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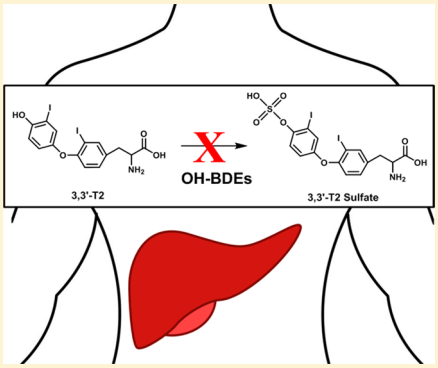
Inhibition of Thyroid Hormone Sulfotransferase Activity by Brominated Flame Retardants and Halogenated Phenolics

Craig M. Butt and Heather M. Stapleton*

Nicholas School of the Environment, Duke University, Durham, North Carolina 27708, United States

Supporting Information

ABSTRACT: Many halogenated organic contaminants (HOCs) are considered endocrine disruptors and affect the hypothalamic−pituitary−thyroid axis, often by interfering with circulating levels of thyroid hormones (THs). We investigated one potential mechanism for TH disruption, inhibition of sulfotransferase activity. One of the primary roles of TH sulfation is to support the regulation of biologically active T3 through the formation of inactive THs. We investigated TH sulfotransferase inhibition by 14 hydroxylated polybrominated diphenyl ethers (OH BDEs), BDE 47, triclosan, and fluorinated, chlorinated, brominated, and iodinated analogues of 2,4,6-trihalogenated phenol and bisphenol A (BPA). A new mass spectrometry-based method was also developed to measure the formation rates of 3,3′-T2 sulfate (3,3′-T2S). Using pooled human liver cytosol, we investigated the influence of these HOCs on the sulfation of 3,3′-T2, a major substrate for TH sulfation. For the formation of 3,3′-T2S, the Michaelis constant (K_m) was 1070 ± 120 nM and the V_{max} was 153 ± 6.6 pmol min^{-1} (mg of protein)$^{-1}$. All chemicals investigated inhibited sulfotransferase activity with the exception of BDE 47. The 2,4,6-trihalogenated phenols were the most potent inhibitors followed by the OH BDEs and then halogenated BPAs. The IC_{50} values for the OH BDEs were primarily in the low nanomolar range, which may be environmentally relevant. *In silico* molecular modeling techniques were also used to simulate the binding of OH BDE to SULT1A1. This study suggests that some HOCs, including antimicrobial chemicals and metabolites of flame retardants, may interfere with TH regulation through inhibition of sulfotransferase activity.



INTRODUCTION

In recent years, there has been considerable attention paid to halogenated organic compounds (HOCs) and their potential as endocrine disruptors.[1] The focus has mainly been on estrogenic and androgenic effects, but there is growing interest in thyroid hormone (TH) regulation.[2] The maintenance of TH homeostasis is complex but critical for normal physical and mental development.[3,4] The thyroid-disrupting effects of HOCs have been attributed to their structural similarity to endogenous THs (i.e., hydroxyl group, halogens on the aromatic rings), which may allow the chemicals to interact with TH enzymes, transporter proteins, and nuclear receptors.

Human studies have shown relationships between HOC exposure and alterations in serum thyroid-stimulating hormone (TSH), thyroxine (T4), and triiodothyronine (T3) concentrations. For example, a higher level of brominated flame retardant exposure has generally been associated with lower TSH levels and higher free and total T4 levels.[5−9] Rodent studies have also shown TH-disrupting effects; however, the effects are typically the opposite of those documented in human studies, with higher levels of exposure to brominated flame retardants generally associated with lower TH levels.[7,10,11]

Several potential mechanisms for TH disruption have been investigated using *in vitro* techniques. HOCs and their metabolites have been shown to competitively bind to TH transporter proteins, transthyretin (TTR),[12,13] and thyroxine-binding globulin (TBG)[14] as well as to the TH-α and -β receptors in mammals.[15,16] Further, some HOCs have been shown to inhibit deiodinase (DI) enzymes,[17,18] including work by our laboratory that investigated DI inhibition by hydroxylated polybrominated diphenyl ethers (OH BDEs), halogenated bisphenol A (BPA) compounds, triclosan, and trihalogenated phenols.[19]

In addition to deiodination, THs undergo phase II metabolism via conjugation of the hydroxyl group with glucuronic acid or sulfate. It has been suggested that the main consequence of TH sulfation is the formation of inactive THs. This is because sulfated THs have increased rates of deiodination compared to those of nonsulfated analogues.[20] For example, using an *in vitro* assay, T4 sulfation increased the level of inner-ring deiodination by ∼200-fold, forming 3,3′,5′-triiodothyronine (rT3) sulfate.[20]

Members of the cytosolic sulfotransferase (SULT) superfamily catalyze a diverse range of endogenous and xenobiotic chemicals.[21] The mechanism involves the transfer of a sulfonate group from the cofactor, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), to the acceptor group of the substrate molecule.

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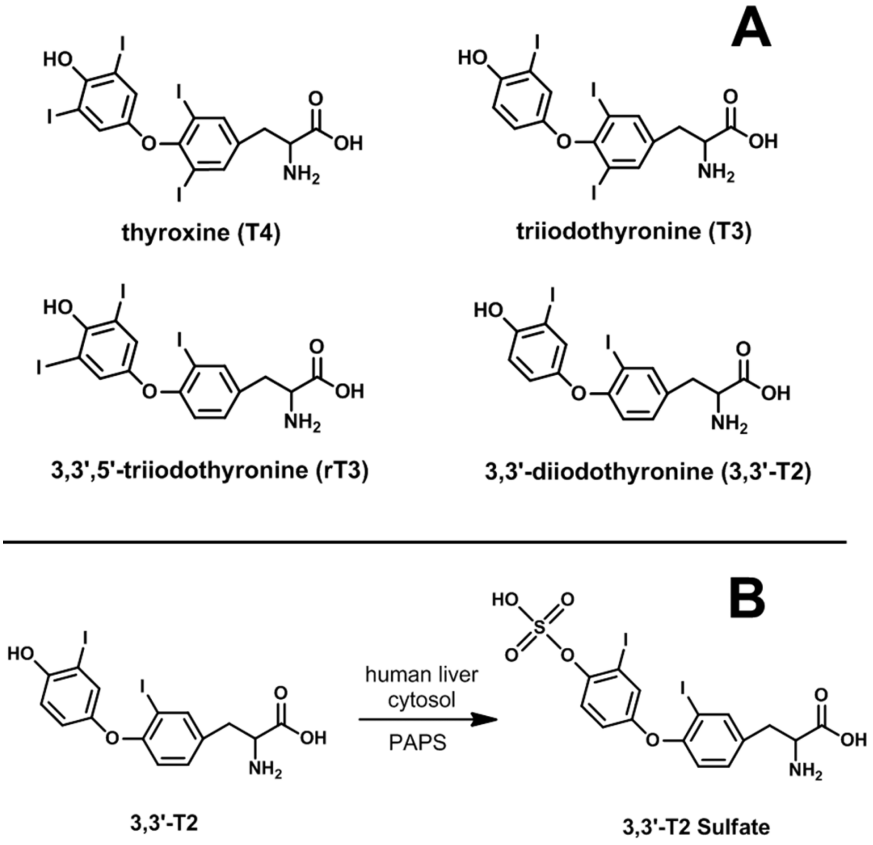

Figure 1. (A) Thyroid hormone structures. (B) Thyroid hormone sulfation reaction investigated herein.

Eight different isozymes (SULT1A1, SULT1A3, SULT1A5, SULT1B1, SULT1B2, SULT1C1, SULT1E1, and SULT2A1) have been shown to perform TH sulfation in humans and are broadly expressed in peripheral tissues.[22,23] In general, there is a substrate preference for 3,3′-diiodothyronine (3,3′-T2), the exception being SULT1E1, which shows equal preference for rT3 and 3,3′-T2.[23]

The SULT enzymes are inhibited by various environmental contaminants, pharmaceuticals and chemicals in the diet, which may ultimately result in impacts on human health.[24] For example, SULT inhibition may slow phase II metabolism, increasing the level of accumulation of toxic chemicals. Further, inhibition of the SULT1E1 isozyme may disrupt normal estrogen and androgen homeostasis.

