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Enantioselectivity in Estrogenic Potential and Uptake of Bifenthrin

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Despite the fact that the biological processes of chiral compounds are enantioselective, the endocrine disruption activity and uptake of chiral contaminants with respect to enantioselectivity has so far received limited research. In this study, the estrogenic potential and uptake of the enantiomers of a newer pyrethroid insecticide, bifenthrin (BF), were investigated. Significant differences in estrogenic potential were observed between the two enantiomers in the in vitro human breast carcinoma MCF-7 cell proliferation assay (i.e., the E-SCREEN assay) and the in vivo aquatic vertebrate vitellogenin enzyme-linked immunosorbent assay (ELISA). In the E-SCREEN assay, the relative proliferative effect ratios of 1*S-cis*-BF and 1*R-cis*-BF were 74.2% and 20.9%, respectively, and the relative proliferative potency ratios were 10% and 1%, respectively. The cell proliferation induced by the two BF enantiomers may be through the classical estrogen response pathway via the estrogen receptor (ER), as the proliferation induced by the enantiomers could be completely blocked when combined with 10^{-6} mol/L of the ER antagonist ICI 182,780. Measurement of vitellogenin induction in Japanese medaka (*Oryzias latipes*) showed that, at an exposure level of 10 ng/mL, the response to 1*S-cis*-BF was about 123 times greater than that to the *R* enantiomer. Significant selectivity also occurred in the uptake of BF enantiomers in the liver and other tissues of J. medaka. These results together suggest that assessment of the environmental safety of chiral insecticides should consider enantioselectivity in acute and chronic ecotoxicities such as endocrine disruption.

Introduction

The adverse effects of man-made endocrine-disrupting chemicals (EDCs) on both humans and wildlife have recently become an issue of great concern (*1*). Endocrine disrupting chemicals are defined as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (*2*). The most studied EDCs are xenoestrogens, which mimic the activity of endogenous estrogens such as 17β-estradiol (E_2), and may influence reproductive development, senescence, and carcinogenesis in humans and wildlife (*3*, *4*).

There are several methods for investigating xenoestrogens, including in vivo assays and in vitro assays. In vivo assays include the uterotrophic assay, the vaginal cornification assay, and the vitellogenin induction assay. In vitro assays include ligand binding assay, recombinant receptor−receptor assay, yeast estrogen screening assay, and cell proliferation assay (*3*, *5*−*9*). The human breast carcinoma MCF-7 cell proliferation assay (i.e., the E-SCREEN assay) is an in vitro assay for testing the estrogenic potential using the proliferative effect of estrogens on their target cells, i.e., the estrogen-dependent MCF-7 human breast carcinoma cell line, as an end point (*10*, *11*). On the other hand, vitellogenin (VTG), a phospholipoprotein and yolk protein precursor, is normally produced in the liver of adult female oviparous vertebrates such as fish (*12*, *13*). While little or no vitellogenin can be detected in the liver of normal male fish, when they are exposed to xenoestrogens, the VTG gene is activated and vitellogenin is synthesized in the liver (*12*). The vitellogenin enzyme-linked immunosorbent assay (ELISA) has been developed based on this interaction and proved to be a simple and sensitive in vivo test to assess environmental estrogens (*12*, *13*). VTG ELISA assay using Japanese medaka (*Oryzias latipes*) is an especially preferred method because J. medaka is a popular laboratory model fish with high sensitivity to hormone administration (*12*, *14*, *15*).

Synthetic pyrethroids (SPs) are among the most widely used insecticides. With the restriction on the use of organophosphorus insecticides, the usage of pyrethroids is expected to further increase (*16*). However, studies are starting to show that pyrethroids possess hormone agonist or antagonist activities. For example, fenvalerate, sumithrin, cypermethrin, and deltamethrin displayed significant estrogenicity in MCF-7 human breast carcinoma cell line and Ishikawa Var-I human endometrial cancer cell line, while fenvalerate and *d-trans*-allethrin significantly antagonized the action of progesterone in T47D human breast carcinoma cell line (*4*, *8*, *17*). Bifenthrin (BF), λ-cyhalothrin, and fenvalerate also induced thyroid hormone regulation dysfunction in rats (*18*, *19*).

