Stereoselective Biotransformation of Permethrin to Estrogenic Metabolites in Fish

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This study investigated the stereoselective biotransformation and resulting estrogenic activity of the pyrethroid insecticide, permethrin (PM). Results of both *in vivo* (male Japanese medaka, vitellogenin (VTG) protein in plasma) and *in vitro* (primary rainbow trout hepatocyte VTG-mRNA expression) assays indicated stereoselective estrogenic activity of PM. 1*S-cis*-PM was observed to have significantly higher activity ($P \leq 0.05$) than the 1*R-cis* enantiomer in both *in vivo* and *in vitro* evaluations. All enantiomers of PM were oxidized to a 4′-hydoxy PM (4OH PM) metabolite and underwent esterase cleavage to 3-phenoxybenzyl alcohol (3-PBOH) and 3-(4′-hydroxyphenoxy)-benzyl alcohol) (3,4′-PBOH). Racemic 4OH PM as well as 3-PBOH, and 3,4′-PBOH possessed significant ($P \leq 0.05$) estrogenicity. 1*S-trans*-PM underwent esterase cleavage more extensively than the corresponding 1*R-trans*-PM. Inhibition studies with ketoconazole confirmed cytochrome P450-catalyzed hydroxylation as well as esterase cleavage of PM for all stereoisomers. These studies indicated stereoselectivity in the estrogenic activity of PM resulting from stereoselective biotransformation of the parent compound to more estrogenic metabolites.

Introduction

Pyrethroids are a group of current-use insecticides extensively used in agriculture, nurseries, and various urban and household pest control, as less-toxic alternatives to the more acutely toxic organophosphorus insecticides that have been banned or restricted (*1*). Despite their hydrophobic characteristics and low aqueous solubility (*2*), pyrethroids have been shown to mobilize via surface runoff or erosion into streams, creeks, and estuaries, and can eventually be deposited into sediments (*3*). Widespread use of pyrethroids has resulted in their detection in surface streams at many locations in California at levels that are acutely toxic to aquatic invertebrates (*4*).

The acute toxicity of pyrethroid insecticides occurs through altering the kinetics of the voltage-gated sodium channels of neurons, which is reportedly similar across species (*5*). The lower acute toxicity to birds and mammals, however, has been attributed to their rapid metabolism and excretion from the body (*6*). Early reports indicate that pyrethroid metabolism and elimination are substantially lower in fish and qualitatively different from that reported in mammals (*7, 8*). Thus, pyrethroids are highly acutely toxic to fish and most other aquatic organisms (*5*).

Because of the presence of multiple asymmetric carbons in their structures, most pyrethroids consist of 4−8 optical isomers. The significance of the overall molecular shape in the mode of action of pyrethroids is evident in the stereospecificity of its insecticidal action (*9*). The stereospecificity also extends to other pyrethroids with a chiral center at the alcohol moiety of the carbon bearing the hydroxyl group (*10*).

Recent investigations reported stereoselective aquatic toxicity and environmental degradation of pyrethroids, including permethrin (PM), bifenthrin, λ-cyhalothrin, and cypermethrin (*11, 12*). Differences in estrogenic potential were observed between bifenthrin enantiomers in the MCF-7 human carcinoma cell proliferation assay, and *in vivo* in Japanese medaka (*Oryzias latipes*) (*13*). Induction of estrogen-responsive genes by PM enantiomers was recently reported in larval and adult zebrafish (*Danio rerio*) (*14*). In addition, PM metabolites 3-phenoxybenzyl alcohol (3-PBOH) and 3-(4′-hydroxyphenoxy)-benzyl alcohol (3,4′-PBOH) have been shown to mimic the interaction of 17-β-estradiol (E2) with estrogen receptors (*15, 16*), while 3-phenoxybenzoic acid (3-PBCOOH) was found to be antiestrogenic (*15*).

Evidence of stereoselective metabolism of some pyrethroids has been reported in rats (*17*). In carp and rainbow trout liver microsomes, *trans*-PM was more susceptible to microsomal esterases than *cis*-PM (*18*). Previous studies also showed significant species differences in PM diastereoisomer metabolism (*7, 17*). For instance, in contrast to rats, limited hydrolysis of *cis*- and *trans*-PM was observed in trout (*Oncorhynchus mykiss*). Instead, both PM stereoisomers were readily converted to a hydroxylated derivative (*7, 8*). These previous studies only evaluated diastereomeric mixtures and did not examine specific enantiomers.

Since little is known regarding the stereoselectivity of biotransformation or estrogenic activity of pyrethroids in fish. This study investigated the stereoselective metabolism and resulting estrogenic activity of PM (Figure 1). Permethrin stereoisomers were investigated for estrogenic activity *in vitro*, using transcriptional measurements of the egg-yolk precursor, vitellogenin, in rainbow trout primary hepatocyte cultures and *in vivo* using vitellogenin protein measurements in male Japanese medaka. To determine whether trout were capable of stereoselective activation of PM to more estrogenic metabolites, *in vitro* biotransformation studies were carried out using trout

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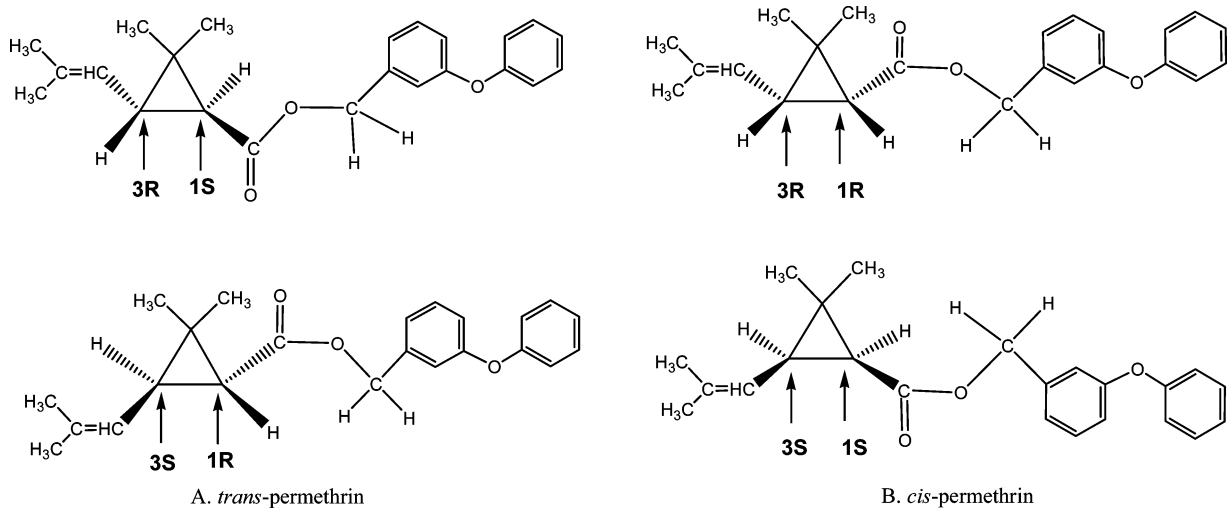

