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Metabolism of Polychlorinated Dibenzo-*p*-dioxins (PCDDs) by Human Cytochrome P450-Dependent Monooxygenase Systems

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Metabolism of polychlorinated dibenzo-*p*-dioxins (PCDDs) by monooxygenase systems dependent on 12 forms of human cytochrome P450 (CYP) was examined with the recombinant yeast microsomes containing each of the human CYP. The metabolites of PCDDs were analyzed by HPLC and GC-MS. Remarkable metabolism by the multiple CYP forms was observed toward dibenzo-*p*-dioxin (DD) and mono-, di-, and trichloroDDs. The metabolism contained multiple reactions such as hydroxylation at an unsubstituted position, hydroxylation with migration of a chloride substituent, and hydroxylation with elimination of a chloride substituent. Although major CYPs in human liver such as CYP2C8, CYP2C9, and CYP3A4 showed no significant metabolism toward the PCDDs, CYP1A1 and CYP1A2 showed high catalytic activity toward DD and mono-, di-, and trichloroDDs. The kinetic parameters K_m^{app} and V_{max} of the CYP1A1-dependent 8-hydroxylation activity of 2,3,7-trichloro-DD (2,3,7-triCDD) were estimated to be 0.30 μM and 51 (mol/min/mol of P450), respectively, suggesting that 2,3,7-triCDD was a good substrate for CYP1A1. However, none of the CYPs showed any detectable activity [<0.01 mol/min/mol of P450)] toward 2,3,7,8-tetraCDD. Substrate-induced absorption spectrum and inhibition studies indicated that CYP1A1 could bind 2,3,7,8-tetraCDD with considerably high affinity. It was strongly suggested that the long half-life (7.1 years) of 2,3,7,8-tetraCDD in humans was due to an extremely low activity of CYPs toward 2,3,7,8-tetraCDD in addition to its chemical stability.

KEYWORDS: Dioxin; PCDD; cytochrome P450; CYP; recombinant yeast

INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are the family consisting of 75 structurally similar chlorinated compounds. They are known as environmental contaminants due to the extreme toxicity of some members of the family. Of these compounds, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin CDD (tetraCDD) is known to be the most toxic one. Recently, the World Health Organization (WHO) established a tolerable daily intake of 1−4 pg of TEQs/kg of body weight (*1*). Human exposure to dioxins appears to be predominantly from meat, fish, and dairy products (*2*). In the past two decades, the metabolism of PCDDs has been studied in vivo using experimental animals (*3*−*9*) and in vitro using liver slices and liver microsomal fractions (*10*). Major metabolites were hydroxylated products, glucuronide conjugates, and sulfate conjugates (*4*, *6*). They involve the initial insertion of a single oxygen atom into the PCDD molecule to form an epoxide, probably by cytochrome P450 (CYP)-dependent monooxygenases. Recently, Hu and Bunce (*10*) studied the metabolism of PCDDs in the microsomal fractions prepared from control and 3-methylcholanthrene-treated rats. They suggested that CYP1A1 and CYP1A2 play an important role in the metabolism of PCDDs. Thus, cytochrome P450-dependent monooxygenases appear to be key enzymes for the metabolism of PCDDs. It is known that the body distribution of PCDDs is strongly species dependent, with the liver and adipose tissue being the major storage sites (*7*, *11*). In addition, the tissue distribution of PCDDs is dose dependent. The liver-to-adipose tissue distribution ratio increases with increasing dose (*12*, *13*). Thus, a remarkable species difference between humans and rats on the half-life of 2,3,7,8-tetraCDD (*3*, *14*, *15*) appears to be based on the difference of tissue distribution of PCDDs, enzymatic properties of cytochromes P450, and their expression levels. To our knowledge, no papers identifying the metabolites of PCDDs by each human CYPs have been published, although studies on the metabolism of PCDDs by human CYPs are essential for human risk assessment of PCDDs.

We have established a yeast expression system for human CYPs and shown its usefulness for the prediction of drug metabolism in humans (*16*−*19*). Addition of a drug to the yeast cell culture or microsomal fraction prepared from the recombinant yeast cells could supply sufficient amounts of metabolites to determine their chemical structures. In this study, the yeast

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expression system was applied to the prediction of the metabolism of PCDDs in humans. The detailed metabolism of various types of PCDDs by human CYPs is demonstrated.

MATERIALS AND METHODS

Materials. Polychlorinated-*p*-dioxins (PCDDs), 2,3,7,8-tetraCDD, 2,3,7-triCDD, and 2,7-DCDD were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). 2,3-DichloroDD (DCDD), 1-monochloroDD (MCDD), and 2-MCDD were purchased from AccuStandard Inc. (New Haven, CT). Dibenzo-*p*-dioxin (DD) was purchased from Wako Pure Chemical Industries, Ltd. Co. (Osaka, Japan). The recombinant yeast microsomes containing each human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were purchased from Sumika Chemical Analysis Service (Osaka, Japan) (*17*, *18*). Specific content of P450 (picomoles per milligram of protein) and standard activity (moles per minute per mole of P450) of each microsomal fraction measured by Sumika Chemical Analysis Service were as followss: CYP1A1, 16.2 pmol/mg of protein and 9.3 mol/min/mol of P450 for 7-ethoxyresorufin O-deethylation; CYP1A2, 40.0 pmol/mg of protein and 2.4 mol/min/mol of P450 for 7-ethoxyresorufin O-deethylation; CYP2A6, 23.1 pmol/mg of protein and 6.8 mol/min/mol P450 for coumarin 7-hydroxylation; CYP2B6, 11.7 pmol/mg of protein and 2.1 mol/min/mol of P450 for 7-ethoxycoumarin O-deethylation; CYP2C8, 145.0 pmol/mg of protein and 3.2 mol/min/mol of P450 for tolbutamide hydroxylation; CYP2C9, 102.0 pmol/mg of protein and 12.4 mol/min/mol of P450 for tolbutamide hydroxylation; CYP2C18, 71.3 pmol/mg of protein and 5.5 mol/min/mol of P450 for tolbutamide hydroxylation; CYP2C19, 29.8 pmol/mg of protein and 16.6 mol/min/mol of P450 for *S*-mephenytoin 4′-hydroxylation; CYP2D6, 12.8 pmol/mg of protein and 4.8 mol/min/mol of P450 for debrisoquin 4-hydroxylation; CYP2E1, 67.1 pmol/mg of protein and 5.5 mol/min/mol of P450 for chlorzoxazone 6-hydroxylation; and CYP3A4, 50.6 pmol/mg of protein and 26.2 mol/min/mol of P450 for testosterone 6β-hydroxylation.