Pyrethroids contain 1−3 asymmetric positions, making them a family of chiral insecticides with 1−4 pairs of enantiomers. Several recent studies focused on the occurrence of enantioselectivity of chiral pesticides, including pyrethroids, in acute aquatic toxicity (*20*−*25*), and environmental fate (*16*, *26*−*30*). However, so far little attention has been given to enantioselectivity in chronic toxicities such as endocrine disruption activity (*31*−*33*). The purpose of this study was to evaluate enantioselectivity in estrogenic potential of pyrethroids using BF as a model compound. Bifenthrin is a newer pyrethroid insecticide and has widespread use in both agricultural and urban environments.

Materials and Methods

Chemicals and Preparation of Bifenthrin Enantiomers. An analytical standard of racemic (*Z*)-*cis*-BF [99.5%, 2-methylbiphenyl-3-ylmethyl-(*Z*)-(1*RS*)-*cis*-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropane carboxylate] was purchased from Sigma (St. Louis, MO) and used for the MCF-7 cell proliferation assay and the medaka VTG ELISA. The [^{14}C] phenyl-labeled BF (radioactive purity > 98%; radioactive specific activity 75.48 kBq/mol) was obtained from FMC (Princeton, NJ) and used for the uptake test in J. medaka.

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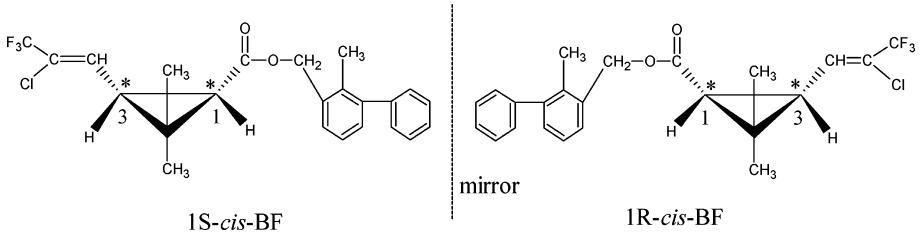

FIGURE 1. Enantiomers of (*Z*)-*cis*-bifenthrin (BF) (* indicates chiral position).

The stereoconfiguration of BF is given in Figure 1. 17β-estradiol (E_2; purity > 98%) was purchased from Sigma and used as the positive control. ICI 182,780 (Tocris, Avonmouth, U.K.), an estrogen receptor (ER) antagonist, was used to test whether the cell proliferation was through the ER pathway. For the cell proliferation assay, all tested chemicals were initially dissolved in ethanol, with the final solvent concentration at <0.1% (v/v). Charcoal-dextran treated fetal bovine serum (CDFBS) was purchased from HyClone (Logan, UT). Other chemicals or solvents used in this study were of cell culture or high-performance liquid chromatography (HPLC) or analytical grade.

The enantiomers of [^{14}C] phenyl-labeled and nonlabeled (*Z*)-*cis*-BF were resolved on a Jasco LC-2000 series HPLC system (Jasco, Tokyo, Japan) equipped with a PU-2089 intelligent quaternary gradient pump, a mobile-phase vacuum degasser, an AS-1559 autosampler with a 100 μL loop, a CO-2060 column temperature-control compartment, a UV-2075 ultraviolet−visible (UV) detector, a variable-wavelength CD-2095 circular dichroism (CD) detector, and a LC-Net II/ ADC data collector. The chromatographic conditions were similar to the methods reported in a previous study (*22*). The detection wavelength of CD detector was 230 nm. Chromatographic data were acquired and processed with the ChromPass software (Jasco, Tokyo, Japan). The resolved enantiomers were individually collected at the HPLC outlet, evaporated to dryness, and used in the following bioassays. Concentrations of the enantiomers were determined on an Agilent 6890N gas chromatograph (GC) equipped with an electron capture detector (ECD), assuming the same response factor for enantiomers as for the racemate of (*Z*)-*cis*-BF.

Experimental Fish. The Japanese medaka (*Oryzias latipes*) (2.5−3.5 cm in total body length) used in this study were purchased from a local supplier. All new fish were observed daily for 10 days for signs of illness and maturity. The healthy adult male fish were kept in glass tanks under a summer photoperiod (14 h light/10 h dark) at 25 ± 1 °C. The dechlorinated water was used and changed three times per week. The water was aerated by continuous air-bubbling. Fish were fed brine shrimp eggs (Red Sun Aquaculture, Tianjin, China) twice a day.

