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Polybrominated Diphenyl Ethers: Structure Determination and Trends in Antibacterial Activity

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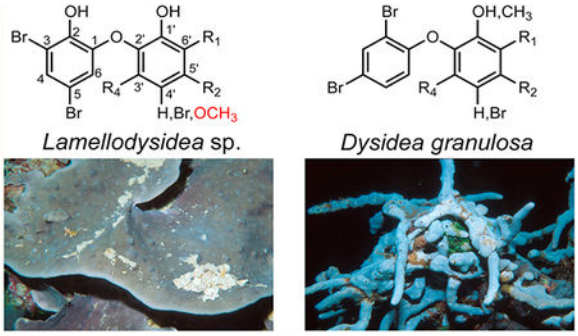
Abstract

Antibacterial-guided fractionation of the Dictyoceratid sponges *Lamellodysidea* sp. and two samples of *Dysidea granulosa* yielded 14 polybrominated, diphenyl ethers including one new methoxy-containing compound (8). Their structures were elucidated by interpretation of spectroscopic data of the natural product and their methoxy derivatives. Most of the compounds showed strong antimicrobial activity with low- to sub-microgram mL^{-1} minimum inhibitory concentrations against drug-susceptible and drug-resistant strains of *Staphylococcus aureus* and *Enterococcus faecium*, and two compounds inhibited *Escherichia coli* in a structure-dependent manner.

Graphical Abstract



The occurrence of bacterial infections by antibiotic-resistant organisms in hospital as well as community settings continues to be a major burden in public health. Knowing that our current arsenal of antibiotics comes almost exclusively from bacterial- and fungal-derived natural products[1] and that high-throughput screening of synthetic small-molecule libraries

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ASSOCIATED CONTENT

Supporting Information

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IR, HRMS, and ^{1}H, ^{13}C, and 2D NMR spectra of new compounds and protocols for biological testing (PDF)

for the discovery of new antibiotics has met with limited success, some experts have proposed continued investigations into natural products libraries for discovery of new antibiotics.[2–4] While screening organic extracts from the NCI Open Repository for antibiotic activity against the model Gram-negative bacterium *Escherichia coli*, we identified three antibacterial extracts that originated from related marine sponges of the family Dysideidae. These included two separate collections of *Dysidea granulosa* and one collection of *Lamellodysidea* sp.

Marine sponges of the Dysideidae family are known for containing a rich array of diverse natural products. Examples include unusual sterols,[5–7] glycolipids,[8] sesterterpenes,[9] and sesquiterpene quinones;[10] the peptidic natural products dysinosins,[11,12] dysideaprolines, and barbaleucamides;[13] and numerous polybrominated and hydroxylated diphenyl ethers (PBDEs).[14–17] Halogenated diphenyl ethers in general have garnered attention from different fields. Revealed by natural products chemists, they represent some of the earliest discoveries of polyhalogenated compounds coming from nature.[18] The distribution of synthetic polychlorinated and polybrominated biphenyls continues to be studied by environmental toxicologists.[19,20] Most recently Agarwal et al. described a biosynthetic scheme that can lead to formation of PBDEs in a widespread marine bacterium.[21] In addition to isolating known PBDEs **1–7** from *D. granulosa* and *Lamellodysidea* sp., we describe the structural assignment of a new PBDE, compound **8** (Figure 1), along with an approach to assign bromination and hydroxylation patterns in substituted diphenyl ethers. Unexpected trends in antibacterial activities were observed for these compounds.

