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Enantioselectivity in Zebrafish Embryo Toxicity of the Insecticide Acetofenate

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Enantioselectivity in separation and toxicology of chiral xenobiotics have become one of the frontier topics interfacing chemistry and toxicology. In this study, enantiomers of insecticide acetofenate (AF) were separated on selected chiral columns by HPLC, and enantioselectivity in developmental toxicity was evaluated using the zebrafish embryo-larval assays. The AF enantiomers were baseline separated on Chiralcel OD, Chiralpak AD, and Sumichiral OA-2500I columns under optimized conditions. Pure enantiomers were obtained on Chiralcel OD. Optical rotatory dispersion (ORD) and circular dichroism (CD) detectors were used to determine the elution order and CD spectra of the enantiomers. The absolute configuration of enantiomers was identified as *S*-(+)-AF and *R*-(−)-AF by the octant rule from force-field calculations and CD spectra. The individual enantiomers were used in 4-day zebrafish embryo-larval bioassays, and a series of developmental end points were measured and compared. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to investigate the expressions of estrogen receptor alpha (ERα) in zebrafish embryo exposed to varying enantiomers. While the enantiomers showed no difference in acute toxicity, significant enantioselectivity was observed in developmental toxicities such as yolk sac edema and pericardial edema. The data of qRT-PCR showed that there was about 3.2-fold induction in the mRNA levels of ERα between fish exposed to (+)-AF and (−)-AF. The results suggest that enantioselectivity may occur at the developmental level even in the absence of selective acute toxicity and should be considered when evaluating ecotoxicological effects of chiral contaminants.

Introduction

A great number of man-made chemicals are chiral, and the significance of enantioselectivity in their fate and effects in the environment have just started to receive attention (*1, 2*). For chiral pesticides, typically only one enantiomer is active against the target pests, while the other enantiomer is inactive but serves as an unwanted burden to the environment (*3*). Studies show that chiral selectivity may occur not only in the biological activity of chiral compounds but also in their microbially mediated environmental fate processes (*4*−*8*). However, most chiral pesticides are produced and released into the environment as racemates, even though their enantiomers can exhibit significant differences in biological activity and toxicity, as well as in environmental behaviors (*9, 10*).

Conventional short-term bioassays, such as 48-h static acute toxicity tests using aquatic invertebrates *Ceriodaphnia dubia* or *Daphnia magna*, have been used for detecting chiral selectivity of pesticides in toxicity profiles (*1, 7*). Recently, we reported enantioselectivity in chronic toxicities such as estrogenic potential using bifenthrin as a model compound (*11*). However, no attention has been given to enantioselectivity in developmental toxicity of chiral xenobiotics. The fish egg assay using zebrafish is a relatively new approach for evaluating lethal and sublethal effects on aquatic vertebrates (*12, 13*). The eggs of zebrafish are transparent, and several nonlethal development end points can be simultaneously determined, providing more insight over simple acute toxicity assays. It is known that many biochemical and molecular mechanisms occur among cells, tissues, and organs during embryogenesis. A great number of pollutants could specifically influence these mechanisms and the possible impact on the tissue differentiation and organization. Previous studies indicated that some environmental endocrine disruptors, such as tetrachlorodibenzo-*p*-dioxin, polychlorinated biphenyls, and dichloro-diphenyl-trichloroethane (DDT), might affect embryo development via estrogen receptor (ER) or aryl hydrocarbon receptor (AhR)-mediated signal pathways (*14, 15*). The signal pathway mediated by ERα through estrogen and estrogen-like chemicals may also play an important role in embryo development (*16*−*18*).

In this study, we used acetofenate (AF, also known as 7504, plifenate, or benzethazet) as a model chiral xenobiotic to evaluate its enantiomer resolution on HPLC columns and enantioselectivity in lethal and developmental effects to zebrafish embryo. AF is an organochlorine pesticide containing one chiral center and is widely used to control mosquitoes and flies both indoors and outdoors in China and other regions of southeastern Asia (*19*). AF displays high efficacy against pests that have developed resistance to DDT or hexachlorocyclohexance and is considered as a good replacement to these legacy organochlorine compounds when used alone or in combination with pyrethroid insecticides. The production of AF in China averages several hundred tons each year. Its application may pose a great chance for human exposure via the respiratory system or cutis. AF has a solubility of 40 mg L^{-1} in water, and it is stable under

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neutral pH. There is little information available on its ecotoxicology, and essentially no information is available about its enantioselectivity potentials. We expect that the procedures developed in this study may be useful to better understand the enantioselectivity in the bioactivity of other chiral chemicals at the sublethal level.

Materials and Methods

Chemicals and Reagents. Acetofenate [AF, 2,2,2-trichloro-1-(3,4-dichlorophenyl)ethyl acetate, 97.3% purity] was purchased from Xinhuo Technology Institute (Baoding, China). Solvents including *n*-hexane, ethanol (EtOH), isopropanol (IPA), and 1,2-dichloroethane (DCE) were HPLC grade from Tedia (Fairfield, OH, USA). The pesticide stock solution was prepared by dissolving AF in *n*-hexane at 2000 mg L^{-1} and stored at 4 °C in the dark. Working solutions were prepared daily by diluting the stock solution to 200 mg L^{-1} with *n*-hexane.