Specific to the focus of this study, some studies have shown disruption of TH sulfotransferase activity by xenobiotics. For example, previous work showed that hydroxylated polychlorinated biphenyls (OH PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and several halogenated phenols inhibit *in vitro* 3,3′-T2 sulfotransferase activity.[25−27] In addition, two BDE congeners were shown to inhibit 3,3′-T2 sulfation in rat liver cytosol, but only after metabolism with CYP-enriched microsomes.[25] Further, Szabo et al.[28] showed increased levels of SULT1B1 mRNA expression in male rat pups that were maternally exposed to a PentaBDE commercial mixture. However, previous work has mostly been performed using rat liver cytosol, and there is a need to improve our understanding of TH sulfotransferase inhibition in human tissues.

In this study, we investigated TH sulfotransferase inhibition by HOCs using a validated *in vitro* assay with a novel detection approach, liquid chromatography tandem mass spectrometry (LC−MS/MS). The 3,3′-T2 reaction is shown in Figure 1. We used 3,3′-T2 as the substrate because it is a primary substrate for multiple SULT isozymes and is a good surrogate for other THs with respect to sulfotransferase inhibition.[29] Our model system was pooled human liver cytosol because the liver is a major site of TH metabolism. We tested several brominated flame retardants and their metabolites as potential TH sulfation inhibitors (chemical structures shown in panels a and b of Figure 2). Further, we explored structure−activity relationships by investigating TH sulfation inhibition by fluorinated, chlorinated, and iodinated analogues. In addition, we tested 14 OH BDEs. Finally, we used *in silico* molecular modeling to simulate the binding of OH BDE to SULT1A1, an important isozyme for TH sulfation.

EXPERIMENTAL PROCEDURES

Chemicals. 3,3′-T2 (>99%), triclosan (Irgasan, >97%), tetrabromobisphenol A (TBBPA, 97%), 4,4′-(hexafluoroisopropylidene)-diphenol (BPA AF, 97%), 2,4,6-tribromophenol (2,4,6-TBP, 99%), 2,4,6-trifluorophenol (2,4,6-TFP, 99%), 2,4,6-trichlorophenol (2,4,6-TCP, 98%), 2,4,6-triiodophenol (2,4,6-TIP, 97%), and adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (>60%) were purchased from Sigma-Aldrich (St. Louis, MO). 3,3′,5,5′-Tetrachlorobisphenol A (TCBPA, 98%) was purchased from TCI America (Portland, OR). 3,3′,5,5′-Tetraiodobisphenol A (TIBPA, 98%) was purchased from Spectra Group Ltd. (Millbury, OH). 2′-OH BDE 3 (2′-OH 4-BDE, 97.5%), 3′-OH BDE 7 (3′-OH 2,4-BDE, 99.3%), 3′-OH BDE 28 (3′-OH 2,4,4′-BDE, 99.6%), 3-OH BDE 47 (3-OH 2,2′,4,4′-BDE, 97%), 5-OH BDE 47 (5-OH 2,2′,4,4′-BDE, 98.0%), 6-OH BDE 47 (6-OH 2,2′,4,4′-BDE, 100%), 4′-OH BDE 49 (4′-OH 2,2′,4,5′-BDE, 97.8%), 4-OH BDE 90 (4-OH 2,2′,3,4′,5-BDE, 99.5%), 5-−OH BDE 99 (5′-OH 2,2′,4,4′,5-BDE, 99.0%), 6′-OH BDE 99 (6′-OH 2,2′,4,4′,5-BDE, 99.3%), 4′-OH BDE 101 (4′-OH 2,2′,4,5,5′-BDE, 99.2%), 3′-OH BDE 154 (3′-OH 2,2′,4,4′,5,6′-BDE, 99.0%), 6-

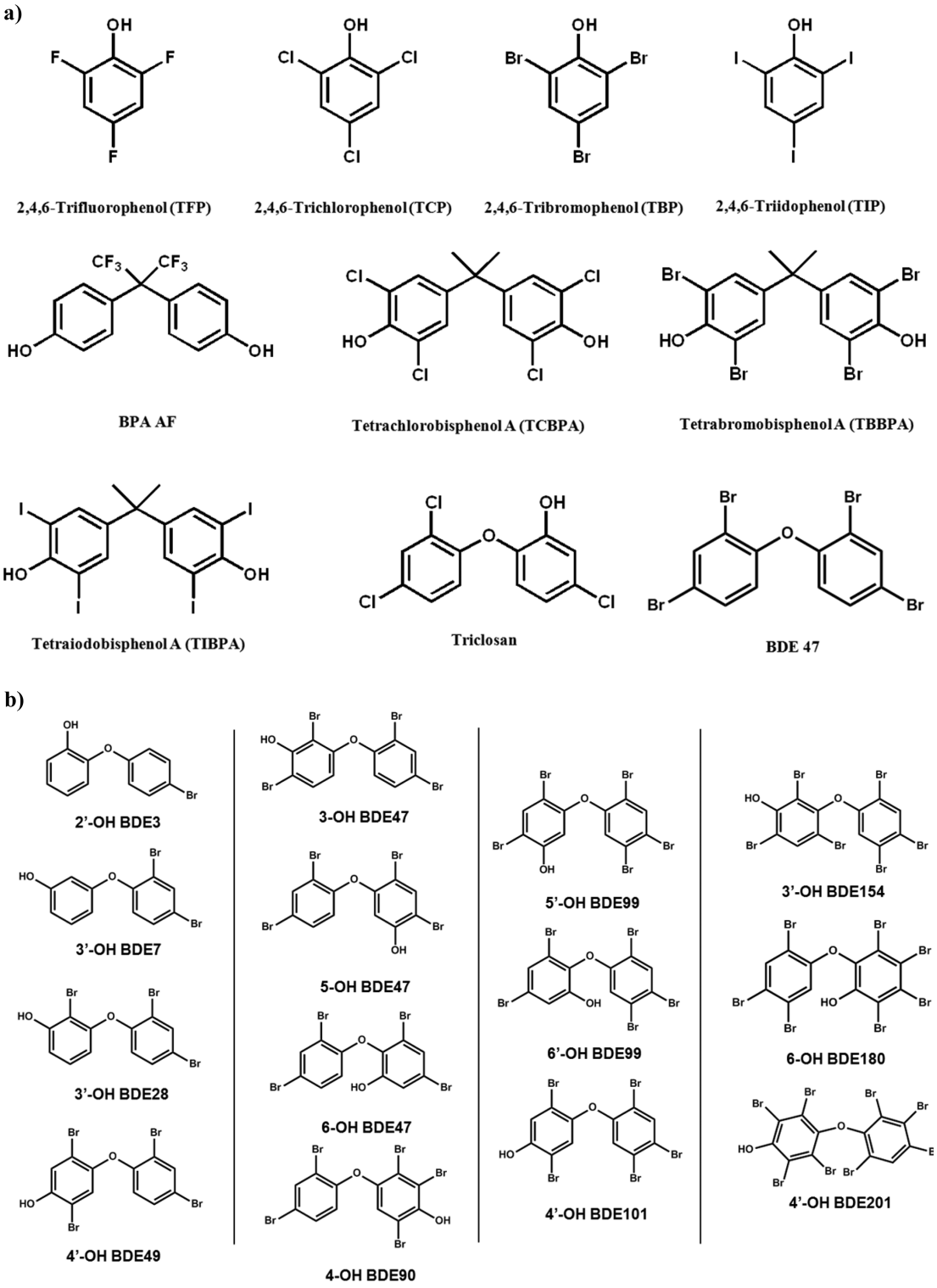

Figure 2. (a) Chemical structures of inhibitors investigated. (b) Chemical structures of inhibitors investigated.