The extracts of *Lamellodysidea* sp. and two collections of *D. granulosa* inhibited the growth of both Gram-positive and Gram-negative bacteria. The Gram-positive bacteria included *Bacillus subtilis* and drug-susceptible and drug-resistant strains of *Staphylococcus aureus* and *Enterococcus faecium*, with *E. coli* representing Gram-negative bacteria. With an emphasis on identifying the compounds responsible for the Gram-negative inhibitory activity, we fractionated these extracts on HP20SS eluting with H_2O to MeOH followed by acetone. LC-MS and NMR analyses of the *E. coli*-active fractions identified a number of known PBDEs, along with a new compound, **8**. Compound **8** was isolated as a white solid, and the low-resolution ESIMS spectrum of **8** showed a distribution of negatively charged ions at *m/z* 620.6, 622.6, 624.6, 626.6, 628.6, and 630.6 having relative intensities of 1:4:6:6:4:1, which indicated the presence of five bromine atoms in the molecule. The molecular formula of $C_{13}H_7Br_5O_4$ was determined by HRESIMS, indicating eight degrees of unsaturation.

The ^{1}H NMR spectroscopic data of compound **8** (Table 1) exhibited a pair of meta-coupled (2.2 Hz) signals at δ_H 6.54 and 7.37 and one methoxy group at δ_H 3.87 (s, 3H). Two broad singlets (δ_H 7.08 and 7.56) in the ^{1}H NMR spectrum were tentatively assigned as hydroxy groups. The presence of one methoxy carbon, two sp^2 methines, and 10 sp^2 nonprotonated carbons was apparent from the ^{13}C NMR and HSQC spectra, consistent with **8** being a polybrominated diphenyl ether.[22] Methylation of **8** to give **8a** confirmed the presence of two hydroxy groups (Table 1).

The locations of the bromines and hydroxy groups in ring A of **8** and **8a** were clear from the chemical shifts, meta-coupling, and HMBC correlations. The HMBC spectra (Figure 2) showed couplings from H-4 to C-2, C-3, C-5, and C-6 and from H-6 to C-1, C-2, C-4, and C-5 in **8** and **8a**. Additionally, the coupling from the methoxy group at δ_H 4.00 to C-2 in derivative **8a** established the positions of the ether and hydroxy groups in ring A and the chemical shifts of C-1 and C-2. These data indicated that the meta-coupled protons were para to the ether and to a brominated carbon, leaving only the H-4/H-6 arrangement possible.

Assignment of the substitution pattern in ring B was more challenging due to the lack of HMBC correlations. In addition, the ^{13}C chemical shifts did not match any known PBDEs. This was likely due to the presence of a methoxy group that affects ^{13}C chemical shifts in PBDEs.[23] We tentatively assigned the position of the naturally occurring $-OCH_3$ group in ring B using a combination of NOEs and distance comparisons from molecular modeling. In selective 1D-NOE spectra of **8** an NOE between the OCH_3 and H-6 was the only one observed (Figure S13, Supporting Information). As shown in Figure 3, this suggests that the $-OCH_3$ group is located *meta* to the ether and attached at C-4′ or C-6′. In the NOE spectra of methylated derivative **8a**, NOEs between the new methoxy group in ring B and H-6 and $-OCH_3$ in ring A were present (Figure 3D); however no NOEs were observed between the two $-OCH_3$ groups in ring B (Supporting Information). This suggested that the hydroxy group in **8** was *para* to the $-OCH_3$ group and thus located at C-1′.

To provide additional support for the location of substituents in ring B of compound **8**, we prepared the methoxy derivatives of known compounds **3–6** by treatment with iodomethane to give compounds **3a–6a**, with **3a** and **5a** being new compounds (Supporting Information), and compared their ^{13}C chemical shifts. Compared to the parent compounds **3–6**, chemical shifts for C-1, C-3, and C-5 in ring A and C-2′, C-4′, and C-6′ in ring B were deshielded by an average of 6.4, 8.3, 4.6, 4.7, 3.2, and 4.0 ppm, respectively. Similarly, the corresponding carbons in **8** and **8a** were deshielded by 5.9, 7.9, 5.0, 6.2, 3.6, and 7.5 ppm (Table 1). These trends in chemical shifts are consistent with the substitution pattern suggested above and confirm the locations of the bromine, hydroxy, and methoxy substituents. We note that these chemical shift changes at positions ortho and para to a methoxy group are seen at both protonated and halogenated carbons, making this a useful approach for assigning these complex substitution patterns. Interestingly, compounds originating from *Lamellodysidea* all contained a 2-hydroxy-3,5-dibromophenyl moiety for ring A, while compounds from *D. granulosa* uniformly contained a 2,4-dibromophenyl moiety, making this chemistry specific for taxonomically distinct marine sponge samples (Table 2).

