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An NMR Strategy for Unraveling Structures of Bioactive Sponge-derived Oxy-polyhalogenated Diphenyl Ethers[⊥]

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Abstract

The over-expression of the Mcl-1 protein in cancerous cells results in the sequestering of Bak, a key component in the regulation of normal cell apoptosis. Our investigation of the ability of marine-derived small molecule natural products to inhibit this protein-protein interaction led to the isolation of several bioactive oxy-polyhalogenated diphenyl ethers. A semi-pure extract, previously obtained from *Dysidea* (*Lamellodysidea*) *herbacea* and preserved in our repository, along with an untouched *Dysidea granulosa* marine sponge afforded 13 distinct oxy-polyhalogenated diphenyl ethers. Among these isolates were four new compounds, **5, 6, 10,** and **12**. The structure elucidation of these molecules was complicated by the plethora of structural variants that exist in the literature. During dereplication, we established a systematic method for analyzing this class of compounds. The strategy is governed by trends in the ^{1}H and ^{13}C NMR shifts of the aromatic rings and the success of the strategy was checked by X-ray crystal structure analysis.

There are several approaches that have been effective in guiding the discovery of anticancer therapeutic leads based on natural product scaffolds. One rich example is represented in the route taken by the Kingston team to further define the pharmacophore of tubulin inhibitors, such as paclitaxel, by combining synthesis with cancer biology studies.[1] A somewhat different tactic was employed in this project that involved the search for marine natural product inhibitors of the Bcl-2 family of proteins. Seven anti-apoptotic family members have been identified to date and include Mcl-1, Bcl-X_L, Bcl-2, Bcl-W, Bcl-B, Bcl-9L, and Bfl-1.[2] It is believed that an important element in the apoptosis regulation mechanism involves the protein-protein interaction (PPI) of Bcl-2 family proteins with the pro-survival BH3-domain binding proteins such as Bak Bax, Bid, Bim, or Noxa. There are several recent findings suggesting that Mcl-1 and Bcl-X_L can be considered druggable targets since inactivation of either suffices for Bak-modulated cell death. A small set of low molecular weight natural products and synthetic compounds has been reported recently as Bcl-2 inhibitors. Significantly, three of the Bcl-2 inhibitors are being pursued in anticancer clinical trials and these include (−) gossypol[3,4] (Phase I and II), ABT-263[5] (Phase II), and obatoclax (Phase II).[6] The presence of multiple aromatic rings with accompanying hydrogen-bonding functionality represents a conserved structural feature prominent in most of the Bcl-2 family inhibitors identified to date.

[⊥]Dedicated to Dr. David G.I. Kingston of Virginia Polytechnic Institute and State University for his pioneering work on bioactive natural products.

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Supporting Information Available: Tables and Figures are provided which include Mass, NMR studies, and X-ray crystallographic information. This material is available free of charge via the Internet.

Several years ago we began a program to discover marine natural products capable of disrupting PPIs,[7] and this effort included screening of compounds that might disrupt the interaction between Mcl-1 and Bak. Our first results were encouraging and represented a proof-of-concept investigation. One aspect of the study involved the testing of the NCI diversity set of 2,240 compounds. The hit rate of 0.76% (based on 17 active compounds, data to be reported elsewhere) indicated that our fluorescent resonance energy transfer (FRET) assay was functional and selective. There were several active samples identified during our screening of marine sponge extracts and semi-purified fractions. We believed that it would be worthwhile to focus on extracts from *Dysidea granulosa* plus the semi-pure compounds obtained from *Dysidea* (*Lamellodysidea*) *herbacea* that exhibited activity in a FRET primary screen. These well-studied genera are characterized by the presence of a symbiotic cyanobacterium, *Oscillatoria spongeliae*.[8–13] This assemblage commonly produces three distinct classes of compounds: sesquiterpenes,[14] polychlorinated peptide derivatives[15,16] and oxy-polyhalogenated diphenyl ethers (O-PHDEs).[17] Early on it was clear that the active principles were members of the latter category. Not only are these compounds prolific, their activity is dynamic, with a broad range of responses such as antibacterial, lipoxygenase inhibition,[18] antifungal,[19–22] antifouling,[23] anti-inflammatory properties,[24] inhibition of a range of enzymes implicated in cancer,[25,26] and the restriction of the assembly of microtubules.[27] There is also evidence for bioaccumulation in marine mammals of synthetic O-PHDEs, widely used as commercial flame retardants.[28] Unexpectedly, the challenge of unequivocally establishing the complete structure of the O-PHDEs we isolated proved to be very challenging. Part of the difficulty is that they have an H/C ratio < 1.[29] To overcome this challenge we have created an NMR data trends and dereplication paradigm. This model and the biological activity data obtained are described below.

Results and Discussion

Extracts from *Dysidea granulosa* and a repository sample from *Dysidea* (*Lamellodysidea*) *herbacea* that inhibited the protein-protein interaction of Mcl-1 and Bak were studied simultaneously. The crude extract from *D. granulosa* had a repeatable IC_{50} of 4.1 μg/mL and the sample mixture from *D.* (*Lamellodysidea*) *herbacea* an IC_{50} of 2.1 μg/mL in the FRET assay used. Purification and structure dereplication indicated that the bioactive constituents were O-PHDEs.

Initially, our plan was to engage in a straightforward dereplication because there are more than 43 known O-PHDEs derived from sponge – cyanobacterium associations. Immediately, we found that an orderly approach had not been previously described. In order to systematize this process, we classified the literature compounds into four structural types (I, II-1, II-2, and III) shown in Figure 1, based on their core formulas. These structure types differ in the number of oxygens present and substitution variations. From these data, it is clear that O-PHDE-producing organisms follow a conserved biogenetic pathway as the ring B 1-position is always oxygenated. Also notable is that the inclusion of chlorine atoms is uncommon and only structure type I includes chlorine as well as bromine. Structure types I and II-1 represent the two most populated categories, with 19 and 21 O-PHDEs reported, respectively.