Chromatographic Separation and Analysis. Enantiomer separation was performed on a Jasco LC-2000 series HPLC system (Jasco, Tokyo, Japan) equipped with a PU-2089 quaternary gradient pump, a mobile phase vacuum degasser, an AS-1559 autosampler with a 100-μL sample loop, a CO-2060 column temperature control compartment, a UV-2075 plus UV/vis detector, a variable-wavelength CD-2095 circular dichroism detector (CD), an OR-2090 PLUS optical rotatory dispersion detector (ORD), and a LC-Net II/ADC data collector. Chromatographic data were acquired and processed with the ChromPass software (Jasco). Chiralcel OD and Chiralpak AD columns were purchased from Daicel Chemical Industries (Tokyo, Japan). Sumichiral OA-2500I column was obtained from Sumika Chemical Analysis Service (Osaka, Japan). The signals of UV and CD detectors were recorded at 230 nm. A volume of 10 μL (~1000 mg L^{-1}) was injected for chiral separation in the normal-phase mode with *n*-hexane as the primary carrier and EtOH, IPA, or DCE (1~5%) as the polar modifier.

After initial method development and on the basis of the separation results, Chiralcel OD was used to prepare the individual enantiomers that were used for subsequent toxicity assays. The resolved enantiomers were manually collected into separate glass vials at the HPLC outlet. The fractions were evaporated to dryness under a stream of nitrogen and redissolved in acetone. The purity and concentration of the recovered enantiomers were determined by chiral HPLC and GC analysis, respectively. For GC analysis, an Agilent 6890N GC equipped with an electron capture detector (ECD) and a HP-5 capillary column were used for concentration quantification. The flow rate of carrier gas (nitrogen) was 1.0 mL min^{-1}. The column temperature was held at 160 °C. The temperature in the inlet was 260 °C, and individual enantiomers were introduced through 1.0 μL injection in the splitless mode. The purity was found to be ≥99.9% for the two enantiomers in this study (the enantiomeric fraction was found to be ≥0.999 for the two enantiomers), and no racemization was observed (Figure S1, Supporting Information).

Chiroptical Detection and Absolute Configuration Determination. The resolution by HPLC along with detection by CD and ORD was used together for distinguishing the enantiomers. The CD spectra were recorded by the stop-flow method at the top of the chromatographic peak with the wavelength ranging from 220 to 420 nm. The CD spectra were obtained with a resolution of 0.2 nm with 10× accumulation. The background CD spectrum of the mobile phase was recorded before the beginning of the CD scanning of the separated enantiomers and was subtracted from the spectrum of each peak. The chromatogram evaluation and data handling were performed using a Borwin 1.50 system controller for Windows (JASCO, Tokyo, Japan). The octant rule was applied to establish the absolute configuration of AF enantiomers. Chem 3D Ultra 8.0 software (CyberChem, Gainesville, FL, USA) was used for calculating the lowest energy molecular conformations.

Acute Fish Toxicity Assay. Zebrafish (*Danio rerio*) were obtained from a local pet store and cultured in the laboratory for more than one month. The fish were kept on a 14 h light:10 h dark cycle and were fed twice daily with *Artemia nauplii*. The feeding was stopped 24-h before exposure. Water was dechlorinated and prefiltered through activated carbon prior to use. The water temperature was maintained at 26 ± 1 °C and the water pH at 7.0 ± 0.3. Enantioselectivity in acute fish toxicity was evaluated by using a procedure consistent with that of the Organization for Economic Co-operation and Development (OECD) (*20*). Briefly, eight fish were exposed to 4 L test solutions containing a given enantiomer (or racemate) over a known concentration range in 10 L glass aquaria. The purchased AF was used as the racemate. The exposure solutions were changed daily. The mortality and survival of the fish was monitored at each 24-h interval until reaching 96-h. The concentration (mg L^{-1}) that caused 50% mortality of the test population (LC_{50}) was determined. Eight untreated fish kept in 4 L dechlorinated water (containing 0.01% solvent acetone) were used as control. All of the tests were considered valid if control mortality was ≤5%. LC_{50} values and 95% confidence limits were determined by probit analysis (ToxCalc v5.0, Tidepool Scientific Software, Mckinleyville, CA).

Fish Embryo Exposure and Toxicity Assay. The embryo collection and preparation techniques followed that of Westerfield (*21*), and the obtained embryos were used for the exposure experiment. The embryo toxicity test procedure was similar to the OECD test guidelines (*22*). Briefly, the eggs were transferred to various exposure chambers containing the test compound with known concentrations at least 60 min after the initial spawning. Fertilized eggs were separated from the nonfertilized ones and placed in 24-well plates (Krackeler Scientific, Albany, NY, USA) using a pipet. Twenty fertilized eggs were placed individually in 2 mL of the respective test solutions containing of 0.5% acetone in reconstituted water according to Westerfield (*21*). The reconstituted water contained 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 4.2 mM $NaHCO_3$ in ultrapure water (18.3 MΩ-cm resistivity) produced by Milli-Q system (Millipore, MA, USA). The remaining four wells of each plate served as internal control that contained only artificial water with 0.5% acetone. Each plate was used as one replicate for one concentration level. Three replicates were used for each concentration treatment. The plates were covered with lids made of the same material as the plate to minimize water evaporation. Incubation was carried out at 27 ± 1 °C and with 24-h light daily, as we have previously done in similar studies (*23*).