We tested compounds **1–14** for antibacterial activities against the panel of drug-susceptible and drug-resistant bacteria and fungi shown in Table 3. Compounds **2–13** showed strong antibacterial effects toward *S. aureus* and *E. faecium* strains, exceeding the potency of control antibiotics oxacillin and vancomycin. Surprisingly and unlike the other compounds, **9** and **11** also inhibited the growth of *E. coli* with MICs of 3.1 and 12.5 μg/mL, respectively. Though previously reported to have broad-spectrum antibacterial activity[24] in our antimicrobial assays, compound **10** did not inhibit the growth of *E. coli*. None of the compounds were active against *P. aeruginosa* or *C. albicans*.

Compounds **1–14**, **3a–6a**, and **8a** were tested for cytotoxicity against a monkey kidney cell line (BSC-1) and a human colorectal tumor cell line (HCT-116).[25] Compounds **1–8**, **3a–6a**, and **8a** were nontoxic at the maximum tested concentration (50 μg/mL). Compounds **9–14** showed some toxicity against the kidney cell line BSC-1 with IC_{50}'s between 7 and 35 μg/mL. Although their ring B structures are similar, rings A of compounds **9–14** lack a hydroxy and contain bromine atoms ortho and para to the ether compared to compounds **2–8**. This difference in conjunction with the cell-based screens suggests that the lack of the hydroxy group on ring A and/or the bromine substitution pattern leads to increased cytotoxicity. To explore whether halogens are required for these activities, we tested the commercially available compound 2,2′-dihydroxydiphenyl ether (**15**) for cytotoxity and antibacterial activity; it was inactive in all assays, as were the permethylated derivatives **3a–6a** and **8a**. The observed structure−activity relationship suggests that ring B needs two bromine atoms and a C-1′ hydroxy group for antibacterial activity. Further, the presence of two phenolic hydroxy groups at C-1′ and C-2 in PBDEs decreases cytotoxity, but also corresponds to a loss in activity against the Gram-negative bacterium *E. coli*.

In summary, we isolated 14 polybrominated and hydroxylated diphenyl ethers from related Dysideidae sponges and demonstrated a simple method employing methylation and associated changes in ^{13}C chemical shifts to assign complex substitution patterns in proton-deficient structures. Because it can be used on compounds of this class that contain both hydroxy and methoxy substitutents, this method augments previous NMR approaches that have been used in PBDE analyses.[23] Although superficially compounds **1–14** appear to be highly similar to one another, their antibacterial spectrum and potencies, as well as cellular cytotoxicities, differed greatly as a function of the presence and pattern of hydroxy and bromine substituents. Given the difficulty of identifying small molecules that inhibit the growth of Gram-negative organisms, these results may provide insights into features that lead to this activity.

EXPERIMENTAL SECTION

General Experimental Procedures.

UV−vis spectra were recorded on an Agilent 8453 spectrophotometer, and IR spectra on a PerkinElmer Frontier FT-IR spectrophotometer (KBr). NMR spectra were recorded at 298 K on a Bruker Avance500 spectrometer equipped with a triple resonance cryoprobe and *z* gradients. Transient NOE spectra were acquired with a 1 s mixing time using a selective 1D double pulsed field gradient spin echo pulse program incorporating perfect-echo excitation, alternating gradient polarity, and zero quantum suppression.[26–29] LRESIMS was carried out on an Agilent 1100 LC system and a 6310 MSD. HRESIMS was carried out on a Waters time-of-flight mass spectrometer model LCT Premier. Semipreparative HPLC (Agilent 1100) was performed using a Waters XBridge preparative C18 column (10 × 250 mm, S-5 μm, 12 nm). All solvents (HPLC grade) and Diaion HP20SS gel were obtained from Sigma-Aldrich.

