



Published in final edited form as:

*Chem Res Toxicol.* 2008 August ; 21(8): 1503–1508. doi:10.1021/tx800133d.

## PENTACHLOROPHENOL AND OTHER CHLORINATED PHENOLS ARE SUBSTRATES FOR HUMAN HYDROXYSTEROID SULFOTRANSFERASE hSULT2A1

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### Abstract

Pentachlorophenol (PCP) is a persistent chemical contaminant which has been extensively investigated in terms of its toxicology and metabolism. Similar to PCP, other chlorinated phenol derivatives are also widely present in the environment from various sources. Even though some of the chlorine-substituted phenols, and particularly PCP, are well known inhibitors of phenol sulfotransferases (SULTs), these compounds have been shown to undergo sulfation in humans. In order to investigate the enzymatic basis for sulfation of PCP in humans, we have studied the potential for PCP as well as the mono-, di-, tri-, and tetra-chlorinated phenols to serve as substrates for human hydroxysteroid sulfotransferase, hSULT2A1. Our results show that all of these compounds are substrates for this isoform of sulfotransferase and the highest rates of sulfation are obtained with PCP, trichlorophenols, and tetrachlorophenols. Much lower rates of sulfation were obtained with isomers of monochlorophenol and dichlorophenol as substrates for hSULT2A1. Thus, the sulfation of polychlorinated phenols catalyzed by hSULT2A1 may be a significant component of their metabolism in humans.

### Introduction

Pentachlorophenol (PCP) is a synthetic molecule that has been used throughout the world for purposes as varied as antimicrobial agent, detergent and wood preservative. Even though its use has been restricted after the 1980s, it is still widely present in the environment, and it is considered as a chemical pollutant of concern (1). The air and the food chain are main pathways for human exposure to this compound, and it is possible to detect significant concentrations of PCP in the plasma of various age groups including newborns (2–4). Acute toxicity of PCP is usually occupationally related with significant damage to various organs, and chronic exposure also results in severe health disorders (5–8). Concentrations of PCP in the blood of exposed individuals can vary widely, and values ranging from 1.5  $\mu\text{g/L}$  to 90  $\mu\text{g/L}$  have been reported (4,8,9). Furthermore, there is a wide tissue distribution for PCP and a relatively long elimination half-life (9–12). Even though PCP is not directly classified as a human carcinogen, it has been shown to be involved in the initiation and/or promotion of carcinogenesis in animal studies (13–14). Moreover, some of the oxidized metabolites of PCP are reactive electrophiles towards DNA and proteins (15–17).

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The biotransformation of PCP has been investigated in a wide range of animal species (9,18–25). In these studies, the unchanged parent molecule and its glucuronide and sulfate conjugates are found, as well as other metabolites. Interestingly, besides the formation of the reactive metabolite, tetrachloro-1,4-benzoquinone, other chlorinated phenols were also identified as metabolites of PCP (21). This was parallel with the identification of PCP as one of the metabolites of hexachlorobenzene (26). In humans, sulfation was found to be a dominant excretion pathway for low concentrations of chlorinated phenols when investigated in saw-mill workers exposed to 2,4,6-tri-, 2,3,4,6-tetra-, and penta-chlorophenol (27).

Even though the chlorinated phenols are subject to sulfation in humans, some of these compounds, particularly PCP, are well known for their characteristic inhibition of the phenol sulfotransferases (i.e., sulfotransferase family 1, or SULT1, enzymes) (28). However, their potential to interact with a human hydroxysteroid sulfotransferase (e.g., the family 2 enzyme, hSULT2A1) has not received attention. hSULT2A1 catalyzes the sulfation of various endogenous molecules (e.g. hydroxysteroids and bile acids) and xenobiotics bearing aliphatic or benzylic alcohol functional groups. Moreover, a few phenols such as 1-hydroxypyrene (29) and certain hydroxylated polychlorinated biphenyls (30) can serve as substrates for this enzyme. Additionally, hSULT2A1 has been reported to catalyze the sulfation of phenols such as estrone (31), raloxifene (32), and 4-hydroxytamoxifen (32), although hSULT1E1 and hSULT1A1 are likely the major SULTs involved for these particular phenols under physiological conditions. While PCP and several related phenols with multiple chlorine atoms clearly inhibit many phenol sulfotransferases (i.e., SULT1 enzymes), we have now investigated the hypothesis that these molecules serve as substrates for hydroxysteroid sulfotransferase hSULT2A1.