The development of embryos from blastula to early larval stages was monitored at specified time points (t = 12, 24, 36, 48, 60, 72, 84, and 96 h) throughout the 96-h exposure experiment. End points used for assessing the effects of AF enantiomers included egg and embryo mortality, malformations, yolk sac edema, pericardial edema, tail not detached, crooked body, and hatching success. No observed effect concentrations (NOECs) were defined as the highest concentration above negative control at which no significant effect was observed. The NOECs were estimated on the basis of the developmental and teratogenic responses of the embryos at different concentrations.

Isolation of RNA and Reverse Transcription. A pool of 5 embryos after 72 h exposure was homogenized in 0.5 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with a homogenizer (Polytron, Kinematica, Littau, Switzerland). Total RNA was isolated according to the manufacturer's instructions. The ratio of absorbance at 260 nm to that at 280 nm as well as the banding patterns on a 2% agarose formaldehyde gel were used to verify the quality of the RNA in each sample. Reverse transcription (RT) was carried out using an M-MLV reverse transcriptase kit (Takara Biochemicals, Dalian, Jiangsu, China). First-strand cDNA synthesis was performed by priming with 500 ng of RNA and 50 pmol of oligo-dT in 10 μL reaction mixture containing 0.5 mM each of dNTP mixture, 1 U/μL of Rnase inhibitor, 5 U/μL M-MLV reverse transcriptase, and 1× M-MLV buffer. The reaction mixture was maintained at 42 °C for 25 min and then heated at 95 °C for 2 min

Table 1. Sequences of Primer Pairs Used in the Real-Time Quantitative PCR

gene name	primer	position	accession number
ERα	forward 5′-CCCACAGGACAAGAGGAAGA-3′ reverse 5′-CCTGGTCATGCAGAGACAGA-3′	780–1011	AF268283
β-Actin	forward 5′-ATGGATGAGGAAATCGCTGCC-3′ reverse 5′-CTCCCTGATGTCTGGGTCGTC-3′	54–160	AF057040

to stop the RT reaction. A portion of 0.5 μL RT products was used directly for quantitative real-time polymerase chain reaction (qRT-PCR).

Real-Time Quantitative PCR. Real-time quantitative PCR was performed on a quantitative PCR instrument (ABI 7300, Foster City, CA, USA). Oligonucleotide primer pairs of ERα and housekeeping genes were used (Table 1). β-Actin transcript was used to standardize the results by eliminating variations in mRNA and cDNA quantity and quality. Each mRNA level was expressed as its ratio to β-Actin mRNA.

The SYBR reaction mixture consisted of modified polymerase (including SYBR green, optimized PCR buffer, $MgCl_2$, and dNTP mix), ROX reference dye, and RNase-free water (Takara Biochemicals, Dalian, Jiangsu, China). The PCR protocol used was as follows: denaturation program (95 °C for 10 s) and amplification and quantification program repeated for 40 cycles (95 °C for 5 s, and then 60 °C for 30 s). Data were collected at the end of each extension step. For the mathematical analysis, the change of fluorescent signal at every cycle was measured. The relative quantification of gene expression among the treatment groups was analyzed by the $2^{-\Delta\Delta Ct}$ method (*24*).

Statistical Analysis. The comparison of data for acute fish toxicity was made between enantiomers and racemate at the same time point. The comparison also was made between different time points of the same analyte using one-way analyses of variance (ANOVA) followed by a multiple comparison test of means (Tukey test). The toxicity data of abnormality and mRNA express for individual enantiomers and racemate were submitted to one-way ANOVA to determine significant differences. The software package used for the statistical analysis was Origin 7.0 (Northampton, MA, USA). The differences were considered statistically significant when p was less than 0.05.

Results and Discussion

Effect of Mobile Phase Composition on Chiral Separation. The resolution of AF enantiomers was evaluated on three commercial columns. Optimization of the chromatographic conditions was made by investigating the effect of the type and concentration of mobile-phase modifier (i.e., EtOH, IPA, and DCE) on the resolution. Figure 1 shows the chromatograms of AF on the three columns under optimal conditions. All of the chromatographic results were highly reproducible. The three columns showed different separation abilities for AF. Excellent baseline separation of AF enantiomers was achieved on Chiralcel OD column using 100% *n*-hexane as the mobile phase at 0.50 mL min^{-1} and 40 °C (Figure 1a). OA-2500I showed baseline separation of AF enantiomers with the addition of any of the modifiers (i.e., EtOH, IPA, or DCE) at 1−5%. To reduce the retention time, *n*-hexane/1,2-dichloroethane (95/5, v/v) as the mobile phase at 0.50 mL min^{-1} and 40 °C was used with the OA-2500I column (Figure 1b). Finally, the Chiralcel AD column showed baseline separation of AF enantiomers using 99/1 *n*-hexane/IPA at 0.40 mL min^{-1} and 25 °C (Figure 1c). The separated enantiomers of AF were collected at the HPLC outlet and were further identified.