NADPH−cyt.*c* reductase activity indicating the contents of yeast NADPH−P450 reductase of each microsome was in the range of 1000−2000 nmol of cyt.*c* reduced/min/mg of protein. Only CYP3A4 microsomes contain human cyt.b_5 at the level of 220 pmol/mg of protein (*18*). The microsomes prepared from the recombinant yeast harboring the plasmid which contains yeast NADPH−P450 reductase gene (*20*) were purchased from Sumika Chemical Analysis Service and used as control microsomes. The microsomes containing human CYP1B1 prepared from recombinant *Sf9* cells were purchased from Daiichi Chemical Co. (Tokyo, Japan). The specific content of P450 and 7-ethoxyresorufin O-deethylation activity were 65 pmol/mg of protein and 4.1 mol/min/mol of P450, respectively. NADPH was from Oriental Yeast (Tokyo, Japan). All other chemicals were of the best commercially available grade.

Measurement of Reduced CO Difference Spectra and Substrate-Induced Difference Spectra. The reduced CO difference spectra were measured with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) according to the following procedure as described previously (*21*−*25*). The substrate-induced difference spectra of the microsomal fraction containing 0.33 μM P450 were measured in the presence of 3.1 μM 2,3,7-triCDD or 2,3,7,8-tetraCDD. The solution contains dimethyl sulfoxide (DMSO) at a final concentration of 2.0%.

Measurement of Catalytic Activity toward PCDDs. The substrates DD, 1-MCDD, and 2-MCDD were dissolved in ethanol, and 2,3-DCDD, 2,7-DCDD, and 2,3,7-TriCDD were dissolved in acetone, whereas 2,3,7,8-tetraCDD was dissolved in DMSO. The reaction mixture contains each PCDD at 0−10 μM with 1% of the organic solvent and the microsomes containing 10 or 33 nM of each human CYP in 50 mM potassium phosphate buffer, pH 7.4. On the metabolism by CYP2E1, DD, 1-MCDD, and 2-MCDD were dissolved in methanol, and 2,3-DCDD, 2,7-DCDD, and 2,3,7-triCDD were dissolved in DMSO. The final concentration of the organic solvents in the reaction mixtures containing CYP2E1 was 0.5%. For the metabolism of 2,3,7,8-tetraCDD, P450 concentration and 2,3,7,8-tetraCDD were adjusted to 0.10 and 0.78 μM, respectively. In the experiments to examine the inhibitory effect of 2,3,7,8-tetraCDD, both substrate and 2,3,7,8-tetraCDD were dissolved in DMSO, and the final concentration of DMSO was adjusted to 1%. The reaction was initiated by the addition of NADPH at a final concentration of 0.5 mM. Aliquots of the reaction mixture were collected after various time intervals and extracted with 4 volumes of chloroform/methanol (3:1). The organic phase was recovered and dried. The resulting residue was solubilized with acetonitrile and applied to HPLC performed on a YMC-Pack ODS-AM (4.6 mm i.d. × 300 mm) (YMC Co., Tokyo, Japan). The mobile phase was acetonitrile/0.01% TFA aqueous solution (50:50, v/v) for 5 min followed by a linear gradient of 50−100% acetonitrile for 15 min and 100% acetonitrile for 10 min. The flow rate was 1.0 mL/min, and the elution was detected by measuring absorption at 227 nm. The column temperature was maintained at 40 °C.

Table 1. Metabolism of PCDDs by 12 Forms of Human Cytochrome P450

	activity (mol/min/mol of P450)						
CYP	DD	1-MC	2-MC	2,3-DC	2,7-DC	2,3,7-TC	2,3,7,8-TC
1A1	1.0	3.4	10.3	18.4	20.0	44.7	–[b]
1A2	5.6	0.2	5.0	1.6	2.2	5.5	–
1B1	–	–	0.6	0.5	1.1	3.5	–
2A6	0.9	–	–	–	–	–	–
2B6	–	–	–	0.8	–	–	–
2C8	–	1.7	–	–	–	–	–
2C9	0.5	–	0.7	3.4	–	–	–
2C18	–	–	–	2.7	–	–	–
2C19	6.3	2.9	3.6	6.7	–	–	–
2D6	1.2	1.7	2.2	2.5	–	–	–
2E1	1.9	0.7	0.7	0.2	–	–	–
3A4	–	–	–	–	–	–	–

[a] Each value represents the total activity including multiple metabolites at a substrate concentration of 10 μM except for 2,3,7,8-tetraCDD (0.78 μM) as described under Materials and Methods. [b] –, not detected (<0.01).

Mass Spectrometric Analysis of the Metabolites. Isolated metabolites from HPLC effluents were subjected to GC-MS analysis using a Finnigan Mat Thermo Quest GC with EI mode. A Chrompack Cp-Sil 24CB-MS (0.32 mm × 30 m) was used as a GC column.

Ah Receptor Assay. The SV40 promoter region of the vector pGV-P (Toyo Ink Manufacturing Co. Ltd., Tokyo, Japan) was replaced with five tandem repeats of XRE (*26*) followed by the nucleotides −164 to +53 of rat glutathione *S*-transferase Ya subunit gene (*27*). The resultant plasmid was introduced into mouse hepatoma Hepa-1clc7 cells. The stable transformant was used for the Ah-receptor assay. After the addition of each PCDD and their metabolites to the cell culture, luciferase activity was measured by using the luciferase assay system (Promega Co., Madison, WI).

Other Methods. The concentrations of PCDDs and their metabolites were estimated by using a molar extinction coefficient of 4.12×10^4 M^{-1} cm^{-1} at 227 nm on the basis of spectral analysis of PCDDs. Protein concentration was determined according to the method of Lowry et al. (*28*), using bovine serum albumin as a standard.