MCF-7 Cells and Cell Proliferation Assay. MCF-7 cells, purchased from the Cell Bank of the Chinese Academy of Science, Shanghai, China (the original source is American Type Culture Collection, ATCC, Manassas, VA), were grown in the seeding medium (Eagle's minimum essential medium (MEM) with phenol red, supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, 0.1 mM of nonessential amino acids, 4 mM of L-glutamine, 0.1 mM of sodium pyruvate, 10 μg/mL of insulin, and 10% of fetal bovine serum) at 37 °C with 5% CO_2 in a humidified Thermo model 3111 forma series II water-jacketed CO_2 incubator (Thermo Electron, Marietta, OH). The overall procedures for the test were based on previously described methods with some modifications (*8*, *10*, *11*). Briefly, MCF-7 cells (1 000 cells/well) were seeded into 96-well plates (Costar, Cambridge, MA) and were allowed to attach for 24 h. Then the medium was replaced with the experimental medium (phenol red-free MEM containing 5% CDFBS) for 2 days to improve the sensitivity of MCF-7 cells to estrogen. After that, the medium was changed to the dosing medium (the experimental medium along with a range of concentration of test compounds). After 3 days, the dosing medium was refreshed. Again 3 days later, cell proliferation was terminated by removing the medium from the wells, adding a thiazolyl blue (Amresco, Solon, OH) solution (5 mg/mL of phosphate-buffered saline, PBS). After 4 h, the solution was removed and 150 μL of dimethyl sulfoxide (DMSO) per well was added. After 10 min of shaking with a micromixer, the absorbance was measured at 490 nm with a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA). Results were calculated as the ratio of each exposure group over the vehicle control (0.1% ethanol, 1-fold) and as the mean ± standard deviation (SD) of five independent measurements performed in four replicates. The results were analyzed using *t*-test with Origin (OriginLab, Northampton, MA). The estrogenicity of BF enantiomers was evaluated (a) by assessing the proliferative effect (PE), which is the ratio between the highest cell yield obtained with the test compound and that with the hormone-free negative control; (b) by determining the relative proliferative effect (RPE), which is 100 times the ratio between the highest cell yield obtained with the xenoestrogens and with E_2; and (c) by measuring the relative proliferative potency (RPP), which is the ratio between the minimal concentration of E_2 needed for maximal cell yield and the minimal dose of the test xenoestrogen needed to achieve its maximal cell yield effect. RPE is calculated as the ratio (PE-1) of the test xenoestrogen over (PE-1) of E_2, multiplied by 100. Thus, RPE indicates whether the chemical being tested induces a proliferation quantitatively similar to the one obtained with E_2. That is, RPE of 100 suggests a full agonist, while RPE significantly lower than 100 is a partial agonist (*10*). The cell proliferation induced by 17β-estradiol (E_2) was included as a positive control to validate the cell line. 1×10^{-12} to 1×10^{-8} mol/L of E_2 induced pronounced proliferation ($p < 0.01$) of MCF-7 cell, and the maximum proliferation was 4.57-fold relative to the negative control (0.1% ethanol) at 1×10^{-10} mol/L (Figure 2). Therefore, 1×10^{-10} mol/L of E_2 was selected as the positive control in the cell proliferation tests by the two enantiomers of BF. The proliferation induced by E_2 was completely inhibited by addition of 1×10^{-6} mol/L of ICI 182,780, a pure antiestrogen (Figure 2). The solvent ethanol was also tested for its ability to affect MCF-7 cell proliferation and was found to have neither estrogenic nor inhibitory activity (data not shown).