Elution Order and Absolute Configurations of AF Enantiomers. Both the OR and CD detectors are available that can be used to characterize enantiomers after elution. ORD detection is based on the refractive index difference between right and left linearly polarized lights, while CD detection is based on an absorption difference between right and left circularly polarized light. Recently, the coupling of HPLC to CD was successfully applied for the determination of absolute configuration and elution order of a number of chiral compounds (*25*).

Because of the low response, 2000 mg L^{-1} of AF in hexane was injected to obtain the ORD signal. The results showed that

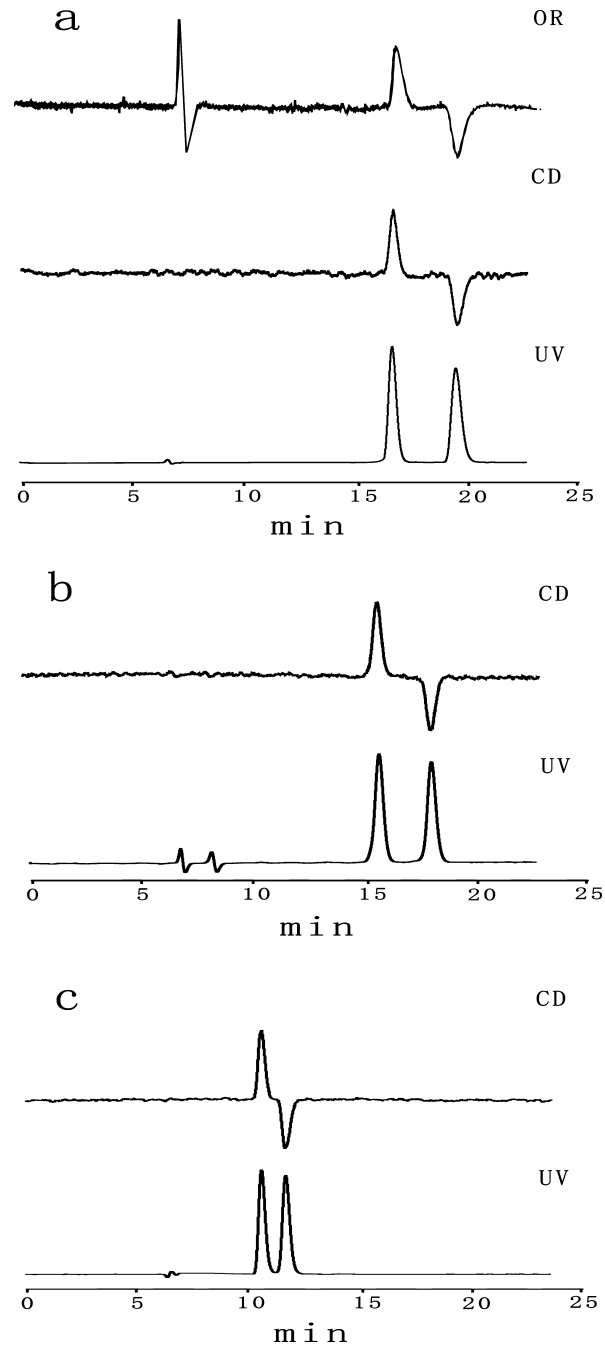

Figure 1. Chiral chromatograms of acetofenate on different columns. (a) Chiralcel OD column with100% *n*-hexane (40 °C and 0.50 mL min^{-1}); (b) OA-2500I column with 95/5 *n*-hexane/1,2-dichloroethane (v/v) (40 °C and 0.50 mL min^{-1}); (c) Chiralpak AD column with 99/1 *n*-hexane/isopropanol (25 °C and 0.40 mL min^{-1}).

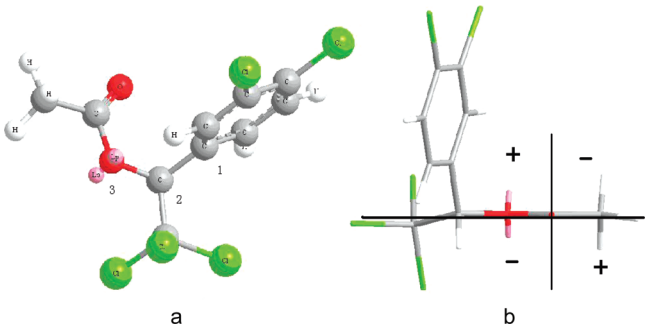

Figure 2. Application of the octant rule.

Table 2. LC_{50} values of Acetofenate Enantiomers (+ or −) and Racemate (±) to Zebrafish (mg L^{-1})[a]

time (h)	(±)	(+)	(−)
24	$1.25 \pm 0.12^{a,A}$	$0.85 \pm 0.14^{b,A}$	$1.16 \pm 0.05^{a,A}$
48	$0.68 \pm 0.06^{a,B}$	$0.85 \pm 0.14^{a,A}$	$1.16 \pm 0.05^{b,A}$
72	$0.61 \pm 0.08^{a,B}$	$0.85 \pm 0.14^{a,A}$	$0.67 \pm 0.07^{a,B}$
96	$0.61 \pm 0.08^{ab,B}$	$0.85 \pm 0.14^{a,A}$	$0.52 \pm 0.08^{b,C}$

[a] Different letters (lowercase) indicate a significant difference ($p < 0.05$) between individual enantiomers or an enantiomer and racemate, while the same letter indicates no significant difference. Different capitalized letters indicate a significant difference ($p < 0.05$) between different time points of the same analyte.

