptomyces* 85E also may block the proliferation of cancer cells.

Previous chemical studies on *Dysidea* species, for example on the most intensively studied species *D. herbacea*, have resulted in the isolation of various brominated diphenyl ethers,[6–9] sesquiterpenes,[10] and polychlorinated alkaloids.[11] The antibacterial, antifungal, and cytotoxic activities of polybrominated diphenyl ethers have been reported.[12,13] Moreover, enzyme inhibition activities related to tumor development including guanosine monophosphate synthetase, inosine monophosphate dehydrogenase, and 15-lipooxygenase were also reported.[14] However, our report here of the general serine/threonine and/or tyrosine kinase inhibitory activities in *Streptomyces* 85E is new.

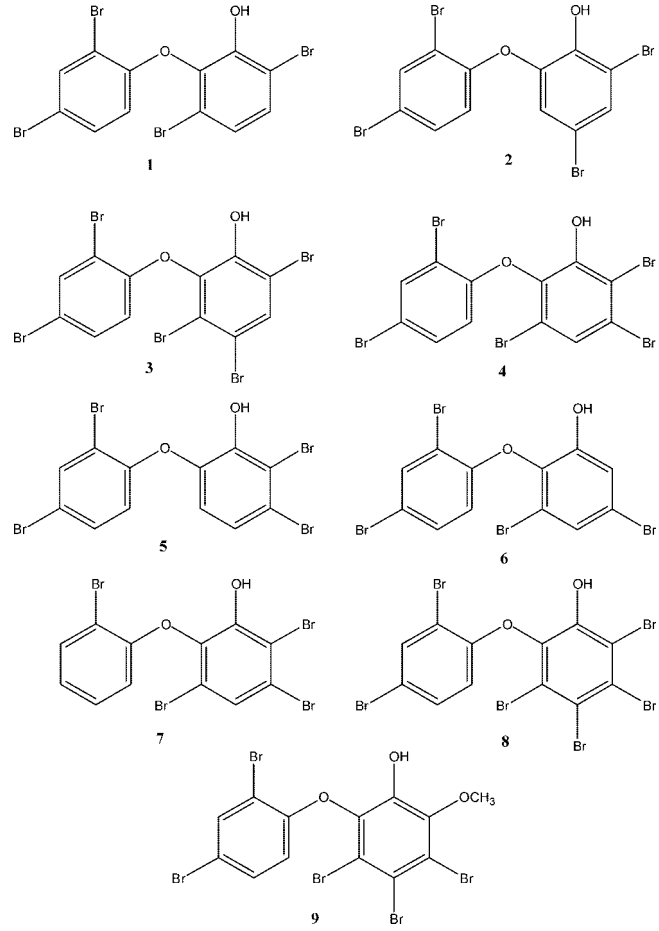

Figure 1. Chemical structures of compounds **1**−**9** isolated from the sponge *Dysidea* sp.

This preliminary result encouraged us to further study *Dysidea* sp. Herein we report the isolation, structure elucidation, and biological activities of compounds **1**−**9** (Figure 1).

Compound **9** was isolated as a white powder. It gave a molecular ion peak at *m/z* 604.6230 [M − H]$^-$ (calcd for $C_{13}H_6Br_5O_3$, 604.6239) in the HRESIMS. The low-resolution ESIMS spectrum of compound **9** showed the pseudomolecular ion peak at *m/z* 604.62, 606.62, 608.62, 610.62, 612.62, and 614.61 with intensities of 1:4:6:6:4:1, which indicated the presence of five bromine atoms in the molecule. The IR data showed the presence of hydroxyl (3378 cm^{-1}) and phenyl ring (1562 and 1465 cm^{-1}) functionalities. This