OH BDE 180 (6-OH 2,2′,3,4,4′,5,5′-BDE, 99.6%), and 4′-OH BDE 201 (4′-OH 2,2′,3,3′,4,5′,6,6′-BDE, 99.3%) were purchased from AccuStandard (New Haven, CT). [$^{13}C_6$]-3,3′-T2 was purchased from Isotec (Miamisburg, OH). 3,3′-T2 sulfate (3,3′-T2S, 98%) was custom synthesized by the Duke University Small Molecule Synthesis Facility (Durham, NC).

Sulfotransferase Inhibition Assays. Sulfotransferase assays were modified from previously described methods.[27] The primary modification in our methods was the use of solid phase extraction (SPE) for sample cleanup and the use of LC−MS/MS for thyroid hormones and sulfated metabolite analysis. Human liver cytosol (pool of 28 donors) was purchased from a commercial source (Invitrogen, Carlsbad, CA) and kept frozen (−80 °C) until it was used. All donors were male and mostly Caucasian, and their ages ranged from 5 to 85 years.

For the investigation of sulfation inhibition, the cytosol was diluted to 0.25 mg of protein/mL in 0.1 M potassium phosphate buffer (pH 7.2) with 50 μM PAPS as the cofactor and 1 μM 3,3′-T2 as the substrate (total volume of 200 μL). Stock solutions of competitors were prepared in DMSO and were added such that the solvent volume

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was 0.5% of the incubation volume. "Active" control samples (no inhibitor) were prepared by spiking with clean DMSO. Buffer blanks were prepared by incubating without the cytosol. Assay reactions were started with the addition of the cytosol. Vials were incubated at 37 °C for 30 min in a shaking water bath, and reactions were stopped by the addition of 0.1 M HCl (800 μL). Samples were then spiked with [$^{13}C_6$]-3,3′-T2 (6.25 ng) as the internal standard. Extracts were cleaned using SampliQ OPT SPE cartridges (Agilent Technologies). The SPE procedures were modified from our previously published methods.[19] Briefly, after being conditioned (3 mL of methanol and 5 mL of water), samples were loaded, and the column was rinsed with 2 mL of water. The thyroid hormones and sulfate conjugate were eluted with 2 mL of methanol, and the extract was reduced to 1 mL under a gentle stream of nitrogen gas.

In addition, potential sulfation of the OH BDE competitors was assessed by monitoring the 4-OH BDE 90 concentrations before and after the 30 min incubation with initial 4-OH BDE 90 concentrations of 86 and 172 nM (n = 3), using the methods described above (i.e., co-incubation with 1 μM 3,3′-T2, 30 min incubation). 4-OH BDE 90 was chosen as the representative compound for the OH BDEs. The 4-OH BDE 90 concentrations were monitored by LC−MS/MS.

3,3′-T2 sulfation kinetics were examined by varying the substrate concentration (10−5500 nM), incubation time (0−90 min), and protein concentration (0−1 mg/mL). The interday variation was determined by performing the assay (1 μM 3,3′-T2 and 0.25 mg/mL protein and a 30 min incubation) on three separate days (n = 3 per day). Samples were extracted and cleaned as described above.

The mechanism of 3,3′-T2 sulfotransferase inhibition (i.e., competitive or noncompetitive inhibition) was investigated by measuring the Michaelis−Menten parameters (3,3′-T2 concentrations of 0, 100, 500, 1000, 2000, and 5000 nM) with varying concentrations of 4-OH BDE 90 (0, 5, 10, and 50 nM). The range of 4-OH BDE 90 concentrations bracketed the calculated IC_{50}.

Instrumental Analysis. Instrumental analysis was performed by liquid chromatography with electrospray ionization tandem mass spectrometry (LC−MS/MS) using conditions modified from our previously published methods.[19] Monitored analytes included 3,3′-T2, 3,3′-T2S, and 3-monoiodothyronine (3′-T1). Specifically, the liquid chromatography gradient program was altered slightly to account for the relatively greater polarity of the 3,3′-T2S. Further, the 3,3′-T2S was analyzed in electrospray ionization negative mode, using the m/z 604 > 524 transition for quantification and m/z 604 > 304 transition for confirmation. MS/MS parameters for 3,3′-T2, 3,3′-T2S, and 3-T1 were optimized using authentic standards. All analyte responses were normalized to the response of [$^{13}C_6$]-3,3′-T2.

***In Silico* Sulfotransferase Docking Simulation.** Molecular docking of 3,3′-T2 and OH BDE compounds to the SULT1A1 binding pocket was simulated using the CDOCKER module in Discovery Studio (Discovery Studio Modeling Environment, version 3.1, Accelrys Software Inc., San Diego, CA). CDOCKER is a CHARMM (Chemistry at Harvard Macromolecular Mechanics) force field-based simulation.[30−33] The SULT1A1 structure, originally determined by crystallography,[34] was obtained from the Protein Data Bank (entry 2D06, Human SULT1A1 complexed with PAP and estradiol). Prior to simulation, the protein was cleaned (i.e., errors in the protein structure were fixed) and validated and the estradiol was removed. Docking was performed using the CDOCKER module, and the interaction energy between the potential ligands and SULT1A1 was calculated.

QA/QC and Data Analysis. Recoveries of 3,3′-T2 and 3,3′-T2S were 84.9% (standard error of 1.2%) and 89.4% (3.0%), respectively, for a 200 nM spike into heat-inactivated cytosol (n = 3). Substrate deiodination was not observed in the buffer blanks, nor was formation of 3,3′-T2S; thus, blank correction was not necessary.

Sulfotransferase inhibition was calculated by comparing the relative response of 3,3′-T2S in the HOC dose treatments to that of the control (clean DMSO only). IC_{50} values were obtained using the "one-site competition" model in SigmaPlot (version 9.01, Systat Software Inc., Chicago, IL).

For the kinetic experiments, the apparent Michaelis constant (K_m) and maximal reaction rate (V_{max}) were obtained by fitting the data to the Michaelis−Menten model in JMP version 10.0 (SAS, Cary, NC). For the investigation of the inhibition mechanism, the Michaelis−Menten parameters were compared in JMP by analyzing the ratio of the parameters using analysis of means.[35] Linear regressions between modeled interaction energies and OH BDE properties were investigated in JMP. The pK_a for OH BDEs was estimated using SPARC (http://ibmlc2.chem.uga.edu/sparc). A multivariate linear regression model was used to assess adjusted associations between predictors (pK_a, OH substitution pattern, number of bromine atoms, and number of bromine atoms adjacent to OH group) and the IC_{50} value (SAS version 9.3). For the OH substitution pattern, congeners were divided into either para or non-para OH BDE. Although the pK_a and number of bromine atoms were highly correlated, the variance inflation factor showed that the parameter estimates were not affected.