the ORD sign was consistent with the CD sign, that is, the resolved enantiomers of AF showed (+) or (−) by both ORD and CD detection (Figure 1a). Considering the superior sensitivity of CD, the elution order of AF enantiomers on the three different columns was established by CD signals observed online at 230 nm. CD spectroscopy has proven to be a powerful method for studying the absolute configuration of chiral structures (*26*). The large number of applications of CD measurements led to the formulation of the octant rule for the correlation of the sign and magnitude of the observed Cotton effect (CE) with the absolute configuration of chiral molecules containing the carbonyl chromophore (*27*). The CD spectra of the two enantiomers were approximately opposite to each other along the wavelength axis (Figure S2a, Supporting Information), and the CD spectrum of (+)-enantiomer of AF showed an intense positive CE at 228 nm and a broad and weak positive CE from 250 to 290 nm. These two bands of CE are ascribed to the substituted phenyl $\pi \rightarrow \pi^*$ and n $\rightarrow \pi^*$ carbonyl chromophores, respectively. Compared to palmarumycins (*28*), the band of the CE of substituted phenyl $\pi \rightarrow \pi^*$ is shifted to a lower wavelength probably due to a stronger electronic effect of the two chlorine atoms (Figure S2b, Supporting Information).

The octant rule was used to establish the absolute configuration of enantiomers of AF. However, the sign of the CE could not be predicted directly by applying the octant rule. Figure 2a shows that the stable configuration of *S*-AF was the geometry optimization based on the MMFF94 force-field calculation (*29*). The carbonyl group of AF could be placed into the origin of the octants (Figure 2b). When the substituted phenyl group is located in the upper right back octant, it should give a positive CE (Figure 2b). Thus, the absolute configuration of *S*-AF is (+)-AF, or *S*-(+)-AF. The absolute configuration of the other enantiomer (−)-AF is *R*-AF, or *R*-(−)-AF.

Enantioselective Acute Toxicity to Zebrafish. Acute toxicity was measured for both enantiomers of AF and its racemate during 96-h semistatic tests, and the LC_{50} values are presented in Table 2. The LC_{50} for (+)-AF showed no change over time during the 96-h exposure. For (±)-AF and (-) AF, LC_{50}s generally decreased over exposure time. Using the 96-h observations for evaluation, (−)-AF was slightly more toxic to zebrafish than (+)-AF. However, analyzed using one-way ANOVA, this difference was not statistically significant at 72-h. This result was different from previous studies where significant differences in acute aquatic toxicity were found between enantiomers of other pesticides (*7, 8*).

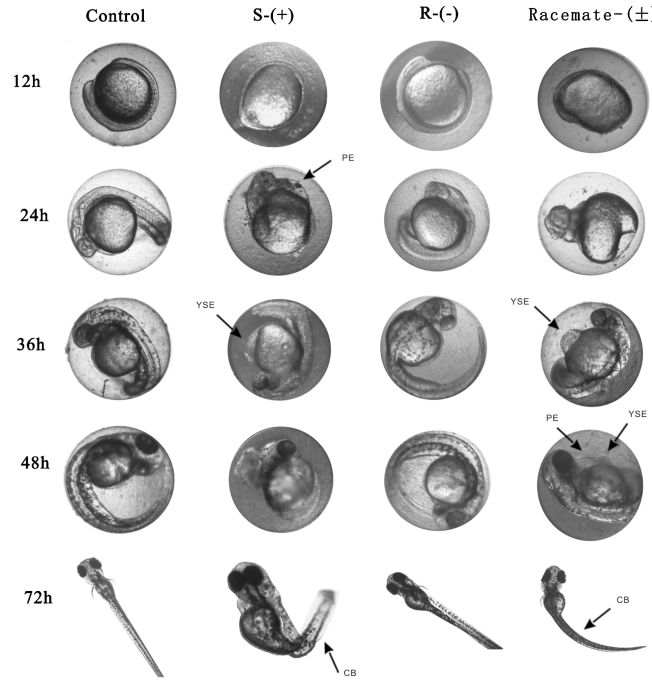

Figure 3. Some of the abnormalities in zebrafish embryos exposed to acetofenate enantiomers and racemate at 1.8 mg L^{-1}. CB = crooked body; PE = pericardial edema; YSE = yolk sac edema.

Enantioselective Embryo Toxicity of AF. Exposure of zebrafish 4-day embryo-larva to AF enantiomers and racemate led to various nonlethal malformations (Figure 3 and Table 3). As the analysis of embryo-larval toxicological results is based on an estimation of the frequency and the intensity of the abnormalities observed, the selection of end points became a key point. In this study, we chose the four most observed end points, yolk sac edema, pericardial edema, crooked body, and average heart rate, for further evaluation. Significant differences were observed in yolk sac edema and pericardial edema between the two enantiomers (Figure 4). Concentrations as low as 1.8 mg L^{-1} caused coagulation of embryos after 24 h exposure to the racemate and (+)-enantiomer of AF (Table 3); however, the (−)-enantiomer of AF did not engender those symptoms even after 72 h exposure. The (+)-enantiomer also induced the occurrence of the crooked body among the larval fish more frequently than the racemate and the (−)-enantiomer (Figure 3 and Table 3). Statistical analysis showed frequent significant differences in yolk sac edema and pericardial edema between the two enantiomers as well as between enantiomers and the racemate (Figure 4). There was a consistent pattern in that the percentages of yolk sac edema and pericardial edema at the different exposure concentrations always followed the order (+)-enantiomer > (±)-racemate > (−)-enantiomer. The NOECs followed the same pattern, in that the (+)-enantiomer was more active than the racemate, while the (−)-enantiomer had less activity at the same concentrations (Table 3). These results are in good agreement with our previous reports about the enantioselective embryo toxicity of lambda-cyhalothrin (*23*); however, AF is much less toxic to the embryo than lambda-cyhalothrin. Therefore, the zebrafish 4-day embryo-larva is a valuable model for identifying systemic targets of chemical virulence factors,

