Probing the Activity Differences of Simple and Complex Brominated Aryl Compounds against 15-Soybean, 15-Human, and 12-Human Lipoxygenase§

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Lipoxygenases (LO) have been implicated in asthma, immune disorders, and various cancers. As a consequence of these broad biological implications, there is great interest in understanding the effects of naturally occurring and environmental contaminants against its activity. On the basis of our earlier studies indicating that polybrominated diphenol ethers are potent inhibitors to mammalian 15-LO, we expanded our structure-activity study to include marine-derived brominated phenol ethers (including a newly discovered tribrominated diphenyl ether), dioxins, and bastadins, as well as the synthetic brominated fire retardants, brominated bisphenol A (BBPA), and polybrominated diphenyl ethers (PBDEs). We report herein the effects of 21 simple and complex organobromine compounds against human platelet 12-LO, human reticulocyte 15-LO, and soybean 15-LO-1.

Introduction

Previously, we determined that marine-derived brominated diphenyl ethers inhibited mammalian 15-lipoxgenase with good effectiveness ($IC_{50} \approx 1-2\ \mu M$);[1] however, it remained unclear if this class of compounds were selective against particular lipoxygenase (LO) isozymes. For the past several years, our labs have discovered over a dozen LO inhibitors from marine sponges with the goal of defining chemical scaffolds for targeted lipoxygenase therapeutics.[2,3] LOs are a class of nonheme iron-containing enzymes that contribute to the eicosanoid pathway[4] by the hydroperoxidation of arachidonic acid (AA). These enzyme products are precursors for the inflammatory mediators, leukotrienes and lipoxins, but are also involved in a variety of human diseases such as asthma,[5] psoriasis,[6] atherosclerosis,[7] and cancer[8,9] and thus are attractive pharmaceutical targets.

Over 1600 organobromine compounds have been isolated from natural sources, and these substances possess a broad range of structural intricacy and biological activity.[10] Some examples include the monocyclic areoplysinin-1, a relatively simple organobromine compound which is a potent antifouling agent. The bicyclic organobromine compound, pentabromopseulilin, was isolated from the marine bacterium *Alteromonas luteoviolaceus* and is the most active member in a group of more than 20 pyrrole antibiotics.[11] The more structurally intricate organobromine scaffold, bastadin, exhibits a plethora of activities, such as moderate antibacterial activity, cytotoxic activity against human tumor cell lines, antiinflammatory activity, and calcium channel activation.[12]

Polybrominated compounds are also synthesized commercially and used in large quantities as fire retardants,[13] such as polybrominated diphenyl ethers (PBDE) and brominated bisphenol A (BBPA). These compounds have broad implications concerning human health due to the increasing levels of PBDEs detected in human blood plasma,[14] adipose tissue,[15] and breast milk.[16,17] Their accumulation in human tissue raises concern because PBDEs have been found to induce murine phospholipase A_2 to release arachidonic acid,[18] potentially affecting the eicosanoid signaling pathway and lipoxygenase in particular. In light of our previous discovery of marine-derived polybrominated LO inhibitors, combined with the potential human health implications of organobromine compounds, we initiated the current investigation in order to examine this class of compounds in more detail and determine what structural features are required for inhibition against human platelet 12-lipoxygenase (12-hLO), human reticulocyte 15-lipoxygenase-1 (15-hLO), and soybean 15-lipoxygenase-1 (15-sLO).

Results and Discussion

The initial investigation of 21 organobromine compounds was to determine if their inhibitory activity was due to reduction of the active site ferric ion. Lipoxygenase inhibitors can be classified as either nonreductive or reductive inhibitors.[19] The nonreductive inhibitors, such as puupehenone and jaspic acid, bind to the protein presumably at the catalytic and/or allosteric sites.[3] The reductive inhibitors reduce the active ferric form of LO to the inactive, ferrous form and typically contain hydroquinone or catecholate moieties, as observed in jaspaquinol[3] and nordihydroguaiaretic acid (NDGA),[20] respectively. These reductive inhibitors are less desirable as pharmaceutical targets due to their susceptibility to undergo chemical modification in the cell.[8] The brominated diphenyl ethers, which are one target of this publication, could potentially be reductive LO inhibitors

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§ This paper is dedicated to C. T. Walsh in recognition of his 60th birthday.

