Interconversion of Hydroxylated and Methoxylated Polybrominated Diphenyl Ethers in Japanese Medaka

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Polychlorinated dibenzofurans (PCDFs), hydroxylated (OH) and methoxylated (MeO), have been widely detected in aquatic environments. However, relationships among these structurally related compounds in exposed organisms are unclear. To elucidate biotransformation relationships among BDE-47, 6-OH-BDE-47, and 6-MeO-BDE-47, dietary accumulation, maternal transfer, and tissue distribution of these compounds and their transformation products were investigated in sexually mature Japanese medaka (Oryzias latipes). In addition, transformation of each compound was determined in vitro using liver microsomes of medaka. OH-PBDEs and MeO-PBDEs were not detected in fish exposed to BDE-47. However, significant concentrations of 6-OH-BDE-47 were detected in medaka or microsomes exposed to 6-MeO-BDE-47. Significant concentrations of 6-MeO-BDE-47 were also measured in fish exposed to 6-OH-BDE-47, but 6-MeO-BDE-47 was not detected in microsomes exposed to 6-OH-BDE-47. Similar patterns of transformation products were observed in medaka eggs from adult fish during exposure. This study presents direct in vivo evidence of biotransformation of 6-MeO-BDE-47 to 6-OH-BDE-47. In addition, this is the first study to demonstrate biotransformation of 6-OH-BDE-47 to 6-MeO-BDE-47. Demethylation of 6-MeO-BDE-47 was the primary transformation pathway leading to formation of 6-OH-BDE-47 in medaka, while the previously hypothesized formation of OH-PBDEs from synthetic BDE-47 did not occur. Biotransformation products formed in adult female medaka were transferred to eggs.

Introduction

Over the course of the past decade, brominated flame retardants (BFRs) have emerged as persistent organic pollutants of concern (1). Among the different classes of BFRs, polychlorinated diphenyl ethers (PCDFs) have received the greatest attention, mostly due to their widespread use, ubiquitous environmental distribution, and bioaccumulation potential (1). Recently, focus has shifted to structural analogues of PBDEs, such as hydroxylated (OH) and methoxylated (MeO) PBDEs, concentrations of which in marine sponges, algae, and mussels in some marine systems can exceed those of PBDEs (2–4). The occurrence of OH-PBDEs is of particular interest since, for some endpoints, they are more potent than PBDEs or MeO-PBDEs (5, 6). Effects of OH-PBDEs on organisms include disruption of thyroid hormone homeostasis, disruption of oxidative phosphorylation, altered estradiol synthesis, and neurotoxicity (5, 7–10). Relatively great ratios of transfer of OH-PBDEs from parent to offspring have been reported for pregnant women (11) and wild fish, such as the Chinese sturgeon (Acipenser sinensis) (12).

Several origins of OH-PBDEs and MeO-PBDEs have been postulated (13, 14). It has been suggested that ortho-substituted OH-PBDEs and MeO-PBDEs are formed from naturally occurring compounds in marine ecosystems (2, 3). Two abundant congeners of MeO-PBDEs, 6-MeO-BDE-47 and 2′-MeO-BDE-68, have been reported to be natural products of marine organisms (3). Similarly, ortho-substituted OH-PBDEs are produced naturally by marine algae or associated microorganisms (2, 4). It has been suggested that MeO-PBDEs are formed via methylation of OH-PBDEs. This pathway was hypothesized on the basis of knowledge of bacterial methylation of phenols in the environment (3, 15, 16). Alternatively, the similarity in structure between these compounds and synthetic PBDEs has led to suggestions that meta-/para-substituted OH-PBDEs and MeO-PBDEs could originate from biotransformation of synthetic PBDEs (17–19). Several in vitro studies have demonstrated that OH-PBDEs can be biotransformation products of PBDEs in fish, rat, and human cells (17–19). However, concentrations of OH-PBDEs detected in laboratory studies were extremely small compared to the ratios of concentrations of OH-PBDEs and MeO-PBDEs to PBDEs in marine organisms (12, 13, 17–19). On the basis of these results, the occurrence of OH- and MeO-PBDEs in wild organisms cannot be explained by formation from synthetic PBDEs alone. This information is consistent with the existence of sources of both OH-PBDEs and MeO-PBDEs other than synthetic PBDEs. On the basis of in vitro exposures using rainbow trout, chicken, and rat microsomes, it was reported that demethylation of naturally occurring MeO-PBDEs is a contributor of OH-PBDEs in wildlife from remote areas (13).