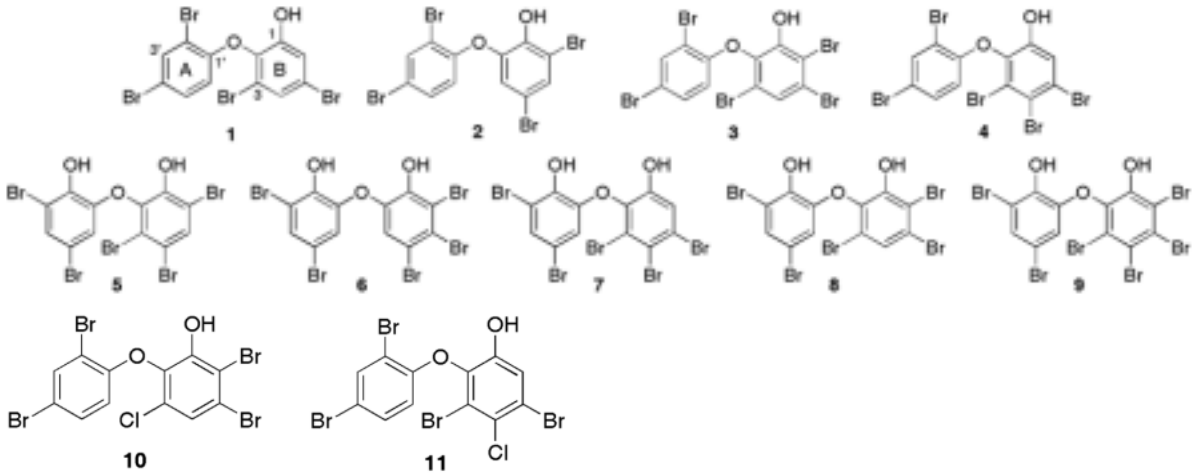
The O-PHDEs of Figure 1 can be further broken down based on substitution into subtypes. All reported permutations of halogens, hydroxyl and proton substitution for rings A and B are shown in Table 1 with the corresponding subtype. The ^{1}H and ^{13}C resonances are largely conserved according to substitution type in this class of compounds and the data accompanying each structure represents an average of those reported in the literature. There are a total of six and 12 different known subtypes for rings A and B, respectively, to date.

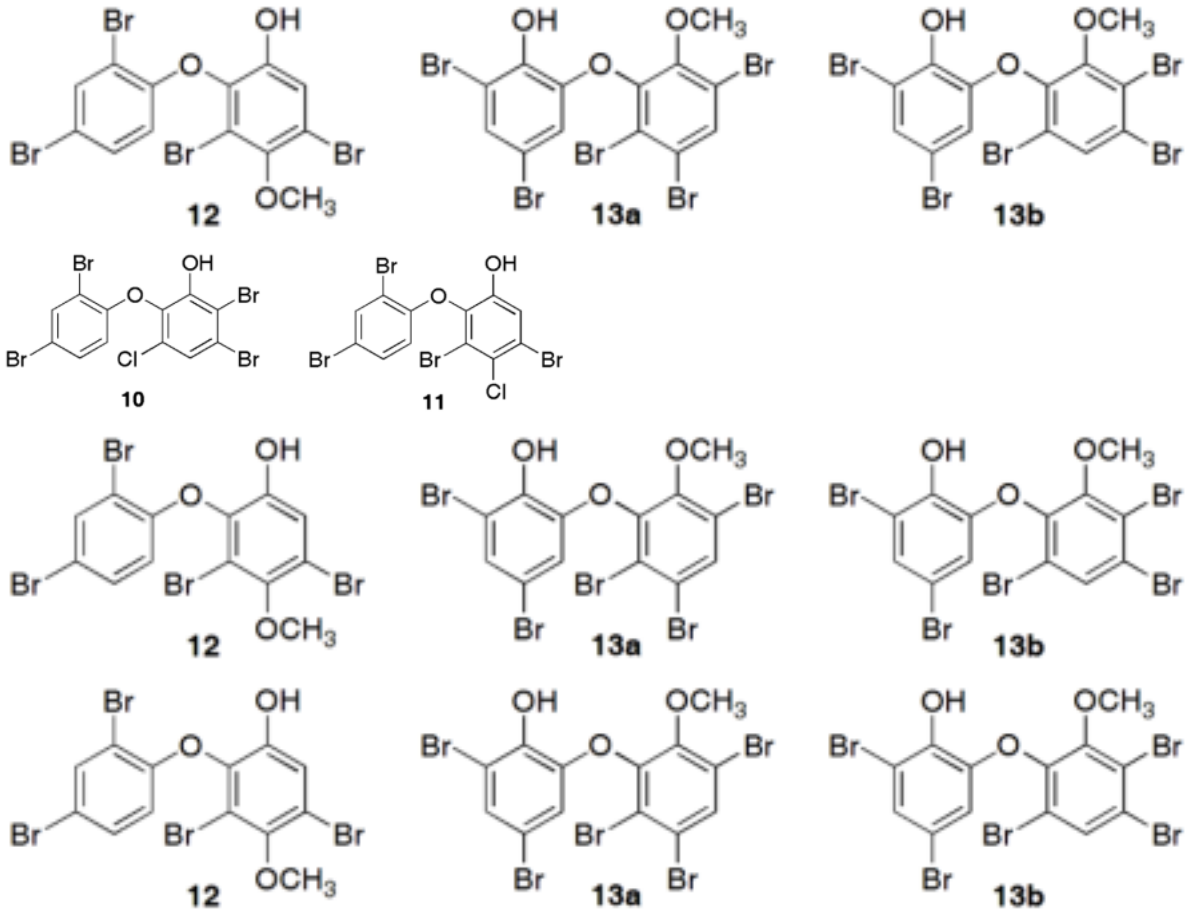
It is important to note that certain O-PHDE types must either be given special consideration or cannot be elucidated using our NMR data trends (Table 1). One important case involves of O-PHDEs that contain a methoxy group, because when present it has an unpredictable but often considerable effect on ^{1}H and ^{13}C NMR chemical shifts. A second case involves molecules that contain ring subtypes B-9 and B-12, whose ^{1}H and ^{13}C NMR chemical shifts are too similar to differentiate. In both situations mentioned above, we suggest that additional data must be used to secure structures.