Animal Material.

Extracts from the three Dysideidae sponge samples used in this study were obtained from the NCI Open Repository. *Lamellodysidea* sp. (NPID no. C024121) was collected at a depth of 43 m in Papua New Guinea in June 2003. *Dysidea granulosa* (NPID no. C024075) was collected in Papua New Guinea at a depth of 10 m, and a separate collection of *D. granulosa* (NPID no. C031381) was collected in June 2010 in the Palau Islands. Taxonomic identifications were made by Michelle Kelly or one of the authors, L.J.B. Voucher specimens are available at the NCI, Frederick, MD, USA, and at the Smithsonian Museum, Washington, DC.

Extraction and Isolation.

Organic extracts from the above three sponges were prepared by extraction with MeOH $-CH_2Cl_2$ and stored at −50 °C until shipment. The organic extract of *Lamellodysidea* sp. (5 g) was chromatographed on a Diaion HP20SS-gel column equilibrated in H_2O and was eluted with a 10% stepwise gradient from H_2O to MeOH. Fractions were combined into three groups, A–C, on the basis of antimicrobial activity. Fraction C (4.5 g) was purified by semipreparative HPLC (5 mL/min, 0–15 min, 50–85%; 15–40 min 85–95% MeCN−H_2O gradient elution) to yield compounds **1** (0.4 mg, t_R 14.5 min), **2** (1.1 mg, t_R 24.0 min), **8** (3.3 mg, t_R 24.5 min), a mixture of compounds with a t_R of 27 min, **4** (10 mg, t_R 27.5 min), **5** (4.2 mg, t_R 29.5 min), and 7 (114 mg, t_R 31.0 min). The mixture eluting at 27 min was resubjected to semipreparative HPLC (5 mL/min, 30 min, 50–70% MeCN−H_2O gradient elution) to give compounds **6** (6.4 mg, t_R 23.5 min) and **3** (15 mg, t_R 25.0 min).

The organic extract (2.19 g) of *D. granulosa* (NPID no. C031381) was dissolved in MeOH and chromatographed on a Diaion HP20SS-gel column eluting with water to MeOH, followed by acetone, to give seven fractions (A–G), which were combined on the basis of their LCMS profiles. The acetone fraction G (1.44 g) was subjected to reversed-phase HPLC (XBridge prep, Waters C18, 5 μm) eluting with a linear gradient of 0.1% TFA in H_2O and 0.05% TFA in MeCN (60–100% in 40 min, flow rate 5 mL/min) to yield compounds **9** (38 mg, t_R 14.5 min), **10** (1.2 mg, t_R 17.3 min), **11** (38.6 mg, t_R 18.0 min), **12** (13.4 mg, t_R 19.0 min), and 13 (1.7 mg, t_R 22.7 min).

The organic extract (2 g) of *D. granulosa* (NPID no. C024075) was dissolved in MeOH and chromatographed on a Diaion HP20SS-gel column as described above. The acetone fraction G (0.35 g) was subjected to reversed-phase HPLC (5 mL/min, 60–80% H_2O−MeCN with 0.01% TFA, gradient elution in 40 min) to yield compounds 9 (20.2 mg, t_R 18.5 min), **10** (6.0 mg, t_R 18.5 min), and **14** (2.1 mg, t_R 37.5 min).