Table 3. Summary of Response Endpoints for Enantioselective Embryo Toxicity of Acetofenate

	24-h exposure			48-h exposure			72-h exposure			96-h exposure		
developmental defects	(±)	(+)	(−)	(±)	(+)	(−)	(±)	(+)	(−)	(±)	(+)	(−)
					malformation (EC_{50}, mg L^{-1})							
yolk sac edema	[a]	[a]	[a]	3.8	2.5	5.4	10.0	3.9	6.8	21.4	3.9	10.0
pericardial edema	[a]	[a]	[a]	5.9	[b]	[b]	4.4	4.9	7.2	3.9	4.9	7.2
					NOECs (mg L^{-1})							
yolk sac edema	[a]	-[a]	-[a]	<5.4	<1.8	<5.4	<5.4	<1.8	<5.4	<1.8	<1.8	<5.4
pericardial edema	[a]	-[a]	-[a]	<5.4	<1.8	<5.4	<5.4	<1.8	<5.4	<1.8	<1.8	<5.4
crooked body	[a]	[a]	[a]	<1.8	<1.8	<10	<1.8	<1.8	<10	<1.8	<1.8	<10.0

[a] Could not be determined because the embryos were still in the pharyngula period. [b] Could not be determined because only one embryo showed pericardial edema.

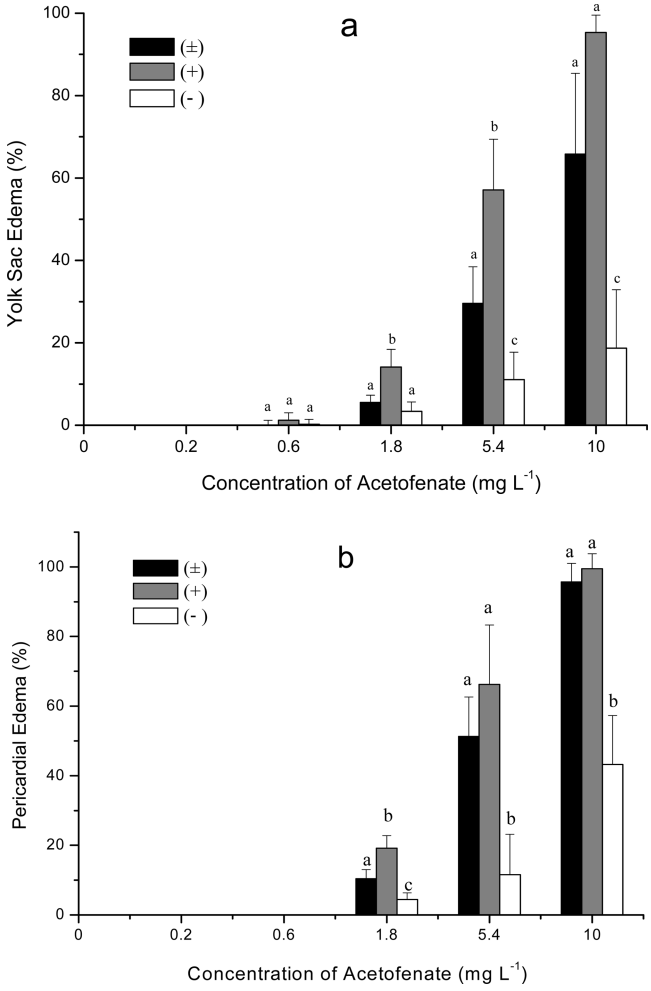

Figure 4. Morphologic abnormalities occurring in zebrafish exposed to different concentrations of acetofenate enantiomers and racemate after 96 h of exposure. (a) Yolk sac edema; (b) pericardial edema. Different letters above adjacent bars indicate a significant difference ($p < 0.05$) between individual enantiomers or between an enantiomer and racemate, while the same letter indicates no significant difference.