A. *trans*-permethrin B. *cis*-permethrin

Figure 1. Structure of permethrin enantiomers. (A) *trans*-Permethrin and (B) *cis*-Permethrin.

hepatic microsomes. Results indicated stereoselective oxidation and hydrolysis of PM to estrogenic metabolites, which may cause the induction of vitellogenin in male fish.

Materials and Methods

Chemicals. Analytical standards of racemic PM (40:60 *cis*/*trans*), PM (97% purity; 3-phenoxybenzyl(1*RS*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate), and *cis*-bifenthrin (97% purity; 2-methylbiphenyl-3-ylmethyl-(1*RS*)-*cis*-3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethylcyclopanecarboxylate) were obtained from FMC (Philadelphia, PA). Pure standards of PM enantiomers (enantiomeric and chemical purity >99%) were provided by U.S. EPA. 3-Phenoxybenzyl alcohol (3-PBOH; 97% purity) was purchased from Sigma-Aldrich (St. Louis, MO). Ketoconazole ((±)-*cis*-1-acetyl-4-(4-[(2-[2,4-dichlorophenyl]-2-[1*H*-imidazol-1-ylmethyl]-1,3-dioxolan-4-yl)-methoxy]phenyl)piperazine) and 3-(4-methoxy phenoxy)-benzaldehyde (97% purity) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents used in this study were of analytical or HPLC grade.

Metabolite Synthesis. 3-(4′-Hydroxyphenoxy)benzyl Alcohol. 3-(4′-Hydroxyphenoxy)benzyl alcohol (3,4′-PBOH), >99% purity, was synthesized using the following procedure. The Ag_2O oxidation (*19*) of commercially available 3-(4-methoxyphenoxy)benzaldehyde afforded 3-(4-methoxyphenoxy)benzoic acid in 80% yield, which was deprotected and subsequently reduced according the standard method (*20*) yielding the desired metabolite 3-(4 (hydroxymethyl)phenoxy)phenol.

Silver I oxide (3.94 mmol) was prepared by the dropwise addition of NaOH (8.75 mmol) in 3.88 mL of H_2O to a boiling solution of 1.49 g of $AgNO_3$ (8.77 mmol) in 11.66 mL of H_2O. The precipitated dark brown Ag_2O was filtered, washed successively with H_2O, acetone, and ether. A suspension of freshly prepared Ag_2O (0.5 g, 2.15 mmol) and 10% NaOH (10 mL) in 10 mL of water was vigorously stirred and was added to 3-(4-methoxyphenoxy) benzaldehyde (0.250 g, 1.09 mmol) in small portions over a 30 min period, keeping the temperature below 30 °C. The mixture was stirred for 1 additional hour, and the silver was removed with a Celite pad and washed with water. The filtrate was acidified with concentrated HCl, and the precipitate was collected and washed with water to afford 210 mg of 3-(4-methoxyphenoxy) benzoic acid as a white solid (80% yield). ^{1}H NMR-for the carboxylic acid: (acetone-d_6, 500 MHz) δ 4.00 (s, 3H, OCH_3), 7.15 (d, 2H, J = 9.0 Hz, ArH), 7.25 (d, 2H, J = 9.0 Hz, ArH), 7.40 (d, 1H, J = 10.2 Hz, ArH), 7.65 (t, 1H, J = 8.0 and 15.5 Hz, ArH), 7.73 (s, 1H, ArH), 7.93 (d, 1H, J = 8.0 Hz, ArH). ^{13}C NMR (acetone-d_6, 125 MHz) δ 53.7, 113.8, 116.3, 119.9, 120.6, 122.3, 128.7, 131.0, 148.1, 155.3, 157.7, 164.9.

3-(2, 2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic Acid. To a solution of permethrin (50 mg, 0.128 mmol), in 5 mL of a CH_2Cl_2/CH_3OH (9:1, v/v) mixture was added a 0.5 N methanolic solution (5 mL) of NaOH. After 5 min of stirring, the solution became cloudy, and a precipitate formed. The mixture was stirred at room temperature for 15 h until all of the ester was consumed. The reaction was monitored by TLC for the disappearance of the permethrin. The solvent was removed by rotary evaporation, and the residue was diluted with water. The aqueous solution was extracted with diethyl ether to remove the water insoluble benzyl alcohol and to remove any remaining unreacted ester. The aqueous phase was cooled, acidified to pH 2−3 with 6 N HCl, and extracted with CH_2Cl_2. The combined organic layers were dried with Na_2SO_4, and the solvent was removed to afford acid as an isomeric mixture as an off-white solid (23.5 mg, 87.5% yield). R_f = 0.18 (25:75 hexanes/chloroform); MS (ESI-) *m*/*z*: [ion] (rel. int. %) 206.6 $[M]^-$ (100), 208.6 $[M + 2]^-$ (70.8), 210.6 $[M + 4]^-$ (12.6).

1,1′-Carbonylbis(3-methylimidazolium) Triflate (CBMIT). Synthesis of CBMIT was performed as previously reported (*39*).

