Four New Bioactive Polybrominated Diphenyl Ethers of the Sponge *Dysidea herbacea* from West Sumatra, Indonesia

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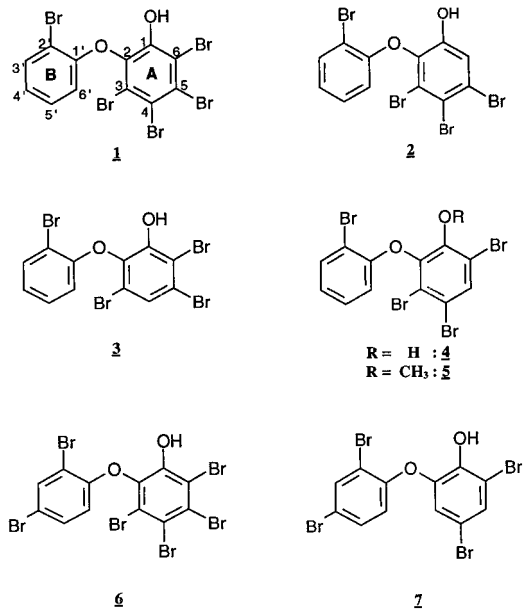
The marine sponge *Dysidea herbacea* collected from Indonesia yielded four new polybrominated diphenyl ether congeners **2**−**5** and the known derivatives **1**, **6**, and **7**. The structures of the new compounds were unambiguously established on the basis of NMR spectroscopic (^{1}H, ^{13}C, COSY, ^{1}H-detected direct and long-range ^{13}C−^{1}H correlations) and mass spectrometric (EIMS) data. All of the compounds were active against the Gram-positive bacteria *Bacillus subtilis* and the phytopathogenic fungus *Cladosporium cucumerinum*. The isolated polybrominated compounds were also active in the brine shrimp lethality test. In the latter bioassay, compounds **1** and **6** were the most active with LC_{50}'s of 0.96 [SE ± 0.19] and 0.94 [SE ± 0.70] μg/mL, respectively.

The marine sponge *Dysidea herbacea* Keller (family Dysideidae, Order Dendroceratida) occurs in two chemotypes;[1] one chemotype contains both polychlorinated amino acid derivatives[2,3] and sesquiterpenes,[4,5] while the second chemotype contains only polybrominated diphenyl ethers.[6,7] Thus, it has been previously argued that this chemical variation in *D. herbacea* is due to different algal or bacterial symbionts associated with the sponge.[8] Recently, it was suggested that the production of polybrominated diphenyl ethers of *D. herbacea* is due to the cyanobacterium (*Oscillatoria spongeliae*) and not by the sponge or a symbiotic heterotrophic bacteria and that these compounds may play a role in the chemical defense of the sponge against potential predators and bacterial invasion.[1,9,10] The polybrominated diphenyl ether derivatives have also been reported to inhibit enzymes implicated in tumor development and arteriosclerotic plaque, which indicates their potential as promising therapeutic agents.[11] In this paper, we describe the isolation and structure elucidation of new polybrominated diphenyl ether derivatives obtained from the marine sponge *D. herbacea*, collected from West Sumatra, Indonesia, and report on their antibacterial, antifungal, and cytotoxic properties.

The marine sponge *D. herbacea* was collected off the shores of the Air island of West Sumatra, Indonesia. The EtOAc soluble material of a crude extract from the sponge was subjected to Sephadex (LH20) column chromatography using methanol as eluent, and 14 fractions were obtained. The polybrominated diphenyl ether congeners were isolated from fractions 9−14, and the last fraction 14 contained the major compound **1**. The known compounds **1**, **6**, and **7** were readily identified from their spectroscopic data and by comparison with published data.[6,8,12−14]