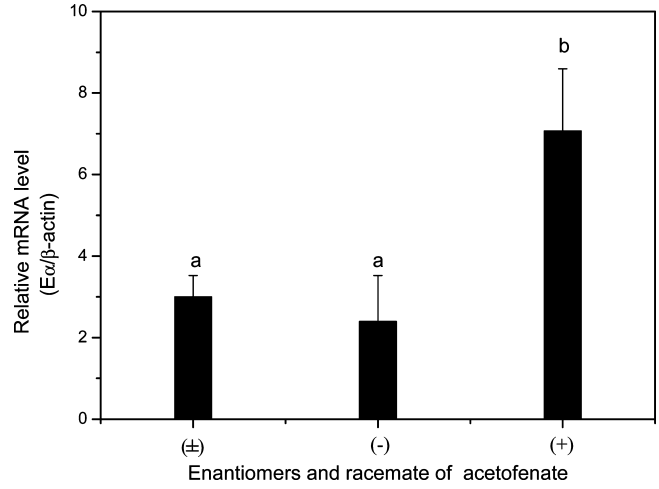

Figure 5. Expressions of ERα in zebrafish embryo after 72 h of exposure to acetofenate enantiomers and racemate. Values were normalized against β-Actin as the housekeeping gene and represent the mean mRNA expression value ± SEM ($n = 5$) relative to those of male controls. Different letters above adjacent bars indicate a significant difference ($p < 0.05$) between individual enantiomers or between an enantiomer and racemate, while the same letter indicates no significant difference.

and this model is useful in the *in vivo* assessment of ecotoxicology at the sublethal level (*30*).

Compared to measuring acute toxicities, the embryo test gives more end points and, as a result, can offer more criteria for identifying enantioselectivity in ecotoxicity. Information about early lethality is necessary for the risk assessment of chemicals; however, the criteria used here cannot inform us about the ability of the other embryos to survive over the key period of hatching (*31*). Indeed, even if live embryos do not show major impairments at 48 h postfecundation, there may be substantial numbers of nonviable larvae that are not detected. Determination of the rate of hatching would be more relevant for assessing the sublethal effect of pollution.

Enantioselectivity of the ERα mRNA Expression in Zebrafish Embyro. The expression of the mRNA of ERα induced by (±), (+), and (−)-AF in 72 h zebrafish embryos was approximately 2.3 to 7.3 times that of the negative control (Figure 5). The most significant induction was exposure to (+)-AF (about 7.3-fold). There was no significant difference in the mRNA levels of ERα between the exposed (−)-AF (2.3-fold) and (±)-AF about (3-fold).

A growing number of environmental toxicants including insecticides, herbicides, and industrial solvents are believed to have deleterious effects on the development of nontarget organisms by disrupting hormone-sensitive processes. The gene expression of ERα was determined in this study to better understand the molecular mechanisms of the enantioselective effect of AF on the development of zebrafish embryos. Endogenous and environmental estrogens have been shown to alter normal processes of cell apoptosis and differentiation via ER-mediated signal pathways (*32*). Results from the present study showed that the AF may activate the ERα signal pathway and that the (+)-AF displayed the strongest induction of ERα mRNA expression. Similar effects were observed in zebrafish embryos after the administration of DDT, polychlorinated biphenyl, and coumestrol (*33*). Such different regulations of ERα by enantiomers of AF may have contributed to the enantioselectivity observed in the effect on zebrafish embryo development.

Conclusions

Our study showed that complete separation of enantiomers of AF may be achieved on several commercially available chiral HPLC columns. By using CD spectra and the octant rule, we were able to deduce the absolute configurations of the resolved enantiomers to be *S*-(−)-AF and *R*-(+)-AF, respectively. No significant difference was observed between the enantiomers in acute toxicity measured as egg and embryo mortality through a 96-h static renewal assay. However, significant enantioselectivity was consistently observed between the two enantiomers when embryo-larval development end points were considered. The (+)-enantiomer was found to be significantly more active than the corresponding (−)-enantiomer in inducing ERα mRNA expression, yolk sac edema, and pericardial edema, formation of crooked body, and alteration in heartbeat rates. This study showed that for chiral xenobiotics, enantioselectivity in sublethal effects is possible even in the absence of an apparent dependence in acute toxicities. The use of zebrafish embryos may be a rapid and expensive approach for revealing the enantioselectivity in the developmental toxicities of chiral compounds.