RESULTS

3,3′-T2 Sulfation Kinetics. The kinetics of 3,3′-T2 sulfation were investigated in pooled human liver cytosol. The 3,3′-T2 sulfation showed typical Michaelis−Menten enzyme kinetics, and the model fit was excellent (r^2 = 0.98) (Figure 3). The apparent K_m for the 3,3′-T2 sulfation reaction

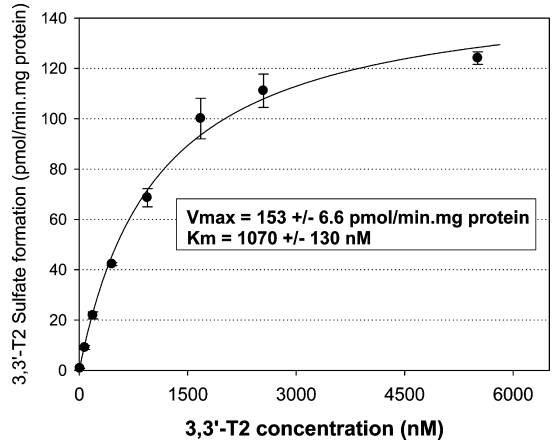

Figure 3. Rate of formation (picomoles per minute per milligram of protein) of 3,3′-T2 sulfate resulting from incubation of 3,3′-T2 in 0.25 mg/mL pooled human liver cytosol for 30 min. The Michaelis constant (K_m) and the maximal reaction rate (V_{max}) were obtained from nonlinear regression analysis. Each data point represented the mean (n = 3), and error bars represent one standard error.

was 1070 ± 130 nM, and the V_{max} was 153 ± 6.6 pmol min^{-1} (mg of protein)$^{-1}$. At physiologically relevant substrate concentrations (i.e., significantly below saturation), the most appropriate parameter for the comparison of metabolic rates is the intrinsic clearance rate (CL_{int}), calculated as the ratio of V_{max} to K_m.[36] In this study, the CL_{int} was 145 μL min^{-1} (mg of protein)$^{-1}$ for 3,3′-T2S formation.

The interday variation was assessed by performing the assay on three separate days (n = 3 per day) using the optimized conditions (1 μM 3,3′-T2 and 0.25 mg/mL protein for a 30 min incubation). The results showed no statistical difference among the three days (ANOVA; p = 0.26).

3-T1 formation was not observed, indicating that deiodination of the 3,3′-T2 did not occur in the sulfation assays. The mean mass balance (n = 3) was 119% (250 nM 3,3′-T2), 107% (500 nM 3,3′-T2), and 93% (1 μM 3,3′-T2).

The 3,3′-T2S formation rate was linear from 0 to 90 min when the 3,3′-T2 (1 μM) and cytosol concentrations (0.25 mg

of protein/mL) were held constant (Supporting Information, Figure S1). Also, when the 3,3′-T2 concentration (1 μM) and incubation time (30 min) were held constant, the 3,3′-T2S formation rate was linear from 0 to 0.50 mg of protein/mL but decreased slightly at 1.0 mg/mL (Supporting Information, Figure S2).

Sulfotransferase Inhibition by Halogenated Phenolic Compounds. The inhibition of thyroid hormone sulfation was investigated by monitoring the formation of 3,3′-T2S from 3,3′-T2 in the presence of varying doses of individual HOCs. A representative chromatogram, using 3′-OH BDE 154 as the inhibitor, is shown in Figure 4. Calculated IC_{50} values are listed in Tables 1 and 2. All compounds tested inhibited 3,3′-T2 sulfation in a dose−response manner with complete suppression of 3,3′-T2 sulfotransferase activity at the largest doses tested. The only exception was BDE 47, which did not inhibit 3,3′-T2 sulfation over the range of concentrations tested (10−1000 nM).

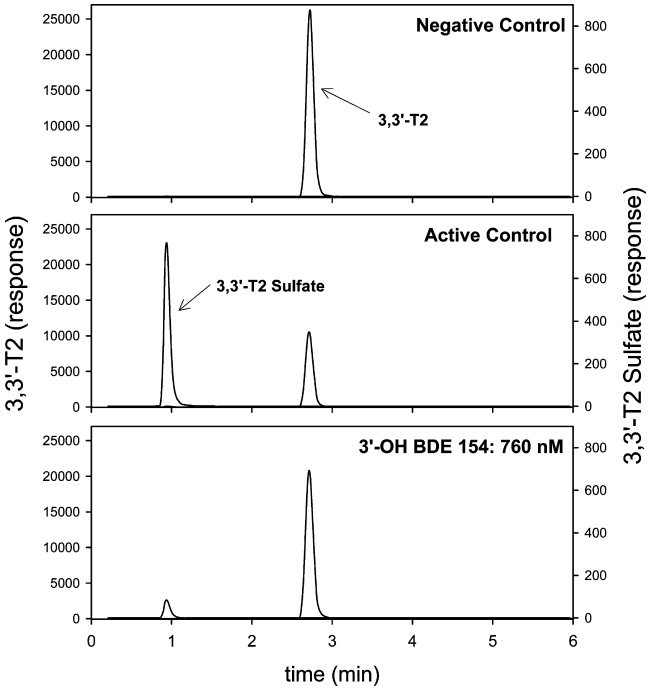

Figure 4. LC−MS/MS chromatograms of negative control (3,3′-T2 only, no cytosol), active control (clean DMSO only), and 760 nM 3′-OH BDE 154. Incubation conditions: 1 μM 3,3′-T2 for 30 min at 37 °C. Peaks were normalized to the [^{13}C]-3,3′-T2 response.

Table 2. IC_{50} Values (nanomolar) for the Inhibition of 3,3′-T2S Formation by Triclosan, Halogenated BPAs, and 2,4,6-Trihalogenated Phenols from the Incubation of 1 μM 3,3′-T2 with Human Liver Cytosol

compound	IC_{50} (nM) (95% confidence interval)
triclosan	1410
BPA-AF	8590 (5900−12,500)
TCBPA	340 (240−490)
TBBPA	460 (260−800)
TIBPA	13220 (8200−21,300)
2,4,6-TFP	4.6 (2.7−7.8)
2,4,6-TCP	4.3 (3.0−6.1)
2,4,6-TBP	8.3 (5.7−12)
2,4,6-TIP	140 (100−190)

The most potent inhibitors were the 2,4,6-trihalogenated phenols. Among the compounds, 2,4,6-TFP (IC_{50} = 4.6 nM), 2,4,6-TCP (IC_{50} = 4.3 nM), and 2,4,6-TBP (IC_{50} = 8.3 nM) all exhibited approximately equal potencies, with 2,4,6-TIP showing less potency (IC_{50} = 140 nM) (Figure 5).

The OH BDEs were the second most potent group of compounds with IC_{50} values ranging from tens to hundreds of nanomolar, with the exception of 6-OH BDE 180 (IC_{50} = 13500 nM). However, the IC_{50} value for 6-OH BDE 180 may be inaccurate because the dose−response curve did not pass through 50% inhibition. Thus, this congener was removed from the data set for the evaluation of structure−activity relationships. Structure−activity trends could be discerned within smaller sets of OH BDEs. For example, within an analogous group of mono- to tetrabrominated OH BDEs, the potency increased with an increasing number of bromines: 2′-OH BDE 3 (IC_{50} = 500 nM) < 3′-OH BDE 7 (IC_{50} = 410 nM) < 3′-OH BDE 28 (IC_{50} = 190 nM) < 3-OH BDE 47 (IC_{50} = 60 nM) (Figure 6). Further, within the group of OH BDE 47

Table 1. IC_{50} Values (nanomolar) and Interaction Energies (kilojoules per mole) for the Inhibition of 3,3′-T2S Formation by OH BDEs from the Incubation of 1 μM 3,3′-T2 with Human Liver Cytosol

compound	structure	IC_{50} (nM) (95% confidence interval)	interaction energy (kJ/mol)	human serum (pmol/g of lipid)
2′-OH BDE 3	2′-OH 4-monoBDE	500 (320−800)	−30.3	
3′-OH BDE 7	3′-OH 2,4-diBDE	410 (220−780)	−36.7	
3′-OH BDE 28	3′-OH 2,4,4′-triBDE	190 (140−250)	−40.3	
3-OH BDE 47	3-OH 2,2′,4,4′-tetraBDE	60 (40−90)	−37.4	0.2,[a] <LOQ to 0.32[b]
5-OH BDE 47	5-OH 2,2′,4,4′-tetraBDE	400 (240−640)	−35.6	3.1[a]
6-OH BDE 47	6-OH 2,2′,4,4′-tetraBDE	130 (70−230)	−34.0	0.6,[a] 1.4−2.4,[b] 0.3[c]
4′-OH BDE 49	4′-OH 2,2′,4,5′-tetraBDE	650 (170−2350)	−43.3	0.6,[a] 0.28−0.96,[b] 0.2[c]
4-OH BDE 90	4-OH 2,2′,3,4′,5-pentaBDE	24 (16−38)	−43.6	<LOQ to 0.70[b]
5′-OH BDE 99	5′-OH 2,2′,4,4′,5-pentaBDE	520 (180−1500)	−34.6	3.4[a]
6′-OH BDE 99	6′-OH 2,2′,4,4′,5-pentaBDE	310 (150−610)	−36.4	0.5[a]
4′-OH BDE 101	4′-OH 2,2′,4,5, 5′-pentaBDE	640 (280−1450)	−45.1	
3′-OH BDE 154	3-OH 2,2′,4,4′,5,6′-hexaBDE	80 (50−140)	−38.2	
6-OH BDE 180	6-OH 2,2′,3,4,4′,5,5′-heptaBDE	13500	−40.9	
4′-OH BDE 201	4′-OH 2,2′,3,3′,4,5′,6,6′-octaBDE	510 (220−1130)	−46.7	

[a]Mean (data from ref 46). [b]Range (data from ref 49). Reference group only. [c]Geometric mean (data from ref 9).