Chart 1



For this series of polybrominated diphenyl ether congeners, an inspection of the multiplicity patterns and the number of protons in the aromatic region indicated their relative distribution between the two ring systems. The occurrence of the 2-bromophenoxy ether ring (ring B, Chart 1) for compounds **2**−**5** was confirmed by direct comparison of the ^{1}H and ^{13}C NMR spectrum with a standard of 2-bromophenol (Fluka). Through-bond heteronuclear (^{1}H-detected one-bond and multiple-bond ^{13}C multiple coherence) correlations have been used to unambiguously establish the assignments and atom

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Table 1. ^{13}C NMR Data of Compounds **2**−**4** in $CDCl_3$

C	2	3	4
1	152.9, s	148.5, s	146.7, s
2	140.3, s	139.0, s	141.0, s
3	121.3, s	116.8, s	120.2, s
4	116.6, s	128.0, d	116.1, s
5	122.0, s	122.1, s	132.8, d
6	121.6, d	113.2, s	109.8, s
1′	151.6, s	152.6, s	152.7, s
2′	111.3, s	111.8, s	111.8, s
3′	133.7, d	134.0, d	134.1, d
4′	123.7, d	124.4, d	124.6, d
5′	128.5, d	128.6, d	128.7, d
6′	114.2, d	114.7, d	114.8, d

connectivities for the protonated aromatic ring systems (Table 1). The magnitude of the long-range couplings, indicated by the intensity of the cross peaks in these spectra, to the carbons carrying oxygen substituents allowed unambiguous determination of the relative positions of the hydrogen atoms in each system. The relative assignments of the carbons carrying oxygen substituents was made by consideration of their substituent chemical shifts and indicated that C-2 is always to high field of C-1 and C-1′ in the compounds considered here.

Compound **2** was obtained as a viscous oil. A cluster of peaks at *m/z* 506, 504, 502, 500, 498 in the EIMS is compatible with the molecular composition of $C_{12}H_6O_2Br_4$ for compound **2**. The ^1H NMR spectrum of **2** showed a broad singlet at δ 7.34 (1H), which indicated the presence of a proton in the phenolic ring system A; subsequently, the broad form of the signal suggested a probable coupling with a hydroxyl group oriented ortho to the proton. In the HMBC spectrum, the presence of a strong correlation of H-6 to C-2 and a weak correlation of H-6 to C-1 confirms that the proton is meta- and ortho-oriented to the oxygen functions, respectively. Therefore, compound **2** is 3,4,5-tribromo-2-(2′-bromophenoxy)phenol.

Compounds **3** and **4** are isomers of **2** and were obtained as an inseparable mixture in a ratio of 3:2, respectively, as observed from the ^1H and ^{13}C NMR spectrum. Overlapping signals appeared in the ^1H NMR spectrum for the 2-bromophenoxy ring system B, but two distinct sets of signals were observed in the ^{13}C NMR spectrum for both of the ring systems, and these allowed differentiation of the two isomers. Further experiments with HMBC established the assignments for both of the isomers. In the ^1H NMR spectrum, the additional singlets at δ 7.55 and 7.75 for isomers **3** and **4**, respectively, suggest the presence of a proton in the phenolic ring as in **2**. Both of these protons are oriented *para* and *meta* to the hydroxyl groups as observed from the cross peaks in the HMBC spectrum, which consequently confirmed that H-5 of isomer **3** is *meta*- and *para*-oriented to the ether and hydroxyl substituents, respectively, while in isomer **4**, the opposite is the case, H-4 is *para*- and *meta*-oriented to the ether and hydroxyl substituents, respectively. Therefore isomer **3** was determined to be 3,5,6-tribromo-2-(2′-bromophenoxy)-phenol, and isomer **4** was 3,4,6-tribromo-2-(2′-bromophenoxy)phenol. For isomers **2**−**4**, the structure of the phenolic ring system A was also confirmed by comparison with published data on tribromophenolic compounds.[12]

Compound **5** is the methoxyl derivative of **4**. A cluster of peaks at *m/z* 520, 518, 516, 514, 512 in the EIMS is compatible with the molecular composition of $C_{13}H_8O_2Br_4$. The methoxyl signal was observed at δ3.86 (s, 3H) in the ^1H NMR spectrum, and the ^1H NMR data are comparable to those of **4**.