RESULTS

Human CYP-Dependent Metabolism of PCDDs. The recombinant yeast microsomal fraction containing each human CYP was examined for the metabolism of PCDDs. The control microsomes with overexpression of yeast NADPH−P450 reductase showed no activity against any of the PCDDs examined in this study. Remarkable metabolism by multiple P450 forms was observed toward DD, 1-MCDD, 2-MCDD, 2,3-DCDD, 2,7-DCDD, and 2,3,7-triCDD as shown in **Table 1**. The metabolism contained multiple reactions such as hydroxylation at an unsubstituted position, hydroxylation with migration of a chloride substituent, and hydroxylation with elimination of a chloride substituent. Major CYPs in human liver such as CYP2C8, CYP2C9, and CYP3A4 showed no significant metabolism toward the PCDDs. On the other hand, CYP2C19 and

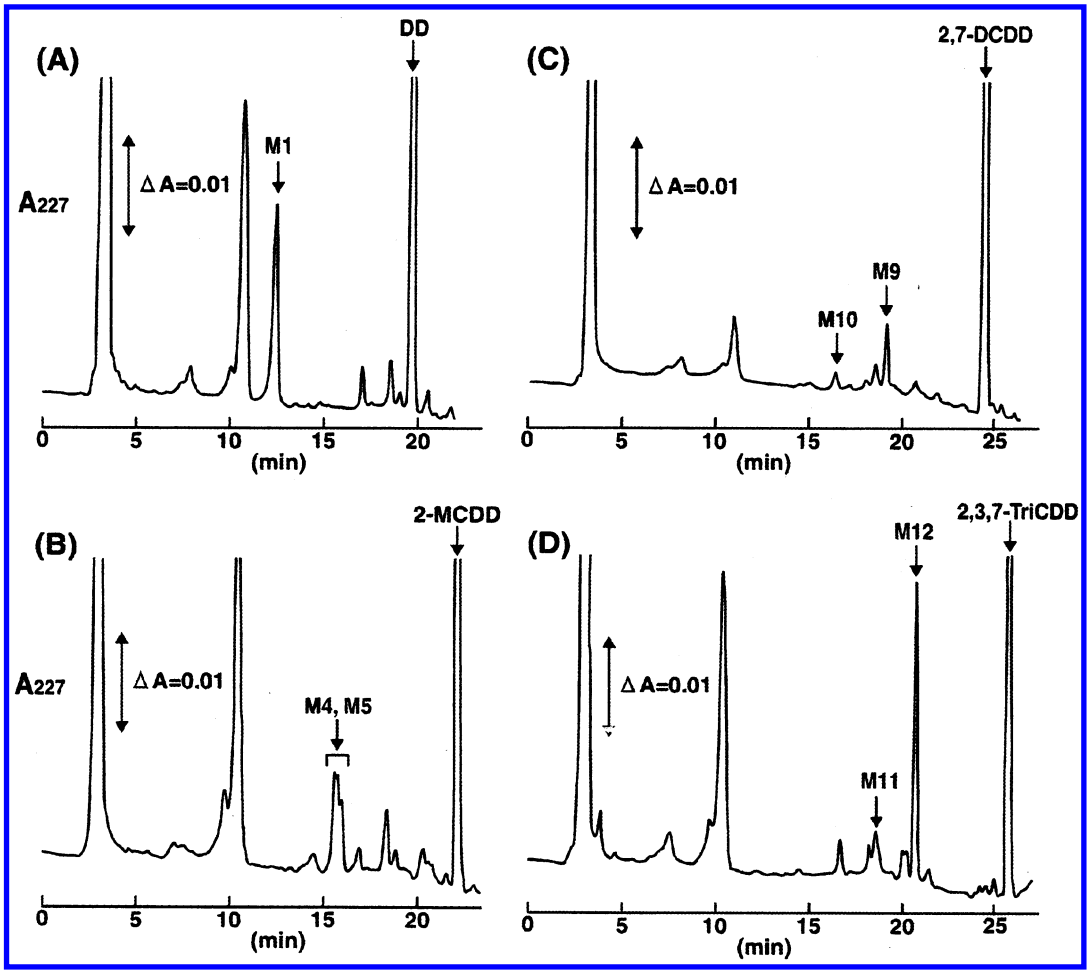

Figure 1. HPLC profiles of PCDDs and their metabolites: DD and its metabolite (**M1**) by human CYP1A2 (A); 2-MCDD and its metabolites (**M4** and **M5**) by human CYP1A1 (B); 2,7-DCDD and its metabolites by human CYP1A2 (C); 2,3,7-triCDD and its metabolites by human CYP1A2 (D). After reaction, the reaction mixture was extracted and analyzed by HPLC as described under Materials and Methods.

CYP2D6, which are known to present genetic polymorphism, showed high catalytic activities toward DD, 1-MCDD, 2-MCDD, and 2,3-DCDD. The CYPs in the CYP1 family, CYP1A1, CYP1A2, and CYP1B1, showed high catalytic activity toward DD and mono-, di-, and trichloroDDs. However, none of the CYPs showed detectable activity toward 2,3,7,8-tetraCDD.

Analysis of Metabolites of DD Produced by Human CYPs. **Figure 1A** shows HPLC analysis of the metabolite of DD by CYP1A2. One major metabolite (**M1**) was observed at a retention time of 12.3 min. The mass spectrum of the metabolite showed molecular ions at *m/z* 200 $(M)^+$, 171 $(M - COH)^+$, and 144 $(M - 2CO)^+$, suggesting that the metabolite was the hydroxylated product of DD. **Table 2** summarizes the relative intensities of the major ion fragments The same metabolite was observed in the reaction mixtures containing CYP1A1, CYP2A6, CYP2C9, CYP2C19, CYP2D6, or CYP2E1.

Analysis of Metabolites of 1-MCDD Produced by Human CYPs. In HPLC analysis, one major peak of a metabolite of 1-MCCD was observed in the metabolism by CYP1A1, CYP1A2, CYP2C8, CYP2C19, CYP2D6, and CYP2E1. However, the retention times of the metabolites were 14.4 min for CYP1A1 and CYP2C8 and 14.9 min for CYP1A2, CYP2C19, CYP2D6, and CYP2E1. The mass spectrum of the metabolite (**M2**) by CYP1A1 showed major molecular ions at *m/z* 234 (M with $^{35}Cl)^+$, 199 $(M - Cl)^+$, and 171 $(M - COCl)^+$, suggesting that the metabolite was a hydroxylated product of 1-MCDD (**Table 1**). On the other hand, the mass spectrum of the metabolite (**M3**) by CYP2C19 showed major molecular ions at *m/z* 234 (M with $^{35}Cl)^+$, 205 $(M - COH)^+$, and 178 $(M - 2CO)^+$. Although the mass spectra of two metabolites were clearly different, the relationship between the fragmentation pattern and the chemical structure has not been elucidated. Methylated compounds of both metabolites showed molecular ions at *m/z* 248 (M with $^{35}Cl)^+$, 233 $(M - CH_3)^+$, and 205 $(M - COCH_3)^+$ (**Table 3**). The presence of the $(M - CH_3)^+$ fragment indicates that the methoxy group is in a lateral (C-2, C-3, C-7, and C-8) position as described by Tulp and Hutzinger (*29*).