*1-(3′,5′,6′-Tribromo-4′-methoxy-1′-hydroxyphenoxy)-3,5-dibromo-2-phenol (**8**):* white solid; UV (MeOH) λ_{max} (log ε) 213 (5.64), 297 (4.57) nm; IR (film) ν_{max} 2918, 2865, 1711, 1415, 1234 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; LRESIMS *m/z* 620.6 $[M - H]^-$; HRESIMS *m/z* 620.6187 $[M - H]^-$ (calcd for $C_{13}H_6Br_5O_4$, 620.6183).

*1-(3′,4′,6′-Tribromo-1′-methoxyphenoxy)-3,5-dibromo-2-methoxybenzene (**3a**):* white solid; UV (MeOH) λ_{max} (log ε) 214 (5.12) nm; IR (film) ν_{max} 2917, 2864, 1701, 1413,

1232 cm^{-1}; ^{1}H and ^{13}C NMR data, Supporting Information; HRESIMS *m/z* 619.6458 $[M + H]^+$ (calcd for $C_{14}H_9Br_5O_3$, 619.6468).

*1-(4′,5′,6′-Tribromo-1′-methoxyphenoxy)-3,5-dibromo-2-methoxybenzene (**5a**):* white solid; UV (MeOH)0020λ_{max} (log ε) 216 (5.34) nm; IR (film) ν_{max} 2917, 2865, 1701, 1510, 1493, 1482 cm^{-1}; ^{1}H and ^{13}C NMR data, Supporting Information; HRESIMS *m/z* 619.6473 $[M + H]^+$ (calcd for $C_{14}H_9Br_5O_3$, 619.6468).

*1-(3′,5′,6′-Tribromo-1′,4′-dimethoxy-1′-phenoxy)-3,5-dibromo-2-methoxybenzene (**8a**):* UV (MeOH) λ_{max} (log ε) 214 (5.68), 295 (4.44) nm; IR (film) ν_{max} 2917, 2865, 1711, 1415, 1234 cm^{-1}; ^{1}H and ^{13}C NMR data, Table 1; HRESIMS *m/z* 649.6567 $[M + H]^+$ (calcd for $C_{15}H_{11}Br_5O_4$, 649.6574).

Methylation of Compounds 3–6 and 8.

Compound 3 (2.0 mg, 3 μmol) was added to a solution of acetone (500 μL) in the presence of K_2CO_3 (0.1 mg) and MeI (400 μL, 6 μM) at rt. The solution was stirred for 1 h, dried under a stream of N_2, and extracted with $CHCl_3$ to give **3a** as a white solid (2.3 mg, 100%). By the same method compounds **4a** (1.7 mg, 95%), **5a** (2.0 mg, 84%), **6a** (3.3 mg, 95%), and **8a** (0.6 mg, 100%) were obtained from **4** (1.5 mg), **5** (2.0 mg), **6** (3.0 mg), and **8** (0.5 mg), respectively.

MIC and Cytotoxicity Determination.

MICs were determined by testing all compounds in duplicate in at least two independent experiments for their antimicrobial activity against laboratory strains listed in Table 3 and as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines.[30] All laboratory bacterial strains were obtained from the American Type Culture Collection. Experimental details for MIC and cytotoxicity data are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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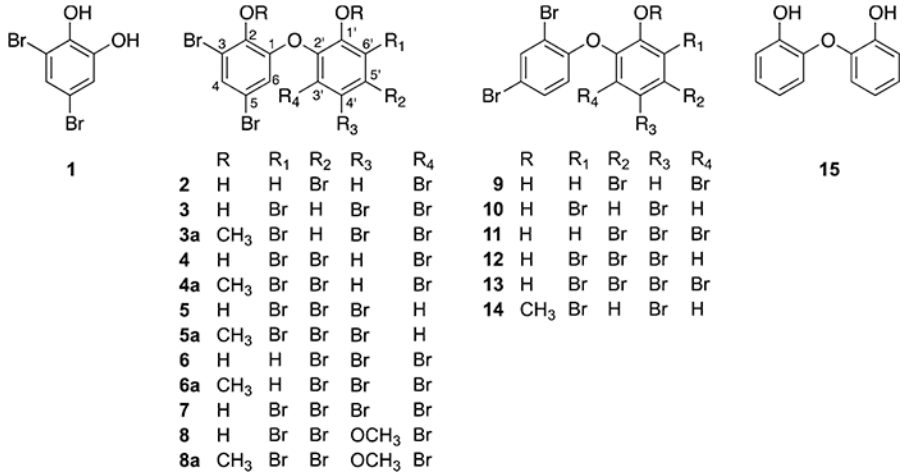

Figure 1.