4-OH-c, *t*-Permethrin. A solution of CBMIT (0.0462 mmol) was transferred via syringe into a suspension of 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (0.0478 mmol, 10 mg) in nitromethane (90 μL). After 5 min, a solution of (4-(3-(hydroxymethyl)phenoxy)phenol (0.0462 mmol, 10 mg) in anhydrous THF (185 μL) was added via syringe. The reaction was quenched with water (0.5 mL) after 17 h, and the mixture was extracted into diethyl ether, washed successively with 5% aqueous Na_2CO_3 and brine, and dried over Na_2SO_4. The organic layer was concentrated by rotary evaporation to yield the crude product, purified by silica gel column chromatography (20:80 ethyl acetate/hexanes). This afforded 4-OH-permethrin as a mixture of *cis*- and *trans*-isomers. Colorless oil (4.2 mg, 22.3% yield). R_f = 0.40 (20:80 ethyl acetate/hexanes); MS (ESI+) *m*/*z*: [ion] (rel. int. %) $[MNa]^+$ 429.2 (100), 431.2 $[MNa + 2]^+$(62.2). ^{1}H NMR was compared to previously reported data (*40*) to verify product as a mixture of *cis*- and *trans*-isomers.

Stereoisomer Preparation and Analysis. Stereoisomers of PM were resolved on an Agilent 1100 Series HPLC (Wilmington, DE, USA) equipped with an online laser polarimeter detector (PDR-Chiral, Lake Park, FL, USA) (*21*). Stereoisomer resolution and isolation were carried out at room temperature on a Chiralcel OJ column (250 mm × 4.0 mm, cellulose tris-(4-methyl benzoate) on a silica gel substrate (Daicel Chemical Industries, Tokyo, Japan) using hexane/isopropanol (96%:4% v/v) as the mobile phase. The flow rate of the mobile phase was 0.8 mL/min. The UV detection wavelength was set at 230 nm for all analyses. The specific rotation of the resolved stereoisomers was determined at 675 nm and using a 50 mm cell path. The rotation sign (+ or −) was directly indicated by a positive or negative peak on the polarimeter. Pure stereoisomers

used for estrogenic activity and metabolic transformation experiments were manually collected at the HPLC outlet and evaporated to dryness under a stream of pure nitrogen, followed by redissolution in acetone (carrier solvent). The purity of the derived stereoisomers was checked with re-analysis on HPLC and was found to be >99% in all cases.

Organisms. Juvenile rainbow trout (*Oncorhynchus mykiss*) (15 ± 2 g; 15 ± 3 cm) were purchased from Jess Ranch Fishing Lakes (Apple Valley, CA) and were held in a flow-through living-stream system (Toledo, OH, USA) with carbon-filtered dechlorinated municipal water at 13−15 °C. The fish were fed daily with commercial fish food (Nelson and Sons Silvercup, Murray, UT). Gonadal morphology was not observed in any of the animals indicating juvenile animals without endogenous expression of vitellogenin (*22*).

Adult male Japanese medaka, >90 day old posthatch (18.8 ± 1.3 mm; 64.8 ± 14.7 mg), were obtained from an ongoing culture at the University of California, Riverside. The fish were kept in glass aquaria in a chemical free room with the temperature maintained at 25 ± 2 °C and a light cycle of 16:8 h of light/dark photoperiods. Dechlorinated water was used for all stock cultures and experiments. Water quality parameters were constantly monitored, and fish were fed live *Artemia* sp. nauplii *ad libitum* twice daily. Fish were not fed during exposure.

Primary Hepatocyte Culture Preparation and Exposure. Primary cultures of rainbow trout hepatocytes were isolated by enzymatic digestion with trypsin followed by mechanical disaggregation and gradient centrifugation with Percoll, as described previously (*23*). Following hepatocyte isolation, the cells were seeded in 48-well plates with a density of 1×10^6 cells/well and allowed to settle for 2 h prior to treatment with PM enantiomers and metabolites. Each treatment had three replicates as well as positive (17-β estradiol) controls and solvent controls (1% acetone). Cells were incubated for 24 h at 18 °C and then resuspended in PBS buffer, centrifuged at 5,200*g* for 5 min, and the pellets washed twice with PBS. Cells used for PCR were immediately processed for total mRNA extraction.

To ascertain that exposure concentrations used in this study were not cytotoxic, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) test was conducted for acetone (carrier solvent), PM, PM stereoisomers, PM metabolites, and 17-β estradiol (positive control), prior to the vitellogenin-mRNA (VTG-mRNA) expression assay. Cell viability was determined by the MTT reduction assay adapted from Mosmann (*24*). Briefly, the isolated hepatocytes were seeded in 96-well plates with a density of 1×10^6 cells/well in 100-μL volumes. The cells were exposed to racemic PM, *cis*/*trans* PM, racemic 4OH PM, and the PM metabolites at 5, 10, 25, 50, and 100 μM for 24 h at 18 °C. MTT solution (5 mg/mL in sterile PBS) was added (20 μL per in each well) 4 h prior to exposure termination. The water-insoluble purple formazan crystals formed during incubation were then dissolved in dimethyl sulfoxide/ethanol (1:1 in v/v). The amount of formazan formed was determined by measuring the absorbance at 595 nm using an ELISA plate reader, and well imperfections were corrected by the measure of the absorbance at 650 nm. Cell viability was determined as a percentage of the control value.

***In Vitro* Measurement of Vitellogenin (VTG) mRNA Expression in Rainbow Trout Hepatocytes.** Vitellogenin (VTG) mRNA was measured by quantitative PCR (qPCR), used previously in our laboratory (*23*). Total mRNA was extracted from cells using QIAshredder and RNeasy Mini RNA Extraction Kit (Qiagen, Valencia, CA) and quantified by qPCR using iScript One-step RT-PCR Kit with SYBR Green from Bio-RAd (Hercules, CA). Thermocycling parameters were as follows: 10 min at 50 °C (cDNA synthesis); 5 min at 95 °C (reverse transcriptase inactivation); and 40 cycles of 10 s at 95 °C and 30 s at 56 °C. A melting curve analysis was carried out between 60 and 95 °C following the amplification reaction to ensure gene specificity. To correct for variations in mRNA and cDNA quantity and quality, all VTG values were normalized against β-actin. Fluorescence data were collected at the end of each cycle every 0.1 °C, and the *Ct* value was determined to be in the linear phase of amplification. All real-time reactions were done in iCycler-MyIQ Single Color Real-Time PCR Detection System (Bio-Rad), and data analysis was done using IQ5 (Bio-Rad).