Table 2. Relative Intensities of Major Ion Fragments of Metabolites of PCDDs

no.	compound	*m/z*	M	M – COH	M – Cl	M – 2CO	M – COCl
M1	DD-OH	200	100	10	–[a]	19	–
M2	1-MCDD-OH	234	100	6	12	4	56
M3	1-MCDD-OH	234	100	16	6	28	22
M4	2-MCDD-OH	234	100	6	31	–	39
M5	2-MCDD-OH	234	100	12	6	18	14
M10	2-MCDD-OH	234	100	12	4	20	20
M6	2,3-DCDD-OH	268	100	4	62	–	62
M7	2,3-DCDD-OH	268	100	3	35	3	35
M8	2,3-DCDD-OH	268	100	12	11	21	24
M9	2,7-DCDD-OH	268	100	2	29	1	52
M11	3,7-DCDD-OH[b]	268	100	11	13	22	21
M12	2,3,7-triCDD-OH	302	100	–	27	–	44

[a] –, not detected. [b] Putative structure of **M11** based on the facts that **M11** is different from any **M6**, **M7**, **M8**, and **M9** by HPLC analysis.

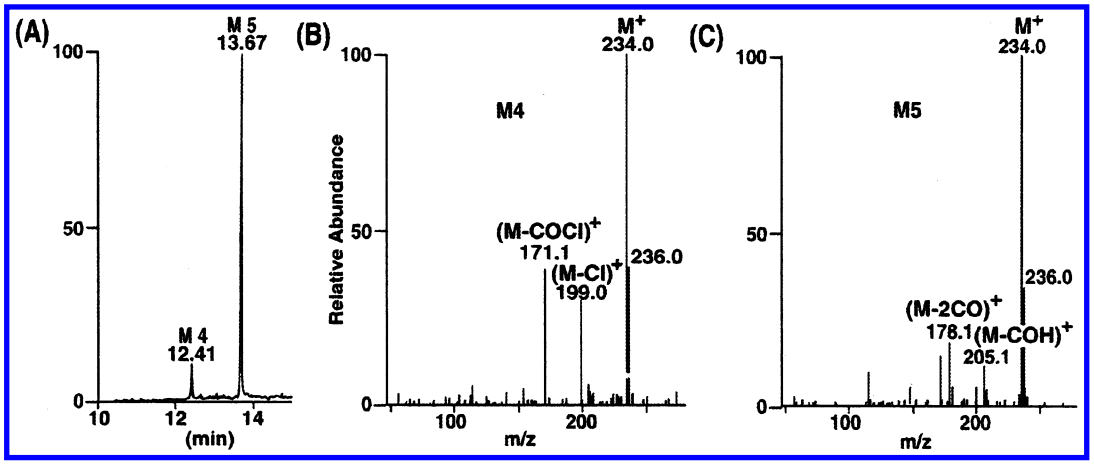

Figure 2. GC profile (*m/z* 233.5–234.5) of the metabolites of 2-MCDD by human CYP1A1 collected from HPLC effluent (A) and mass spectra of metabolites designated **M4** (B) and **M5** (C).

Table 3. Relative Intensities of Major Ion Fragments of the Methylated Metabolites and Putative Positions of OCH_3 Group

no.	compound	*m/z*	M	M – CH_3	M – $COCH_3$	position[a]
M2	1-MCDD-OCH_3	248	100	51	42	2,3,7,8
M3	1-MCDD-OCH_3	248	100	48	42	2,3,7,8
M4	2-MCDD-OCH_3	248	100	70	69	3,7,8
M5	2-MCDD-OCH_3	248	100	54	60	3,7,8
M(6,7,8)	2,3-DCDD-OCH_3[b]	282	100	95	51	7
M(6,7,8)	1,3-DCDD-OCH_3[b]	282	100	48	45	2
M9	2,7-DCDD-OH	282	100	48	48	3
M12	2,3,7-triCDD-OCH_3	316	100	69	60	8

[a] The presence of molecular ion $(M - CH_3)^+$ means that the OCH_3 group is in a lateral (C-2, C-3, C-7, and C-8) position. [b] The relationship between MS data and putative chemical structures might be reversed.

Analysis of Metabolites of 2-MCDD Produced by Human CYPs. Figure 1B shows the HPLC profile of the metabolites of 2-MCDD by CYP1A1. A broad peak at ~16 min including small peaks was observed. The metabolites at the broad peak were collected from HPLC effluents and analyzed by GC-MS. **Figure 2A** shows the GC profile of the metabolites. The mass spectrum of the metabolite (**M4**) by CYP1A1 showed molecular ions at *m/z* 234 (M with $^{35}Cl)^+$, 199 $(M - Cl)^+$, and 171 $(M - COCl)^+$ (**Figure 2**). On the other hand, the mass spectrum of metabolite **M5** showed molecular ions at *m/z* 234 (M with $^{35}Cl)^+$, 205 $(M - COH)^+$, and 178 $(M - 2CO)^+$ (**Figure 2**). Methylated compounds of both metabolites showed molecular ions at *m/z* 248 $(M)^+$, 233 $(M - CH_3)^+$, and 205 $(M - COCH_3)^+$ (**Table 3**). These results suggest that **M4** and **M5** contain a hydroxyl group at a distinct site in a lateral position.

Analysis of Metabolites of 2,3-DCDD Produced by Human CYPs. The HPLC profile of the metabolites of 2,3-DCDD by CYP1A1 showed a broad peak at ~17.5 min (data not shown). A similar HPLC pattern was observed in the metabolism of CYP1A2, CYP1B1, CYP2B6, CYP2C9, CYP2C18, CYP2C19, CYP2D6, and CYP2E1. The metabolites by CYP1A1 were collected from HPLC effluents and analyzed by GC-MS. The GC profile of the metabolites indicated the presence of three distinct metabolites designated **M6**, **M7**, and **M8** with the same molecular mass (**Table 2**). However, only two metabolites were detected after methylation. The methoxy group of both metabolites was in a lateral position, suggesting that one of the metabolite would arise via an NIH shift after enzymatic formation of an epoxide as described by Petroske et al. (*30*) (**Table 3**). It is noted that only two distinct metabolites are possible even if the NIH shift is considered. Further studies are needed to reveal the chemical structure of the third metabolite.