The substituent in the 3-position of ring B must also be given special consideration. A cross ring effect, which has been noted previously,[30] occurs when a bromine is located at C-3, influencing the proton shift at H-6′. Higher δ_H and δ_C values at the 6′-position ($\delta_H \sim 6.8 – 7.0$ and $\delta_C \sim 121$) are observed when the 3-position is occupied by a proton and lower values ($\delta_H \sim 6.4 – 6.5$ and $\delta_C \sim 115 – 117$) when a bromine is in the 3-position. In this case, assigning the structure of ring B must be accomplished first to properly consider this difference. It is essential to point out that a $\delta_H \sim 6.4 – 6.5$ can be assigned unambiguously to the 6′-position; no other O-PHDE aromatic protons occur so far upfield. Solving the constitution of ring B first, and anticipating changes for the proton of the 6′-position allows for this dichotomy to be reliably overcome.

Once an unknown O-PHDE is assigned to a structure type and it is determined that the O-PHDE contains no methoxy groups, Table 1 may be applied. Analysis of the spin system for each ring, A and B, is also useful step. For example, four protons can be attributed to an ABCD or AA′XX′ system, as seen in subtypes A-1 and A-2. More complicated is the case when three protons populate the ring. Several ABX systems, subtypes A-3, A-4, B-1, B-2, and B-3, are possible and they can be distinguished by the magnitude of the expected *J* values. The same strategy can be used to differentiate the two-spin system containing rings. The 1-position OH of ring B, which is conserved biogenetically, provides the anchor point to construct ring B and sometimes represents the best starting point before proceeding to analyze ring A.

A total of 13 O-PHDEs were isolated in this study (**1 – 13b**). These are divided into three new compounds (**5**, **6**, and **10**) devoid of methoxy substituents, one with a methoxy group (**12**) and not assignable using Table 1, and one structure we show definitively to be misassigned previously (**13a** revised to **13b**). Although we will not detail the identification of the known compounds (**1 – 4, 7 – 9** and **11**),[17,21,30–38] it is important to point out that using Table 1 to solve the structures greatly expedited the recognition that they were already known. Further evidence that our approach was effective was shown by the X-ray crystallographic data shown in Figure 2 confirming the dereplicated structures for **4** and **7**.





The first new metabolite to be analyzed was **5**, isolated from impure compound sample obtained from *D.* (*Lamellodysidea*) *herbacea*. It was found to have at least 12 carbons (^{13}C NMR), five protons, three of which were aromatic (^{1}H NMR), and five bromines (molecular ion cluster analysis). This data, together with the ESIMS data afforded the molecular formula $C_{12}H_5O_3Br_5$, making it a member of structure type II-1 (Figure 1). Two of the aromatic protons were *meta*-coupled (2.2 Hz) and the isolated proton belonged to ring B, as no A rings with a single hydrogen have been discovered to date. The observed proton at δ_H 7.77 clearly designated this as ring subtype B-11, with a bromine at C-3 (Table 1). For ring A, the *meta*-coupled protons were observed at δ_H 7.39 and 6.55, identifying that the other half of the structure as ring subtype A-5, with the shift at δ_H 6.55 being attributed to H-6′. Thus, compound **5** was a combination of A-5 and B-11. This result was also verified by X-ray crystallographic analysis result shown in Figure 2.

The second new compound **6** ($C_{12}H_5O_3Br_5$) was isomeric to **5,** had a molecular formula of $C_{12}H_5O_3Br_5$, and, analogous to **5**, had three aromatic protons as two separate spin systems. The isolated proton at δ_H 7.24 was indicative of ring type B-9 or B-12, but as mentioned previously, we were unable to distinguish between the two. The two *meta*-coupled A-ring protons (δ_H = 7.03 and 7.48) could be attributed to either ring type A-6 or A-5 with a possible cross-ring deshielding adjustment described previously from the ring type A-3 (3 and 6′-positions = H).[30,32] This situation represents a limitation of our strategy, but the choices were narrowed to four alternatives (A-5/B-9, A-5/B-12, A-6/B-9 and A-6/B-12). The final structure was solved as ring types A-5 and B-9 through X-ray crystallographic analysis.

The accurate mass of compound **10** gave the molecular formula $C_{12}H_5O_2ClBr_4$. We proceeded with the structure elucidation under the assumption that substituting chlorine for bromine would have little effect on ^1H and ^{13}C NMR values of non-chlorinated positions, as noted previously,[31] and focused on using our NMR data trends to place hydrogen atoms and generic halogen atoms (Br or Cl). A single aromatic proton at δ_H 7.55 and δ_C 127.9 established ring B as subtype B-10, with a halogen at C-3. The ABX system ^1H and ^{13}C NMR values matched chemical shifts for ring subtype A-3 very well, securing the A-3/B-10 structure. This molecular framework is identical to compound **3**, but **3** contains only bromine atoms. Compounds **4** and **11**, also isolated in this study, similarly differ only by the substitution of a chlorine for a bromine, and **11** was verified by a crystal structure.[31] The carbon chemical shift of the chlorinated 4-position is δ_C 127.8 in compound **11** while the identical position in **4** is δ_C 119.5; this downfield chemical shift can be attributed unambiguously to the presence of the chlorine atom. A similar downfield shift was observed in compounds **10** and **3** (from δ_C 117.0 to 122.9) for C-3, indicating that the chlorine is located at the 3-position in compound **10**.