***In Vivo* Measurement of VTG Protein Expression in Medaka.** An initial range finding test was performed to estimate the range of concentrations at which VTG production occurs in male fish without lethality. The 96 h-LC50 of PM to adult Japanese medaka was 39.5 μg/L. Upon the basis of this value and other studies evaluating the estrogenic activity of permethrin and bifenthrin in fish (*13, 14*), the exposure concentration that was used for the *in vivo* exposure was 10 μg/L, and the duration of exposure was 8 days. The exposure protocol was based on standard methods with slight modification (*25*). Adult male Japanese medaka were held in 500-mL glass jars (3 fish per jar, three replicates per treatment) under static conditions for 8 days. On the last day of exposure, the fish were anesthetized with 1 g/L tricaine methane sulfonate (MS-222), and the livers were excised and pooled from each individual replicate and frozen at −80 °C until VTG analysis. The VTG protein level in the liver was quantified by an enzyme-linked immunosorbent assay (ELISA) using commercially available kits for Japanese medaka (BiosenseTM, Bergen, Norway). The VTG concentrations were normalized to the total protein in the liver homogenate determined with the Coumassie blue protein assay (Pierce Biotechnology, Rockford, IL). The limit of detection for the assay was 0.1 ng/μg protein. VTG production was compared between PM enantiomers to determine enantioselectivity.

Microsomal Biotransformation. Microsomal fractions were prepared from livers dissected from 5 rainbow trout and pooled (*26*). The excised liver was weighed and rinsed with ice cold 1.15% KCl and homogenized in 1:5 w/v cold homogenization buffer (100 mM KH_2PO_4/K_2HPO_4 buffer at pH 7.4, containing 100 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethyllsulfonylfluoride (PMSF), 1 mM dithiothreitol (DTT), and 0.1 mM 1,10-phenanthroline). The homogenate was centrifuged at 1500*g* for 15 min, the fatty layer removed, and the obtained supernatant centrifuged at 12,000*g* for 20 min. The 12,000-*g* supernatant was further centrifuged at 100,000*g* for 60 min to obtain microsomal fractions. Microsomal pellets were resuspended in a small volume of homogenization buffer containing 20% w/v glycerol. Protein concentrations were measured by the Bradford method, using the Coomasie Plus-200 Protein Assay reagent (Pierce, Rockford, IL, USA) and the bovine serum albumin (BSA) standard.

Hepatic microsomal activity was determined on the basis of the method by Godin et al. (*27*) and Glickman and Lech (*7*) with some modifications. A 250-μL reaction volume containing 250 μg of microsomal protein, 100 μM substrate (racemate and enantiomers), 1 mM NADPH, and 100 mM Tris-HCl buffer (pH 7.4) was incubated at 25 °C for 60 min. Previous studies indicated that this was in the linear range for catalytic activity (*7*). The reaction was quenched by the addition of equal volumes of ice-cold acetonitrile following the addition of internal standards (20 μM *cis*-bifenthrin and 10 μM 3-(4-methoxyphenoxy)-benzaldehyde). Following centrifugation, the supernatant was transferred to HPLC glass vials and analyzed for metabolic products by HPLC/UV at $\lambda = 230$ nm. Negative controls omitted NADPH or included boiled microsomal protein. Cytochrome P450 inhibition experiments were conducted with coincubation with 500 μM ketoconazole added immediately after NADPH addition and incubated for 5 min prior to the addition of individual PM enantiomers.

Reverse-phase HPLC-UV analysis of the products was based on the method of Choi et al. (*28*). Analysis was performed on an Agilent 1100 Series HPLC (Wilmington, DE, USA) equipped with a reverse-phase C-18 HPLC column (Dionex Acclaim 120; 4.6 × 250 mm, 5 μm). The mobile phase consisted of solvents A (90% acetonitrile and 10% water) and B (100% water adjusted to pH 1.7 with 85% phosphoric acid). The analytes were eluted using the following gradient program: 0 min (50% A; 50% B), 6 min (75% A; 25% B); 7 min (100%A; 0% B); 11 min (100% A; 0% B); 12 min (50% A; 50%B); and 20 min (50% A; 50% B) at a flow rate

of 1 mL/min. Analytes were detected at 230 nm and quantified using external standards.

Identification of the hydroxylated derivative was conducted on Waters ACQUITY Ultra Performance Liquid Chromatography (UPLC) System (Waters, Milford, MA, USA) using an Acquity UPLC BEH C18 column (100 mm × 2.1 mm, i.d., 1.7 μm particle size) from Waters (Milford, MA, USA). Mass spectrometric detection was carried out using an ACQUITY TQD tandem quadrupole mass spectrometer (Waters, Milford, MA, USA). The mobile phase consisted of ammonium acetate (solvent A) and methanol (solvent B). The gradient conditions were 0 min (80%A; 20% B); 0.5 min (50%A; 50%B); 9 min (0% A; 100% B); 10 min (0% A; 100% B); 11 min (80%A; 20% B); and 15 min (80% A; 20%B) at a flow rate of 0.2 mL/min. The ESI parameters were capillary voltage of 3.0 kV, source temperature of 120 °C, and desolvation temperature of 350 °C. The cone and desolvation gas flows were 10 L/h and 600 L/h, respectively, and were obtained from an in-house nitrogen source. Data acquisition was performed using MassLynx 4.0 software with the QuanLynx program (Waters). The analysis was done in negative mode, and the data were acquired in full-scan at *m/z* 70−450. The accurate molecular mass measurement was further confirmed at UCR Mass Spectrometry Facility (Riverside, CA, USA) using Agilent 6210 ESI-TOF (Agilent Corp, Santa Clara, CA, USA), operated in negative ESI mode. Quantification was made using external standards.

Data Analysis. Data were presented as the mean ± standard error of the mean (SEM). Statistical differences between treatments ($p < 0.05$) were determined using analysis of variance (ANOVA). Prior to performing ANOVA, the experimental data were checked for the homogeneity of variance using Bartlett's test. Treatment differences were assessed with Bonferroni's Multiple Comparison Test (GraphPad Prism v.4.03; GraphPad Software Inc., San Diego, CA, USA).

Results

Chiral Separation and Identification. Permethrin consists of two diastereomers (*cis*- and *trans*-) and four stereoisomers (1*R*-*cis*-, 1*S*-*cis*-, 1*R*-*trans*-, and 1*S*-*trans*-) (Figure 1). Optimal resolution and isolation of PM stereoisomers was achieved on a Chiralcel OJ column using hexane/isopropanol (96:4% v/v) as the mobile phase. Assignment of absolute configurations to the resolved peaks was made by comparing chromatograms with enantiopure standards. Elution order for the PM stereoisomers was 1*S*-(+)-*cis*, 1*R*-(−)-*cis*, 1*S*-(+)-*trans*, and 1*R*-(−)-*trans* (*12, 41*).