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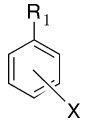
Table 1. LO Inhibition Activity (μM ± SD) of Simple Bromophenols (**1a**−**g**)



compd	R_1	X	cLog P	12-hLO: IC_{50} (μM)	15-hLO: IC_{50} (μM)	15-sLO: IC_{50} (μM)
1a	OH	2-Br	2.35	>100	>100	>100
1b	OH	3-Br	2.63	>100	>100	>100
1c	OH	4-Br	2.59	>100	55 ± 6	48 ± 4
1d	CH_2OH	4-Br	1.97	>100	>100	>100
1e	OCH_2CH_3	4-Br	3.59	>100	>100	>100
1f	OH	2,4-Br	3.22	>100	34 ± 4	>100
1g	OH	2,6-Br	3.36	>100	>100	>100

due to their phenolic moieties. We probed this possibility and determined that on a kinetic time scale (less than 2 min), reduction of the ferric form of 15-sLO was not observed by fluorescence spectroscopy for any of the 21 organobromine compounds studied. However, fluorescence spectroscopy did reveal partial reduction of the active site ferric iron with many inhibitors (**1a**−**7b**) after 20 min of incubation, suggesting that these phenolic compounds could reduce the active site iron, but on a time scale that is kinetically irrelevant (i.e. greater than 2 min). This was further supported by EPR experiments of 15-sLO and compound **2a**,[21] a micromolar inhibitor of both soybean and human lipoxygenases (IC_{50} (15-sLO) = 7 ± 3 μM, IC_{50} (12-hLO) = 0.7 ± 0.2 μM, and IC_{50} (15-hLO) = 1.8 ± 0.4 μM). Upon addition of 5 equiv of **2a** to 15-sLO, the ferric signal was unchanged. The sample was then allowed to incubate for 20 min at 22 °C and subsequently showed a 40% decrease in the ferric EPR signal (data not shown). The EPR result, combined with the fluorescence data, lead us to conclude that reductive inactivation of 15-sLO does occur slowly with some phenolic inhibitors, but it is not a relevant factor on a kinetic time scale for any of the compounds studied. Furthermore, there was no change in the EPR signal of 15-sLO upon addition of **1c**, **2a**, **3a**, and **5a**, demonstrating that their phenolic moieties do not chelate to the active site iron atom.

The bioactivity studies of the three lipoxygenase enzymes began with seven simple brominated phenols (**1a**−**g**), which in general showed no inhibition against 12-hLO, 15-hLO, and 15-sLO, except for two mild inhibitors (Table 1). The compound, 4-bromophenol (**1c**), weakly inhibited 15-sLO and 15-hLO, but did not inhibit 12-hLO, while 2,4-dibromophenol (**1f**) was a weak inhibitor of 15-hLO, but did not inhibit either 15-sLO or 12-hLO. These results suggest that *p*-bromination of simple phenols increases their potency. Nevertheless, these simple bromophenols are poor inhibitors against LO in general and more extended dicyclic structures are required for potent lipoxygenase activity (*vida infra*).

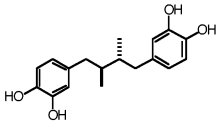
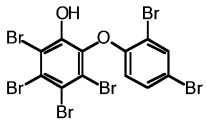
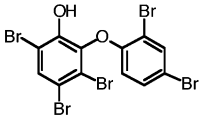
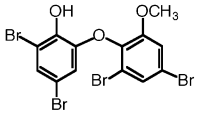
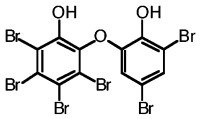
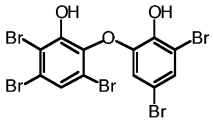
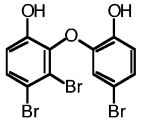
We next investigated the brominated diphenyl ethers (**2a**−**3c**),[21−23] which possess more complex structural features with regards to their degrees of hydroxylation, methoxylation, and bromination (Table 2). The compound, 3,4-dibromo-2-(5′-bromo-2′-hydroxyphenoxy)phenol (**3c**), is a novel marine natural product, with unique bromination relative to the phenol. These brominated diphenyl ethers (**2c**−**3c**), in general, were poor inhibitors of 15-sLO relative to the human enzymes except for the monohydroxylated diphenyl ethers, **2a** and **2b**, which affected moderate potency ($IC_{50} \approx 8$ μM for both). Small structural changes to **2a** and **2b,** such as the addition of a hydroxyl and/or the bromination position, abolish 15-sLO inhibition entirely, as seen for **2c**, **3a**-**3c**. Specifically, the positioning of the *p*-bromide for the simple phenols inhibition (*vida supra*) is not observed for the more complex brominated diphenyl ethers.