As noted above, our NMR trends strategy is not reliable for instances where a methoxy group is present. Such was the case with compound **12**, and caution was exercised during elucidation. The molecular formula was established, via HRESIMS, as $C_{13}H_7O_3Br_4$. The isolated proton of ring B, based on Table 1, appears to occupy C-6, but this was a tentative assignment, and we recognized it would need corroboration. The HMBC correlations provided the additional essential data and consisted of those observed from the isolated proton to the oxygenated carbon of the ether (C-2, δ_C 139.2), the carbon bearing the methoxy (C-4, δ_C 149.1), the phenolic carbon (C-1, δ_C 146.7), and one brominated carbon (C-5, δ_C 115.3). The intensity of the three bond correlations, to ether C-2 and methoxy C-4, together with the deshielding effect seen across the ring (the 3-position is brominated) confirmed that the proton was located at the 6-position. The ABX system of ring A, which lacked a methoxy group, was easily correlated to the ^1H and ^{13}C NMR chemical shifts of ring subtype A-3, clinching the structure of novel compound **12**. Interestingly, this compound does not correlate to any of the previously described O-PHDE structure types (Figure 1) and so we designate this additional structure type as II-3.

When known compound **13a** was isolated, containing the troublesome methoxy group, it was essential to double-check the published structural assignment. The isolated proton of ring B (δ_H 7.76) resembled that of ring subtype B-11, but no HMBC correlation was seen to the methoxy-bearing carbon, as drawn three bonds away. We revised the structure, placing the isolated proton and the methoxy group *para* to each other. The original ^1H NMR analysis[37] for **13a** was atypically run in acetone-d_6 (isolated proton at δ_H 7.93), which does not match the large body of literature NMR values for O-PHDEs, primarily run in $CDCl_3$. This revision to a structure correctly described previously as **13b**,[17,33] was confirmed by X-ray crystallographic analysis.

The Mcl-1 FRET assay data consisting of IC_{50} results shown in Table 4 were used to screen fractions obtained during the purification steps leading to the pure compounds subsequently isolated. Initially we were optimistic that the 4.1 μg/mL IC_{50} activity observed for the *D. granulosa* partition fraction, 05406XFD, would translate into the isolation of compounds with sub μg/mL activity. Another initially promising observation was that the repository sample coded as 88098F whose IC_{50} was 2.1 μg/mL was a mixture of six O-PHBEs including **5–9** and **13b**. Disappointingly, none of the isolated compounds possessed anticipated sub μg/mL activity. Only three compounds (**1, 2, 4**), of the ten compounds tested twice, exhibited significant IC_{50} values < 10 μg/mL, with compound 1 showing the most important IC_{50} of 2.1 μg/mL. Further unexplained is the lack of follow-up activity of the compounds isolated from active sample 88098F. The absence of significant activity for the pure O-PHDEs from 88098F

suggests that the strongest inhibition of 2.1 μg/mL may be the result of a synergetic effect from combination of O-PHDEs.

There have been more than nine previous studies dealing with sponges containing O-PHDEs. This record is summarized in Table 5 and shows that the genera *Dysidea* and *Phyllospongia* (Order Dictyoceratida) are reliable sources of such compounds. The literature also contains cogent discussions about the involvement of the symbiont, *O. spongeliae*, in elaborating halogenated secondary metabolites, especially O-PHDEs.[39–41] The report of **1**, **2** and **4** from certain *Dysidea* and *Phyllospongia* species containing the symbionts indicate to us that the isolation of O-PHDEs can provide preliminary insights into the taxonomy of source sponges. In this context, we believe that the taxonomy of the unidentified Great Barrier Reef Australian sponge, a source of **1** and the unusually functionalized monochlorinated metabolite **11**, should be re-examined.[31] In this study, we obtained **1** and **11** from a sponge collection firmly identified as *D. granulosa*. Thus, we suggest this unidentified sponge could be a *D. granulosa* specimen.

Our first foray into investigating new small molecule inhibitors of the protein-protein interaction between Mcl-1 and Bak was encouraging. The purified O-PHDE compounds ultimately showed modest responses in the Mcl-1/Bak FRET screen. The data obtained also validated our approach of a parallel investigation of crude extracts alongside selected semi-pure samples taken from our repository. Other ongoing projects within our consortium will ultimately provide additional active chemotypes in this screen. The initially unanticipated challenge of unraveling the structures of $C_{12}-C_{13}$ containing O-PHDE compounds motivated us to create a mini NMR data trends (Tables 1) that should be of use to others. Early on, we found that the dereplication-structure elucidation of O-PHDEs was challenging because of a paucity of hydrogen atoms (H/C ratio is often < 1) populating the diphenyl ether core. Employing Table 1 as a prime tool alongside an analysis of the ^{1}H NMR coupling patterns in the phenyl rings A and B provides a concise approach. However, there are limitations as our NMR trends strategy cannot be extended to PDHEs containing methoxy substituents, yet it is reliable for compounds where Br has been replaced by Cl.

Experimental Section

General Experimental Procedures

The NMR spectra were recorded in $CDCl_3$, CD_3OD, DMSO-d_6 and acetone-d_6 on a Varian 500 and 600 MHz for ^{1}H and 125 MHz for ^{13}C. High and low-resolution mass measurements were obtained respectively from a FAB and an ESI-TOF mass spectrometers. Semi preparative HPLC was performed by a 5μ C_{18} column by using a single wavelength (λ = 254 nm) for compound detection. X-ray crystallographic data were recorded on a Bruker APEX-II area detector diffractometer (Bruker AXS Inc.) with Mo-K(α) radiation. Specific details are given in the crystallographic data summary (See Supporting Information).

Animal Material

Dysidea granulosa (coll. no. 05406, 400 g wet weight) was collected in November, 2005 at Pocklington reef (10°43.586′ S: 155°51.911′ E) using SCUBA in Milne Bay, Papua New Guinea. Collection depth was from 60 to 30 ft. The taxonomic identification of *D. granulosa* was performed by Dr. Nicole J. de Voogd, curator of the Natural History Museum in Leiden, Netherlands. *Dysidea* (*Lamellodysidea*) *herbacea* (coll. no. 88098) was collected from Vim Levu, Fiji in 1989.[17] Semi-pure fractions of this previously extracted specimen were in our repository and used in this study. The recent re-classification of the family Dysideidae has led to the formation of the novel genus *Lamellodysidea*,[42] which is marked by its massive, lamellate to digitate morphology, and thin basal plate. This newly proposed taxonomic nomenclature is synonymous with many sponges previously designated as *D. herbacea*, and

is not yet widely recognized in the literature. Thus, we have chosen to indicate these sponges as *Dysidea* (*Lamellodysidea*) *herbacea*.