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was also confirmed by an OH proton observed at δ 10.64 in ^{1}H NMR (DMSO-d_6). Comparison of its ^{1}H and ^{13}C NMR data with values for the known compound 3,4,5,6-tetrabromo-2-(2′,4′-dibromophenoxy)phenol (**8**),[11,12] also isolated during this investigation, indicated that **9** was a brominated diphenyl ether analogue.[6–8] The signal at δ 3.84 (acetone-d_6) with integration of three indicated the presence of a methoxyl group in the molecule. A doublet (J = 2.5 Hz) at δ 7.83 (H-3′), a doublet of doublets (J = 9.0, 2.5 Hz) at δ 7.41 (H-5′), and a doublet (J = 9.0 Hz) at δ 6.65 (H-6′) were observed as an ABX system of a 2′,4′-dibrominated B ring,[8] suggesting that the three bromines and two oxygenated substituents were all present in ring A. The ^{13}C NMR spectrum indicated 13 signals including one methoxy (δ 61.3), three methines (δ 136.5, 132.6, 117.2), and nine quaternary carbons (δ 153.8, 149.6, 147.9, 140.4, 119.7, 115.5, 115.1, 113.4, 113.3) based on an APT experiment. In the HMBC experiments, the methoxyl group (δ 3.84) exhibited a cross-peak with a signal at δ149.6 (C-6). The position of this methoxy was established from the following observation. The IR spectrum of **1** showed a sharp absorption band at 3400 cm^{-1} for a hydroxyl group, whereas the IR spectrum of **9** exhibited a broader and lower frequency absorption band at 3378 cm^{-1} (presumably due to the formation of a hydrogen bond of OH to the oxygen of the methoxyl group). Therefore, the methoxyl group in the molecule of **9** could be assigned to the C-6 position. In addition, a methoxyl group substitution of ring A decreases the resonances of ortho and para substitutions in ring A and increases the resonance of meta substitution in ring A. This inference was consistent with a comparison of ^{13}C NMR data of 3,4,5-tribromo-2-(2′,4′-dibromophenoxy)phenol[9] with compound **9**. From an analysis of all the data described above, compound **9** was assigned as 3,4,5-tribromo-6-methoxy-2-(2′,4′-dibromophenoxy)phenol.

Eight known compounds, 3,6-dibromo-2-(2′,4′-dibromophenoxy)phenol (**1**),[7,16] 4,6-dibromo-2-(2′,4′-dibromophenoxy)phenol (**2**),[8] 3,4,6-tribromo-2-(2′,4′-dibromophenoxy)phenol (**3**),[15] 3,5,6-tribromo-2-(2′,4′-dibromophenoxy)phenol (**4**),[15] 5,6-dibromo-2-(2′,4′-dibromophenoxy)phenol(**5**),[9]3,5-dibromo-2-(2′,4′-dibromophenoxy)phenol (**6**),[15] 3,5,6-tribromo-2-(2′-bromophenoxy)phenol (**7**),[12] and 3,4,5,6-tetrabromo-2-(2′,4′-dibromophenoxy)phenol (**8**),[12,13] were isolated from *Dysidea* sp. and identified by comparison with published data.

An organic extract of *Dysidea* sp. was found to show inhibitory activity in the HFI assay, giving a 22 mm bald and clear zone of inhibition at a concentration of 80 μg/disk. This preliminary result encouraged us to study *Dysidea* sp. for protein kinase inhibitors. Following the bioassay-guided fractionation of *Dysidea* sp., isolates **1**−**9** were obtained. These compounds were evaluated individually for their inhibitory activities in the HFI assay.[4] Compounds **1**−**9** all exhibited inhibitory activities against *Streptomyces* 85E as bald and clear zones of inhibitions at a concentration of 20 μg/disk (Table 1). Compounds **1**, **2**, **5**, and **9** showed moderate activity and gave a 10 mm bald zone of inhibition at 2.5 μg/disk. Compounds **6** and **7** exhibited significant inhibitory activities and gave a 20 mm clear zone of inhibitions at 20 μg/disk as well as a 12–13 mm clear zone of inhibitions at 2.5 μg/disk.

It is hypothesized that natural products that inhibit hyphae formation in *Streptomyces* 85E may block the proliferation of cancer. In the human breast cancer MCF-7 cells, proliferation can be blocked in numerous ways, including serum deprivation and pharmacological inhibition of specific kinase and steroid hormone pathways.[17,18] Thus, compounds **1**−**9** were further evaluated for their antiproliferative activity using a MCF-7 human adenocarcinoma breast cancer cell line. For the antiproliferation assay,[19] compound **6** was the most active, with an IC_{50} value of 2.84 μM. Small structural changes, such as the bromination position and number of substituents, may affect the activity. In general, the biological activity decreases with an increase in the number of bromine substituents in the A ring.