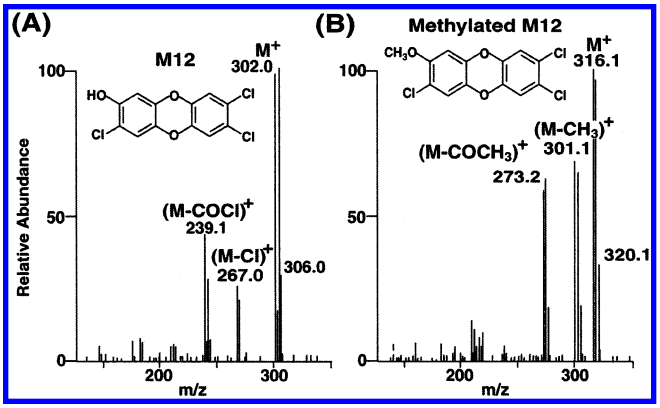

Figure 3. Mass spectra of the metabolite (**M12**) of 2,3,7-triCDD by human CYP1A2 (A) and of the methylated compound of **M12** (B). Putative structures of **M12** and the methylated **M12** are presented.

Analysis of Metabolites of 2,7-DCDD Produced by Human CYPs. HPLC analysis showed a metabolite of 2,7-DCDD designated **M9** by CYP1A1 (data not shown). The mass spectra of **M9** and its methylated compound suggested that **M9** was the hydroxylated product of 2,7-DCDD at the C-3 position (**Tables 2** and **3**), although the possibility of the hydroxylated product with migration of a chloride substituent could not be discarded. On the other hand, CYP1A2 showed another metabolite (**M10** in **Figure 1C**). The mass spectrum of **M10** was quite similar to that of **M5**. These results suggest that the metabolite contains a hydroxyl group with elimination of a chloride substituent of 2,7-DCDD, namely, 7-hydroxy-2-MCDD.

Analysis of Metabolites of 2,3,7-TriCDD Produced by Human CYPs. Figure 1D shows the HPLC profile of the metabolites of 2,3,7-triCDD by CYP1A2, indicating the presence of two metabolites designated **M11** and **M12**. Although the mass spectrum of **M11** was quite similar to that of **M8**, the metabolites **M11** and **M8** were clearly separated by HPLC. The mass spectra of **M12** and its methylated compound suggested that **M12** was the hydroxylated product of 2,3,7-triCDD at the C-8 position (**Figure 3**). On the metabolism of 2,3,7-triCDD by CYP1A1 and CYP1B1, the 8-hydroxylated product of 2,3,7-triCDD (**M12**) was observed as a metabolite, but **M11** was not detected (data not shown).

Substrate-Induced Difference Spectra of CYP1A1. Addition of 2,3,7-triCDD or 2,3,7,8-tetraCDD to the microsomal fraction containing CYP1A1 induced a typical type I spectrum

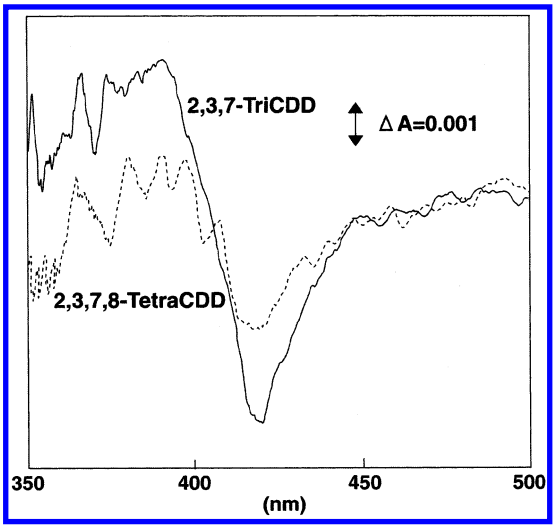

Figure 4. 2,3,7-TriCDD- (—) and 2,3,7,8-tetraCDD-induced (- - -) difference spectra of CYP1A1. The difference spectra of the microsomal fraction containing 0.33 μM P450 were measured in the presence of 3.1 μM 2,3,7-triCDD or 2,3,7,8-tetraCDD as described under Materials and Methods.

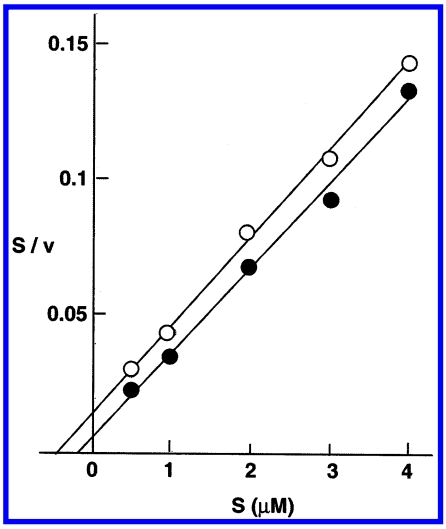

Figure 5. Hanes–Woolf plots (S/v vs S) of CYP1A1-dependent 8-hydroxylation of 2,3,7-triCDD in the presence (○) and absence (●) of 2,3,7,8-tetraCDD (0.78 μM). The units of S and v are μM and (mol/min/mol of P450), respectively.