Extraction and Isolation

Samples were preserved in the field according to our standard laboratory procedures[43] and stored at 4 °C until extraction was performed. The sponges were extracted with hexanes (XFH), dichloromethane (XFD), and methanol (XFM) using an accelerated solvent extractor (ASE). The Mcl-1 active dichloromethane extract (05406XFD, 317 mg) was fractionated using several rounds of semi preparative reversed-phase HPLC. Isocratic conditions were employed with acetonitrile and water, each containing 0.1% formic acid. The fractionation (70:30 CH_3CN-H_2O) afforded 25 fractions. To accumulate more material, an additional dichloromethane extraction (05406XFMFD, 400 mg) from the methanol extract (XFM) was necessary. Fractions H6 (5.2 mg), H8 (80.4 mg), H10 (11.7 mg), H18 (2.9 mg) provided, respectively, **12, 1, 2** and **3**. Fraction H12 (20.0 mg) and H14 (8.2 mg) were each purified using 40:60 CH_3CN-H_2O and gave, respectively, **4** (3.8 mg), accompanied by **11** (5.0 mg) and **10** (3.8 mg). Compounds **5** (7.0 mg), **6** (7.0 mg), 7 (7.0 mg), **8** (199 mg), **9** (250 mg), and **13b** (3.8 mg) were purified (70:30 CH_3CN-H_2O) from a Mcl-1 active semi-pure fraction found in our repository (88098F), previously extracted from *D.* (*Lamellodysidea*) *herbacea*.

2-(2′,4′-Dibromophenoxy)-3,5-dibromophenol (1): white powder; ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H 7.79 (1H, d, *J* =2.4 Hz, H-3′), 7.35 (1H, d, *J* = 2.4 Hz, H-4), 7.30 (1H, dd, *J* = 9.0, 2.4 Hz, H-5′), 6.45 (1H, d, *J* = 9.0 Hz, H-6′), 7.22 (1H, d, *J* = 2.4 Hz, H-6), 5.56 (1H, brs, OH); ^{1}H NMR (DMSO-d_6, 600 MHz) δ_H 10.85 (1H, brs, OH), 7.89 (1H, d, *J* = 2.4 Hz, H-3′), 7.41 (1H, dd, *J* = 9.0, 2.4 Hz, H-5′), 7.40 (1H, d, *J* = 2.4 Hz, H-4), 6.46 (1H, d, *J* = 9.0 Hz, H-6′), 7.17 (1H, d, *J* = 2.4 Hz, H-6); ^{13}C NMR (DMSO-d_6, 125.7 MHz) δ_C 152.6 (C-1′), 152.1 (C-1), 138.4 (C-2), 135.1 (C-3′), 131.6 (C-5′), 125.1 (C-4), 119.5 (C-6), 118.7 (C-3), 118.0 (C-5), 115.9 (C-6′), 113.9 (C-4′), 111.6 (C-2′); ESIMS [M - H]$^-$ *m/z* 496.2 (18), 498.2 (73), 500.2 (100), 502.2 (65), 504.2 (17).

2-(2′,4′-Dibromophenoxy)-4,6-dibromophenol (2): white powder; ^{1}H NMR and ^{13}C NMR (see Table 2); ESIMS [M - H]$^-$ *m/z* 496.2 (18), 498.2 (73), 500.2 (100), 502.2 (65), 504.2 (17).

2-(2′,4′-Dibromophenoxy)-3,5,6-tribromophenol (3): white power; ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H 7.78 (1H, d, *J* =2.1 Hz, H-3′), 7.55 (1H, s, H-4), 7.29 (1H, dd, *J* = 9.0, 2.1 Hz, H-5′), 6.42 (1H, d, *J* = 9.0 Hz, H-6′); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ_C 152.4 (C-1′), 148.4 (C-1), 138.9 (C-2), 136.3 (C-3′), 131.6 (C-5′), 128.2 (C-4), 122.4 (C-5), 117.0 (C-3), 116.0 (C-4′), 116.0 (C-6′), 113.7 (C-6), 112.9 (C-2′); ESIMS [M - H]$^-$ *m/z* 574.1 (11), 576.1 (51), 578.1 (100), 580.1 (97), 582.1 (43), 584.1 (6); X-Ray analysis (see Supporting Information).

2-(2′,4′-Dibromophenoxy)-3,4,5-tribromophenol (4): white powder; ^{1}H NMR and ^{13}C NMR (see Table 2); ESIMS [M-H]$^-$ *m/z* 574.1 (11), 576.1 (51), 578.1 (100), 580.1 (97), 582.1 (43), 584.1 (6); X-ray analysis (see Supporting Information).

2-(3′,5′-Dibromo-2′-hydroxyphenoxy)-3,4,6-tribromophenol (5): white powder recrystallized from acetonitrile as colorless needles; ^{1}H NMR and ^{13}C NMR (see Table 3); ESIMS [M - H]$^-$ *m/z* 590.5 (11), 592.5 (51), 594.5 (100), 596.5 (96), 598.7 (45), 600.7 (10); X-ray analysis (see Supporting Information).

2-(3′,5′-Dibromo-2′-hydroxyphenoxy)-4,5,6-tribromophenol (6): white powder recrystallized from chloroform (+ 1 drop DMSO) as colorless needles; ^{1}H NMR and ^{13}C NMR (see Table 3); ESIMS [M - H]$^-$ *m/z* 590.5 (11), 592.5 (51), 594.5 (100), 596.5 (96), 598.7 (45), 600.7 (10); X-ray analysis (see Supporting Information).