Table 1. Kinase Inhibitory Activity and Antiproliferative Activity of Compounds **1–9**

	zone of inhibition observed (mm)[a,b,c] concentration (μg) per disk				
compound	20	10	5	2.5	antiproliferative activity IC_{50}[d,e] (μM)
1	14C	13C	11B	10B	6.89 ± 0.12
2	13C	13B	12B	10B	8.90 ± 7.41
3	12C+3B	12C+2B	15B	13B	8.73 ± 1.01
4	14B	13B	11B	10B	7.52 ± 0.85
5	13C	14B	13B	10B	7.23 ± 1.75
6	20C	17C	16C	13C	2.84 ± 1.04
7	20C	18C	13C	12C	6.96 ± 2.81
8	15C	13C	12B	12B	9.48 ± 2.00
9	12B	12B	11B	10B	8.69 ± 2.18

[a] Diameter of disk alone is 6 mm. Stock solutions were prepared in either DMSO or methanol. No zones of inhibition were observed with MeOH or DMSO as negative controls. [b] All compounds were tested at 20 μg/disk. Active compounds will be retested again at lower concentrations (20−2.5 μg/disk). B indicates bald phenotype and C indicates clear phenotype. [c] Kinase inhibitory activity of compounds were tested on the growth and sporulation of *Streptomyces* 85 E. [d] 50% cell survival after 2 days of incubation. The IC_{50} values are given as the mean ± SD (n = 3) of three repeated experiments. [e] Assays were carried out with the MCF-7 breast human adenocarcinoma cell line as described in the Experimental Section.

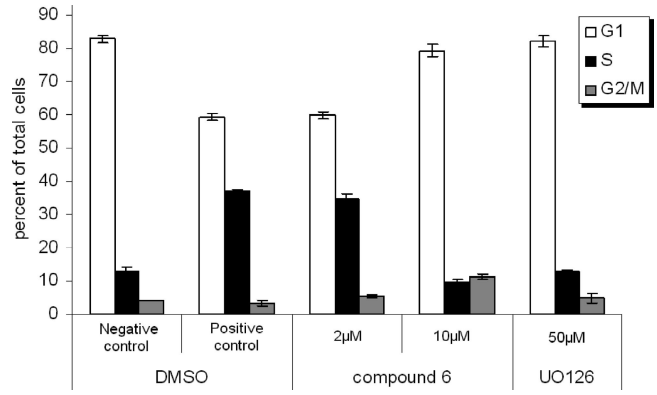

Figure 2. G1 phase arrest effects of compound **6** in MCF-7 cells were determined by cell cycle assay. Cells were prepared and treated as described in the Experimental Section. Compound **6** (at 2 or 10 μM), UO126 (50 μM), or DMSO vehicle was added. The percent of cells in G1, S, and G2/M phases are shown; error bars indicate one standard deviation (n = 3). This experiment was repeated once with similar results.

Compound **6** was further evaluated for cancer cell growth inhibition by performing a cell cycle analysis[20] in the MCF-7 cell line (Figure 2). As indicated in Figure 2, culturing MCF-7 cells in serum-free media resulted in G_1 phase cell cycle arrest, with over 80% of cells in G_1 phase. Treatment with serum for 24 h resulted in a decrease in the number of cells in G_1 phase to ~60%, with a corresponding increase in the number of cells in S phase, indicating that serum treatment reversed the G_1 arrest. This effect was blocked when cells were exposed to compound **6** at 10 μM prior to serum treatment, suggesting that **6** blocks one or more required signal transduction pathways necessary for the initiation of DNA synthesis in MCF-7 cells, rather than blocking DNA replication or metaphase directly. No effect was found when cells were treated with the vehicle as a positive control. We observed no indications of cell death (cells detaching from substrate, cells with sub-G_1 phase DNA content) with treatment with compound **6** at the indicated concentrations; however, at higher concentrations, cytotoxicity was observed (data not shown). Proliferation in MCF-7 cells is controlled both by the steroid hormone estrogen through the estrogen receptor and by peptide growth factors that act by binding membrane-bound