## Materials and Methods

### Chemicals and Biochemicals

2-Mercaptoethanol, dehydroepiandrosterone (DHEA), PAPS, potassium phosphate, methylene blue, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,3-dichlorophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 2,6-dichlorophenol, 3,4-dichlorophenol, 3,5-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4-trichlorophenol, 2,4,5-trichlorophenol and pentachlorophenol were purchased from Sigma Chemical Co. (St. Louis, MO). PAPS was further purified by a previously described procedure (33) to a purity greater than 99% as judged by HPLC. 2,3,6-Trichlorophenol, 2,3,5-trichlorophenol, 3,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol, 2,3,5,6-tetrachlorophenol and 2,3,4,5-tetrachlorophenol were obtained from Supelco (Bellefonte, PA). Each of the chlorinated phenols was of the highest purity commercially available from the manufacturer. Sucrose, chloroform and anhydrous sodium sulfate were from Fisher Scientific (Pittsburgh, PA). Tris-HCl was obtained from RPI (Mt. Prospect, IL). DE52 was purchased from Whatman (Fairfield, IL). Hydroxyapatite (Bio-Gel HT) was from Bio-Rad Laboratories (Hercules, CA), and Tween 20 was obtained from J.T. Baker Chemicals (Philipsburg, NJ).

### Expression and Purification of Human SULT2A1

Expression of hSULT2A1 in *Escherichia coli* cells and the preparation of cell extract were carried out according to the previously described procedure (30). The cell extract obtained (1 g protein) was applied to a DE52 anion exchange column (2.5 × 17 cm) equilibrated with buffer A (50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 1 mM DTT, 10% (v/v) glycerol and 0.05% (v/v) Tween 20). After removing the proteins which did not bind to this column by washing with buffer A, the protein was eluted with a linear gradient formed between 250 mL of buffer A and 250 mL of buffer A containing 0.5 M potassium chloride. The fractions containing hSULT2A1 were combined and concentrated by ultrafiltration (YM10 membrane;

Millipore Corporation, Bedford, MA). The concentration of potassium chloride was then reduced through successive dilution and concentration by ultrafiltration, with the dilutions carried out using the same buffer to be employed for the subsequent hydroxyapatite chromatography step (i.e., buffer B: 10 mM potassium phosphate, 0.25 M sucrose, 1 mM DTT and 0.05% (v/v) Tween 20, pH 6.8). The resulting protein (0.4 g) was applied to a column of hydroxyapatite (2.5 × 9.0 cm) equilibrated with buffer B. Buffer B was used to wash the column in order to remove all proteins that did not bind to the column, and elution was then carried out with a linear gradient formed between 200 mL of buffer B and 200 mL of buffer B containing 0.4 M potassium phosphate. The fractions containing hSULT2A1 activity were pooled and concentrated by ultrafiltration. In order to prepare this mixture for the following hydroxyapatite chromatography the phosphate concentration was then elevated to 30 mM by successive dilution/concentration with buffer C (buffer C: 30 mM potassium phosphate, 0.25 M sucrose, 1 mM DTT and 0.05% (v/v) Tween 20, pH 6.8). A new column (2.5 × 9.0 cm) of hydroxyapatite equilibrated with buffer C was prepared and the concentrated protein (0.1 g) from the first hydroxyapatite column was then applied to the second hydroxyapatite column. Proteins that did not bind to the column were removed by washing with buffer C, and elution of SULT2A1 was carried out with a linear gradient formed between 75 mL of buffer C and 75 mL of buffer C containing 0.3 M potassium phosphate. The fractions containing hSULT2A1 with the highest specific activity were analyzed by SDS-PAGE, and 36 mg of purified hSULT2A1 was obtained. The resulting purified enzyme was judged to be homogeneous by SDS-PAGE with Coomassie Blue staining. At each step of purification, protein content was determined by the modified Lowry method (34). The subunit relative molecular mass of the homogeneous protein was found to be 33.7 kDa, and the kinetic behavior of the protein with DHEA as substrate was completely consistent with the previously reported data for the native enzyme isolated from human liver (31). A summary of the purification (Table S1) and figures showing results at each step in the purification (Figure S1–Figure S4) are included in the Supporting Information for this article (available at <http://pubs.acs.org>).