***In Vivo* Estrogenic Activity of Permethrin Stereoisomers.** To determine stereoselectivity for estrogenic activity, vitellogenin (VTG) production in adult male Japanese medaka livers was compared following 8 days of exposure to sublethal concentrations of PM stereoisomers (Figure 2). All PM stereoisomers showed the ability to induce the production of VTG at levels significantly higher than that in controls ($p \leq 0.05$). Statistically significant differences ($p \leq 0.05$) in the relative estrogenic potential between enantiomers were also observed, with 1*S*-*cis*- and 1*S*-*trans*-PM eliciting, respectively, 2.5 and 1.3 times greater responses than their respective corresponding antipodes.

***In Vitro* Estrogenic Activity of Permethrin Stereoisomers.** Treatment of juvenile rainbow trout primary hepatocyte cultures with individual PM stereoisomers resulted in significant increases in VTG-mRNA expression in the primary hepatocytes cultures ($p \leq 0.05$) (Figures 2 and 3). The 1*S*-*cis*-PM treatment resulted in approximately 2-fold higher VTG-mRNA expression levels compared to that with 1*R*-*cis*-PM. The same trend was observed for the *trans*-PM enantiomers, although the difference was not statistically significant ($p > 0.05$) (Figure 2). Between the 4 stereoisomers, treatment with 1*S*-*cis*-PM resulted in 2-, 1.2-, and 1.4-fold higher VTG-mRNA expression than that with 1*R*-*cis*-, 1*S*-*trans*-, and 1*R*-*trans*-PM, respectively.

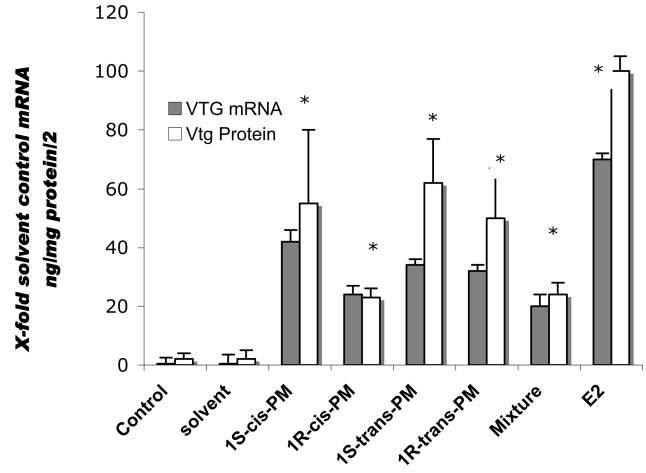

Figure 2. Vitellogenin protein concentrations in the livers of adult male Japanese medaka (*Oryzias latipes*) following 8 days of exposure to permethrin (PM) enantiomers (10 ug/L) and expression of vitellogenin-mRNA in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 50 μM permethrin. Values are the mean ± SEM; $n = 3$ for medaka; and $n = 9$ for hepatocytes. * Indicates significant difference from the control ($p \leq 005$).

***In Vitro* Estrogenic Activity of Permethrin Metabolites.** To determine whether fish are capable of stereoselectively activating PM to more estrogenic compounds, PM metabolites previously reported as estrogenic in mammalian cell lines (4OH PM, 3-PBOH, and 3,4′-PBOH) were investigated for their estrogenic activity in the primary trout hepatocyte assay. A concentration-related increase in VTG-mRNA expression level was observed for 4OH PM and 3,4′-PBOH (Figure 3). In contrast, significant VTG-mRNA expression was only observed at 10 μM for 3-PBOH. VTG-mRNA expression observed after treatment with 50 μM 3,4′-PBOH was 3-fold higher than the 1*S*-*cis*-PM enantiomer at the same concentration (Figures 2 and 3).

***In Vitro* Biotransformation of Permethrin Stereoisomers.** Stereoselective formation of three metabolites was observed following the incubation of PM in rainbow trout liver microsomes (Table 1). *trans*-PM underwent ester clevage more extensively than *cis*-PM. However, the hydroxylated derivative of the intact ester was the predominant metabolite that was produced (Table 1). In addition, *cis*-PM diastereomers were observed to be more susceptible to hydroxylation, forming more than twice the amount of hydroxylated product (M4) than *trans*-PM diastereomers (Table 1).

Identification of the hydroxylated product (M4) was accomplished by accurate-mass measurements on LC-MS (TOF) and LC-MS/MS (TQD) (Figure 4A and B). In all samples, the $[M - 1]^-$ ion was the most abundant and gave the characteristic two-chlorine isotope cluster (*m/z* 405:407 at 1:0.7 ratio), consistent with the expected molecular mass (*m/z* 405) and structure of the hydroxylated parent compound. The retention times were consistent with those of the parent stereoisomers (*trans* eluted before *cis* on the C18 column), further suggesting that the stereochemistry was likely conserved during the hydroxylation process.

Of the four stereoisomers, 1*S*-*cis*-PM had the highest NADPH-dependent metabolic conversion with the hydroxylated metabolite (M4) constituting 99% of the total metabolite profile. Formation of M4 from 1*S*-*cis*-PM was approximately 25 times higher than the formation from 1*R*-*cis*-PM, indicating a significant stereoselectivity toward the 1*S*-*cis*-PM for the hydroxy-