Compounds isolated from *Lamellodysidea* sp. (**1–8**), two collections of *Dysidea granulosa* (**9–14**), synthetic methylates **3a–6a** and **8a**, and a commercial 2,2′-dihydroxydiphenyl ether (**15**).

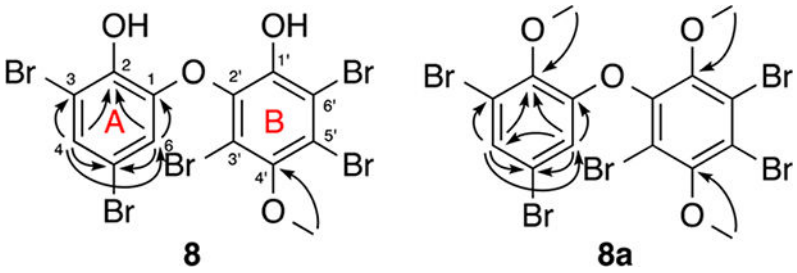

Figure 2.
HMBC correlations used in structure determination of compounds **8** and **8a**.

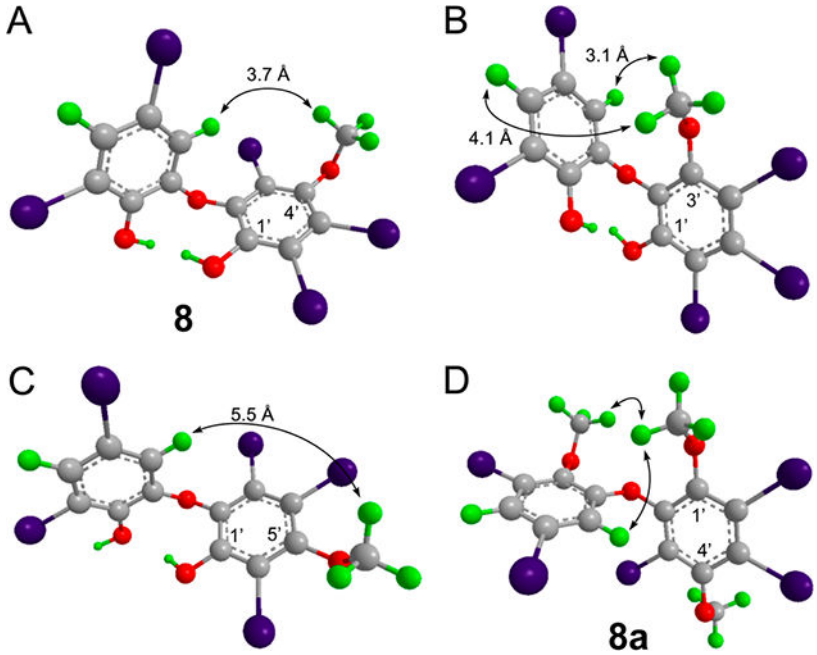

Figure 3.

Interproton distances of three possible isomers of **8** and NOEs observed for **8a**. Coordinates for the three possible structures of compound **8** were energy minimized using Chem3D, and interproton distances between H-4 and H-6 of ring A and the $-OCH_3$ group in ring B were measured in each and compared to the experimental NOE data (A–C). The isomer shown in B (1′-hydroxy-3′-methyoxy) predicts NOEs that are not observed, and the isomer in C (1′-hydroxy-5′-methyoxy) shows a distance greater than 5.5 Å for an NOE that is present. Panel D shows the NOEs observed for methylated **8a**, in agreement with experimental data. 1D NOE spectra are shown in Figure S13.