### Assay of Chlorinated Phenols as Substrates of Human SULT2A1

The potential of all chlorinated phenols to serve as substrates for hSULT2A1 was investigated using a standard methylene blue assay (35,36). Rates of sulfation were determined at 10  $\mu$ M and 50  $\mu$ M concentrations of each chlorinated phenol using the following procedure. Each 400  $\mu$ L of reaction mixture contained 0.25 M potassium phosphate buffer at pH 7.0, 0.2 mM PAPS, 7.5 mM 2-mercaptoethanol, chlorinated phenol, and 2.5 % (v/v) ethanol as co-solvent for chlorinated phenols. The reactions were initiated by the addition of 32  $\mu$ g of purified hSULT2A1 and incubated at 37°C for 10 min. Then, the reactions were terminated by the addition of 0.5 mL methylene blue reagent and 1.5 mL chloroform. The chlorinated phenol-dependent formation of a product organic sulfate that formed a paired ion with methylene blue in the chloroform phase was measured at 651 nm.

In order to make a relative comparison of the degree of sulfation that accounted for any day-to-day experimental variability in enzyme activity, the rate of sulfation of dehydroepiandrosterone (DHEA) at 50  $\mu$ M concentration was determined using the same procedure described above. For each set of experiments, sulfation of the chlorinated phenol derivative (at 10  $\mu$ M and 50  $\mu$ M) was analyzed together with the sulfation of 50  $\mu$ M DHEA. The overall standard deviation of all hSULT2A1-catalyzed DHEA-sulfation reactions was 13% of the mean.

Although the methylene blue assay has been routinely used for the determination of SULT activities, and it has been successfully applied to various substrates including steroids (35), phenols (37), amines (38), and benzylic alcohols (39), confirmation of the assay methodology was obtained by the comparison of the rates of sulfation of representative chlorinated phenols

(i.e., 3,5-dichlorophenol, 3,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol, and pentachlorophenol) at 50  $\mu\text{M}$  using an HPLC method for the determination of substrate-dependent formation of PAP catalyzed by the SULT (36). These assays were carried out at 37°C and at pH 7.0 with the amounts of enzyme and other components directly scaled to a 30  $\mu\text{L}$  reaction mixture for comparison to the methylene blue assay. At the end of the 10 min incubation time, the reactions were terminated by the addition of 30  $\mu\text{L}$  methanol and the substrate-dependent formation of PAP from PAPS was determined by HPLC as previously described (36). The rates of hSULT2A1-catalyzed sulfation of 3,5-dichlorophenol, 3,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol, and pentachlorophenol in HPLC assays were found to be  $7.8 \pm 0.2$ ,  $36.2 \pm 1.4$ ,  $20.2 \pm 1.0$ , and  $46.9 \pm 0.7$  nmoles of product/min/mg hSULT2A1, respectively. These results were consistent with those obtained for the product sulfates using the methylene blue assay for 3,5-dichlorophenol, 3,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol, and pentachlorophenol (i.e.,  $7.2 \pm 0.2$ ,  $38.2 \pm 0.7$ ,  $21.6 \pm 0.2$ , and  $48.0 \pm 0.9$ , respectively).

Control experiments under the assay conditions used for these studies indicated the dependence of the hSULT2A1-catalyzed reaction product formation on both time and protein concentration for both PCP and for 3,5-dichlorophenol as representative substrates. There was no detectable sulfation of the co-solvent (i.e., 2.5% v/v ethanol) in control experiments without DHEA or a chlorinated phenol. Unless otherwise noted, assays were done in duplicate and the means  $\pm$  standard errors are reported.

### Solubility of Chlorinated Phenols in the Assay

In order to assess solubility of compounds under the experimental conditions, a previously described light scattering method was employed at 400 nm using a Perkin-Elmer LS55 Luminescence Spectrometer (40).

## Results

### Investigation of Chlorinated Phenols as Substrates of hSULT2A1

In analyzing the potential for chlorinated phenols to serve as substrates for hSULT2A1, we first determined their solubility at 10  $\mu\text{M}$  and 50  $\mu\text{M}$  concentrations under the specific conditions of assay (Materials and Methods). All of the compounds were found to be soluble at these concentrations (data not shown). The hSULT2A1-catalyzed formation of sulfates from chlorinated phenols was analyzed by the methylene blue paired ion extraction method, and the rates of sulfation were compared using the rate of sulfation of 50  $\mu\text{M}$  DHEA as reference.