The diphenyl ethers (**2a**−**3c**) were markedly more potent against both human enzymes than 15-sLO, with increased potency against 12-hLO relative to 15-hLO. Interestingly, both the monohydroxy- and dihydroxydiphenyl ethers manifest the same potency trends, in which increasing bromination increased potency. This increase in potency relative to increased bromination could be due to multiple factors: an increase in hydrophobicity, a dependency on bromide position, or an increase in size. The hydrophobicity (i.e. cLog P) correlates well with IC_{50} values within each subclass (**2a**−**c** and **3a**−**c**); however, it does not cross-correlate between the two subclasses: the mono- and diphenolic inhibitors. For example, **2a** and **3a** have similar IC_{50} values but different cLog P values. The position of bromination could also be a factor; however, no clear trend is observed between the mono- and diphenolic compounds in this investigation. Increased size due to increased bromination is the strongest correlation with potency, but it is unclear how size affects binding since both enzymes have previously been shown to accept far larger inhibitors, such as puupehenones.[3] Since none of these factors completely explain the data, the inhibitor potency appears to be due to as yet undefined factors. Molecular docking studies are currently underway to model the sites in both human enzymes and assess possible binding scenarios for these inhibitors.

The next class of LO inhibitors are the monohydroxydioxins (**4a** and **4b**).[24,25] These compounds were very potent against 15-hLO, similar to the monohydroxylated diphenyl ethers; however, they were poor inhibitors toward 12-hLO (Table 3). The bromination position appears not to be a factor because **4b** has comparable bromination to **2b**, yet has a larger IC_{50} value. Considering that their cLog P values are similar to the other potent brominated inhibitors against 12-hLO (such as **2c** and **3c**), this lowered inhibitor potency toward 12-hLO is most likely due to the rigid three-ring structure of the dioxin scaffold.

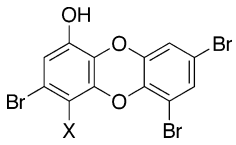
The synthetic fire retardants are the next class of LO inhibitors to be investigated and can be divided into two subgroups, the phenolic (BBPAs) and the nonphenolic brominated diphenyls (PBDEs). The diphenolic fire retardants (**5a** and **5b**) were good inhibitors of both 12- and 15-hLO and have similar potency to **2b** and **3b**, indicating that the isopropylidene bridge and the position of the phenol relative to the bridge is not critical for micromolar inhibition (Table 4). BBPA fire-retardants (**5a** and **5b**) were not inhibitors to 15-sLO, which correlates well with the previous results of poor inhibition by nonbrominated BPA against 15-sLO.[26] The brominated diphenyl ethers (**6a** and **6b**) are industrial mixtures of brominated isomers, typically used in toxicology studies.[18] These fire retardant mixtures were poor inhibitors with respect to both human enzymes (IC_{50} > 100 μM for both 12-hLO and 15-hLO), which suggests the free phenols are required for inhibition. It

Table 2. LO Inhibition Activity (μM ± SD) of Marine-Derived Polybrominated Phenol Ethers (**2a**−**c**)[1,21,22] and Diphenol Ethers (**3a**−**c**)[23]

Compound	Structure	cLog P	12-hLO IC_{50} (μM)	15-hLO IC_{50} (μM)	15-sLO IC_{50} (μM)
NDGA[a]		3.92	5.1 ± 1	0.11 ± 0.01	0.18 ± 0.02
2a		8.21	0.7 ± 0.2	1.8 ± 0.4	7 ± 3
2b		7.34	6 ± 2	5 ± 1	9 ± 3
2c		6.42	12 ± 2	10 ± 1	>100
3a		7.67	0.41 ± 0.03	0.79 ± 0.07	>100
3b		6.77	6.2 ± 0.8	2.2 ± 0.4	>100
3c		5.26	47 ± 8	11 ± 1	>100

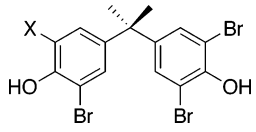
[a] Whitman, S., Gezginci, M., Timmermann, B. N., Holman, T. R. *J. Med. Chem.* **2002**, *45*, 2659−2661.