2-(3′,5′-Dibromo-2′-hydroxyphenoxy)-3,4,5-tribromophenol (7): white powder re-crystallized from acetonitrile as colorless needles; ^{1}H NMR and ^{13}C NMR (see Table 3); ESIMS [M - H]$^{-}$ *m/z* 590.5 (11), 592.5 (51), 594.5 (100), 596.5 (96), 598.5 (45), 600.5 (10); X-ray analysis (see Supporting Information).

2-(3′,5′-Dibromo-2′-hydroxyphenoxy)-3,5,6-tribromophenol (8): white powder; ^{1}H NMR (CD_3OD-d_4, 600 MHz) δ_H 7.54 (1H, s, H-4), 7.34 (1H, d, *J* = 2.1 Hz, H-4′), 6.48 (1H, d, *J* = 2.1 Hz, H-6′); ^{13}C NMR (DMSO-d_6, 125.7 MHz) δ_C 150.5 (C-1), 146.0 (C-1′), 143.9 (C-2′), 138.8 (C-2), 128.3 (C-4′), 126.4 (C-4), 122.2 (C-5), 116.3 (C-3), 115.5 (C-6′), 114.5 (C-6), 111.1 (C-3′), 109.3 (C-5′); ESIMS [M - H]$^{-}$ *m/z* 590.5 (11), 592.5 (51), 594.5 (100), 596.5 (96), 598.5 (45), 600.5 (10).

2-(3′,5′-Dibromo-2′-hydroxyphenoxy)-3,4,5,6-tetrabromophenol (9): white powder; ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H 7.29 (1H, d, *J* = 2.4 Hz, H-4′), 6.41 (1H, d, *J* = 2.4 Hz, H-6′); ^{13}C NMR (DMSO-*d*6, 125.7 MHz) δ_C 149.0 (C-1), 145.7 (C-1′), 143.9 (C-2′), 139.4 (C-2), 128.3 (C-4′), 125.5 (C-3), 120.2 (C-4), 117.3 (C-5), 115.6 (C-6), 115.5 (C-6′), 111.1 (C-3′), 109.5 (C-5′); ESIMS [M - H]$^{-}$ *m/z* 667.8 (5), 669.8 (31), 671.8 (76),673.8 (100), 675.8 (73), 677.8 (29), 679.8 (5).

2-(2′,4′-Dibromophenoxy)-5,6-dibromo-3-chlorophenol (10): white powder; ^{1}H NMR and ^{13}C NMR (see Table 2); ESIMS [M - H]$^{-}$ *m/z* 530.7 (14), 532.7 (61), 534.7 (100), 536.7 (80), 538.7 (31), 540.7 (4); HRFABMS [M-H]$^{-}$ m/z 534.6613 (calcd for $C_{12}H_4O_2Cl^{79}Br_2{}^{81}Br_2$ 534.6592).

2-(2′,4′-Dibromophenoxy)-3,5-dibromo-4-chlorophenol (11): white powder; ^{1}H NMR and ^{13}C NMR (see Table 2); ESIMS [M-H]$^{-}$ *m/z* 530.7 (14), 532.7 (61), 534.7 (100), 536.7 (80), 538.7 (31), 540.7 (4); HRFABMS [M-H]$^{-}$ m/z 534.6613 (calcd for $C_{12}H_4O_2Cl^{79}Br_2{}^{81}Br_2$ 534.6592).

2-(2′,4′-Dibromophenoxy)-3,5-dibromo-4-methoxyphenol (12): green gum; ^{1}H NMR and ^{13}C NMR and ^{13}C NMR (see Table 2); ESIMS [M - H]$^{-}$ *m/z* 526.7 (17), 528.7 (68), 530.7 (100), 532.7 (66), 534.7 (17); HRFABMS [M - H]$^{-}$ m/z 530.7054 (calcd for $C_{12}H_7O_3Cl^{79}Br_2{}^{81}Br_2$ 530.7088).

2-(3′,5′-Dibromo-2′-hydroxyphenoxy)-3,5,6-tribromoanisol (13b): white powder re-crystallized from acetone as colorless prisms; ^{1}H NMR (acetone-d_6, 600 MHz) δ_H 7.93 (1H, s), 7.43 (1H, d, *J* = 2.1 Hz, H-4′), 6.78 (1H, d, *J* = 2.1 Hz, H-6′), 9.30 (1H, brs, OH), 3.83 (3H, s, OCH_3); ^{13}C NMR (acetone-d_6, 125.7 MHz) δ_C 155.6 (C-1), 146.9 (C-2), 145.9 (C-1′), 144.7 (C-2′), 133.0 (C-4), 130.0 (C-4′), 123.4 (C-5), 122.2 (C-6), 118.0 (C-3), 117.2 (C-6′), 111.8 (C-5′), 111.8 (C-3′), 62.2 (OCH_3); ^{1}H NMR and ^{13}C NMR in $CDCl_3$ (see Table 3); ESIMS [M - H]$^{-}$ *m/z* 604.6 (7), 606.6 (46), 608.6 (100), 610.6 (89), 612.6 (51), 614.6 (11); X-ray analysis (see Supporting Information).