As illustrated in Table 1 and Table 2, all of the chlorinated phenols were substrates with a relative activity range of 0.3–161% when compared with the rate of sulfation of 50  $\mu\text{M}$  DHEA. The velocities at 50  $\mu\text{M}$  were higher than the ones at 10  $\mu\text{M}$  for all tested compounds. The highest relative activity was observed with 2,3,4,5-tetrachlorophenol (161% relative activity) and the monochlorinated phenols were the weakest substrates (around 0.3–4% relative activity). Even though variabilities were seen among the members of each group of compounds, there was a general tendency of increased sulfation with increased chlorination of the phenol.

Only the monochlorophenols were not sulfated at 10  $\mu\text{M}$ , but these compounds were sulfated at very low rates when present at 50  $\mu\text{M}$ . The range of relative activities for dichlorophenols was found to be 5–17% of the rate for DHEA, and 3,5-dichlorophenol showed the highest relative ability to serve as a substrate in this group. Trichlorophenols exhibited rates of sulfation 5–10 times greater than dichlorophenols at both 10 and 50  $\mu\text{M}$  levels. Among the trichlorophenols, 2,3,5-trichlorophenol, 2,4,5-trichlorophenol, and 3,4,5-trichlorophenol were sulfated at the highest rates. Rates of sulfation for tetrachlorophenols suggest that steric effects

of two chlorine atoms *ortho* to the phenolic hydroxyl may decrease the rate of catalysis, since 2,3,4,5-tetrachlorophenol showed the highest relative rate of sulfation.

## Discussion

It has long been known that PCP and several other chlorinated phenols are inhibitors of phenol sulfotransferases (i.e., *SULT1* enzymes) in both in vitro and in vivo studies (28,41–46), and that their effects on these enzymes differ from their interactions with hydroxysteroid sulfotransferases (*SULT2* enzymes). For example, studies with female rat hepatic cytosol indicated that PCP inhibited sulfation of 4-nitrophenol (a substrate for *SULT1* enzymes), but had little effect on the sulfation of 5-hydroxychrysene and DHEA (substrates for hydroxysteroid sulfotransferases) (41). Likewise, in vivo studies in the rat have indicated that while PCP was an excellent inhibitor of the sulfation of *SULT1* substrates such as 1-naphthol (43), acetaminophen (45), and harmol (46), there was no inhibition of the sulfation of DHEA at two different doses administered by i.v. infusion (47). Such differences in the effects of PCP on *SULT1* and *SULT2* enzymes were even more prominent at a high dose infusion of DHEA (50 mg/kg), where an increase in DHEA-sulfate serum concentrations was observed with PCP-treatment (47). Although in vivo studies on the effects of PCP on the sulfation of 5-hydroxymethylchrysene (41) and DHEA (41,47) in the rat did not address the possible formation of PCP-sulfate, sulfation has been shown to be a significant pathway for excretion of low doses of PCP in humans (27). Thus, when our results on the ability of human hydroxysteroid sulfotransferase *hSULT2A1* to catalyze sulfation of PCP are considered along with previous studies on urinary excretion of PCP-sulfate, it is reasonable to conclude that *hSULT2A1* contributes to the sulfation of PCP in humans. Human sulfotransferases such as *hSULT2B1a* and *hSULT2B1b* might also contribute to this observed sulfation, but the ability of these *SULTs* to catalyze the formation of PCP-sulfate remains to be investigated.

After the identification of PCP as a substrate for *hSULT2A1* we also analyzed the substrate specificity of this enzyme with other chlorinated phenols. Similar to PCP, mono-, di-, tri-, and tetrachlorophenol derivatives are also widely present in the environment. Some of these chlorinated phenols are metabolites of hexachlorobenzene or PCP, and some of them are produced for industrial purposes (48–49). Our studies with two concentrations (i.e., 10  $\mu\text{M}$  and 50  $\mu\text{M}$ ) of chlorinated phenols indicated variations in their abilities to serve as substrates for *hSULT2A1*. Monochlorophenols have been previously shown to be substrates for phenol sulfotransferases (i.e., *SULT1* enzymes) (50). Our studies with the hydroxysteroid sulfotransferase *hSULT2A1* indicated that these compounds are only very weak substrates of this enzyme. Thus any contribution of *hSULT2A1* in the sulfation of these monochlorophenols is expected to be very low. Dichlorophenols were better substrates in comparison to monochlorophenols, since sulfation was observed at concentrations of both 10  $\mu\text{M}$  and 50  $\mu\text{M}$ . However, their relative activities were still low in comparison to DHEA and to other higher chlorinated phenol derivatives. Moreover, it is possible that some lower chlorinated phenols might potentially serve as substrates for *SULT1* enzymes, and these structure-activity relationships will be the subject of future studies as well.