Table 3. LO Inhibition Activity (μM ± SD) of Marine-Derived Polybrominated Dioxins (**4a**,**b**)[24,25]



compd	X	cLog P	12-hLO: IC_{50} (μM)	15-hLO: IC_{50} (μM)	15-sLO: IC_{50} (μM)
4a	H	6.66	30 ± 11	0.8 ± 0.1	>100
4b	Br	7.42	50 ± 14	0.9 ± 0.1	>100

Table 4. LO Inhibition Activity (μM ± SD) of Synthetic Brominated Fire Retardants (BBPAs) (**5a**,**b**)

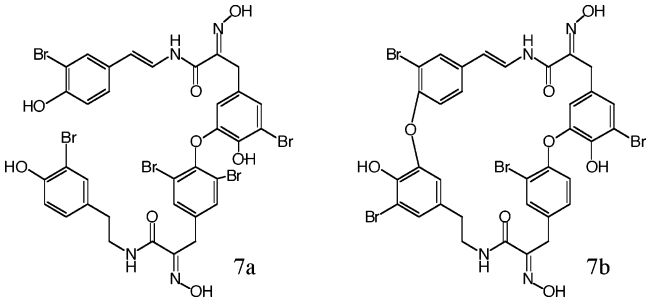


compd	X	cLog P	12-hLO: IC_{50} (μM)	15-hLO: IC_{50} (μM)	15-sLO: IC_{50} (μM)
5a	H	6.12	7 ± 3	5 ± 2	>100
5b	Br	6.81	10 ± 5	4 ± 1	>100

should be noted that hydroxylated PBDE's are the primary metabolites of PBDEs in rodents,[27,28] which could increase their potency toward lipoxygenase, as seen for the hydroxylated compounds of this study.

The marine-derived bastadins (**7a**, **7b**)[29] are the most structurally complex of the brominated natural products in this study. They were weak inhibitors toward 15-sLO, but potent inhibitors against the human enzymes (Table 5). The linear bastadin 2 (**7a**) was a submicromolar inhibitor of 12-hLO, while the macrocyclic bastadin 7 (**7b**) was ~6-fold less potent. Significantly, 15-hLO was inhibited by both **7a** and **7b** with micromolar potency and showed no difference between linear and macrocyclic bastadins. These data translate into a selectivity preference where the linear **7a** was ~5-fold more selective for 12-hLO than for 15-hLO, but the macrocyclc **7b** demonstrated no selectivity. Interestingly, **7a** and **7b** are some of the largest LO inhibitors (FW > 1000 g/mol) studied in our labs. This could account for their selectivity due to the fact that 12-hLO has a longer active site than 15-hLO[30] and would require more molecular contacts, consistent with the modeled substrate binding in the two enzymes.[30] Even though compounds **7a** and **7b** are large, complex inhibitors,

Table 5. LO Inhibition Activity (μM ± SD) of Marine-Derived Bastadins (**7a**,**b**)[29]



compd	cLog P	12-hLO: IC_{50} (μM)	15-hLO: IC_{50} (μM)	15-sLO: IC_{50} (μM)
7a	9.10	0.4 ± 0.1	2.0 ± 0.4	59 ± 45
7b	10.21	2.3 ± 0.6	4 ± 1	27 ± 12

their structural intricacy does not enhance their potency relative to either **2a** or **3a**.

The overall selectivity of the 21 organobromine compounds against 12-hLO and 15-hLO tends toward favoring 15-hLO inhibition over that of 12-hLO, a common feature in the marine-derived compounds we have isolated previously[2,3] (Table 6). The most noteworthy compounds in this regard are the dioxins (**4a**, **4b**), which inhibit 15-hLO more effectively than 12-hLO, 38-fold and 56-fold, respectively. This selectivity is similar to that observed for the rigid, tetracyclic puupehenones derivatives from our previous publications and is comparable to the redox inhibitor, NDGA (12-hLO/15-hLO = 46).[20] Finally, the selectivity of these compounds with respect to 15-hLO versus 15-sLO indicates that there is little similarity between their inhibition activity, thus supporting the conclusion that 15-sLO should not be used as a 15-hLO mimic.

In summary, these SAR inhibitor results allow us three general conclusions. First, these brominated phenols can reduce the active site iron; however, this property is not responsible for the inhibition potency because it is irrelevant on the kinetic time scale. Second, the brominated diphenyl ethers (**2a**, **3a**) are potent inhibitors to human LOs and increasing their rigidity, such as in the dioxins (**4a**, **4b**), can increase their potency and selectivity toward 15-hLO. Finally among the synthetic brominated fire retardants, the phenolic BBPAs (**5a**, **5b**) are human LO inhibitors, while the nonphenolic PBDEs (**6a**, **6b**) are not, indicating a requirement of hydroxylation for potency.