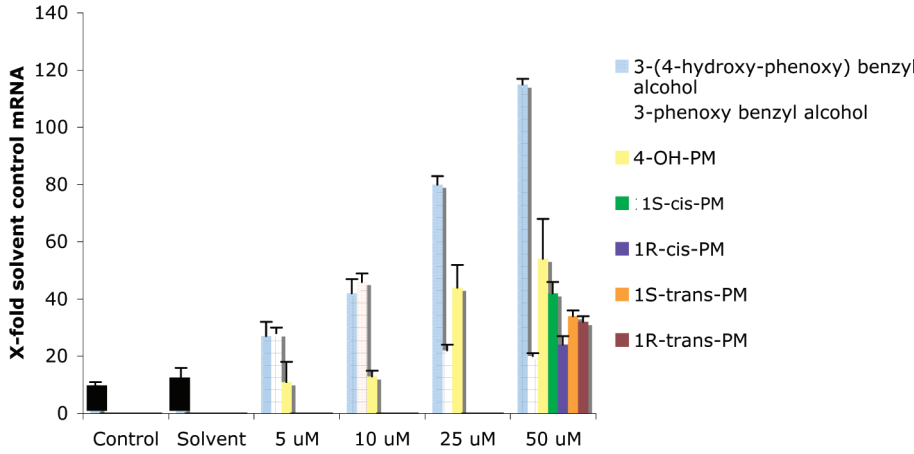

Figure 3. Expression of vitellogenin-mRNA (VTG-mRNA) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes following treatment with 4-hydroxy PM, 3-phenoxybenzyl alcohol, and 3-(4-hydroxy-3-phenoxy)-benzyl alcohol. * Indicates significant difference from the control ($p \leq 0.01$). VTG-mRNA expression in the positive control (E2; 3.6×10^{-2} μM) = 142.5 ± 5.1-fold relative to the solvent control.

Table 1. Stereoselective Biotransformation of Permethrin in Trout (*Oncorhynchus mykiss*) Liver Microsome and the Effects of the CYP Inhibitor Ketoconazole on Biotransformation

substrate	metabolites[a]	− NADPH	+ NADPH	+ ketoconazole (500 μM)
		1*S-cis*-PM		
	M1	BDL	3.27 ± 0.15	0.39 ± 0.23
	M2	4.28 ± 1.31	0.64 ± 0.35	1.55 ± 0.89
	M3	BDL	BDL	BDL
	M4	BDL	711.77 ± 24.25	BDL
	% conversion	3.92 ± 1.76	53.93 ± 2.56	0.91 ± 0.67
		1*R-cis*-PM		
	M1	BDL	1.99 ± 0.44	BDL
	M2	4.45 ± 0.09	11.95 ± 0.19	4.16 ± 0.17
	M3	BDL	BDL	BDL
	M4	BDL	28.09 ± 0.04	BDL
	% conversion	6.79 ± 3.03	10.40 ± 1.46	1.38 ± 0.58
		1*S-trans*-PM		
	M1	0.72 ± 0.64	21.54 ± 2.15	0.80 ± 0.01
	M2	46.65 ± 0.79	51.86 ± 0.93	4.05 ± 0.08
	M3	16.49 ± 0.47	22.65 ± 0.43	BDL
	M4	BDL	57.01 ± 0.67	BDL
	% conversion	5.00 ± 0.78	13.74 ± 0.90	6.44 ± 1.94
		1*R-trans*-PM		
	M1	BDL	4.57 ± 2.20	0.86 ± 0.17
	M2	5.72 ± 0.33	10.71 ± 0.36	4.36 ± 0.33
	M3	BDL	ND	BDL
	M4	BDL	27.55 ± 0.78	BDL
	% conversion	0.45 ± 0.18	7.36 ± 2.09	2.51 ± 2.26

[a] M1, 3,4′-PBOH; M2, 3-PBOH; M3, 3-PBCOOH; M4, OH-PM; Data are presented as the mean ± SEM ($n = 3$) in pmol/min/mg protein; BDL, below the detection limit (<0.3 pmol/min/mg protein); M4 is estimated from the calibration curve generated from the parent compound standards.

lation of PM. In contrast, the NADPH-catalyzed cleavage of the *cis*-PM enantiomers was enantioselective for 1*R-cis*-PM (Table 1).

1*S-trans*-PM was observed to be the most susceptible to ester cleavage among the PM stereoisomers. Although the *trans*-PM stereoisomers were observed to be more susceptible to ester cleavage than the *cis*-stereoisomers, hydroxylation in trout liver microsomes was also greater than ester cleavage. Between the two *trans*-PM enantiomers, 1*S-trans*-PM was more extensively hydroxylated than 1*R-trans*-PM (Table 1).

In general, metabolite formation and enantiomer conversion were significantly inhibited with ketoconazole when individual enantiomers were coincubated with ketoconazole. The greatest inhibition was observed with 1*S-cis*-PM where a 98% reduction in conversion was observed presumably due to the diminished formation of M4.

Discussion

The adverse effect of endocrine disrupting chemicals (EDCs) in aquatic environments has become a major issue in environmental research and policy (*29*). Estrogenic agents have received the most attention primarily because of the relatively simple methods of measuring exposure and bioavailability in oviparous aquatic vertebrates in which estrogens activate the hepatic synthesis of vitellogenin (VTG). VTG is the major precursor in egg-yolk proteins, which provides energy reserves for embryonic development. 17β-Estradiol (E2) is the feminization hormone of female fish and stimulates the liver to produce VTG (*30*). The male fish liver also has the receptor for E2, which may be stimulated by estrogens initiating the transcription and subsequent translation of VTG. Thus, VTG induction in adult male and juvenile fish has been used as a biomarker to assess exposure to estrogenic contaminants in aquatic environments (*29, 30*).

In this study, all PM stereoisomers showed the ability to induce the production of VTG at levels significantly higher than the control. A 123-fold difference in VTG induction in adult male medaka was observed among *cis*-bifenthrin enantiomers (*13*). In the present study, enantioselectivity was observed for *cis*-PM enantiomers but not for *trans*-PM enantiomers. In the MCF-7 cell proliferation assay, a 3.6 times higher proliferative effect was observed for 1*S-cis*-bifenthrin relative to the 1*R-cis* enantiomer (*13*). In addition, stereoselective induction of the hepatic estrogen responsive gene transcription in male adult zebrafish (*Danio rerio*) was observed following a 2-day exposure to PM enantiomers (*14*). Expression of VTG1 and VTG2 in zebrafish was 2.6 and 1.8 times higher, respectively, than its antipode following exposure to (−)-*trans*-PM.

In addition to the *in vivo* medaka response, the present study also utilized juvenile rainbow trout primary hepatocyte cultures in evaluating the enantiomer-specific estrogenic potential of PM. Significant increases in VTG-mRNA expression was observed in the primary hepatocytes cultures ($p \leq 0.05$) following a 24-h treatment with PM stereoisomers. Results from both *in vivo* and *in vitro* studies indicated stereoselective estrogenic activity with the 1*S* enantiomer of both *cis*- and *trans*-PM. The magnitude of enantioselectivity observed in the present study was comparable to those reported in the livers of zebrafish (*14*).