All isolated compounds were tested for their antibacterial and fungicidal activities and their response in the brine shrimp lethality test (Table 2). All compounds were found to be active against the Gram-positive bacteria *B. subtilis*. No inhibition was observed for the Gram-negative bacteria *E. coli*. As a guide to the sensitivity of *B. subtilis* to the isolated polybrominated diphenyl ether derivatives, the minimum inhibitory concentrations (MICs) for each of the compounds were determined. Compound **1** was found to be the most active with a MIC of 0.20 μg/mL (0.34 nmol), followed by the isomeric mixture of **3** and **4** then by compound **6**, whereas compounds **2**, **7**, and **5** were less active with MICs of 6.25 μg/mL (12.45 nmol), 25 μg/mL (49.80 nmol), and 104 μg/mL (201.55 nmol), respectively. The activity of compound **1** toward *B. subtilis* was the same as that of the standard antibiotic gentamycin.

All compounds except **5** were active against the fungus *C. cucumerinum*. The isomeric mixture **3** and **4** was the most active at concentrations of 50 and 25 nmol, causing inhibition zones of 16 and 8 mm in diameter, respectively, followed by compounds **1**, **2**, and **6**, and compound **7** with the weakest activity (Table 2).

For the brine shrimp lethality test, compounds **1** and **6** were found to be the most active with LC_{50}'s of 0.96 and 0.94 μg/mL, respectively, followed by the tribromophenol derivatives **2**−**4**, while compounds **7** and **5** showed only very weak activities (Table 2).

From the results of these bioassays, preliminary statements can be made on the structure−activity relationship between the different polybrominated diphenyl ether derivatives. In general, the derivatives with the 2′-bromophenoxy ether ring system are more active than their 2′,4′-dibromophenoxy ether congeners. In compound **2**, the absence of a bromine substituent at C-6, which is ortho to the hydroxyl group of the phenolic ring system, decreases the bioactivity. In the case of the brine shrimp lethality test, the biological activity is directly proportional to the number of bromine substituents. Methylation of the hydroxyl group, as in **5**, results in a weakening or in the loss of activity in all the bioassays performed.

Experimental Section

General Experimental Procedures. ^1H NMR and ^{13}C NMR spectra (chemical shifts in ppm) were recorded on Bruker ARX 400 NMR and AVANCE DMX 600 NMR spectrometers, respectively. Mass spectra (EIMS) were measured on a Finnigan MAT 8430 mass spectrometer. UV spectra were recorded in MeOH. Percent purity of isolated compounds was analyzed by HPLC. For HPLC analysis, samples were injected into an HPLC system (Gynkotek, Germany) coupled to a photodiode-array detector. For each run, the separation column was equilibrated with 50% A (H_2O adjusted to pH 2 in phosphoric acid) in B (MeOH) for 10 min, and then the separation was achieved by a linear gradient from 50% A to 100% B in 40 min, followed by an isocratic segment of 100% B for the last 5 min. Routine detection was at 254 nm. The separation column (125 × 4 mm, i.d.) was prefilled with Eurospher C-18 (Knauer, Germany).

Table 2. Bioactivities of the Compounds Isolated from *Dysidea herbacea*

	serial dilution assay *B. subtilis*	zone of inhibition fungicidal activity against *C. cucumerinum*		brine shrimp lethality test *A. salina*
compd no.	MIC μg/mL (nmol)	dose = 50 nmol (mm diam)	dose = 25 nmol (mm diam)	LC_{50} (μg/mL ± SE)
1	0.20 (0.34)	14.0	7.0	0.96 ± 0.19
2	6.25 (12.45)	13.0	8.0	3.30 ± 0.51
3/4	1.56 (3.11)	16.0	8.0	3.20 ± 0.35
5	104.00 (201.55)			26.25 ± 0.42
6	3.13 (4.73)	8.0	5.0	0.94 ± 0.70
7	25.00 (49.80)	3.5	1.5	8.66 ± 1.32

Solvents were distilled prior to use, and spectral grade solvents were used for spectroscopic measurements. TLC was performed on precoated TLC plates with Si gel 60 F_{254} (Merck, Darmstadt, Germany). The compounds were detected from their UV absorbance at 254 nm.