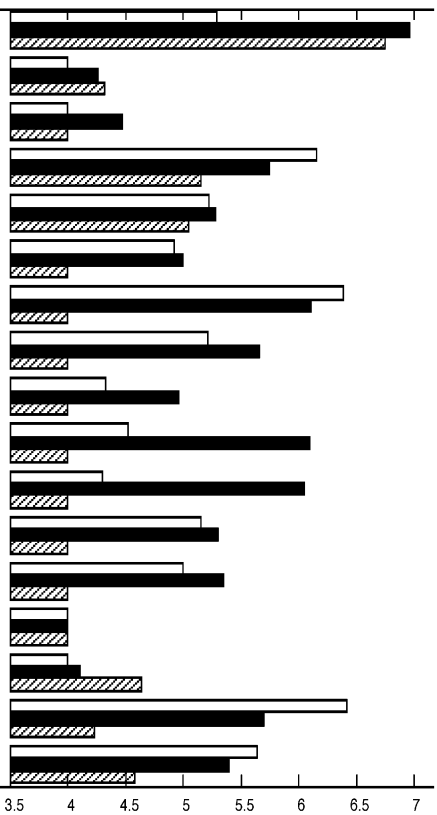
Experimental Section

Materials. Linoleic acid (LA) and AA were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were reagent grade or better and were used without further purification.

Expression and Purification of Lipoxygenases. 12-hLO and 15-hLO were expressed and purified as described previously.[3] Briefly, both His-tagged enzymes were purified in one

Table 6. Selectivity of Biologically Active Compounds (**1c**, **1f**, **2**a−**7b**)

Compound	12-hLO/15-hLO	15-sLO/15-hLO	Concentration[a] -log(IC_{50}) (M)
NDGA[b]	46	1.6	
1c	> 2	1	
1f	> 3	> 3	
2a	0.4	4	
2b	1	2	
2c	1	> 10	
3a	1	> 130	
3b	3	> 45	
3c	4	> 10	
4a	38	> 130	
4b	56	> 110	
5a	1	> 20	
5b	2	> 20	
6a	≈ 1	≈ 1	
6b	≈ 1	0.3	
7a	0.2	30	
7b	0.6	7	



[a] Concentration is expressed as −log(IC_{50}) in molar units. Open bars represent 12-hLO activity, solid bars represent 15-hLO activity, and hatched bars represent 15-sLO activity. [b] Whitman, S., Gezginci, M., Timmermann, B. N., Holman, T. R. *J. Med. Chem.* **2002**, *45*, 2659−2661.

step and stored in 20% glycerol at −80 °C, or significant inactivation occurs. 15-sLO was purified as previously published.[31]

Lipoxygenase Assay. The IC_{50} determination for all three enzymes was performed as previously described with the following modifications.[2] Under separate reaction conditions (12-hLO, 25 mM HEPES, pH 8; 15-hLO, 25 mM HEPES, pH 7.5; 15-sLO, 100 mM borate, pH 9.2), substrate was added (AA for 12-hLO and LA for 15-hLO/15-sLO). Reactions were initiated by adding the appropriate enzyme, and reaction rates were monitored at 234 nm at room temperature (22−23 °C) with constant stirring with a rotating magnetic stir-bar. Inhibitors were dissolved in MeOH to a concentration of 1 mg/mL. Control reactions were performed by adding an equivalent amount of solvent to the reaction and NDGA was regularly evaluated and found to be consistent with previous results.[20] The data were fit to a simple hyperbolic curve with the program KaleidaGraph (Synergy) and IC_{50} calculated.

Purification of Marine-Derived Compounds. Pure compounds **2a** and **2b** were obtained from a 100 g portion of *Psammocina* sp. extract (Papua New Guinea, pooled collections) that was partitioned with CH_2Cl_2, hexane, and MeOH. The CH_2Cl_2 partition (49 g) was fractionated using silica gel with serial elutions of 100% toluene, EtOAc, and finally MeOH. The bioactive fractions were combined and further fractionated using silica gel chromatography with hexane, CH_2Cl_2, and MeOH, then subjected to HPLC with a gradient of 70%-100% ACN in H_2O (with 0.1% formic acid in both solvents) yielding **2a** (1.5 mg) and **2b** (0.5 mg). Structures of **2a** and **2b** were determined based on comparison with published spectral data (^1H and ^{13}C NMR).[21,22] Compounds **2c, 3a**, **4a**, **4b**, **7a**, **7b** were obtained from the Marine Natural Products Repository at UCSC; analytical data matches that in the literature.[1,23−25,29] All marine natural products used in this study were HPLC purified and determined to be greater than 95% pure based on their HPLC traces and NMR and mass spectra.