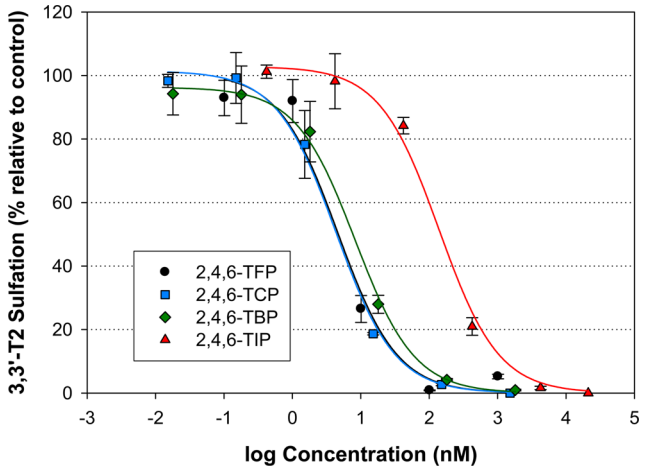

Figure 5. Inhibition of 3,3′-T2S formation resulting from the incubation of human liver cytosol with 1 μM 3,3′-T2 and various trihalogenated compounds. Data points represent the mean (n = 3), and error bars represent one standard error.

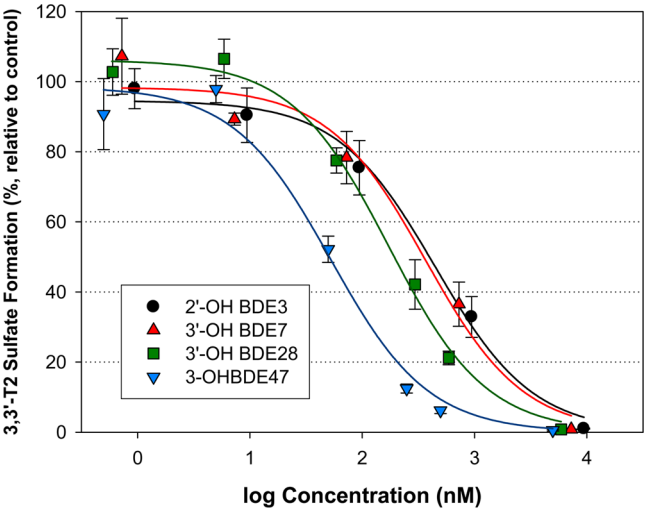

Figure 6. Inhibition of 3,3′-T2S formation resulting from the incubation of human liver cytosol with 1 μM 3,3′-T2 and mono-, di-, tri- and tetra-OH-substituted BDEs. Data points represent the mean (n = 3), and error bars represent one standard error.

compounds, potency varied by OH substitution: 3-OH BDE 47 (IC_{50} = 60 nM) > 6-OH BDE 47 (IC_{50} = 130 nM) > 5-OH BDE 47 (IC_{50} = 400 nM). As stated earlier, BDE-47 did not exhibit sulfotransferase inhibition. When considering the entire OH BDE data set, no trends were obvious (Supporting Information, Figure S3). Notably, the para OH BDEs were outliers in the IC_{50} versus the number of bromine atoms and the IC_{50} versus pK_a relationships. To adjust for the OH substitution pattern, a multivariate linear regression model was developed using the pK_a, the OH substitution pattern, and the number of bromine atoms. Starting with pK_a as the predictor, we found inclusion of OH substitution resulted in a statistically significant model (r^2 = 0.46; p = 0.04). The model was further improved by including the number of bromine atoms as a predictor. Although the pK_a and number of bromine atoms are highly correlated, multicollinearity analysis, using the variance inflation factor, showed that the parameters were not affected. Inclusion of the number of bromine atoms adjacent to the OH group did not improve the model, and thus, this parameter was not incorporated. The overall model was as follows: IC_{50} (nanomolar) = 230 × pK_a + 97.8 × number of bromine atoms + 320(para OH) − 1770 (r^2 = 0.64; p = 0.02). The experimentally measured IC_{50} values versus the model-predicted IC_{50} values are shown in Figure 7.

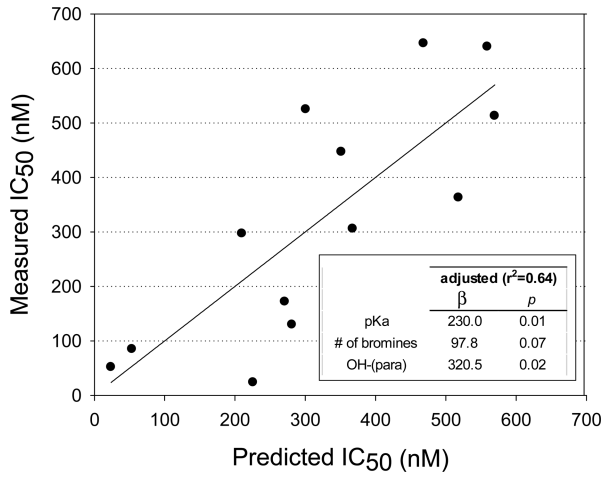

Figure 7. Experimentally measured vs model-predicted IC_{50} values using the multivariate linear regression model: IC_{50} (nanomolar) = 230 × pK_a + 97.8 × number of bromine atoms + 320(para OH) − 1770 (r^2 = 0.64; p = 0.02). The regression line shows the 1:1 relationship between measured and predicted values.

Similar to the 2,4,6-trihalogenated phenols, the halogenated bisphenol A compounds did not show a clear trend with halogen size. The relative potency decreased in the following order: TCBPA (IC_{50} = 340 nM) ≈ TBBPA (IC_{50} = 406 nM) > BPA-AF (IC_{50} = 8590 nM) > TIBPA (IC_{50} = 13220 nM) (Figure 8).

The type of inhibition (competitive vs noncompetitive) was investigated by calculating the Michaelis−Menten parameters using varying concentrations of 4-OH BDE 90 (Figure 9). 4-OH BDE 90 was tested because it was the most potent OH BDE compound in the sulfotransferase inhibition assays. For comparison between 4-OH BDE 90 concentrations, the 3,3′-

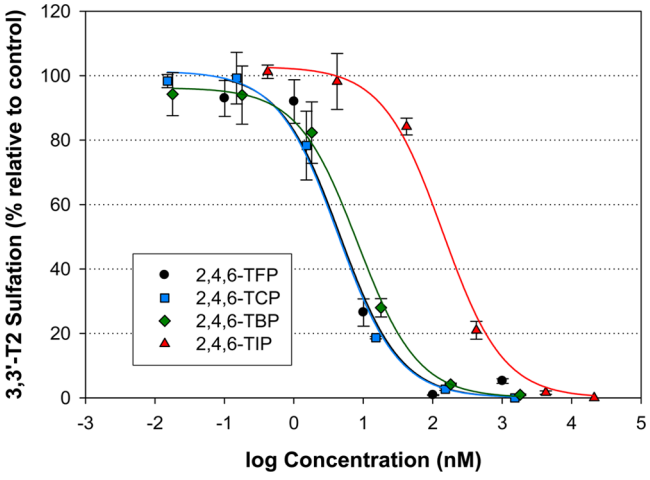

Figure 8. Inhibition of 3,3′-T2S formation resulting from the incubation of human liver cytosol with 1 μM 3,3′-T2 and various halogenated BPA compounds. Data points represent the mean (n = 3), and error bars represent one standard error.