While the results of in vitro studies provided insight into biotransformation relationship(s) between PBDEs, MeO-
Materials and Methods

Preparation of Diet. Commercial fish food (Nutrafin Basix Staple Food, Rolf C. Hagen Inc. Canada) was ground with a mortar and pestle, weighed into a glass flask, and spiked with known amounts of each target compound (BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47) dissolved in 150 mL of acetone. The flask was shaken for 30 min to ensure thorough mixing of the food and chemicals. Following mixing, the contents of the flasks were concentrated to dryness in a rotary evaporator. The resulting spiked food was air-dried for 3 days prior to use.

Preparation of Diet. Commercial fish food (Nutrafin Basix Staple Food, Rolf C. Hagen Inc. Canada) was ground with a mortar and pestle, weighed into a glass flask, and spiked with known amounts of each target compound (BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47) dissolved in 150 mL of acetone. The flask was shaken for 30 min to ensure thorough mixing of the food and chemicals. Following mixing, the contents of the flasks were concentrated to dryness in a rotary evaporator. The resulting spiked food was air-dried for 3 days prior to use.

Feeding Study. Prior to initiation of exposure, 5-month-old medaka (mean weight: 0.60 ± 0.08 g, 8 females and 4 males per tank) were randomly assigned to 10 L tanks (4 females and 2 males per tank) and allowed to acclimate to the experimental tanks for 3 days prior to the initiation of exposure. Two replicate tanks were used for each exposure. Each day, approximately 50% of the water volume in the tanks was replaced. Water temperatures were maintained at 23–24 °C during the course of the experiment. Other details of the animals are provided in the Supporting Information.

Medaka were fed diets of food spiked with BDE-47, 6-MeO-BDE-47, 6-OH-BDE-47, or the carrier solvent acetone alone (vehicle control). This method provided an effective non-invasive means of administering the chemicals without stressing the fish. Dietary exposure was deemed a more realistic route of exposure than intraperitoneal injections (20). Fish were fed approximately 2% of their average body weight per day, half the daily food was provided in the morning and the other half in the afternoon. All fish displayed vigorous eating behavior, and the food was completely consumed in each tank.

Medaka were exposed to each target chemical for 14 days, and eggs were collected each morning (day 0–14) during the exposure period. After collected eggs from each individual tank were rinsed with Nanopure (Barnsted) water and gently dried on Kimwipes, the mass of each egg was determined, and then, eggs from each exposure were composited and stored separately at −20 °C until analysis. On day 14, following egg collection, six female fish were sampled from each treatment tank and sacrificed for the determination of target chemicals. The liver was dissected from each fish, and both liver and the liver-free residual carcass were weighed and stored at −20 °C until analysis.

To confirm the results of the in vivo feeding study, transformation of BDE-47, 6-MeO-BDE47, and 6-OH-BDE-47 was investigated using microsomes isolated from livers of female medaka obtained from the same breeding colony as those animals used in the feeding study, according to the method of Kennedy and Jones (21). The details of the methods used for microsomal incubations are described elsewhere (13) and provided in the Supporting Information.

Sample Extraction and Cleanup. The methods used to quantify 6-OH-BDE-47 have been published previously (22), and details of the chemicals, instrumental analysis, quality assurance, and quality control are provided in the Supporting Information. For this study, quantifications of BDE-47 and 6-MeO-BDE-47 were incorporated in the same method. Liver (∼0.02 g), egg (∼0.2 g), fish food (∼0.2 g), and a microsomal incubation mixture (250 µL) were homogenized and transferred into amber tubes. After spiking with surrogate recovery standards, 2 mL of Nanopure water (18 MΩ), 50 µL of hydrochloric acid (HCl, 37%), and 3 mL of 3-propanol were added to the samples. Samples were extracted three times with 3 mL of hexane/methyl tert-butyl ether (MTBE; 1:1; v/v). To remove residual acid, extracts were washed four times with 4 mL aliquots of pure water. Samples were concentrated and dried under nitrogen. For whole fish (about 0.6 g), the extraction process was the same as described above, except that the amount of each solvent used was 10-fold greater to accommodate the greater mass.