FRET Assay

A fluorescence resonance energy transfer (FRET) assay system (LANCE) was used for identification of Mcl-1 inhibitors. Europium-labelled anti-GST (glutathione S-transferase) was used as the donor fluorophore and APC (allophycocyanin)-labelled streptavidin was used as the acceptor fluorophore. Biotinylated Bak (Anaspec) peptide bound to Mcl-1 (Novartis) was co-incubated with potential Mcl-1 inhibitors and the signals were read on the EnVision multi-label reader at 320 nm wavelength excitation, 665 nm primary emission, 615 nm secondary emission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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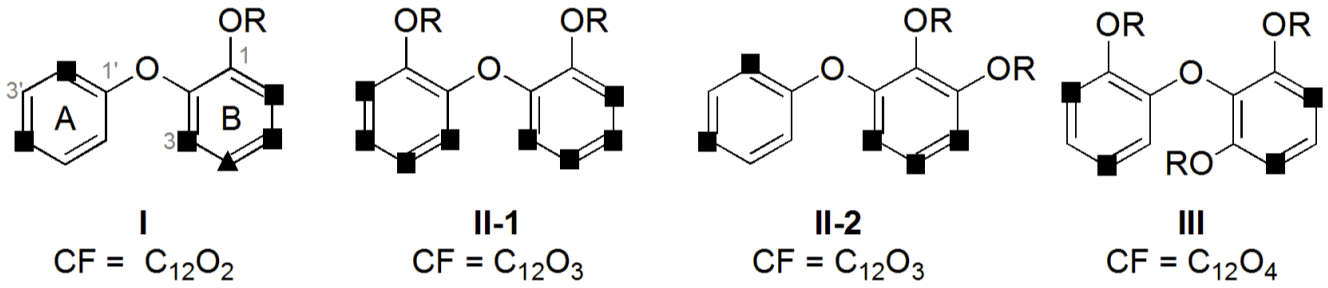


Figure 1.
Structure type of sponge-derived O-PHDEs based on core formulas (CF) and substitution pattern.
Possible halogenation sites: ■ Br, ▲ Br or Cl
Core formula (CF) based on diphenyl ether and attached oxygens
R = H or CH_3

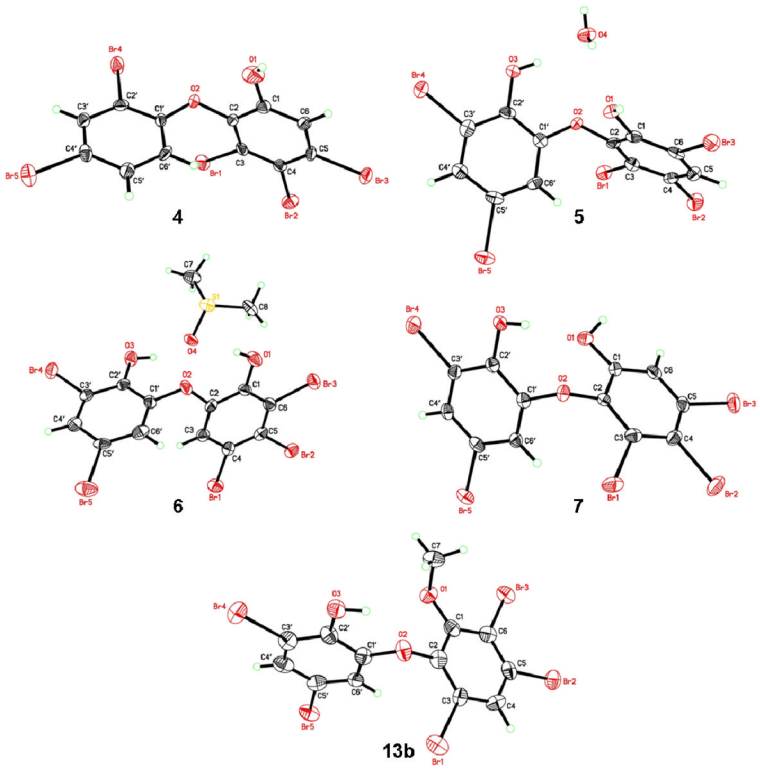
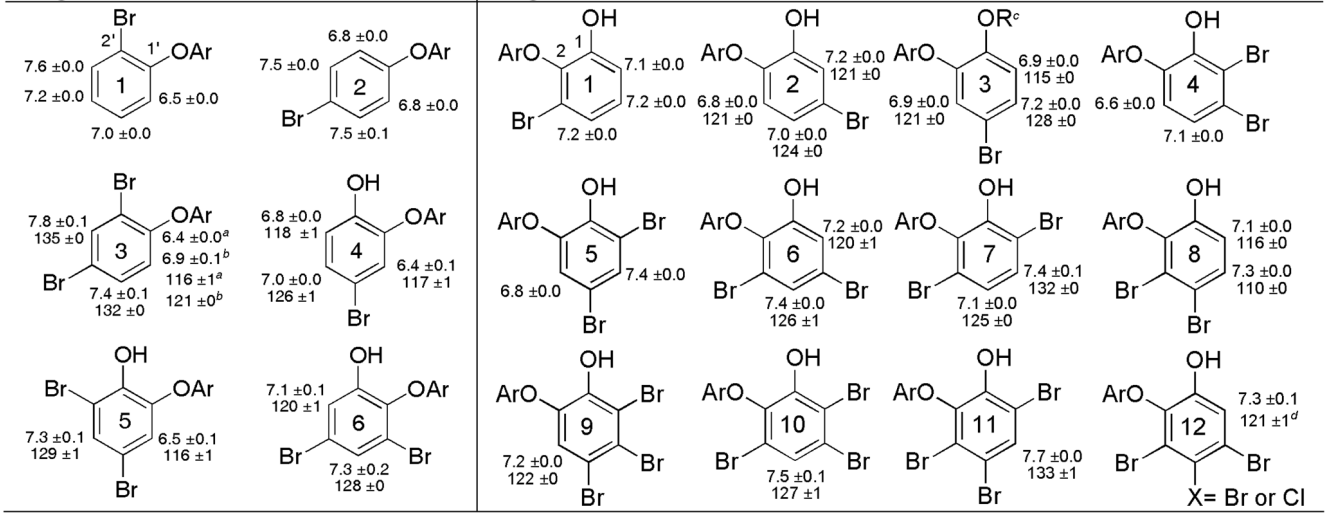


Figure 2.
X-ray crystal structures of O-PHDEs **4–7** and **13b**; hydroxyl groups in compounds **5** and **6** are involved in hydrogen bonding with water and dimethyl sulfoxide molecules respectively (see Supporting Information). Thermal displacement ellipsoids are depicted at 50% propability level.

Table 1

NMR (^{1}H and ^{13}C) Data Trends at Protonated Positions of the Rings A and B from Known Sponge-derived O-PHDEs

Ring A	Ring B
	

[a] Values for the 6′-position (^{1}H and ^{13}C) are for 3-position = Br.