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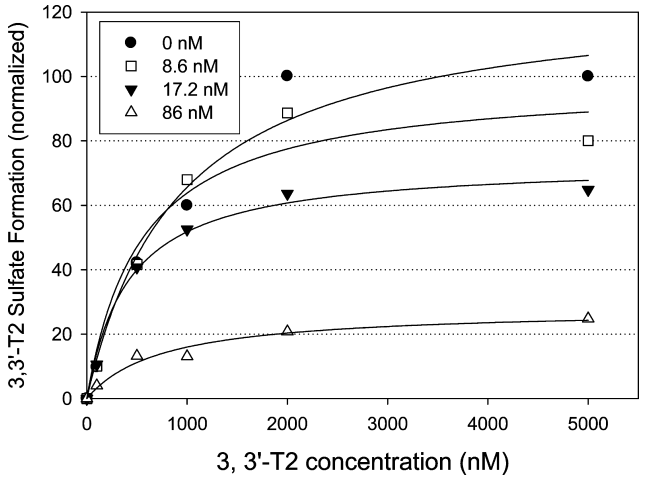

Figure 9. 3,3′-T2 sulfate formation rate (data points normalized to the formation rate at 5000 nM S and 0 nM 4-OH BDE 90) resulting from incubation of 3,3′-T2 with varying concentrations of 4-OH BDE 90 (0, 8.6, 17.2, and 86 nM) in 0.25 mg/mL pooled human liver cytosol for 30 min. Best fit lines were obtained using nonlinear regression analysis. Each data point represents the mean (n = 2), and error bars were not included for the sake of clarity.

T2S formation rates were normalized to the rate obtained when the 3,3′-T2 concentration was 5000 nM. The V_{max} rate was statistically lower with increasing 4-OH BDE 90 concentrations, but the apparent K_m did not vary with 4-OH BDE 90 concentration. These results suggest that the inhibition mechanism for 4-OH BDE 90 was noncompetitive, but it is not known if this is valid for the remaining OH BDEs.

We also investigated the potential sulfation of OH BDEs to determine if inhibition was reflective of substrate competition. To test this, we monitored the concentration of 4-OH BDE 90 at the beginning and end of the incubation period. If the OH BDE was being sulfated, the concentration would be significantly decreased at the end of the incubation period. However, the 4-OH BDE 90 levels remained steady during the 30 min incubation, indicating the 4-OH BDE 90 was not sulfated during our competition experiments. The initial and final 4-OH BDE 90 concentrations were 87 nM (standard error of 6.3 nM) and 91 nM (standard error of 9.1 nM), respectively, in one experiment using a dosing level of 86 nM and 140 nM (standard error of 10 nM) and 145 nM (standard error of 6 nM), respectively, with a dosing level of 172 nM.

***In Silico* Sulfotransferase Docking Simulation.** We investigated potential interactions between the OH BDEs and SULT1A1 using *in silico* modeling. Docking the 3,3′-T2 with SULT1A1 showed that the molecule was positioned with the OH group toward the active site, adjacent to the PAP cofactor, forming hydrogen bonds with Lys[106] and His[108] (Figure 10). All OH BDEs adopted a "flexed" structure within the binding pocket. All para-substituted OH BDEs exhibited similar positioning and hydrogen bonding with both Lys[106] and His[108]. With the exception of the mono-, di-, and tribrominated OH BDEs, ortho- and meta-substituted OH BDEs did not form hydrogen bonds with Lys[106] and His[108]. Further, some of the ortho- and meta-substituted OH BDEs demonstrated non-optimal docking by orienting with the OH group positioned away from the active site (5-OH BDE 47, 6-OH BDE 47, 6′-OH BDE 99, and 6-OH BDE 180).

Interaction energies were determined for 3,3′-T2 and the individual OH BDEs (Table 1). Lower interaction energies

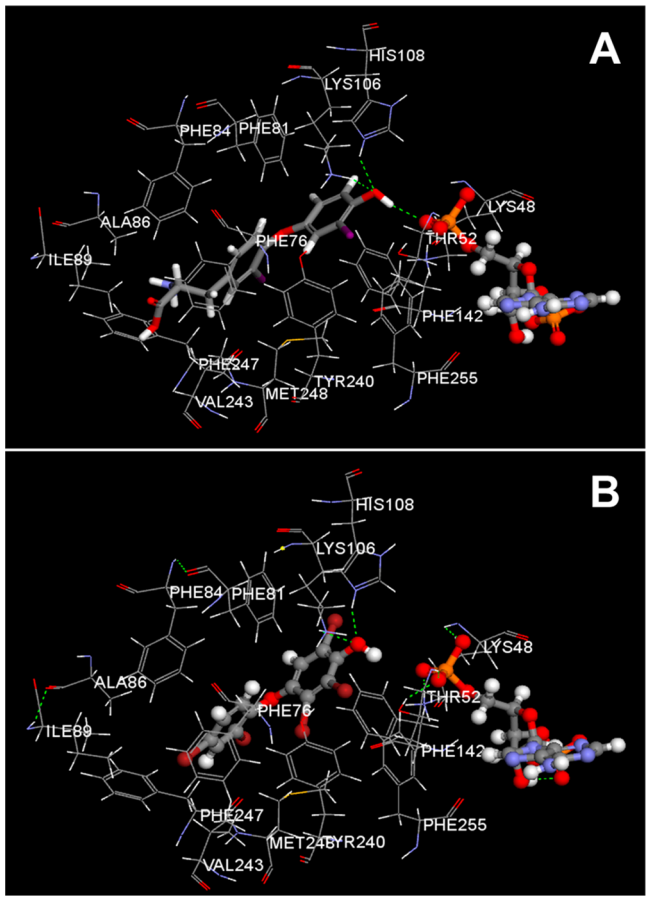

Figure 10. SULT1A1 docking to 3,3′-T2 (A) and 4-OH BDE90 (B), and the PAP cofactor. Green dashed lines represent hydrogen bonds. Carbon is colored gray, oxygen red, nitrogen blue, phosphate orange, iodine purple, bromine dark red, and hydrogen white.

were generally associated with an increased number of bromines (p < 0.05) (Supporting Information, Figure S4). Also, OH BDEs with para OH substitutions had lower interaction energies compared to those of ortho OH and meta OH substitutions (Supporting Information, Figure S4). Further, OH BDEs with an increased number of bromine atoms adjacent to the OH group showed lower interaction energies, although there was considerable overlap between treatments (Supporting Information, Figure S4). Finally, there was a positive relationship with pK_a (p < 0.01) (Supporting Information, Figure S4).

Comparison of Simulated Interaction Energies and Experimental IC_{50} Values. Overall, there was a significant positive relationship between the calculated interaction energy and experimental IC_{50} values (r^2 = 0.46; p < 0.05) (Supporting Information, Figure S5). However, three para OH-substituted BDEs did not fit the model (4′-OH BDE 49, 4′-OH BDE 101, and 4′-OH BDE 201). These compounds were less potent (higher IC_{50} values) than predicted by the model. 6-OH BDE 180 was excluded from the relationship because the IC_{50} value may have been inaccurate because the dose–response curve did not pass through 50% inhibition.

DISCUSSION

In our experiments, we used mass spectrometry techniques to monitor the formation of 3,3′-T2S, using an authentic standard for identification and quantification. As we are aware, this is the

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first direct measurement of a sulfated thyroid hormone metabolite. Earlier studies quantified TH sulfate formation using radioactivity counting. Mass spectrometry techniques are unambiguous and presumably are superior to radioactivity counting.