Dried residues were dissolved in 200 µL of aqueous sodium bicarbonate (100 mM/L, pH adjusted to 10.5 with sodium hydroxide), and 200 µL of dansyl chloride (1 mg/mL in acetone) was added. After vortex mixing for 1 min, the samples were incubated at 60 °C for 5 min. Once the samples cooled, 1 mL of pure water and 3 × 3 mL of hexane were added, and the organic solvent layer was removed and transferred onto a silica gel column (60–100 mesh size) for fractionation. The silica gel column was wet packed with 4 g of silica gel and 4 g of sodium sulfate. After application of the extract, the column was eluted with 15 mL of hexane/dichloromethane (DCM; 1:1, v/v) and, then, 20 mL of DCM. The first fraction was evaporated to dryness and reconstituted with 50 µL of nonane for high-resolution gas chromatography combined with high-resolution mass spectrometry (HRGC-HRMS) analysis of PBDEs (BDE-28, BDE-47, BDE-99, BDE-100, BDE-119, BDE-154, and BDE-153) and MeO-PBDEs (6-MeO-BDE-17, 4-MeO-BDE-17, 2′-MeO-BDE-68, 6-MeO-BDE-47, 5-MeO-BDE-47, and 4′-MeO-BDE-49). The second fraction was evaporated to dryness and reconstituted with 50 µL of acetonitrile/water (60:40) for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of OH-PBDEs (3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4′-OH-BDE-49, and OH-triBDEs).

Results

Purity of Stock Solutions and Food Preparations. 6-OH-BDE-47 was not present as an impurity in stock solutions of BDE-47 or 6-MeO-BDE-47. A negligible con-
concentration of 6-MeO-BDE-47 (4.3 ng/g) was detected as an impurity (0.002%) in the 6-OH-BDE-47 stock standard solution (Table 1). BDE-47 was detected as an impurity in both stock solutions of 6-OH-BDE-47 (0.12%) and 6-MeO-BDE-47 (0.36%). Concentrations of the parent compounds in prepared food were 900, 8000, and 2100 ng/g dry weight for 6-OH-BDE-47, 6-MeO-BDE-47, and BDE-47, respectively. 6-OH-BDE-47 was not present as an impurity in either the BDE-47 or the 6-MeO-BDE-47 spiked food. Concentrations of BDE-47 in the 6-MeO-BDE-47 and 6-OH-BDE-47 food preparations were 28.3 and 15 ng/g, respectively. Trace amounts of 6-MeO-BDE-47 (0.1–0.2 ng/g) were detected in the control (acetone spiked), 6-OH-BDE-47, and BDE-47 food preparations. The presence of 6-MeO-BDE-47 and BDE-47 as impurities in stock solutions and prepared food did not affect conclusions drawn from the studies.

Concentrations and Distributions of Target Compounds in Medaka. Of all the screened compounds, only BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47 were detected in tissues from medaka exposed separately to individual chemicals for 2 weeks (Figure 1, Figure S1 in Supporting Information). Concentrations of BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47 were 71 ng/g ww; liver-free residual carcass: 9.3 ng/g ww; liver: 25 ng/g ww; liver-free residual carcass: 14 ng/g ww; liver-free residual carcass: 9.4 ng/g ww; and liver-free residual carcass: 9.4 ng/g ww.

FIGURE 1. Concentrations of BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47 in liver (Liver) and liver-free residual carcass (Whole Fish-Liver) tissue from female Japanese medaka after 14 days of dietary exposure to feed spiked with solvent (control), BDE-47, 6-MeO-BDE-47, or 6-OH-BDE-47. The numbers above the error bars are the average concentrations of target compounds (ng/g ww).

to 6-OH-BDE-47 were significantly greater than concentrations in medaka that were unexposed or exposed to BDE-47 (p < 0.01, Figure 1 b). Comparable concentrations of BDE-47 were observed in female medaka exposed to 6-MeO-BDE-47 (liver: 25 ± 19 ng/g ww; liver-free residual carcass: 14 ± 5.6 ng/g ww) and 6-OH-BDE-47 (liver: 19 ± 20 ng/g ww; liver-free residual carcass: 9.4 ± 10 ng/g ww; Figure 1c).

Distributions of each of the three chemicals either as exposed compound or transformation product were different among liver, egg, and whole fish (Table S2 in Supporting Information). Concentration ratios of 6-OH-BDE-47 between liver and whole fish (L/F) were 6.1–6.9, which was greater than those of 6-MeO-BDE-47 (1.2) or BDE-47 (1.1). Concentration ratios between egg and liver (E/L) were 0.74–0.76 and 0.93 for 6-MeO-BDE-47 and BDE-47, respectively, which were comparable to those between egg and whole fish (E/F) of 6-MeO-BDE-47 (0.62 and 0.72) and BDE-47 (0.92). However, greater E/F ratios (0.34 and 1.8) compared to E/L ratios (0.23 and 0.59) were observed for 6-OH-BDE-47. Significantly greater maternal transfer ratios were observed for 6-OH-BDE-47 (0.59–1.8) as a parent compound than as a transformation product (0.23–0.34).