Measurement of Liver Vitellogenin Level. Bifenthrin enantiomers were dissolved in ethanol (1.0 mg/mL) and diluted to 10 ng/mL in rearing water immediately prior to use. Adult male medaka fish were exposed to the above testing solutions under static conditions for 10 d. The vitellogenin synthesis level in livers of medaka was quantified using the medaka vitellogenin ELISA kit (Biosense Laboratories, Bergen, Norway). The fish were kept on ice for 1−2 min, and the livers were extracted, weighed, and then homogenized in 250 μL of the ice-cold dilution buffer included in the ELISA kit. The homogenized liver samples were immediately centrifuged (8000×*g*, 10 min, 4 °C) and the supernatant was stored at −80 °C until use. The samples were measured within

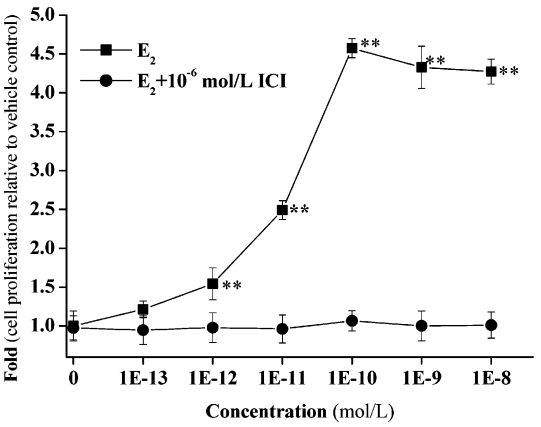

FIGURE 2. Proliferation of MCF-7 cells grown in 5% CDFBS-supplemented medium exposed to E_2 and combined with 10^{-6} mol/L of ICI 182,780. Results are presented as mean ± SD of five independent assays (indicates $p < 0.01$ by *t*-test).**

1 week according to the manufacturer's protocol. Briefly, the samples were thawed on ice, and then the dilutions of standard and samples were prepared. Dilution buffer (100 μL) was added to the nonspecific binding (NSB) wells, and diluted standards and liver samples (100 μL) were added to the remaining wells, followed by incubation at room temperature for 1 h. After washing three times with 200 μL of washing buffer per well, 100 μL of the diluted detecting antibody was added to all wells and incubated at room temperature for 1 h. Subsequently, the plates were washed with 200 μL of washing buffer per well for three times, and 100 μL of the diluted horseradish peroxidase (HRP)-labeled secondary antibody was added to all wells and incubated at room temperature for 1 h. After washing the plates five times with 200 μL of washing buffer per well, 100 μL of *o*-phenylenediamine (OPD) peroxidase substrate solution was added to all wells and incubated in the dark at room temperature for 30 min. A 50 μL aliquot of 2 M H_2SO_4 per well was used to stop the reaction. After 5 min, the absorbance was read at 490 nm with a Bio-Rad model 680 microplate reader. The data were analyzed using the Origin software according to the methods provided by the manufacturer of the test kit, and the *t*-test was used to indicate the significant differences ($n = 5$).

Uptake of Bifenthrin Enantiomers by J. Medaka. ^{14}C-labeled BF enantiomers were dissolved in acetone (1.0 mg/L) and diluted to 10 ng/mL in rearing water before use. Half of the adult male medaka fish were exposed to the above testing solution of 1*R-cis*-bifenthrin, and the other half of the fish were exposed to that of 1*S-cis*-BF under static conditions for 4 d, respectively. At 1, 2, 3, and 4 d after the exposure was begun, the fish were rinsed with deionized water, from which the livers were extracted. The liver tissue and the remaining whole fish were then dried at room temperature and combusted on an OX500 biological pxidizer (R.J. Harvey, Hillsdale, NJ). A LS 5000TD liquid scintillation counter (LSC; Beckman, Fullerton, CA) was used to measure the released ^{14}C activity, from which the uptake of BF enantiomers was determined (*34*).

Results and Discussion

Enantiomer Separation and Isolation. Baseline separation of (*Z*)-*cis*-BF enantiomers was achieved at a reduced flow rate of the mobile phase at 0.4 mL/min (Figure 3). From the previous studies (*22*, *23*) and Figure 3, it may be concluded that the 1*R-cis*-BF is dextral (+) at 675 nm and sinistral (−) at 365 nm and has a negative (−) Cotton effect at 230 nm on the CD detector, while the 1*S-cis*-BF is sinistral (−) at 675 nm and dextral (+) at 365 nm and has a positive (+) Cotton effect at 230 nm on the CD detector. The purity of the enantiomers was determined to be >99% as analyzed by GC−ECD.