Table 4. Kinetic Parameters for 2,7-DCDD 3-Hydroxylation Activity and 2,3,7-TriCDD 8-Hydroxylation Activity by Human CYP1A1 and CYP1A2

CYP	substrate	K_m^{app} [a] (μM)	V_{max}[a] (mol/min/mol of P450)
1A1	2,7-DCDD	0.24 ± 0.11	20.5 ± 6.5
1A1	2,3,7-TriCDD	0.30 ± 0.08	50.8 ± 2.7
1A2	2,7-DCDD	0.06 ± 0.01	2.2 ± 0.2
1A2	2,3,7-triCDD	0.23 ± 0.04	3.9 ± 0.7

[a] K_m^{app} and V_{max} values represent the mean ± SD from three separate experiments.

as shown in **Figure 4**, indicating a change of the heme iron of CYP1A1 from a low-spin state to a high-spin state upon binding of 2,3,7-triCDD and 2,3,7,8-tetraCDD. These results are consistent with the fact that human CYP1A1 purified from the recombinant yeast cells used in this study is a low-spin state based on the absolute spectrum (Dr. S. Imaoka, personal communication). The magnitude of absorbance difference between 390 and 420 nm for 2,3,7,8-tetraCDD was ~45% of that for 2,3,7-triCDD. The dissociation constant K_d for 2,3,7-triCDD was estimated to be 0.65 μM. It should be noted that 2,3,7,8-tetraCDD bound to the substrate-binding pocket of CYP1A1, whereas no detectable activity of CYP1A1 was observed toward 2,3,7,8-tetraCDD. On the other hand, no spectral change of CYP1A2 was observed with the addition of 2,3,7-triCDD and 2,3,7,8-tetraCDD, probably due to a high-spin form of CYP1A2 even in the absence of a substrate.

Kinetic Analysis of 3-Hydroxylation of 2,7-DCDD and 8-Hydroxylation of 2,3,7-TriCDD by CYP1A1 and CYP1A2. The apparent Michaelis constant designated K_m^{app} and V_{max} for the CYP1A1-dependent 3-hydroxylation activity toward 2,7-DCDD were estimated to be 0.24 μM and 20.5 mol/min/mol of P450, respectively. These values for the CYP1A1-dependent 8-hydroxylation activity toward 2,3,7-triCDD were estimated to be 0.30 μM and 50.8 mol/min/mol of P450, respectively (**Table 4**). On the other hand, the K_m^{app} and V_{max} values for the CYP1A2-dependent 3-hydroxylation activity toward 2,7-DCDD were estimated to be 0.06 μM and 2.2 mol/min/mol of P450, respectively, and those for the CYP1A2-dependent 8-hydroxylation activity toward 2,3,7-triCDD were 0.23 μM and 3.9 mol/min/mol of P450, respectively. These results indicate that both 2,7-DCDD and 2,3,7-triCDD are good substrates for CYP1A1 and CYP1A2.

Inhibitory Effect of 2,3,7,8-TetraCDD to 8-Hydroxylation of 2,3,7-TriCDD. Hanes−Woolf plots (S/v vs S) of the CYP1A1-dependent 8-hydroxylation of 2,3,7-triCDD in the presence and absence of 2,3,7,8-tetraCDD (0.78 μM) indicated the competitive inhibition by 2,3,7,8-tetraCDD (**Figure 5**). The apparent K_i value of 2,3,7,8-tetraCDD for the CYP1A1-dependent 8-hydroxylation of 2,3,7-triCDD was estimated to be 0.61 μM. These results strongly suggest that 2,3,7,8-tetraCDD binds to the substrate-binding pocket of CYP1A1 with considerably high affinity.

Ah Receptor Assay. Addition of 8-hydroxy-2,3,7-triCDD to the cell culture at a final concentration of 50 nM showed no effect on the luciferase activity, whereas 50 nM 2,3,7-triCDD clearly demonstrated a positive effect. It was indicated that the magnitude of the effect of 8-hydroxy-2,3,7-triCDD was <10% of that of 2,3,7-triCDD and 0.001% of that of 2,3,7,8-tetraCDD. These results indicated that 8-hydroxylation of 2,3,7-triCDD was a detoxication reaction.

DISCUSSION

To our knowledge, this is the first identification of metabolites of PCDDs by human CYPs. Remarkable metabolism by multiple P450 forms was observed toward dibenzo-*p*-dioxin (DD) and mono-, di-, and trichloroDDs. The metabolism contained multiple reactions such as hydroxylation at an unsubstituted position, hydroxylation with migration of a chloride substituent (NIH shift), and hydroxylation with elimination of a chloride substituent. From the results summarized in **Table 3**, the hydroxyl group is predominantly added to a lateral position of PCDDs in CYP-dependent metabolism. These results are quite similar to those obtained in rats as reported by Tulp and Hutzinger (*4*). Major CYPs in human liver such as CYP2C8, CYP2C9, and CYP3A4 did not show high catalytic activity toward PCDDs. The fact that CYP2C19 and CYP2D6 showed high activities toward DD, 1-MCDD, 2-MCDD, and 2,3-DCDD is pharmacologically interesting, because they are polymorphic with significant percentages of populations deficient. It was noted that CYP1A1 showed multiple metabolites of 2,3-DCDD,