Accumulation in Eggs. Each of the exposure compounds and their transformation products were detected in eggs collected during the exposure period. Daily rates of egg mass production were 0.17 ± 0.05, 0.14 ± 0.04, 0.19 ± 0.03, and 0.18 ± 0.06 g ww for control, BDE-47-exposed, 6-MeO-BDE-47-exposed, and 6-OH-BDE-47-exposed during the exposure period, respectively. The slopes of the accumulation trend lines, as a function of exposure duration during the initial 6 days of the exposure, were 1.94, 1.44, and 0.73 for 6-MeO-BDE-47, BDE-47, and 6-OH-BDE-47, respectively (Figure 2). Accumulation of 6-OH-BDE-47 and 6-MeO-BDE-47 in eggs of female Japanese medaka during the course of a 14-day dietary exposure to feed spiked with BDE-47, 6-MeO-BDE-47, or 6-OH-BDE-47. C_{egg} is the concentration of target compounds in fish eggs, and C_{food} is the concentration in dosing food. At each of the sampling time points, all eggs from the individual experimental tanks were collected for analysis.
Accumulation of Exposed Compounds and Biotransformation Products. Significant concentrations of 6-OH-BDE-47 were measured in medaka exposed to 6-MeO-BDE-47 but not BDE-47 (Figures 1a and S1 and Table S1 in Supporting Information). Previous studies have indicated that an important prerequisite for exposure studies is ensuring standards and prepared exposure media are free of impurities that could lead to false results (23, 24). The analysis of purity suggested that 6-OH-BDE-47 was not a trace contaminant in any of the stock solutions or media employed in this study. Thus, 6-OH-BDE-47 measured in fish and eggs from these treatment groups originated from biotransformation of exposed parent compounds. This is consistent with results from previous in vitro studies (13), further supporting the hypothesis that 6-MeO-BDE-47 is a contributor to formation of 6-OH-BDE-47.

6-MeO-BDE-47 was also observed to be formed from 6-OH-BDE-47 in medaka. Detection of trace concentrations of 6-MeO-BDE-47 in all prepared food was not surprising (Table 1), since the ingredients of the commercial fish food include fish, plankton, and shrimp, which could contain MeO-PBDEs of natural origin (3). Assuming that the dosing level did not influence the food accumulation factors, the accumulation factors of fish exposed to parent 6-MeO-BDE-47 were used to calculate concentrations of impurity (6-MeO-BDE-47) accumulated in fish in other treatments. Concentrations of 6-MeO-BDE-47 in liver-free residual carcass were calculated to be 0.06 ± 0.02 ng/g ww with dosing concentrations of 0.2 ng/g in food, which is similar to those of fish exposed to BDE-47 (0.1 ± 0.03 ng/g ww) and in controls (0.06 ± 0.02 ng/g ww). In addition, concentrations of 6-MeO-BDE-47 in medaka exposed to 6-OH-BDE-47 (liver-free residual carcass: 12.01 ± 3.58 ng/g ww) were significantly greater than those in unexposed and medaka exposed to BDE-47 (p < 0.01, Figure 1b). A second feeding study was performed with 6-OH-BDE-47 in order to verify this observation. Consistent with the first study, significant concentrations of 6-MeO-BDE-47 compared to the control group were observed. This result confirmed that 6-MeO-BDE-47 is formed as a biotransformation product of 6-OH-BDE-47. Previous studies have suggested that some MeO-PBDEs are formed via methylation of OH-PBDEs (3, 16). The results presented here are the first experimental evidence of in vivo transformation of a MeO-PBDE from an OH-PBDE. To further confirm this observation, in vitro transformation of 6-OH-BDE-47 by microsomes isolated from female medaka was assessed (Table S1, Supporting Information). Consistent with previous in vitro studies (13), biotransformation of 6-OH-BDE-47 to 6-MeO-BDE-47 was not observed (Table S1, Supporting Information). Taken together, these results suggest that 6-OH-BDE-47 can be transformed to 6-MeO-BDE-47 in Medaka, but the conversion does not occur in the hepatic microsomal fraction under the conditions of this experiment.