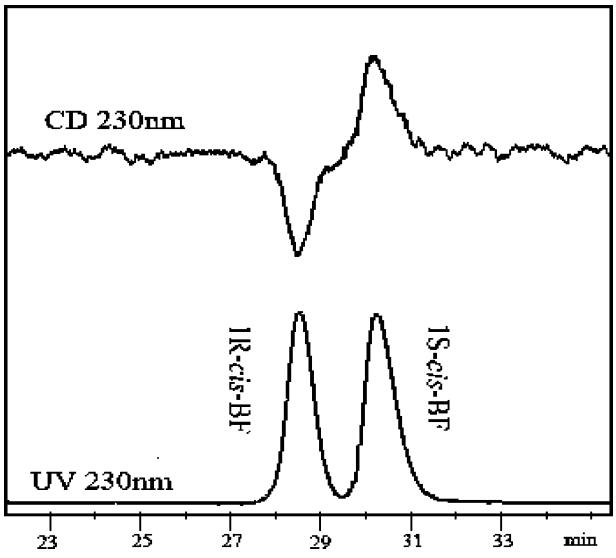

FIGURE 3. HPLC chromatogram showing enantiomeric separation of (*Z*)-*cis*-bifenthrin on a Sumichiral OA-2500-I column (detection wavelength 230 nm).

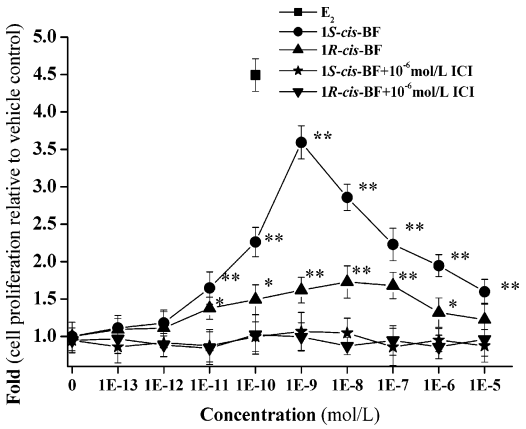

FIGURE 4. Proliferation of MCF-7 cells grown in 5% CDFBS-supplemented medium exposed to enantiomers of (*Z*)-*cis*-bifenthrin and combined with 10^{-6} mol/L of ICI 182,780. Results are presented as mean ± SD of five independent assays (* indicates $p < 0.05$ and ** indicates $p < 0.01$ by *t*-test).

TABLE 1. Estrogenic Effect of Enantiomers of Bifenthrin (BF) Measured by the E-SCREEN Assay

compound	concentration[a] (mol/L)	PE[b]	RPE[c] (%)	RPP[d] (%)
E_2	1×10^{-10}	4.49	100	100
1*S-cis*-BF	1×10^{-9}	3.59	74.2	10
1*R-cis*-BF	1×10^{-8}	1.73	20.9	1

[a] The lowest concentration needed for maximal cell proliferation. [b] The proliferative effect (PE) calculated as the ratio of the maximal cell yield of the test compound to the cell yield of the negative control. [c] The relative proliferative effect (RPE) calculated as the ratio (PE-1) of the test chemical over (PE-1) of E_2 (×100). [d] The relative proliferative potency (RPP) calculated as the ratio between E_2 and xenoestrogen doses needed to produce maximal cell proliferation (×100).

Enantioselectivity in in vitro E-SCREEN Assay. The dose−response relationships of cell proliferation induced by the BF enantiomers and the combined effects with 10^{-6} mol/L of ICI 182,780 are shown in Figure 4. From Table 1, PE values of 1*S-cis*-BF and 1*R-cis*-BF were 3.59 and 1.73, respectively. In comparison with the PE of E_2 synchronously (4.49), RPE