[b] values for the 6′-position (^{1}H and ^{13}C) are for 3-position = H.

[c] R = CH_3

[d] ^{13}C value for only X = Br.

Table 2

Structures Types I and II-3, Ring Type A-3: ^{1}H and ^{13}CNMR Data of the O-PHDEs **2, 4**, and **10–12** Recorded in $CDCl_3$ (^{1}H, 600 MHz and ^{13}C, 125.7 MHz)

	2[a]		**4**		**10**		**11**[a]		**12**	
pos#	**δ_H (int., m., *J* [Hz])**	**δ_C**	**δ_H (int., m., *J* [Hz])**	**δ_C**	**δ_H (int., m., *J* [Hz])**	**δ_C**	**δ_H (int., m., *J* [Hz])**	**δ_C**	**δ_H (int., m., *J* [Hz])**	**δ_C**
1		144.6		149.2		147.4		148.6		146.7
2		143.5		139.7		139.4		139.9		139.2
3	7.80 (1H, d, 2.1)	119.9		121.3[b]		122.9		118.9		113.5
4		112.1		119.5	7.55	127.9		127.8		149.1
5	7.45 (1H, d, 2.1)	130.4		123.1		120.1		120.7		115.3
6		111.0	7.44 (1H, s)	120.9		116.0	7.44 (1H, s)	120.8	7.31 (1H, s)	119.9
1′		151.7		151.9		152.4		152.0		152.7
2′		115.9		112.9		112.9		112.9		112.8
3′	7.81 (1H, d, 2.4)	136.7	7.79 (1H, d, 2.4)	136.5	7.78 (1H, d, 2.1)	136.4	7.80 (1H, d, 2.4)	136.5	7.79 (1H, d, 2.4)	136.4
4′		118.4		116.5		116.2		116.5		116.2
5′	7.43 (1H, dd, 2.4, 8.7)	132.3	7.31 (1H, dd, 2.4, 9.0)	131.8	7.29 (1H, dd, 2.4, 8.7)	131.6	7.30 (1H, dd, 2.4, 9.0)	131.8	7.30 (1H, dd, 2.4, 9.0)	131.8
6′	6.89 (1H, d, 8.7)	121.8	6.41 (1H, d, 9.0)	116.0	6.43 (1H, d, 8.7)	116.0	6.42 (1H, d, 9.0)	116.0	6.43 (1H, 9.0)	116.1
OH	nd[c]		5.63 (1H, brs)		5.96 (1H, brs)		5.56 (1H, brs)		5.34 (1H, brs)	
OCH_3									3.86 (3H, s)	61.2

[a] The ^{13}C data in previous literature was either absent or not assigned.

[b] The ^{13}C data for position-3 was previously mis-reported.[36]

[c] nd = not detected.

Table 3

Structure Type II-1, Ring Type A-5: ^{1}H and ^{13}C NMR Data of the Sponge-derived O-PHDEs **5 – 7** and **13b**

	5[a]		**6**[b]		**7**[b]		**13b**[b]	
pos#	**δ_H (int., m., *J* [Hz])**	**δ_C**	**δ_H (int., m., *J* [Hz])**	**δ_C**	**δ_H (int., m., *J* [Hz])**	**δ_C**	**δ_H (int., m., *J* [Hz])**	**δ_C**
1		148.5		145.1		153.7		152.7
2		140.7		143.0		139.6		145.1
3		119.4	7.24 (1H, s)	122.8		121.3		117.2
4		113.7		115.3		119.3	7.76 (1H, s)	132.3
5	7.77 (1H, s)	132.9		123.5		123.4		123.5
6		111.6		128.3	7.45 (1H, s)	121.1		121.7
1′		145.9		144.1		149.1		145.1
2′		144.2		144.1		142.9		142.7
3′		111.3		111.5		111.2		110.8
4′	7.39 (1H, d, 2.2)	128.3	7.48 (1H, d, 1.8)	131.2	7.42 (1H, 1.8)	130.2	7.38 (1H, d, 1.8)	129.6
5′		109.2		112.3		112.4		112.1
6′	6.55 (1H, d, 2.2)	115.6	7.03 (1H, d, 1.8)	121.8	6.65 (1H, d, 1.8)	117.3	6.57 (1H, d, 1.8)	117.1
OH	nd[c]		5.96 (1H, brs)		6.04 (1H, brs)		5.96	
OH	nd[c]		6.07 (1H, brs)		6.43 (1H, brs)			
OCH_3							3.81	61.8

[a] NMR in $CDCl_3$+1 drop CD_3OD (^{1}H, 600 MHz) and DMSO-d_6 (^{13}C, 125.7 MHz)

[b] NMR in $CDCl_3$ (^{1}H, 600 MHz and ^{13}C, 125.7 MHz)

[c] nd = not detected

Table 4

Mcl-1 Inhibition Data for a Semi-pure Extracts of *D. granulosa*, a Repository Fraction of *D.* (*Lamellodysidea*) *herbacea*, and Pure Compounds

sample	IC_{50} (μg/mL)
05406XFD[a]	4.1±0.6
88098F[b]	2.1±0.2
1[b]	2.4±0.1
2[b]	8.9/>10
3	>10
4[b]	7.3/>10
5	>10
6	>10
7	>10
8[b]	>10
9[b]	>10
13b	>10

[a] Quadruplicate runs.

[b] Duplicate runs.

Table 5

The O-PHDEs Isolated in this Study from *Dysidea granulosa*, Also Observed Previously from Other Sponges

compound	*Dysidea* species	*Dysidea Herbacea*[a]	*Dysidea Chlorea*[a]	*Dysidea fragilis*	*Phyllospongia dendyi*	Unidentified sponge
1	✓17, 30, 44	✓32				✓31
2	✓17, 19, 26, 30, 44	✓17	✓32		✓27	
3		✓36				
4	✓17, 30, 36	✓32		✓35		
12						✓31

[a]Sponges recently reclassified into the novel genus *Lamellodysidea*[42]