Our experiments used human liver cytosol, which is known to contain five SULT isozymes that are capable of TH sulfation (e.g., SULT1A1, SULT1A3, SULT1B1, SULT2A1, and SULT1E1).[23] The use of liver cytosol is the strength of our study because it is more representative of actual *in vivo* conditions, as opposed to the testing of individual SULT isozymes. Assessing TH inhibition in individual SULT isozymes may provide additional mechanistic information and should be the focus of future research. However, the principal finding of our study is maintained: some HOCs, including metabolites of brominated flame retardants, are potent inhibitors of TH sulfation.

In the absence of competitors, 3,3′-T2 was readily sulfated. The formation of 3-T1 was not observed, indicating that 3,3′-T2 was not deiodinated. This was not surprising because the assays were not optimized for DI activity (i.e., there was no dithiothreitol cofactor, and we used cytosol instead of microsomes).

The K_m value was very similar to that previously reported for human liver cytosol using radiolabeled techniques, but the V_{max} value was approximately 2-fold lower.[27,37] The lower V_{max} value in this study may reflect differences in the source of the cytosol, as ours was derived from a pool of multiple donors and therefore may reflect interindividual variability.

Over the course of these experiments, some day-to-day variability in 3,3′-T2S formation rates was observed. Using the same standard conditions (1 μM 3,3′-T2, 30 min incubation, 0.25 mg/mL protein) in the kinetic experiments (and in the time- and protein-variable experiments), the level of 3,3′-T2S formation ranged from approximately 70 to 80 pmol min^{-1} (mg of protein)$^{-1}$. Assessment of the interday variation showed no statistical differences in activity between days. These results suggest that any difference between activity rates was most likely due to analytical systematic bias.

Here we investigated the inhibition of thyroid hormone sulfotransferase activity using 3,3′-T2 as the substrate. Previous work has shown that the sulfotransferase inhibition activity toward 3,3′-T2 was similar to that of T3 when using OH PCBs as the inhibitors.[29] These results suggest that 3,3′-T2 can be used as a surrogate to investigate general thyroid hormone sulfotransferase inhibition activity.

This study expands upon previous research showing that some HOCs inhibit TH sulfation.[25−27] Only one study used human liver cytosol,[27] whereas another study used a purified SULT isozyme.[26] To the best of our knowledge, TH sulfotransferase inhibition by OH BDEs has not been previously reported. However, OH BDEs were shown to inhibit estradiol sulfotransferase activity in recombinant human SULT1E1.[38]

Because OH BDEs are hydroxylated compounds, they are also likely substrates for sulfation. To assess the potential for OH BDE sulfation, we monitored changes in the concentration of 4-OH BDE 90 during our experiments. The 4-OH BDE 90 concentrations did not decrease during the incubations, indicating that the OH BDEs were not sulfated under the conditions used in our experiments. 4-OH BDE 90 was assumed to be representative of the entire suite of OH BDEs, because it was not practical to assess all of the OH BDEs individually.

The presence of an OH group was necessary for 3,3′-T2 sulfotransferase inhibition. For example, BDE 47 did not show inhibition, but all three OH BDE 47 congeners did inhibit TH sulfation. This trend is consistent with a previous study that did not show inhibition with nonhydroxylated PCBs but did show inhibition with OH PCBs.[25] Further, the same study showed that BDE 47 and BDE 99 were TH sulfotransferase inhibitors only after incubation with CYP-enriched microsomes.[25] Although not specifically monitored, presumably the incubation resulted in the formation of OH BDE metabolites that were ultimately responsible for the TH sulfotransferase inhibition. Interestingly, incubation of the Bromkal 70-5-DE mixture (primarily pentaBDE congeners) did not result in TH sulfotransferase inhibition.[25]

It was shown that the para OH BDEs were outliers in the overall IC_{50} trends (i.e., in relationships with pK_a and the number of bromine atoms). Univariate models were not predictors of the IC_{50} values, and thus, a multivariate linear regression model was developed to adjust for the OH substitution pattern, the number of bromine atoms, and the pK_a. The developed model was a good predictor of the experimental IC_{50} values and explained 64% of the total variation (Figure 7).

Here we also investigated the influence of halogen substitution by testing fluorinated, chlorinated, brominated, and iodinated analogues of 2,4,6-trihalogenated phenol and BPA. Also, triclosan, which is a hydroxylated trichlorinated diphenyl ether, was tested and compared to 3′-OH BDE 28. In general, there were no consistent trends in 3,3′-T2 sulfotransferase inhibition potency. Among the trihalogenated phenols, the fluorinated, chlorinated, and brominated analogues exhibited approximately equal potency, but the iodinated analogue was 20−40-fold less potent. Interestingly, a previous *in vitro* study with human liver cytosol also showed that 2,4,6-TIP was a less potent 3,3′-T2 sulfotransferase inhibitor than 2,4,6-TBP.[27] Conversely, increasing potency was associated with increasing halogen size (I > Br > Cl > F) of 2,4,6-trihalogenated phenol for estradiol sulfation with recombinant human estrogen sulfotransferase.[39] These trends may suggest differing binding pocket characteristics for the various SULT isozymes. Similar to the trihalogenated phenols, the iodinated BPA (TIBPA) was also least potent among the BPA analogues, which showed decreasing relative potencies: TCBPA ≈ TBBPA > BPA-AF > TIBPA. It should be noted that BPA-AF is not an identical analogue to the other halogenated BPA compounds; BPA-AF has the halogens on the bridge methyl groups, whereas the other BPA compounds have the halogens on the phenol groups. Finally, triclosan was approximately 7-fold less potent than 3′-OH BDE 28, which is the structurally similar brominated analogue.

The compounds investigated in this study were more potent sulfotransferase inhibitors than deiodinase inhibitors.[19] In general, IC_{50} values for sulfotransferase were in the nanomolar range but were in the micromolar range for deiodinase inhibition. These results suggest that sulfotransferase inhibition may be a more sensitive end point for monitoring thyroid hormone disruption resulting from HOC exposure. For example, in this study, the trihalogenated phenols had IC_{50} values between 4 and 140 nM for sulfotransferase inhibition but T4 → T3 deiodinase inhibition IC_{50} values were between 10 and 6200 μM. An exception was TIBPA, which showed

approximately similar potency for sulfotransferase and deiodinase inhibition. These trends may also indicate different binding pocket requirements between the SULT and DI enzymes.

The mechanism of sulfotransferase inhibition was investigated by calculating the Michaelis−Menten parameters with varying concentrations of 4-OH BDE 90. 4-OH BDE 90 was tested because it was identified as the most potent OH BDE compound in the sulfotransferase inhibition assays. It was assumed that 4-OH BDE 90 was an appropriate surrogate for the remaining OH BDEs, but further testing is needed for verification.