receptor tyrosine kinases; fetal bovine serum contains both steroid hormones and peptide growth factors. Interestingly, both pathways intersect at the level of MAPK activation, and the MEK1/2 inhibitor UO126, which blocks MAPK activation, also blocks serum-dependent cell cycle reentry in MCF-7 cells. Future studies will examine the ability of compound **6** to inhibit cell cycle entry after stimulation with activators of specific signal transduction pathways, such as 17β-estradiol and TGF-α, to gain insight into the molecular target or targets of the compound's antiproliferative activity.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Shimadzu UV–visible spectrophotometer in MeOH. The FT-IR spectra were recorded on a Perkin-Elmer BX FT-IR spectrometer. 1D and 2D NMR experiments were recorded in acetone-d_6 on an INOVA Unity (500) Varian spectrometer. Mass spectra and high-resolution MS spectra were taken with a BioTOF II ESI mass spectrometer. Reversed-phase HPLC was carried out on a Beckman Coulter Gold-168 system equipped with a photodiode array detector using an Alltech semi-preparative Econosil C_{18} column (10 μm, 22 × 250 mm) run with a flow rate of 6.0 mL/min. Cell density was tested on a Labsystems Multiskan MCC/340 plate reader. Cell distributions were analyzed on a FACSCaliber flow cytometer (Beckton Dickinson Bioscience, CA). Cell cycle analyses were estimated using ModFit LT software.

Animal Material. The sponge *Dysidea* sp.[10] (Dysideidae) was collected at a depth of 1 m in the Federated States of Micronesia in June 1992. The sponge was frozen immediately and shipped to NCI (National Cancer Institute, Frederick, MD). A voucher specimen is deposited at the National Museum of Natural History, Smithsonian Institution, Washington, DC.

Extraction and Isolation. Following aqueous extraction, the material was lyophilized and extracted subsequently with CH_2Cl_2−MeOH (1:1) and MeOH. The combined extracts were evaporated *in vacuo* and stored at −30 °C. An organic extract (5.0 g) derived from 139 g wet weight of the initial collection was received. This organic extract was chromatographed over Si gel and eluted with a gradient of $CHCl_3$−MeOH to afford a series of fractions. The first and second fractions exhibited inhibitory activities in the hyphae formation assay in *Streptomyces* 85E at 80 μg/disk. These active fractions were combined and further chromatographed over Si gel using medium-pressure liquid chromatography (MPLC), eluted with *n*-hexane−EtOAc in a gradient to give 11 fractions. Fraction six, which showed inhibitory activity in the hyphae formation inhibition (HFI) assay, was chromatographed sequentially on Sephadex LH-20 eluting with $CHCl_3$−MeOH (1:1) and C_{18} RP-HPLC eluting isocratically with CH_3CN−H_2O (85:15) to yield five semipure subfractions. Final purification of compound **1** (2.1 mg) (CH_3CN−H_2O, 65:35, t_R 90.2 min), compound **2** (12.9 mg) (CH_3CN−H_2O, 75:25, t_R 55.5 min), compound **3** (7.2 mg) (CH_3CN−H_2O, 75:25, t_R 65.98 min), compound **4** (13.8 mg) (CH_3CN−H_2O, 75:25, t_R 69.9 min), compound **5** (2.2 mg) (CH_3CN−H_2O, 75:25, t_R 46.8 min), compound **6** (10.3 mg) (CH_3CN−H_2O, 70:30, t_R 70.8), compound **7** (1.0 mg) (CH_3CN−H_2O, 65:35, t_R 86.0 min), compound **8** (9.6 mg) (CH_3CN−H_2O, 80:20, t_R 53.6 min), and compound **9** (5.1 mg) (CH_3CN−H_2O, 70:30, t_R 76.7 min) was obtained with C_{18} RP-HPLC eluting isocratically with CH_3CN−H_2O.