As illustrated in Table 2, the rates of sulfation seen for many of the trichloro-, and tetrachlorophenols were comparable to those seen for DHEA. Two compounds, 2,3,5-trichlorophenol and 2,3,4,5-tetrachlorophenol, exhibited higher rates of sulfation in comparison to DHEA under these conditions. As suggested for pentachlorophenol, our results with tri- and tetra-chlorophenols indicated a possible detoxication pathway of these compounds involving the contribution of *hSULT2A1*. This potential for excretion through metabolic sulfation assumes additional importance, since some of these compounds are also known as inhibitors of phenol sulfotransferases such as those involved in regulation of estrogen concentrations (51).

The overall increase in the rate of sulfation with increasing number of chlorine atoms may be primarily related to the increasing lipophilicity of the compounds. Indeed, according to the crystal structures of this enzyme, the substrate binding pocket of hSULT2A1 is composed of hydrophobic amino acid residues (52). However, this is not sufficient to explain the different rates of sulfation for compounds within each group. Thus, steric interactions between the active site residues of the enzyme and specific chlorine atoms on the phenol must also play a role. In general, the presence of two chlorine atoms *meta* to the phenolic hydroxyl was found to be important for higher rates of sulfation. Indeed, all of the compounds that indicated the highest relative activities among the members of individual groups (3,5-dichlorophenol, 2,3,5-trichlorophenol and 2,3,4,5-tetrachlorophenol) have this *di-meta* chlorine substitution. Nevertheless, it should be emphasized that, in addition to these initial observations, more detailed kinetic analyses of each of the 19 chlorinated phenols will be needed to establish firm structure-activity relationships for substrates and to assess the extent to which substrate inhibition may be occurring for individual congeners. While the current studies clearly show the ability of a large number of chlorinated phenols to serve as substrates for hSULT2A1, the presence of substrate inhibition for some, but perhaps not all, substrates of hSULT2A1 would complicate analysis of structure-activity relationships. Additional studies will also be necessary to assess the potential for each of the chlorinated phenols to compete with DHEA as a substrate for hSULT2A1. This will require analysis of multiple concentrations of both DHEA and the chlorinated phenol, since the potential for such interactions would be dependent upon kinetic parameters of both the chlorinated phenol and DHEA.

In summary, we have determined that PCP and related chlorinated phenols, many of which are known as inhibitors of phenol sulfotransferases, are substrates for the human hydroxysteroid sulfotransferase hSULT2A1. The importance of the contribution of this enzyme towards the detoxication of these molecules under in vivo conditions will be the subject of future investigations.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

This study was supported by the National Institutes of Health through research grants R01 CA038683 from the National Cancer Institute and P42 ES013661 from the National Institute of Environmental Health Sciences. Support from the NIEHS through P30 ES05605 is also acknowledged. The contents of this paper are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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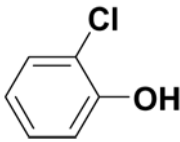
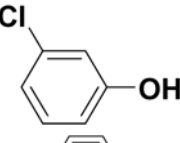
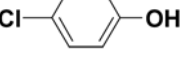
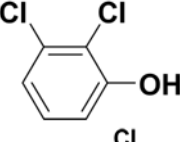
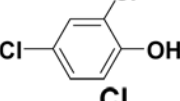
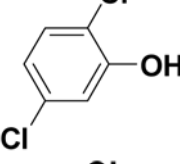
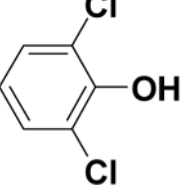
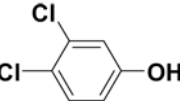
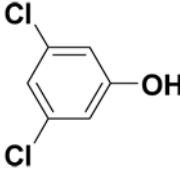