This study showed that increasing 4-OH BDE 90 concentrations resulted in decreasing V_{max} rates, but the K_m concentration did not change, suggesting that the mechanism employed noncompetitive inhibition. Using crystallography, it was shown that 4,4′-OH 3,3′,5,5′-chlorobiphenyl binds to the human estrogen sulfotransferase binding pocket, suggesting competitive inhibition.[40] Consistent with our *in vitro* results, noncompetitive inhibition was shown for 3-OH benzo(*a*)-pyrene sulfation in human liver cytosol with triclosan as the inhibitor[41] and for estradiol sulfonation in recombinant estrogen sulfotransferase with OH PCBs as the inhibitor.[39] Conversely, competitive inhibition was shown for 3,3′-T2 sulfation in rat liver cytosol using 4-OH 2,3,3′,4′,5-CB as the inhibitor.[26] Previous studies have shown that there are two binding sites for *p*-nitrophenol within the SULT binding pocket,[42] and it has been postulated that the HOC inhibitor binds to the catalytic site and TH binds to the secondary site, resulting in slower sulfation of the endogenous TH.[41]

***In Silico* Sulfotransferase Docking Simulation.** The docking simulations used the SULT1A1 binding pocket as the model for our *in vitro* assays with human liver cytosol. Selection of SULT1A1 was justified by the dominant expression of this isozyme in the liver, as well as the relatively low K_m value, as compared to those of the other SULTs in the liver. As noted earlier, the human liver expresses five isozymes that are capable of TH sulfation: SULT1A1, SULT1A3, SULT1B1, SULT2A1, and SULT1E1.[23] It was beyond the scope of this study to quantify the individual SULT expression in our pooled liver cytosol, but previous work, using immunoblotting techniques, showed the human liver SULT profile is dominated by expression of SULT1A1 (53%) followed by SULT2A1 (27%), SULT1B1 (14%), and SULT1E1 (6%).[43] SULT1A3 was not expressed in the liver cytosol. Further, with respect to reaction with 3,3′-T2, SULT1A1 has the lowest K_m value. Specifically, K_m values were 0.12 μM for SULT1A1,[37] 31−35 μM for SULT1A3,[37] 3.0 for SULT2A1,[44] and 3.5−9.7 μM for SULT1E1.[22,44] The K_m value for SULT1B1 has not been published. Finally, it was shown that 3,3′-T2 sulfation inhibition, by various phenols as the inhibitors, was correlated between human liver cytosol and recombinant SULT1A1.[27] This further indicates that SULT1A1 is an important isozyme for TH sulfation in the human liver.

Although the binding pocket is highly conserved between isozymes, slight differences in the amino acid sequence result in substrate binding specificity.[45] Further, SULT1A1 has been shown to have a very high affinity for 3,3′-T2. As mentioned above, SULT1A1 has been shown to be the dominant SULT expressed in the human liver, but other SULTs are present. Therefore, our docking simulations may not completely capture the variation in SULT binding that occurs in the liver or in our pooled human liver cytosol samples.

As determined by crystallography, the binding pocket of SULT1A1 is L-shaped and is comprised of hydrophobic residues (i.e., Phe, Val, Ala, Ile, Tyr, and Met).[42] The catalytic residues, His^{108} and Lys^{106}, are positioned near the PAPS binding site. For sulfation to occur, the OH group must be positioned near the sulfur group of the PAPS and adjacent to the histidine residue.[45] Consistent with previous *in silico* modeling,[42] our modeling showed that the hydroxyl group on 3,3′-T2 was positioned within the catalytic site, forming hydrogen bonds with His^{108} and Lys^{106}.

The simulations also calculated the interaction energy between the SULT1A1 binding pocket and OH BDEs. Lower interaction energies are associated with higher binding affinities. Our results found that lower interaction energies were associated with the para-substituted OH BDEs. These compounds showed hydrogen bonding between the OH group and the catalytic His^{108} and Lys^{106} residues, and the OH group was close to the PAP, similar to that of endogenous 3,3′-T2. This was expected because 3,3′-T2 also has a para-substituted OH group, and thus, these OH BDEs most closely resembled the endogenous ligand. Conversely, the ortho- and meta-substituted OH BDEs had higher (less negative) interaction energies and did not show hydrogen binding with catalytic residues. Also, the OH group in most of these compounds was not positioned near the PAP. In addition, more brominated OH BDEs were associated with lower interaction energies, consistent with the very hydrophobic architecture of the binding pocket. Increasing the number of bromines adds size and hydrophobic character to the molecule, which could result in steric hindrance restrictions in the binding pocket. However, this was not observed in our simulations as the compound with the largest number of bromine atoms (4′-OH BDE 201) also had the lowest calculated interaction energy.

In general, there was a positive relationship between the calculated interaction energy and the OH BDE potency, as determined by the *in vitro* inhibition experiments (Supporting Information, Figure S5). The exceptions were the para-substituted OH BDEs in which three of the four compounds were less potent than predicted by the IC_{50}−interaction energy relationship. It is not known why these OH BDEs were outliers, and further investigation is warranted. The general agreement between modeled and experimental results is suggestive of a competitive inhibition mechanism that is inconsistent with our experimental results. However, this discussion assumes that SULT1A1, or a structurally similar isozyme, was the dominant SULT in the pooled human liver cytosol. Although previous research has shown that SULT1A1 is the dominant SULT expressed in the human liver, this was unconfirmed in our pooled liver cytosol, and thus, it may not be possible to draw associations between the modeling and experimental data.

Environmental Significance. The study contributes to a growing body of literature that demonstrates HOCs may be endocrine disruptors of the thyroid hormone system. Specifically, the study showed that several HOCs, including brominated flame retardants, hydroxylated metabolites of PBDEs, halogenated BPA chemicals, and the antimicrobial triclosan, can interfere with thyroid hormone sulfation. The IC_{50} values calculated here for the halogenated phenols and OH BDEs were in the low nanomolar range. While it is difficult to directly compare these *in vitro* measures to *in vivo* exposure levels, it is worth noting that some of these OH BDEs have been measured in human serum at levels of up to 0.06 nM (lipid-normalized levels reported in Table 1), much lower than

our IC_{50} values.[9,46] Further, it is not known how the *in vitro* IC_{50} values in our experiments can be extrapolated to *in vivo* effects. The primary role of TH sulfation appears to be the regulation of biologically active T3. T4S is not metabolized to T3S via outer-ring deiodination but forms rT3S via inner-ring deiodination. Also, sulfated THs are more readily deiodinated than their nonsulfated analogues, further promoting the formation of inactive THs. Therefore, the *in vivo* consequence of sulfotransferase inhibition may be to increase the amount of circulating, biologically active T3. Relatively high levels of T3S have been measured in cord blood, suggesting that TH sulfation is critical for regulating TH levels in the fetus.[47] Further, TH sulfotransferase activity has been measured in astrocytes from rat brain tissues.[48] Thus, future research should focus on TH sulfotransferase inhibition in these tissues.

■ ASSOCIATED CONTENT

Supporting Information

Chemical structures of inhibitors investigated, 3,3′-T2S formation as a function of time and protein concentration, calculated interaction energy as a function of the number of bromines, OH substitution and the number of adjacent bromine atoms, and calculated interaction energy versus experimental IC_{50} values. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Nicholas School of the Environment, Duke University, Box 90328, Levine Science Research Center, Room A220, Durham, NC 27708. E-mail: heather.stapleton@duke.edu. Telephone: (919) 613-8717. Fax: (919) 684-8741.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HOC, halogenated organic compound; TH, thyroid hormone; TSH, thyroid-stimulating hormone; T4, thyroxine; T3, triiodothyronine; TTR, transthyretin; TBG, thyroxine-binding globulin; DI, deiodinase; OH BDE, hydroxylated polybrominated diphenyl ether; rT3, 3,3′,5′-triiodothyronine; SULT, cytosolic sulfotransferase; PAP, 3′-phosphoadenosine 5′-phosphosulfate; 3,3′-T2, 3,3′-diiodothyronine; OH PCB, hydroxylated polychlorinated biphenyl; PCDD, dibenzo-*p*-dioxin; PCDF, dibenzofuran; LC−MS/MS, liquid chromatography and tandem mass spectrometry; TBBPA, tetrabromobisphenol A; BPA AF, 4,4′-(hexafluoroisopropylidene)diphenol; 2,4,6-TBP, 2,4,6-tribromophenol; 2,4,6-TFP, 2,4,6-trifluorophenol; 2,4,6-TCP, 2,4,6-trichlorophenol; 2,4,6-TIP, 2,4,6-triiodophenol; TCBPA, 3,3′,5,5′-tetrachlorobisphenol A; TIBPA, 3,3′,5,5′-tetraiodobisphenol A; 3,3′-T2S, 3,3′-T2 sulfate; SPE, solid phase extraction; 3′-T1, 3-monoiodothyronine

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