was determined to be 74.2% for 1*S*-*cis*-BF and 20.9% for 1*R*-*cis*-BF. In addition, by comparing with the concentration needed for maximal cell proliferation of E_2 (1×10^{-10} mol/L), RPP was calculated to be 10% for 1*S*-*cis*-BF and 1% for 1*R*-*cis*-BF. Therefore, the estrogenic potential of 1*S*-*cis*-BF was greater than that of 1*R*-*cis*-BF. The proliferation induced by BF enantiomers was completely blocked by 10^{-6} mol/L of ICI 182,780 (Figure 4). These results suggest that BF enantiomers acted through the classical estrogen response pathway via the estrogen receptor (*4*, *8*). Bifenthrin having the structural components similar to estrogen metabolites, may have contributed to its estrogenic response (*4*). On the other hand, the open phenyl group of BF could undergo hydroxylation via a cytochrome P450 monooxygenase-mediated conversion and generate metabolic byproducts in situ by the MCF-7 cells. These metabolic byproducts are possible EDCs. The enantioselectivity of BF in inducing estrogenic activity may be attributed to the different stereoconfigurations of BF enantiomers when interacting with enzymes or other naturally occurring chiral biomolecules.

Enantioselectivity in in vivo Vitellogenin ELISA Assay. No fish mortality was observed during the liver vitellogenin synthesis induced by BF enantiomers under the test conditions. The liver vitellogenin level of the adult male medaka fish placed in BF-free dechlorinated water (containing 0.001% solvent ethanol) for 10 d was the negative control and was below the detection limit as measured with the medaka vitellogenin ELISA kit. Both 1*S*-*cis*-BF and 1*R*-*cis*-BF showed the ability to induce the production of VTG. However, there was a drastic difference between the two enantiomers in their relative estrogenic potential for inducing VTG production. The average level of VTG in the adult male medaka exposed to 1*S*-*cis*-BF for 10 d was 1532 ng/mg, while that with exposure to 1*R*-*cis*-BF was only 12.45 ng/mg. Therefore, at the given exposure level (10 ng/mL), the response to 1*S*-*cis*-BF was about 123 times greater than that to the *R* enantiomer based on medaka vitellogenin induction. As with the MCF-7 cells, the VTG expression effects observed by the BF enantiomers may be due to the in situ formation of metabolic intermediates.

Previous studies showed enantioselectivity in estrogenic activity of the enantiomers of *o*,*p*′-Dichloro-Diphenyl-Trichloroethane (DDT), suggesting that the *R*-*o*,*p*′-DDT was a more active estrogen mimic in rats (*31*, *32*). Hoekstra et al. (*33*) used a yeast-based assay to assess the enantiomer-specific transcriptional activity of *o*,*p*′-DDT with the human estrogen receptor (hER) and found that the *R* enantiomer was the active estrogen mimic, whereas the hER activity of *S*-*o*,*p*′-DDT was negligible. DDT and analogs have been banned for use in most parts of the world for several decades. This study suggests that enantioselectivity in endocrine disruption may also occur among chiral pesticides that have current use.

Enantioselectivity in Uptake in J. Medaka. Significant differences were noted in the uptake of BF enantiomers by J. medaka (Figure 5). The uptake of BF in the liver was generally greater than that in the other tissues. For example, the ratios of 1*R*-*cis*-BF in the liver to that in the other tissues were 12.8, 10.1, 10.5, and 9.5 after 1, 2, 3, and 4 d exposure, respectively. The ratios of the 1*S* enantiomer in the liver to that in the other tissues were 9.6, 5.1, 4.3, and 5.5 after 1, 2, 3, and 4 d exposure, respectively (Table 2). Under the same conditions, uptake of 1*R*-*cis*-BF in both the liver and the other tissues was consistently greater than that of 1*S*-*cis*-BF. For example, the ratios of 1*R*-*cis*-BF to 1*S*-*cis*-BF in liver tissues were 5.0, 4.0, 4.6, and 5.2 after 1, 2, 3, and 4 d exposure, respectively. The ratios of 1*R*-*cis*-BF to 1*S*-*cis*-BF in the other fish tissues were 3.7, 2.0, 1.9, and 3.0 after 1, 2, 3, and 4 d exposure, respectively (Table 2).