Comparable concentrations of BDE-47 were observed in female medaka exposed to 6-MeO-BDE-47 and 6-OH-BDE-47 (Figure 1c), which is likely due to BDE-47 impurities in the stock standard solutions. Using the method described above, concentrations of BDE-47 in liver-free residual carcass were calculated to be 7.0 ± 1.9 and 2.7 ± 1.0 ng/g ww for dosing concentrations of 28.3 and 15 ng/g ww in food, respectively. The calculated concentrations were comparable to those in fish exposed to 6-MeO-BDE-47 (liver-free residual carcass: 13.5 ± 5.6 ng/g ww) and 6-OH-BDE-47 (9.4 ± 10.0 ng/g ww). The fact that significant concentrations of neither 6-OH-BDE-47 nor 6-MeO-BDE-47 were detected in female medaka exposed to BDE-47 is consistent with some previous in vitro observations that some PBDE congeners are not biotransformed into either OH-PBDEs or MeO-PBDEs in rainbow trout, Chinese sturgeon, salmon, chicken, beluga whale, or rat microsomes (12, 13, 25, 26).

Significant differences in distributions of parent compounds and their biotransformation products were observed among tissues. The relatively large L/F ratios for 6-OH-BDE-47 are consistent with its preferential accumulation in liver compared to the other compounds studied. This result is also consistent with the tissue distribution of each target compound examined in Chinese green sturgeon, in which the greatest concentrations of 6-OH-BDE-47 were found in liver, while 6-MeO-BDE-47 and BDE-47 preferentially accumulated in adipose tissue (12). The fact that the E/L ratio for 6-OH-BDE-47 was greater than the E/F ratio is also mainly due to the preferential accumulation of 6-OH-BDE-47 in liver. The greater maternal transfer ratios (E/L and E/F ratios) for 6-OH-BDE-47 as a transformation product could be a result of the more than 10-fold lesser concentrations of 6-OH-BDE-47 formed as a transformation product. These results are consistent with reports that maternal transfer ratios of brominated flame retardants in zebrafish are dose dependent, with greater ratios observed in individuals exposed to greater concentrations of the parent material (27).

Accumulation of Exposed Compounds and Biotransformation Products in Eggs. Chemical-specific accumulation trends were observed for each exposed compound over the course of the exposure period (Figure 2). In contrast to 6-OH-BDE-47, relatively great assimilation efficiencies were observed for 6-MeO-BDE-47 and BDE-47 as indicated by the
steep slopes for accumulation during the initial 6 days of exposure. Accumulation of BDE-47 did not reach steady state, and previous studies have reported that, when zebrafish (Danio rerio) were exposed to BDE-47, eggs required 60 days to reach steady state (27). On the basis of the slow assimilation rate and large concentration ratios between fish and feed, it can be inferred that the depuration rate of BDE-47 is likely less than that of 6-MeO-BDE-47 (28).

The observation that both 6-OH-BDE-47 and 6-MeO-BDE-47 occurred in eggs as biotransformation products of 6-MeO-BDE-47 and 6-OH-BDE-47, respectively, while neither transformation product was detected in eggs collected from medaka exposed to BDE-47 is consistent with profiles of transformation products in liver and liver-free carcass (Figure 1). This observation is consistent with the hypothesis that OH-PBDEs are formed from MeO-PBDEs but not from PBDEs. It is also consistent with OH-PBDEs being precursors of MeO-PBDEs in vivo. However, the times required to reach steady state for 6-MeO-BDE-47 and 6-OH-BDE-47 as biotransformation products in eggs were different (Figure 3). The linear increase in concentration of 6-MeO-BDE-47 in eggs through biotransformation could be due to larger biotransformation ratios (M/P ratio) and/or maternal transfer of 6-MeO-BDE-47 as a transformation product (Table S2 in Supporting Information). Biotransformation products detected in eggs were likely generated in female medaka and subsequently transferred to the eggs. This conclusion is based on the fact that M/P ratios in medaka eggs were significantly less than those in liver and liver-free carcass. This result is consistent with the results of previous studies that have also demonstrated that the biotransformation capacity of fish eggs is less than that of later life stages (27, 29).