The phenolic functionality enhances binding to the estrogen receptor, suggesting that phenolic metabolites of PM may be

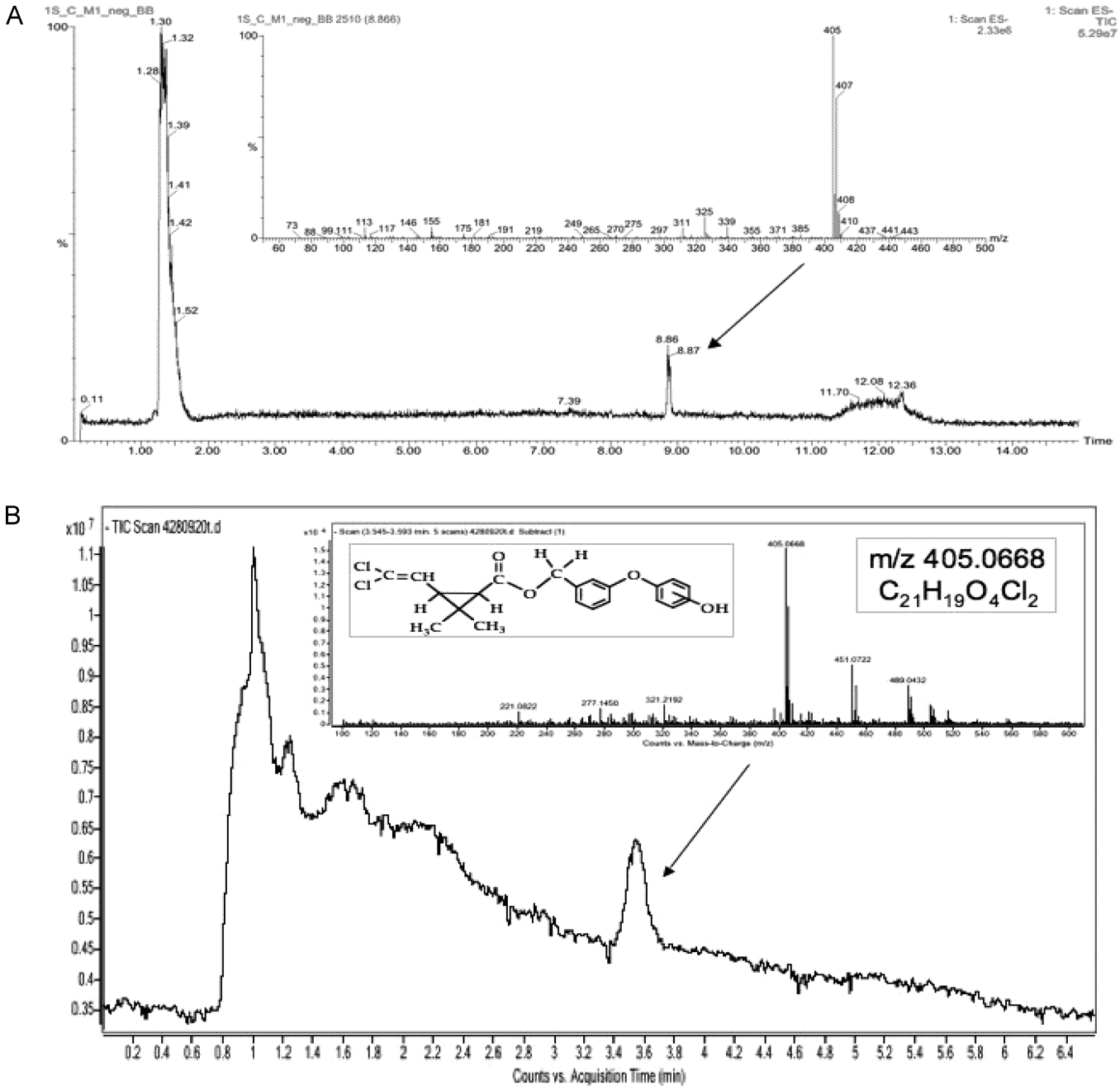

Figure 4. HPLC trace and (A) LC-TQD-MS/MS and (B) LC-TOF-MS spectra of the major metabolite from 1*S-cis*-PM biotransformation in trout liver microsome. Most probable structure of the metabolite is indicated; the major metabolite collected from 1*S-trans*-PM biotransformation is identical to the figure shown here.

more effective estrogen mimics than either 3-PBOH or the parent PM stereoisomers (*31*). *In vitro* estrogenic activity was observed with 4-OH PM, 3-PBOH, and 3,4′-PBOH (*15, 16*). Consistent with these studies, VTG-mRNA expression following 3,4′-PBOH treatment was dramatically higher than the 1*S-cis*-PM enantiomer at the same exposure concentration. While concentration-dependent increases were observed with 3,4′-PBOH and 4OH PM, VTG-mRNA expression was observed only at the 10 μM exposure concentration for 3-PBOH and not at higher concentrations. One potential explanation may be the subsequent oxidation of 3-PBOH to the antiestrogenic 3-PBCOOH (*15*). Oxidation of 3-PBOH to 3-PBCOOH was observed in the present study at high substrate concentrations and also in a previous study with human hepatic microsomes, where one or more isoforms of alcohol dehydrogenase (ADH) was shown to catalyze 3-PBOH oxidation to 3-PBCOOH (*28*). ADH has also been reported in primary hepatocyte culture from rainbow trout (*32*).

Although metabolites from ester cleavage were formed after microsomal incubations with each stereoisomer, the predominant metabolite formed from the most estrogenic stereoisomers (1-*S*) was the 4′-hydroxylated PM (4OH PM). The relatively high acute toxicity of PM to many fish species is likely due to reduced clearance brought about by the significantly lower esterase activity in fish compared to mammals. In mammals and fish, *trans*-Permethrin has been observed to be qualitatively more susceptible to hepatic microsomal esterases than *cis*-PM (*18*). However, in contrast to mammals where hydrolytic cleavage is the major detoxification pathway, very little quantitative hydrolysis of PM diastereomers has been observed in fish (*7*).