Animal Material. The gray-green sponge *D. herbacea* was collected by snorkelling off the shores of Air island of West Sumatra, Indonesia. The sponge grows in shallow water on coral rubble and dead coral branches as a partly encrusting, partly thinly bladed erect form, with irregularly flattened digitate outgrowths. The surface is smooth and slippery. In places there are fine, faint conules. A convergent system of subdermal canals is visible. Consistency is cartilaginous, but rather fragile. The skeleton is a fairly meshed system of thin spongin fibers completely filled with sand grains and specule debris. The size of the smallest meshes are about 250 × 400 μm, while many are larger. There is no clear distinction between primary and secondary fibers and their thickness varies between 30 and 50 μm. The ectosomes are covered by a thin coat of foreign material that is mostly spicule debris. The samples were immersed in methanol immediately after collection and transported to the University of Würzburg, Germany. A voucher fragment is kept in 70% methanol under the registration number ZMA POR.10983 in the Zoological Museum, Amsterdam.

Extraction and Isolation. The sample of *D. herbacea* (ca. 500 g, wet weight) was extracted successively with acetone and MeOH (500 mL × 3 for each). The total extract was evaporated under reduced pressure and was partitioned between EtOAc (200 mL × 5) and H_2O (100 mL). The organic fraction was taken to dryness (ca. 10 g) and chromatographed over a Sephadex LH20 column using methanol as eluent, and 14 fractions were obtained. The polybrominated diphenyl ether congeners were isolated from fractions 9−14. Fraction 9 afforded the pure compounds **5** (2.3 mg, 0.0005%) and **7** (13.7 mg, 0.0027%) and a mixture (3:2, 17.6 mg, 0.0035%) of **3** and **4**. Fraction 10 yielded **2** (5.4 mg, 0.0011%) and was purified by column chromatography on RP-18 Lobar (CH_3CN:H_2O, 70:30). Fraction 13 contained **6** (11.2 mg, 0.0024%) and was further purified on RP-18 Lobar (MeOH:H_2O, 70:30). The last fraction 14 yielded the major compound **1** (ca. 2.5 g, 0.5%). The identity of the fractions was confirmed by HPLC and UV spectra recorded online.

3,4,5-Tribromo-2-(2′-bromophenoxy)phenol (2): viscous oil; percent purity 92%; UV λ max (MeOH) 209 nm ($C_{12}H_6O_2Br_4$); EIMS (70 eV) *m/z* 506 (16), 504 (68), 502 (100), 500 (72), 498 (18) $[M]^+$, 425 (5), 423 (24), 421 (24), 419 (9) $[M - Br]^+$, 344 (30), 342 (58), 340 (30) $[M - Br_2]^+$; ^1H NMR ($CDCl_3$) δ 6.50 (dd, 1H, J = 1.4, 8.2 Hz), 6.94 (ddd, 1H, J = 1.3, 7.7, 7.7 Hz), 7.16 (ddd, 1H, J = 1.5, 7.6, 8.3 Hz), 7.34 (brs), 7.61 (dd, 1H, J = 1.6, 7.9).