**Naturally Occurring Concentrations and Relationships.** On the basis of the results of the controlled laboratory study, the relationship among PBDEs, OH-PBDEs, and MeO-PBDEs was further investigated by comparing concentrations in organisms collected worldwide (Table 2). When all the compounds were quantified, PBDEs, OH-PBDEs, and MeO-PBDEs were almost always codetected in aquatic animals with BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47 as the predominant congeners. Concentrations of PBDEs have a wider range (0.2–2210 ng/g ww), and similar concentration variations were observed for MeO-PBDEs and OH-PBDEs. Concentrations of OH-PBDEs were generally greater than those of MeO-PBDEs in blood. This could be due to binding of OH-PBDEs to plasma transport proteins, including the thyroxine transport proteins (e.g., transthyretin (TTR), thyroxine binding globulin (TBG)) (11, 30). The fact that both MeO-PBDEs and OH-PBDEs were detected with relatively great concentrations in liver suggested that liver would be the suitable tissue for investigations of the two groups of chemicals.

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*Concentrations are reported as ng/g. Glaucous gulls (larus hyperboreus), polar bear (Ursus maritimus), bald eagle (Haliaeetus leucocephalus), beluga whale (Delphinapterus leucas), tuna (Katsuwonus pelamis), five albatross species (Thalassarche chlororhynchos, Phoebetria palpebrata, Thalassarche chrysostoma, Thalassarche cauta, and Thalassarche melanocephalus), and Chinese sturgeon (Acipenser sinensis). A Average percentage of 6-MeO-BDE-47/2MeO-PBDEs. B Average percentage of 6-OH-BDE-47/2OH-PBDEs. C Average percentage of BDE-47/2PBDEs.
also suggested that natural accumulation in the aquatic environment could be another important source of 6-OH-BDE-47 (12). In addition, herein, we report for the first time the \textit{in vitro} biotransformation of 6-OH-BDE-47 to 6-MeO-BDE-47. The interconversion of 6-OH-BDE-47 and 6-MeO-BDE-47 in Japanese medaka was consistent with the code- 

\section*{Acknowledgments}

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\section*{Supporting Information Available}

Detailed information on chemical, animals, \textit{in vitro} biotransformation, instrumental analysis, and data analysis. Concentration ratios (liver/whole fish, egg/whole fish, and egg/ liver) of exposed chemicals and metabolites in female Japanese medaka after a 14 days dietary exposure. Chromatographic profiles of OH-Tetra-BDEs detected in prepared food and medaka livers after 14-day exposure. This material is available free of charge via the Internet at http://pubs.acs.org.

\section*{Literature Cited}


(10) Morgado, I.; Hamers, T.; Vander Vennet, L.; Power, D. M. Disruption of thyroid hormone binding to sea bream recombinant tran- 


SUPPORTING INFORMATION

Interconversion of Hydroxyalted and Methoxylated Polybrominated Diphenyl Ethers in Japanese Medaka

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This supporting information includes:
- Chemicals and Standards
- Animals
- In vitro Biotransformation
- Instrumental Analysis
- Quality Assurance and Quality Control (QA/QC)
- Data Analysis
- Table S1
- Table S2
- Figure S1

Summary of the number of pages, figures, and tables

- Number of pages: 5
- Number of figures: 1
- Number of tables: 2
MATERIALS AND METHODS

Chemicals and Standards

MeO-PBDEs (6-MeO-BDE-17, 4-MeO-BDE-17, 2’-MeO-BDE-68, 6-MeO-BDE-47, 5-MeO-BDE-47 and 4’-MeO-BDE-49) and OH-PBDEs (3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4’-OH-BDE-49 and 6’-OH-BDE-17) were synthesized in the Department of Biology and Chemistry, City University of Hong Kong, Hong Kong, China. PBDEs (BDE-28, BDE-47, BDE-99, BDE-100, BDE-119, BDE-154 and BDE-153) and $^{13}$C-PBDEs were obtained from AccuStandard (New Haven, Connecticut, USA). All compounds were determined to be $\geq98\%$ pure by high-resolution gas chromatograph interfaced to a high-resolution mass spectrometer (HRGC-HRMS). Dichloromethane (DCM), n-hexane, nonane, methyl tert-butyl ether (MTBE), acetone, acetonitrile and methanol were pesticide residue grade and were obtained from OmniSolv (EM Science, Lawrence, KS, USA). Silica gel (60-100 mesh size), formic acid, hydrochloric acid (37%, A.C.S. reagent), 2-propanol and dansyl chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male and female wild-type *O. latipes* were obtained from a stock maintained at the aquatic culture unit at the US Environmental Protection Agency Mid-Continent Ecology Division (Duluth, MN, USA). Individuals were maintained at the Aquatic Toxicology Research Facility (ATRF) in the Toxicology Centre, University of Saskatchewan (Saskatoon, SK, Canada), by use of previously described methods. Medaka were cultured in flow-through tanks under conditions that facilitated breeding (23-24 °C; 16:8 light/dark cycle)
and were fed once daily to satiety. All protocols were approved by the University of Saskatchewan Animal Research Ethics Board (U of S-AREB).