whereas Tulp and Hutzinger (*4*) observed only one metabolite of 2,3-DCDD in rats. One of the metabolites was considered to be 2-hydroxy-1,3-DCDD arising via an NIH shift after enzymatic formation of an epoxide (**Table 3**). Although 2,3-DCDD was a good substrate for multiple P450 species, 2,7-DCDD was metabolized by only the CYP1 family. These results indicated that the presence of a chloride substituent in both rings of PCDDs greatly diminished the activity of CYPs except for the CYP1 family. Both CYP1A1 and CYP1A2 showed high catalytic activity toward DD and mono-, di-, and trichloroDDs. From the contents of CYP1A1, CYP1A2, and CYP1B1 in human liver, a major CYP for the metabolism of 2,7-triCDD and 2,3,7-tetraCDD in human liver appears to be CYP1A2. Surprisingly, the activity of CYP1A1 toward PCDDs increased the number of chloride substituents from 0 to 3. The K_m^{app} (= 0.30 μM) and V_{max} (= 50.8 mol/min/mol P450) values for the 2,3,7-triCDD 8-hydroxylation indicate that 2,3,7-triCDD is a good substrate for CYP1A1. It should be noted that 8-hydroxy-2,3,7-triCDD (= 2-hydroxy-3,7,8-triCDD) is one of the major metabolites of 2,3,7,8-tetraCDD found in bile of 2,3,7,8-tetraCDD-treated dogs (*6*). The Ah receptor assay demonstrated that the magnitude of the effect of 8-hydroxy-2,3,7-triCDD was <10% of that of 2,3,7-triCDD and 0.001% of that of 2,3,7,8-tetraCDD. These results indicated that 8-hydroxylation of 2,3,7-triCDD was a detoxication reaction. Remarkable metabolism showing hydroxylation with elimination of a chloride substituent was observed in the CYP1A2-dependent metabolism of 2,7-DCDD and 2,3,7-triCDD. Recently, Yanagita et al. (*31*) reported dehalogenation of chlorinated ethylenes and ethanes by rat CYP1A2 and its mutants under anaerobic conditions. To examine the possibility of the elimination of a chloride substituent by reduced heme iron of CYP1A2 without monooxygenation, metabolism of 2,7-DCDD or 2,3,7-triCDD by CYP1A2 was observed under N_2 gas. However, no metabolites were observed, suggesting that the elimination of a chloride substituent is closely linked to the monooxygenase activity of CYP1A2. The major metabolites of 2,3,7,8-TCDD in the rats and dogs were determined to be 2-hydroxy-1,3,7,8-tetraCDD and 2-hydroxy-3,7,8-triCDD (= 8-hydroxy-2,3,7-triCDD in this paper) (*32*). However, we have not detected a metabolite of 2,3,7,8-tetraCDD by any of the CYPs. From the detection limits, the catalytic activity of CYPs toward 2,3,7,8-tetraCDD is <0.01 mol/min/mol of P450. On the other hand, the substrate-induced difference spectra (**Figure 4**) demonstrated the binding of 2,3,7,8-tetraCDD to the substrate-heme pocket of CYP1A1. In addition, 2,3,7,8-tetraCDD showed competitive inhibition toward 8-hydroxylation of 2,3,7-triCDD with the apparent K_i value of 0.61 μM. Thus, it is strongly suggested that CYP1A1 can bind 2,3,7,8-tetraCDD, although it shows no detectable catalytic activity toward 2,3,7,8-tetraCDD. We have not observed spectral change of CYP1A2 by the addition of 2,3,7,8-tetraCDD, probably due to the high-spin form of CYP1A2 even in the absence of 2,3,7,8-tetraCDD. However, CYP1A2-dependent 2,3,7-triCDD 8-hydroxylation was also significantly inhibited by 2,3,7,8-tetraCDD (data not shown), suggesting that CYP1A2 could bind 2,3,7,8-tetraCDD. These results appear to be consistent with the finding that CYP1A2 is the sequestering protein for 2,3,7,8-tetraCDD in mice liver (*33*).

It was strongly suggested that dibenzo-*p*-dioxin (DD) and mono-, di-, and trichloroDDs examined in this study were nontoxic for humans because of the remarkable metabolism by human CYPs in addition to their low affinity for Ah receptor. On the other hand, the extreme toxicity of 2,3,7,8-tetraCDD appears to depend on its undetectable metabolism by human CYPs in addition to its high affinity for Ah receptor. Tai et al. (*34*) reported that human CYP1A1 showed much lower activity toward 2,3,7,8-tetrachlorodibenzofuran (TCDF) than rat CYP1A1. Thus, it might be possible to assume that a remarkable species difference between humans and rats in the half-life of 2,3,7,8-TCDD is mainly due to the difference of enzymatic properties of the CYP1 family, although further analysis is needed.

SAFETY

2,3,7,8-TetraCDD is known to be extremely toxic. Thus, appropriate measures should be used to minimize exposure during handling of 2,3,7,8-tetraCDD.

ABBREVIATIONS USED

P450 or CYP, cytochrome P450; PCDD, polychlorinated dibenzo-*p*-dioxin; MCDD, monochlorodibenzo-*p*-dioxin; DCDD, dichlorodibenzo-*p*-dioxin; triCDD, trichlorodibenzo-*p*-dioxin; tetraCDD, tetrachlorodibenzo-*p*-dioxin.

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LITERATURE CITED

(1) van Leeuwen, F. X. R.; Feely, M.; Shrenk, D.; Larson, J. C.; Farland, W.; Younes, M. Dioxins: WHO's tolerable daily intake (TDI) revisited. *Chemosphere* **2000**, *40*, 1095−1101.

(2) Huwe, J. K. Dioxins in foods: A modern agricultural perspective. *J. Agric. Food Chem.* **2002**, *50*, 1739−1750.

(3) Rose, J. Q.; Ramsey, J. C.; Wentzler, T. H.; Hunmmel, R. A.; Gehring, P. J. The fate of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin following single and repeated oral doses to the rat. *Toxicol. Appl. Pharmacol.* **1976**, *36*, 209−226.

(4) Tulp, M. Th. M.; Hutzinger, O. Rat metabolism of polychlorinated dibenzo-*p*-dioxins. *Chemosphere* **1978**, *9*, 761−768.

(5) Poiger, H.; Buser, H. R.; Weber, H.; Zweifel, U.; Schlatter, C. Structure elucidation of mammalian TCDD-metabolites. *Experientia* **1982**, *38*, 484−486.

(6) Wroblewski, V. J.; Olson, J. R. Hepatic metabolism of 2,3,7,8-tetrachlorobibenzo-*p*-dioxin (TCDD) in the rat and guinea pig. *Toxicol. Appl. Pharmacol.* **1985**, *81*, 231−240.

(7) Van den Berg, M.; Jongh, J.; Poiger, H.; Olson, J. R. The toxicokinetics and metabolism of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) and their relevance for toxicity. *Crit. Rev. Toxicol.* **1994**, *24*, 1−74.

(8) Hu, K.; Bunce, N. J. Metabolism of polychlorinated dibenzo-*p*-dioxins and related dioxin-like compounds. *J. Toxicol. Environ. Health B* **1999**, *2*, 183−210.

(9) Huwe, J. K.; Freil, V. J.; Larson, G. L.; Wiener, C. Metabolism and disposition oof 1,4,7,8-tetrachlorodibenzo-*p*-dioxinin rats. *Chemosphere* **1998**, *37*, 1885−1893.

(10) Hu, K.; Bunce, N. J. Metabolism of polychlorinated dibenzo-*p*-dioxins by rat liver microsomes. *J. Biochem. Mol. Toxicol.* **1999**, *13*, 307−315.

(11) Pircle, J. L.; Wolfe, W. H.; Patterson, D. G. Estimates of the half-life of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in Vietnam veterans of operation Ranch Hand. *J. Toxicol. Environ. Health* **1989**, *27*, 165−171.