3,4-Dibromo-2-(5′-bromo-2′-hydroxyphenoxy)phenol (3c). Pure compounds **3b** and **3c** were obtained from the sponge identified in the field as belonging to the genus *Phyllospongia* (Solomon Islands, coll. no. 94034) following the published extraction scheme.[32] A 0.5 g portion of the CH_2Cl_2 partition extract was fractionated using Sephadex LH-20 with 100% MeOH, then further separated using C_{18} HPLC with a gradient of 50% to 100% MeOH in H_2O (0.1% trifloroacetic acid in both solvents) to yield **3b** (8.8 mg) and **3c** (7.3 mg). The structure of **3b** was determined based on comparison with published spectral data (^1H and ^{13}C NMR).[23] The structure of **3c** was determined by its physical properties: red solid. UV (MeOH) λ_{max} (log ϵ) 207 (4.7), 287 (3.7); ^1H and ^{13}C NMR data see Supporting Information. TOFMS: *m*/*z* 435/437/439/441 [M − H] (calcd for $C_{12}H_7Br_3O_2$).

Purification of Synthetic Brominated Fire Retardants. BBPA (BA-59P, Lot #2008JM17B) and PBDE mixtures (DE−69, Lot #21220B04A and DE-71, Lot #2525DI01A) were a gift from Great Lakes Chemical Corporation (West Lafayette, IN). A 0.5 g sample of BA-59P was purified by HPLC with a gradient of 75% to 100% MeOH in H_2O (0.1% formic acid in both solvents) to yield **5a** (4.8 mg) and **5b** (8.7 mg). Structures of **5a** and **5b** were verified by ^1H and ^{13}C NMR, in comparison to predicted data derived from simulation using Advanced Chemical Dictionary, Inc. software. DE-69 and DE-71 were a mixture of pentabrominated (**6a**) and octabrominated (**6b**) PBDE isomers, respectively. Due to the low solubility of pure compounds under assay conditions, these mixtures were used without further purification and are designated as compounds **6a** and **6b**.

Spectroscopic Characterization of the Ferric and Ferrous Soybean Lipoxygenase. The fluorescence of the resting, ferrous form of 15-sLO has been reported previously.[33,34] Briefly, the ferrous signal was obtained by excitation at 280 nm and monitored at 328 nm on a Perkin-Elmer LS50B Spectrometer. Assays were 2 mL in volume and constantly stirred with a rotating magnetic bar. The ferrous signal was obtained by addition of 700 nM 15-sLO to an appropriate volume of 100 mM borate (pH 9.2) and was converted to the active ferric form by addition of 1 equiv of 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (HPOD), quenching the fluorescence by 30%. The change in fluorescence was then monitored upon addition of 5-fold excess inhibitor dissolved in MeOH. Increasing signal was attributed to the reduction of the ferric enzyme to the ferrous form via the inhibitor and verified by adding the known reductant, NDGA. A blank was performed by addition of MeOH and no increase in fluorescence was observed. The reduction of the active site was also examined by EPR in frozen solution at 4.3 K (data not shown). The procedure of Nelson[35] was followed with the following modifications. Addition of 1 equiv of HPOD (≈150 μM) to the resting enzyme produced a signal at $g \approx 6$. The solution was thawed and 5 equiv of inhibitor, dissolved in DMSO, was added. The solution was mixed and quickly refrozen in liquid nitrogen and the spectrum taken. If no reduction was seen, then the sample was thawed, allowed to incubate for 20 min at 22 °C, and refrozen and the spectrum remeasured. A decrease in the signal at $g \approx 6$ was ascribed to reduction of the ferrous iron.

cLog P Determination. The cLog P was calculated using the atom fragmentation method,[36] available from Daylight Chemical Information Systems, Inc. via the worldwide web.[37]

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Supporting Information Available: ^1H and ^{13}C NMR spectra for 3,4-dibromo-2-(5′-bromo-2′-hydroxyphenoxy)phenol (**3c**). This material is available free of charge via the Internet at http://pubs.acs.org.

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