In vitro Biotransformation

To confirm the results of the in vivo feeding study, transformation of BDE-47, 6-MeO-BDE47, and 6-OH-BDE-47 was investigated using microsomes isolated from female medaka liver according to the method of Kennedy and Jones (19). The details of the methods used for microsomal incubations are described elsewhere (11). Briefly, reactions were performed in 0.1 M NaH$_2$PO$_4$ buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM dithiothreitol (DTT), and 100 µM NADPH. The final reaction volume was 250 µL and contained 125 µL of microsomes and 10 µL of exposure chemicals. The concentration of the chemical in the final reaction mixture was 2 µg/mL. Incubations without chemicals and without microsomes were used as negative controls to assess the presence of background contaminants and the possibility of non-enzyme mediated changes in chemical structure. After incubation, the samples were extracted immediately for quantification of residues and their transformation products.

Instrumental Analysis

Identification and quantification of BDE-47 and 6-MeO-BDE-47 were performed using a Hewlett-Packard 5890 series II high-resolution gas chromatography (HRGC) interfaced to a Micromass® Autospec® high-resolution mass spectrometry (HRMS) (Micromass®, Beverly, MD). Chromatographic separation was achieved by use of a DB-5MS fused silica capillary
column for all target compounds (30 m length, 0.25 mm ID, 0.1 μm film, Agilent, Carlsbad, CA) and helium was used as the carrier gas. The mass spectrometer was operated in a Selective Ion-Monitoring (SIM) mode. Resolution of all reference peaks in all time windows was greater than 7,000. The injector temperature was held at 285 °C and the ion source was kept at 285 °C. The electron ionization energy was 37 eV and the ion current was 750 μA. The temperature program was from 110 °C (10 min) to 250 °C at a rate of 25 °C/min, then increased to 260 °C at a rate of 1.5 °C/min, and then to 323 °C (15 min) at a rate of 25 °C/min.

Quantification of 6-OH-BDE-47 was conducted using an Agilent 1200 series high performance liquid chromatography system (Santa Clara, CA, USA) connected to an API 3000 triple-quadrupole tandem mass spectrometry (MS/MS) system (PE Sciex, Concord, ON, Canada). An XBridge C18 column (100 × 2.1 mm, 3.5 μm particle size) from Waters (Milford, MA, USA) was used for chromatographic separation at room temperature. Injection volume was 20 μL. The mobile phase, consisting of acetonitrile (Solvent A) and 0.1% formic acid in water (Solvent B), was used with a gradient elution of A:B = 60:40 (0-1 min) to 95:5 (1-15 min) and 95:5 (15-22 min) at a flow rate of 0.25 mL/min. Analytes were detected using a mass spectrometer equipped with a turbo ion spray source operated in the positive multi-reaction monitoring (MRM) mode. All the source and instrument parameters were optimized by infusing the purified dansyl derivatives of analytes into the mass spectrometer. Optimal MS conditions were: ion spray voltage 3750 V, curtain gas (N₂) 8, nebulizer gas (N₂) 12, collision gas (N₂) 10, turbo ion spray probe temperature 475 °C.
Quality Assurance and Quality Control (QA/QC).

To avoid cross contamination among treatments, equipment was changed after processing each treatment. To confirm the purities of the chemicals used in this study, concentrations of target compounds were analyzed in spiked food and stock standard solutions (Table 1). To avoid sample contamination during chemical analysis, all equipment was rinsed repeatedly with acetone and hexane. A laboratory blank was incorporated in the analytical procedures for every batch of 10 samples. Concentrations of all congeners were quantified by the internal standard isotope-dilution method using mean relative response factors determined from standard calibration runs. BDE-47 and 6-MeO-BDE-47 were quantified in sample extracts relative to $^{13}$C-BDE-47, and 6-OH-BDE-47 was quantified relative to 6’-OH-BDE-17. Recoveries of $^{13}$C-BDE-47 and 6’-OH-BDE-17 were $82\pm 26\%$ and $72\pm 28\%$, respectively, in all samples, and the concentrations of the analytes were recovery-corrected. The method detection limits (MDL) were defined as three times of the standard deviation in the blank samples, in which BDE-47 was detected. The MDLs for the other compounds, which were not detected in blank samples, were set to the instrumental minimum detectable amounts. Detection limits were 0.02 ng/g ww, 0.05 ng/g ww, and 1.6 ng/g ww for 6-OH-BDE-47, 6-MeO-BDE-47 and BDE-47, respectively, in analyzed samples. For those result less than the MDL, half of the MDL was assigned to avoid missing values in statistical analyses, and details of the data analysis were provided in the supporting information.
**Data Analysis**

All statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, IL, USA). Results are reported as mean ± standard deviation (SD). Differences between two groups were analyzed by the non-parametric Wilcoxon signed rank sum test. Differences in concentrations of target compounds among treatments were compared using one-way analysis of variance (ANOVA). Levene’s test was used to check the equality of variances (the value of significance is less than 0.05). Where variances were equal, data were analyzed by the F test. Where the equality of variances could not be assumed, Welch’s and Brown-Forsythe’s robust tests were used to perform one-way ANOVA analysis. Multiple paired comparisons were used to determine which means differed from one another. Tukey’s Honestly Significant Differences (HSD) was used where variances were presumed to be equal, and the Games-Howell test was used where equality of variances could not be assumed. Results were considered significant when $p$ values were less than 0.05.
Table S1. Concentrations of target compounds after metabolism with medaka microsomes exposed to BDE-47, 6-MeO-BDE-47, and 6-OH-BDE-47 (ng/mL). The dosing concentrations for all chemicals were 2 μg/mL.

<table>
<thead>
<tr>
<th>Exposed Chemicals</th>
<th>Analyzed Chemicals</th>
<th>BDE-47</th>
<th>6-MeO-BDE-47</th>
<th>6-OH-BDE-47</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-MeO-BDE-47</td>
<td>&lt;1.6</td>
<td>710±72</td>
<td>62.8±9.9</td>
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</tr>
<tr>
<td>6-OH-BDE-47</td>
<td>&lt;1.6</td>
<td>&lt;0.05</td>
<td>680±110</td>
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<tr>
<td>BDE-47</td>
<td>620±185</td>
<td>&lt;0.05</td>
<td>&lt;0.02</td>
<td></td>
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</table>
Table S2. Concentration ratios (liver/whole fish, egg/whole fish and egg/liver) of exposed chemicals and metabolites in female Japanese medaka after a 14 d dietary exposure to feed spiked with either BDE-47, 6-MeO-BDE-47 or 6-OH-BDE-47.

<table>
<thead>
<tr>
<th>Exposure groups</th>
<th>6-OH-BDE-47</th>
<th></th>
<th>6-MeO-BDE-47</th>
<th></th>
<th>BDE-47</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exposed chemical</td>
<td>Metabolite</td>
<td>Exposed chemical</td>
<td>Metabolite</td>
<td>Exposed chemical</td>
<td>Metabolite</td>
</tr>
<tr>
<td>Liver/Whole fish (L/F)</td>
<td>6.9±7.6</td>
<td>1.2±0.5</td>
<td>1.2±0.7</td>
<td>6.1±5.9</td>
<td>1.1±0.5</td>
<td>-</td>
</tr>
<tr>
<td>Egg/ Whole fish (E/F)</td>
<td>1.8±0.93</td>
<td>0.72±0.22</td>
<td>0.62±0.41</td>
<td>0.34±0.12</td>
<td>0.92±0.23</td>
<td>-</td>
</tr>
<tr>
<td>Egg/ Liver (E/L)</td>
<td>0.59±0.44</td>
<td>0.76±0.40</td>
<td>0.74±0.71</td>
<td>0.23±0.35</td>
<td>0.93±0.31</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure S1. LC-MS/MS MRM chromatographic profiles of OH-Tetra-BDEs detected in prepared food and medaka livers after 14-day exposure. Standard: OH-Tetra-BDEs standard solution (5 ng/ml), 3-OH-BDE-47 (1), 5-OH-BDE-47 (2), 6-OH-BDE-47 (3), 4′-OH-BDE-49 (4); C-F: control food; M-F: 6-MeO-BDE-47 spiked food; P-F: BDE-47 spiked food; O-F: 6-OH-BDE-47 spiked food; C-L: liver of medaka in control group; M-L: liver of medaka exposed to 6-MeO-BDE-47; P-L: liver of medaka exposed to BDE-47; O-L: liver of medaka exposed to 6-OH-BDE-47.