(12) Poiger, H.; Schlatter, C. Pharmacokinetics of 2,3,7,8-TCDD in man. *Chemosphere* **1986**, *15*, 1489−1494.
(13) Abraham, K.; Weberruss, U.; Wisemuller, T.; Hagenmainer, H.; Krowke, R.; Neupert, D. Comparative studies on absorption and distribution in the liver and adipose tissue of PCDDs and PCDFs in rats and marmoset monkeys. *Chemosphere* **1989**, *19*, 887−895.
(14) Kochiba, R. J.; Keyers, D. G.; Beyer, J. E.; Carreon, R. M.; Wade, C. E. Results of a two year chronic toxicity and oncogeneity study of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats. *Toxicol. Appl. Pharmacol*. **1978**, *46*, 279.
(15) Abraham, K.; Krowke, R.; Neupert, D. Pharmacokinetics and biological activity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. I. Dose-dependent tissue distribution and induction of hepatic ethoxyresorufin *O*-deethylase in rats following a single injection. *Arch. Toxicol*. **1988**, *62*, 359−409.
(16) Oeda, K.; Sakaki, T.; Ohkawa, H. Expression of rat liver cytochrome P-450MC cDNA in *Saccharomyces cerevisiae*. *DNA* **1985**, *4*, 203−210.
(17) Imaoka, S.; Yamada, T.; Hiroi, T.; Hayashi, K.; Sakaki, T.; Yabusaki, Y.; Funae, Y. Multiple forms of human P450 expressed in *Saccharomyces cerevisiae*. *Biochem. Pharmacol*. **1996**, *51*, 1041−1050.
(18) Hayashi, K.; Sakaki, T.; Kominami, S.; Inouye, K.; Yabusaki, Y. Coexpression of genetically engineered fused enzyme between yeast NADPH-P450 reductase and human cytochrome P450 3A4, and human cytochrome b_5 in yeast. *Arch. Biochem. Biophys*. **2000**, *381*, 164−170.
(19) Sakaki, T.; Inouye, K. Practical application of mammalian cytochrome P450. *J. Biosci. Bioeng*. **2000**, *90*, 583−590.
(20) Murakami, H.; Yabusaki, Y.; Sakaki, T.; Shibata, M.; Ohkawa, H. Expression of cloned yeast NADPH-cytochrome P450 reductase gene in *Saccharomyces cerevisiae*. *J. Biochem*. **1990**, *108*, 859−865.
(21) Inouye, K.; Mae, T.; Kondo, S.; Ohkawa, H. Inhibitory effects of vitamin A and vitamin K on rat cytochrome P4501A1-dependent monooxygenase activity. *Biochem. Biophys. Res. Commun.* **1999**, *262*, 565−569.
(22) Kondo, S.; Sakaki, T.; Ohkawa, H.; Inouye, K. Electrostatic interaction between cytochrome P450 and NADPH-P450 reductase: Comparison of mixed and fused systems consisting of rat cytochrome P4501A1 and yeast NADPH-P450 reductase. *Biochem. Biophys. Res. Commun.* **1999**, *257*, 273−278.
(23) Inouye, K.; Mizokawa, T.; Saito, A.; Tonomura, B.; Ohkawa, H. Biphasic kinetic behavior of rat cytochrome P4501A1-dependent monooxygenation in recombinant yeast microsomes. *Biochim. Biophys. Acta* **2000**, *1481*, 265−272.
(24) Inouye, K.; Saito, A.; Orita, M.; Tonomura, B.; Imaishi, H.; Ohkawa, H. Inhibitory effects of 1,4-naphthoquinone derivatives on rat cytochrome P4501A1-dependent monooxygenation in recombinant yeast microsomes. *J. Biochem.* **2000**, *127*, 1041−1046.
(25) Inouye, K.; Kondo, S.; Yamamura, M.; Nakanishi, D.; Sakaki, T. Inhibitory effects of detergents on rat CYP1A1-dependent monooxygenase: Comparison of mixed and fused systems consisting of rat CYP1A1 and yeast NADPH-P450 reductase. *Biochem. Biophys. Res. Commun*. **2001**, *280*, 1346−1351.
(26) Sogawa, K.; Fujisawa-Sehara, A.; Yamane, M.; Fujii-Kuriyama, Y. Location of regulatory elements responsible for drug induction in the rat cytochrome P-450c gene. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8044−8048.
(27) Rushmore, T. H.; King, R. G.; Paulson, K. E. Regulation of glutathione S-transferase Ya subunit gene expression: Identification of a unique xenobiotic-responsive element controlling inducile expression by planar aromatic compounds. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3826−3830.
(28) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **1951**, *193*, 265−275.
(29) Tulp, M. Th. M.; Hutzinger, O. Identification of hydroxylated chlorodibenzo-*p*-dioxins, chlorodibenzofyrans, chlorodiphenyl ethers, and chloronaphthalenes as their methyl ethers by gas chromatography mass spectrometry. *Biomed. Mass Spectrom*. **1978**, *5*, 224−231.
(30) Petroske, E.; Feil, V. J.; Larsen, G. L. Identification of NIH-shifted metabolites of 1,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat by NMR comparison with synthesized isomers. *Chemosphere* **1997**, *34*, 1549−1555.
(31) Yanagita, K.; Sagami, I.; Shimizu, T. Distal site and surface mutations of cytochrome P450 1A2 markedly enhance dehalogenation of chlorinated hydrocarbons. *Biochem. Biophys. Res. Commun*. **1997**, *346*, 269−276.
(32) Mason, G.; Safe, S. Synthesis, biologic and toxic effects of the major 2,3,7,8-tetrachlorodibenzo-*p*-dioxin metabolites in the rat. *Toxicology* **1986**, *41*, 153−159.
(33) Diliberto, J. J.; Burgin, D. E.; Birnbaum, L. S. Effects of CYP1A2 on disposition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin 2,3,4,7,8-pentachlorodibenzofuran, and 2,2′,4,4′,5,5′-hexachlorobiphenyl in CYP1A2 knockout and parental (C57BL/6N and 129/Sv) strains of mice. *Toxicol. Appl. Pharmacol*. **1999**, *159*, 52−64.
(34) Tai, H. L.; McReynolds, J. H.; Goldstein, J. A.; Eugster, H. P.; Sengstag, C.; Alworth, W. L.; Olson, J. R. Cytochrome P4501A1 mediates the metabolism of 2,3,7,8-tetrachlorodibezofuran in the rat and human. *Toxicol. Appl. Pharmacol*. **1993**, *123*, 34−42.

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