3,4,5-Tribromo-6-methoxy-2-(2′,4′-dibromophenoxy)phenol (9): white powder; UV (MeOH) λ_{max} (log ϵ) 216 (4.71), 292 (3.77) nm; IR (KBr) ν_{max} 3378, 1562, 1465, 1421, 1389, 1228, 1068, 1042, 968, 922, 808 cm^{-1}; ^{1}H NMR (CD_3)$_2$CO, 500 MHz) δ 7.83 (1H, d, J = 2.5 Hz, H-3′), 7.41 (1H, dd, J = 9.0, 2.5 Hz, H-5′), 6.65 (1H, d, J = 9.0 Hz, H-6′), 3.84 (3H, s, OCH_3); (DMSO-d_6, 500 MHz) δ10.64 (1H, br s, OH), 7.90 (1H, d, J = 2.5 Hz, H-3′), 7.40 (1H, dd, J = 9.0, 2.5 Hz, H-5′), 6.50 (1H, d, J = 9.0 Hz, H-6′), 3.74 (3H, s, OCH_3); ^{13}C NMR ((CD_3)$_2$CO, 125 MHz) δ 153.8 (C-1′), 149.6 (C-6), 147.9 (C-1), 140.4 (C-2), 136.5 (C-3′), 132.6 (C-5′), 119.7 (C-4), 117.2 (C-6′), 115.5 (C-5), 115.1 (C-4′), 113.4 (C-3), 113.3 (C-2′), 61.3 (OCH_3); HRESIMS m/z 604.6230 [M − H]$^-$ (calcd for $C_{13}H_6Br_5O_3$, 604.6239).

Hyphae Formation Inhibition (HFI) Assay in *Streptomyces* 85E. The HFI assay was performed by a method previously reported.[4] The mycelia fragments of *Streptomyces* 85E were spread on minimal medium ISP 4 agar plates to produce a bacterial lawn. The impregnated paper disks (with a dose of 80 μg/disk) were applied directly on the surface of agar plates seeded with *Streptomyces* 85E. The growth inhibition zone was recorded after 30 h of incubation at 30 °C. Two types of phenotypes were observed: a clear zone of inhibition and/or bald phenotype around the disk. The sporulation inhibitor surfactin was used as a positive control, and a solvent was used as a negative control, respectively. An inhibition zone of greater than 9 mm is considered active. Fractions and compounds **1**−**9** were tested at 80 μg/disk on 6 mm paper disks. Active compounds were tested at lower concentrations (20, 10, 5, 2.5 μg/disk).

Antiproliferation Assay.[18] A human breast cancer cell line (MCF-7) was obtained from ATCC and routinely cultured in IMEM medium supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin in a humidified incubator at 37 °C with 5% CO_2. To perform the antiproliferation assay, cells were plated in 96-well microtiter plates containing test compounds dissolved in DMSO at the indicated concentrations. Negative controls received normal growth media and the same amount of DMSO. Plates were incubated for 2 days after treatment, then fixed in 1% glutaraldehyde in PBS for 15 min and stained with 0.1% crystal violet in PBS for 15 min. The bound dye was solubilized in 0.5% Triton x-100. A plate reader was used to measure the staining intensity of each well by subtracting the absorbance at 405 nm from the absorbance at 560 nm. Percent growth was calculated from the formula % growth = (absorbance$_{sample}$ − absorbance$_{day0}$)/(absorbance$_{DMSO\ control}$ − absorbance$_{day0}$) × 100. The IC_{50} value was measured graphically from a dose–response curve using five drug concentrations (the assays were performed in triplicate).

Cell Cycle Analysis.[19] MCF-7 cells were plated at 1 × 10^5 cells per well in six-well tissue culture dishes, and then were cultured in phenol red-free IMEM containing no serum or antibiotics for 2 days to initiate G_1 phase cell cycle arrest. Cells were then treated with test compounds or DMSO at the indicated concentrations in serum-free, phenol-red free, IMEM. After 30 min treatment, FBS was added to the treatment wells and positive controls to a final concentration of 5%. Negative controls were maintained in serum-free media. Cells were incubated for 24 h, then washed with PBS, trypsinized, and fixed with ice cold 80% EtOH. Fixed cells were centrifuged at 1500 rpm for 5 min, and then the cell pellets were suspended in propidium iodide stain and permeabilization buffer. Cell distributions were analyzed on a FACSCaliber flow cytometer.

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