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## Abbreviations

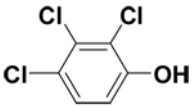
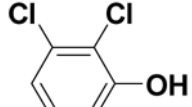
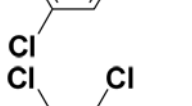
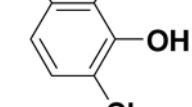
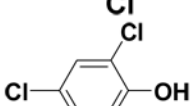
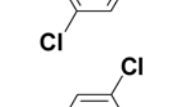
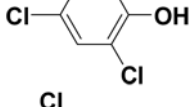
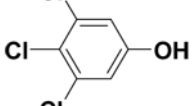
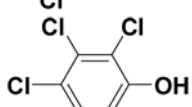
DE52, Diethylaminoethyl cellulose anion exchange column; DHEA, dehydroepiandrosterone (5-androsten-3 $\beta$ -ol-17-one); DTT, dithiothreitol; hSULT2A1, human hydroxysteroid sulfotransferase 2A1; PAP, adenosine 3'5'-diphosphate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PCP, pentachlorophenol; SULT, sulfotransferase; Tris-HCl, [tris (hydroxymethyl)aminomethane] hydrochloride..

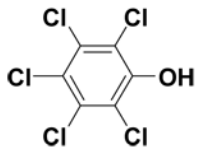
**Table 1**  
Monochlorophenols and dichlorophenols as substrates for hSULT2A1.

Molecule	Structure	Sulfation at 10 $\mu$ M (nmol/min/mg)	Sulfation at 50 $\mu$ M (nmol/min/mg)	Relative activity (%) <sup>a</sup>
2-Chlorophenol		0	0.2 $\pm$ 0.1	0.3
3-Chlorophenol		0	1.4 $\pm$ 0.7	2.3
4-Chlorophenol		0	1.6 $\pm$ 0.5	3.8
2,3-Dichlorophenol		0.6 $\pm$ 0.2	4.2 $\pm$ 0.5	9.8
2,4-Dichlorophenol		1.1 $\pm$ 0.3	2.7 $\pm$ 0.3	5.4
2,5-Dichlorophenol		1.7 $\pm$ 0.1	6.9 $\pm$ 0.2	13.3
2,6-Dichlorophenol		0.3 $\pm$ 0	2.5 $\pm$ 0.5	5.8
3,4-Dichlorophenol		0.3 $\pm$ 0.2	2.9 $\pm$ 0.2	6.9
3,5-Dichlorophenol		2.3 $\pm$ 0.4	7.2 $\pm$ 0.2	17.1

<sup>a</sup>Relative activity is expressed as a percentage based on the ratio of the rate of sulfation of the chlorinated phenol at 50  $\mu$ M to the rate of sulfation of 50  $\mu$ M DHEA under the same assay conditions.

**Table 2**  
Tri-, tetra-, and pentachlorophenols as substrates for hSULT2A1.

Molecule	Structure	Sulfation at 10 $\mu$ M (nmol/min/mg)	Sulfation at 50 $\mu$ M (nmol/min/mg)	Relative activity (%) <sup>a</sup>
2,3,4-Trichlorophenol		10.1 $\pm$ 1.0	30.9 $\pm$ 0.3	55.2
2,3,5-Trichlorophenol		14.6 $\pm$ 0.8	47.7 $\pm$ 0.7	106.4
2,3,6-Trichlorophenol		10.5 $\pm$ 0.7	28.4 $\pm$ 0.8	65.9
2,4,5-Trichlorophenol		14.4 $\pm$ 1.3	46.0 $\pm$ 1.1	81.7
2,4,6-Trichlorophenol		9.3 $\pm$ 0.1	26.2 $\pm$ 0.2	44.7
3,4,5-Trichlorophenol		12.1 $\pm$ 1.0	38.2 $\pm$ 0.7	89.3
2,3,4,5-Tetrachlorophenol		16.0 $\pm$ 0.5	67.1 $\pm$ 1.7	160.9
2,3,4,6-Tetrachlorophenol		10.1 $\pm$ 1.5	21.6 $\pm$ 0.2	51.2
2,3,5,6-Tetrachlorophenol		4.0 $\pm$ 0.3	12.9 $\pm$ 2.6	29.5

Molecule	Structure	Sulfation at 10 $\mu\text{M}$ (nmol/min/mg)	Sulfation at 50 $\mu\text{M}$ (nmol/min/mg)	Relative activity (%) <sup>a</sup>
Pentachlorophenol		13.2 $\pm$ 0.2	48.0 $\pm$ 0.9	91.2

<sup>a</sup>Relative activity is expressed as a percentage based on the ratio of the rate of sulfation of the chlorinated phenol at 50  $\mu\text{M}$  to the rate of sulfation of 50  $\mu\text{M}$  DHEA under the same assay conditions.