Table 1.

^{1}H (500 MHz) and ^{13}C NMR (126 MHz) Data for 8 and 8a[a]

	8		8a		
position	**δ_C, type**	**δ_H (*J* in Hz)**	**δ_C, type**	**δ_H (*J* in Hz)**	**$\Delta\delta_C(\delta_{8a}-\delta_8)$**
1	144.9, C		150.8, C		5.9
2	142.7, C		145.5, C		2.8
3	111.3, C		119.2, C		7.9
4	116.3, CH	7.37, d (2.2)	116.9, CH	7.40, d (2.2)	0.6
5	111.8, C		116.8, C		5.0
6	129.5, CH	6.54, d (2.2)	129.4, CH	6.49, d (2.2)	−0.1
1′	145.1, C		148.5, C		3.4
2′	138.8, C		145.0, C		6.2
3′	119.2, C		119.4, C		0.1
4′	149.0, C		152.6, C		3.6
5′	113.4, C		113.5, C		0.2
6′	114.2, C		121.7, C		7.5
−OH		7.08, br s			
−OH		7.56, br s			
1′-OCH_3			61.6, CH_3	3.79, s	
4′-OCH_3	61.1, CH_3	3.87, s	61.0, CH_3	3.91, s	
2-OCH_3			61.3, CH_3	4.00, s	

[a]Chemical shifts (δ) are referenced to residual $CDCl_3$ (^{1}H: 7.26/^{13}C: 77.16 ppm).

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Table 2.

Occurrence of Compounds in Three Dysideae Sponges

sponge	NPID number[a]	collection site	compounds
Lamellodysidea sp.	C024121	Papua New Guinea	**1–8**
Dysidea granulosa	C031381	Palau	**9–13**
Dysidea granulosa	C024075	Papua New Guinea	**9**, **10**, and **14**

[a]Corresponds to the NCI Open Repository Natural Products ID number.

Table 3.

Minimum Inhibitory Concentrations and Cytotoxicity of Compounds 1–14, 3a–6a, 8a, and 15[a]

	S. aureus ATCC 29213	*S. aureus* ATCC 43300	*E.faecium* ATCC 29212	*E.faecium* ATCC 51299	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 28517	Bsc-1
1	100	>50	12.5	25	>100	>50	>50	>50
2	1.6	1.25	3.1	1.6	50	>50	>50	>50
3	1.6	0.31	3.1	3.1	100	>50	>50	>50
4	0.78	0.16	1.6	0.78	50	>50	>50	>50
5	0.31	0.16	1.6	0.78	50	50	>50	>50
6	0.31	0.078	0.39	0.39	25	50	>50	>50
7	0.39	0.16	0.78	0.39	50	50	50	>50
8	0.78	0.39	3.1	1.56	>50	>50	>50	>50
3a	>50	>50	50	>50	>50	>50	>50	>50
4a	>50	>50	50	50	>50	>50	>50	>50
5a	>50	>50	>50	>50	>50	>50	>50	>50
6a	>50	>50	>50	>50	>50	>50	>50	>50
8a	3.1	3.1	13	13	>50	>50	>50	>50
9	0.042	0.08	1.2	1.2	3.1	>50	>50	7.0
10	0.78	0.19	0.8	0.8	>100			32
11	0.14	0.015	0.4	0.4	12.5			8.8
12	3.7	0.4	1.2	1.2	>100			15
13	1.6	0.8	33	33	>100			29
14	3.7	0.4	11	11	>100			35
15	50	50	>50	>50				
oxacillin	0.13	>32		32				
gentamicin					0.5	1		
vancomycin		1	2					
amphotericin B							<7.8	

[a]MICs and cytotoxity data are reported in μg/mL.