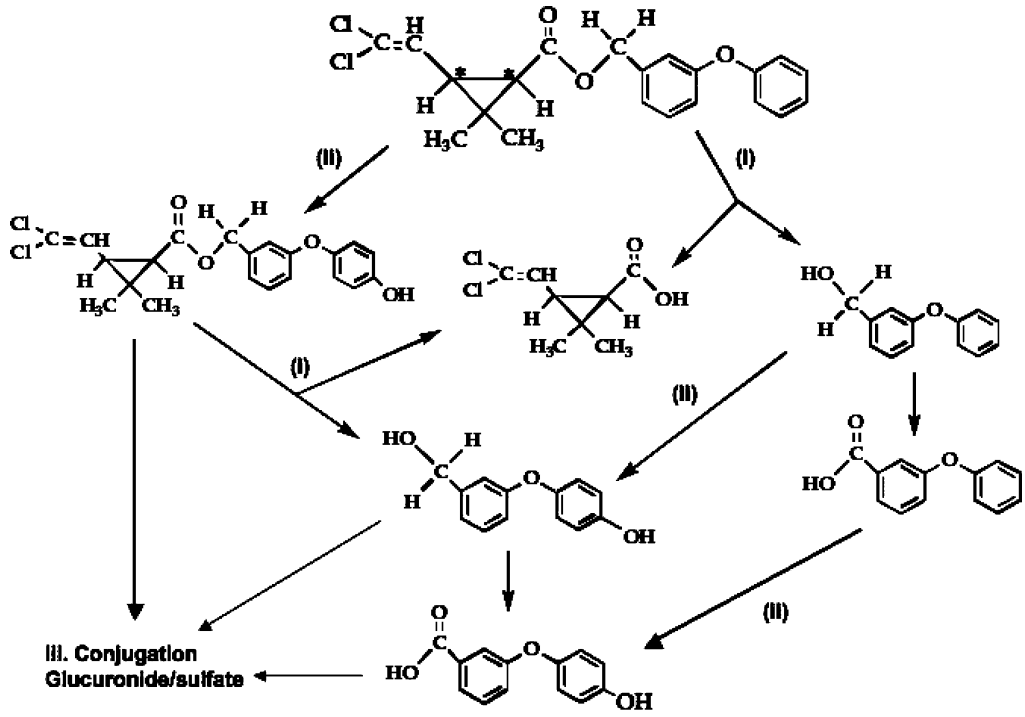

Figure 5. Proposed biotransformation pathway of permethrin in fish ((i) ester hydrolysis; (ii) CYP-catalyzed hydroxylation; (iii) glucuronide/sulfate conjugation); * indicates a chiral center.

Consistent with these results, ester cleavage does not appear to be the major biotransformation pathway for the stereoisomers of PM in trout liver microsomes but rather oxidation.

Previous studies with trout liver microsomes indicated a strong preference for hydroxylation at the 4′ position of the alcohol moiety of *trans*-PM as opposed to other sites (*7, 18*). It is likely that stereoselective cleavage of the hydroxylated ester occurred in the present investigation, as evidenced by the greater than 2-fold higher levels of 3,4′-PBOH produced from *trans*-PM over *cis*-PM stereoisomers indicating hydroxylation at the 4′-position of the alcohol moiety of the parent PM compound (*7, 18*). The significant inhibition of 4OH PM formation by ketoconazole is consistent with a cytochrome P450 (CYP) catalyzed hydroxylation reaction. In addition, the formation of the ester cleavage products was also diminished by ketoconazole. CYP has been shown to catalyze NADPH-dependent cleavage of bulky esters (*33*). Since ketoconazole is a mechanism-based inhibitor for CYP, inhibition of microsomal carboxylesterases is unlikely. Stereoselectivity of CYP has not been previously evaluated in piscine CYPs but is clearly evident in mammals particularly with CYP2 family members (*33*). CYP2C19 had the highest clearance of PM in studies with heterologously expressed human CYPs (*34*). Additional studies should help identify the isoforms responsible for each reaction in rainbow trout and help to better understand the potential for mixture interactions with other CYP inhibitors (PBO) and substrates.

In the present study, consistently greater biotransformation of the 1*S*-*cis*- and 1*S*-*trans*-PM was observed over that of their corresponding antipodes. In addition, enantiomer-specific hydroxylation (1*S*-*cis*-PM) and esterase (1*S*-*trans*-PM) activities were also observed. Recent studies reported similar observations of enhanced oxidative biotransformation, consistent with the clearance of the 1*S* enantiomers of PM diastereomers in human liver microsomes (*28, 34*). In contrast, in rat/mouse hepatic microsome hydrolysis predominates (*35*). Although stereoselective biotransformation of PM has not been studied previously in fish, significant stereoselectivity was observed in the uptake of *cis*-bifenthrin (*cis*-BF) enantiomers in medaka liver, showing greater uptake of 1*R*-*cis*-BF over 1*S*-*cis*-BF, which may be attributed to either the preferential uptake of the 1*R*-*cis*-enantiomer or the stereoselective metabolism of 1*S*-*cis*-bifentrhin (*13*). Taken together, the differences in the metabolic pathway between PM stereoisomers may contribute to stereoselective reproductive toxicity not just in fish (Figures 3−5) but quite possibly in humans as well (*35*).

Commercially available PM is usually a mixed product containing *cis* and *trans* isomers at respective ratios of 40:60 or 25:75. *cis*-PM is reportedly more acutely toxic to mammalian species than the *trans* isomer, with 1*R*-*cis*-PM having equal insecticidal toxicity but greater mammalian toxicity than 1*R*-*trans*-PM (*35, 36*). Recent environmental degradation studies on PM stereoisomers showed significant enrichment of the 1*S*-*cis*-PM in field soils and sediments due to enhanced mineralization of the 1*R*-enantiomer of both *cis*- and *trans*-PM (*12*). Thus, the stereoisomer with the higher estrogenic potential was more persistent and may potentially move into the aquatic environment. Typical PM concentrations in surface water average in the low ng/L concentrations with rare occurrences in low μg/L in runoff events (*37*). With approximate BCFs of 2800 (*38*), accumulation to body burdens capable of eliciting estrogenic responses in fish is plausible and deserves further study.

The results of the present study showed that the insecticidally inactive 1*S*-*cis*- and 1*S*-*trans*- stereoisomers of PM are mainly responsible for the estrogenic potency of *cis*- and *trans*-PM, respectively. In addition, our results also show significant differences in metabolic pathways between PM stereoisomers in fish, which may contribute to the differences in the estrogenic activity between stereoisomers.

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