Mixture (3:2) of 3,5,6-tribromo-2-(2′-bromophenoxy)phenol (3) and **3,4,6-tribromo-2-(2′-bromophenoxy)phenol (4):** viscous oil; percent purity 95%; UV λ max (MeOH) 220 and 222 nm ($C_{12}H_6O_2Br_4$); EIMS (70 eV) *m/z* 506 (16), 504 (68), 502 (100), 500 (68), 498 (18)) $[M]^+$, 425 (14), 423 (38), 421 (38), 419 (14) $[M - Br]^+$, 344 (36), 342 (70), 340 (36) $[M - Br_2]^+$; ^1H NMR ($CDCl_3$)**(3)** δ 6.54 (dd, 1H, J = 1.4, 8.2 Hz), 7.00 (ddd, 1H, J = 1.7, 7.7, 7.7 Hz), 7.20 (ddd, 1H, J = 2.0, 7.5, 8.2 Hz), 7.55 (s), 7.65 (dd, 1H, J = 1.9, 7.9 Hz). ^1H NMR ($CDCl_3$) **(4)** δ 6.54 (dd, 1H, J = 1.4, 8.2 Hz), 7.00 (ddd, 1H, J = 1.7, 7.7, 7.7 Hz), 7.20 (ddd, 1H, J = 2.0, 7.5, 8.4 Hz), 7.65 (dd, 1H, J = 1.9, 7.9 Hz), 7.75 (s).

3,5,6-Tribromo-1-(2′-bromophenoxy)-2-benzene methyl ether (5): viscous oil; percent purity 90%; UV λ max (MeOH) 226 nm ($C_{13}H_8O_2Br_4$); EIMS (70 eV) *m/z* 520 (16), 518 (64), 516 (100), 514 (70), 512 (18) $[M]^+$, 424 (14), 420 (46), 422 (44), 418 (14) $[M - CH_3Br]^+$, 358 (22), 356 (42), 354 (22) $[M - Br_2]^+$; ^1H NMR ($CDCl_3$) δ 3.86 (s), 6.40 (dd, 1H, J = 1.2, 8.3 Hz), 6.93 (ddd, 1H, J = 1.2, 7.7, 7.8 Hz), 7.14 (ddd, 1H, J = 1.6, 7.5, 8.2 Hz), 7.60 (dd, 1H, J = 1.5, 7.8 Hz), 7.74 (s).

Serial Dilution Assay. Quantitative assays of the active metabolites against selected test organisms involved utilization of a 2-fold serial dilution in liquid medium (Müller-Hinton-Bouillon). Twofold serial dilutions of each of the active compounds (1000 μg in 1 mL solvent) in Müller-Hinton-Bouillon (0.5 mL) were inoculated with a culture containing 10^3 microorganisms and incubated at 37 °C for 24 h. The concentration of the test compounds in the initial hole of the microtiter plate was 125 μg/mL. Cultures of *B. subtilis* 168 were used in the assay. Growth inhibition was judged by comparison with growth in control holes prepared without test compound, which were set up at the same time as the test holes. The concentration of the tube at highest dilution, that was free from growth, was recorded as the minimum inhibitory concentration (MIC value in μg/mL).

Bioautographic Detection of Fungicidal Activity. Spores of *C. cucumerinum* were cultivated on carrot-nutrient agar and were inoculated into a liquid yeast culture medium as previously described.[15,16] Si gel TLC plates were spotted with the isolated compounds at concentrations of 50 nmol and 25 nmol, and the plates were sprayed with a suspension of spores of *C. cucumerinum* in liquid yeast culture medium. The fungitoxic compound was observed as a clear white spot of inhibition in a dark layer of the mycelia covering the TLC plate after the inoculated plates were incubated for 2 days at 25 °C.

Brine Shrimp Lethality Test.[17,18] Eggs of *Artemia salina* (Dohse, Aquaristik GmbH, Bonn, Germany) were hatched in a small tank filled with artificial sea water, which was prepared with a commercial salt mixture (Sera Sea-Salt, Aquaristik GmbH, Bonn, Germany) and distilled water. After 48 h, the 20 phototropic nauplii were transferred to each sample vial using a pipet, and artificial sea water was added to make 5 mL. The percent deaths at each dose and control were determined. LC_{50}s were calculated from the dose−response curve by probit analysis.

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