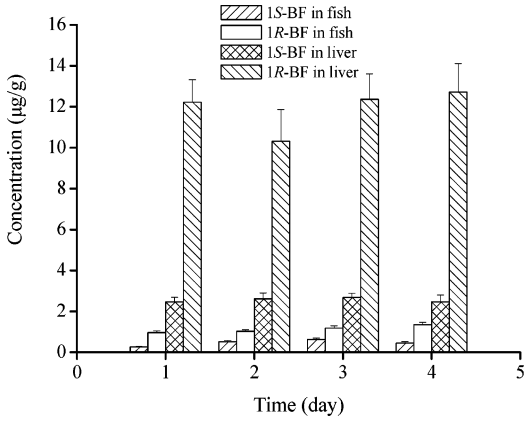

FIGURE 5. Uptake of the enantiomers of bifenthrin in Japanese medaka ($n = 5$). 1*S*-BF in liver and 1*R*-BF in liver indicated the uptake of 1*S*-*cis* bifenthrin and 1*R*-*cis* bifenthrin in the fish liver tissues, respectively. 1*S*-BF in fish and 1*R*-BF in fish indicated the uptake of 1*S*-*cis*-bifenthrin and 1*R*-*cis*-bifenthrin in the fish tissues excluding liver, respectively.

TABLE 2. Uptake of the Enantiomers of Bifenthrin in Japanese Medaka ($n = 5$)

day	*R*/*S* (fish)[a]	*R*/*S* (liver)[b]	liver/fish (*R*)[c]	liver/fish (*S*)[d]
1	3.7	5.0	12.8	9.6
2	2.0	4.0	10.1	5.1
3	1.9	4.6	10.5	4.3
4	3.0	5.2	9.5	5.5

[a] *R*/*S* (fish)-ratio of 1*R*-*cis*-bifenthrin to 1*S*-*cis*-bifenthrin in fish tissues excluding the liver. [b] *R*/*S* (liver)-ratio of 1*R*-*cis*-bifenthrin to 1*S*-*cis*-bifenthrin in the liver tissue of J. medaka. [c] Liver/fish (*R*)-ratio of 1*R*-*cis*-bifenthrin in the liver to that in the other tissues. [d] Liver/fish (*S*)-ratio of 1*S*-*cis*-bifenthrin in the liver to that in the other tissues.

Several recent studies addressed the occurrence of enantioselectivity in aquatic toxicity and biodegradation of BF and other pyrethroids. For instance, Liu et al. (*22*) found that 1*R*-*cis*-BF was 18 and 22 times more active than the 1*S*-*cis* enantiomer in causing acute toxicity to aquatic invertebrates *Ceriodaphnia dubia* and *Daphnia magna*, respectively. The 1*S*-*cis* enantiomer of BF was found to be preferentially degraded in sediments, and the deviation in enantiomer ratio from the original value increased with the age of the sediments (*20*, *21*). In this study, both the in vitro mammalian cell proliferation assay and the in vivo aquatic vertebrate vitellogenin synthesis assay showed clear enantioselectivity between BF enantiomers in inciting estrogenic activity. In fact, as 1*S*-*cis*-BF uptake into the liver of J. medaka was relatively less but the induction of the liver vitellogenin synthesis was substantially greater than that of the 1*R* enantiomer, the absolute estrogenic potential of 1*S*-*cis*-BF could be much stronger than that of its *R* counterpart compared to what was shown through the in vivo ELISA test.

Results from this study suggest that enantiomers of BF possess estrogenic properties and that the estrogenic activity is enantioselective. This is the first report on the enantioselectivity in endocrine-disruption activity of synthetic pyrethroids. Further in vitro and in vivo studies are needed to better understand the effects of such xenoestrogens on the endocrine system in both humans and wildlife. It must also be noted that while 1*R*-*cis*-bifenthrin has higher acute aquatic toxicity, 1*S*-*cis*-BF displays higher estrogenic potential. Therefore, a general rule with regard to the direction of enantioselectivity may not exist between acute and chronic toxicities. Studies so far suggest that monitoring of the racemate concentration will give an inadequate or misleading basis for assessing the environmental risk of chiral pesticides

(*20*). Given the widespread use of pyrethroids, a more comprehensive understanding of the significance of enantioselectivity in both acute and chronic ecotoxicities is imperative for improving risk assessment and regulation of these pesticides (*20*). Development of enantiomer-enriched pesticide products should consider the selection of the enantiomer with a high potency toward the target organisms but a low adverse effect to nontarget species.

Acknowledgments

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