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Supporting Information Available: Chromatograms of separated enantiomers of acetofenate on Chiralcel OD. CD and UV spectra of separated enantiomers of acetofenate. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Liu, W. P., Gan, J. Y., Schlenk, D., and Jury, W. A. (2005) Enantioselectivity in environmental safety of current chiral insecticides. *Proc. Natl. Acad. Sci. U.S.A. 102*, 701–706.
(2) Garrison, A. W. (2006) Probing the enantioselectivity of chiral pesticides. *Environ. Sci. Technol. 40*, 16–23.
(3) Richardson, S. D., and Ternes, T. A. (2005) Water analysis: Emerging contaminants and current issues. *Anal. Chem. 77*, 3807–3838.
(4) Lewis, D. L, Garrison, A. W., Wommack, K. E., Whittemore, A., Steudler, P., and Melillo, J. (1999) Influence of environmental changes on degradation of chiral pollutants in soils. *Nature 401*, 898–901.
(5) Wong, C. S., Garrison, A. W., Smith, P. D., and Foreman, W. T. (2001) Enantiomeric composition of chiral polychlorinated biphenyl atropisomers in aquatic and riparian biota. *Environ. Sci. Technol. 35*, 2448–2454.
(6) Vetter, W., Smalling, K. L., and Maruya, K. A. (2001) Interpreting nonracemic ratios of chiral organochlorines using naturally contaminated fish. *Environ. Sci. Technol. 35*, 4444–4448.
(7) Liu, W. P., Gan, J. Y., and Qin, S. J. (2005) Separation and aquatic toxicity of enantiomers of synthetic pyrethroid insecticides. *Chirality 17*, S127−S133.
(8) Zhou, S. S., Lin, K. D., Yang, H. Y., Li, L., Liu, W. P., and Li, J. (2007) Stereoisomeric separation and toxicity of a new organophosphorus insecticide chloramidophos. *Chem. Res. Toxicol. 20*, 400–405.
(9) Ward, T. J., and Hamburg, D. M. (2004) Chiral separations. *Anal. Chem. 76*, 4635–4644.
(10) Gasparrini, F., Misiti, D., and Villani, C. (2001) High-performance liquid chromatography chiral stationary phases based on low-molecular-mass selectors. *J. Chromatogr. A 922*, 35–50.
(11) Wang, L. M., Liu, W. P., Yang, C. X., Pan, Z. Y., Gan, J. Y., Xu, C., Zhao, M. R., and Schlenk, D. (2007) Enantioselivity in estrogenic potential and uptake of bifenthrin. *Environ. Sci. Technol. 41*, 6124–6128.
(12) Hill, A. J., Teraoka, H., Heideman, W., and Peterson, R. E. (2005) Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol. Sci. 86*, 6–19.
(13) Beis, D., and Stainier, D. Y. R. (2006) In vivo cell biology: Following the zebrafish trend. *Trends Cell Biol. 16*, 105–112.
(14) Mortensen, A. S., and Arukwe, A. (2007) Targeted salmon gene array (SalArray): A toxicogenomic tool for gene expression profiling of interactions between estrogen and aryl hydrocarbon receptor signaling pathways. *Chem. Res. Toxicol. 20*, 474–488.
(15) Bevan, C. L, Porter, D. M., Prasad, A., Howard, M. J., and Henderson, L. P. (2003) Environmental estrogens alter early development in *Xenopus laevis*. *Environ. Health Prespect. 111*, 488–496.
(16) Okada, A., Ohta, Y., Inoue, S., Hiroi, H., Muramatsu, M., and Iguchi, T. (2003) Expression of estrogen, progesterone and androgen receptors in the oviduct of developing, cycling and pre-implantation rats. *J. Mol. Endocrinol. 30*, 301–315.
(17) Tsutsumi, O. (2000) Effects of endocrine disruptors on preimplantation embryo development. *Nippon Rinsho. 58*, 2464–2468.
(18) Takai, Y., Tsutsumi, O., Ikezuki, Y., Hiroi, H., Osuga, Y., Momoeda, M., Yano, T., and Taketani, Y. (2000) Estrogen receptor-mediated effects of a xenoestrogen, bisphenol A, on preimplantation mouse embryos. *Biochem. Biophys. Res. Commun. 270*, 918–921.
(19) Liu, W. P. (2006) *Pesticide Environmental Chemistry*, pp 26−28, Chemical Industry Press, Beijing.
(20) OECD (1992). Guideline for Testing of Chemicals: Fish, Acute Toxicity Test.
(21) Westerfield, M. (1993) *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish Danio (Brachydanio) rerio*, University of Oregon Press, Portland, OR.
(22) Braunbeck, T., and Lammer, E. (2006) *Background Document on Fish Embryo Toxicity Assays*, OECD, Dessau, Germany.
(23) Xu, C., Wang, J. J., Liu, W. P., Sheng, G. D., Tu, Y. J., and Ma, Y. (2008) Separation and aquatic toxicity of enantiomers of the pyrethroid insecticide lambda-cyhalothrin. *Environ. Toxicol. Chem. 27*, 174–181.
(24) Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔ*C*t method. *Methods 25*, 402–408.
(25) Jenkins, A. L., and Hedgepeth, W. A. (2005) Analysis of chiral pharmaceuticals using HPLC with CD detection. *Chirality 17*, 24–29.
(26) Gawronski, J. (1996) Determination of Absolute and Relative Configuration by Chiroptical Methods, in *Methods of Organic Chemistry (Houben-Weyl)* (Helmchen, R. W., Hoffmann, R. W., Mulzer, J., and Schaumann, E., Eds.) Vol. 1, pp 499−533, Thieme, Stuttgart, Germany.
(27) Lightner, D. A. (1994) The Octant Rule, in *Circular Dichroism, Principles and Applications* (Nakanish, K., Berova, N., and Woody, R. W., Eds.) pp 259−299, VCH Publishers, New York.
(28) Bringmann, G., and Busemann, S. (1997) Quantumchemical calculation of CD spectra: the absolute configuration of palmarumycins CP3 and C2. *Tetrahedron 53*, 655–1664.
(29) Halgren, T. A. (1996) Merck molecular force field 0.1. Basis, form, scope, parameterization, and performance of MMFF94. *J. Comput. Chem. 17*, 490–519.
(30) Fraysse, B., Mons, R., and Garric, J. (2006) Development of a zebrafish 4-day embryo-larval bioassay to assess toxicity of chemicals. *Ecotoxicol. Environ. Saf. 63*, 253–267.
(31) Ensenbach, U. (1998) Embryonic development of fish-a model to assess the toxicity of sediments to vertebrates. *Fresenius Environ. Bull. 7*, 531–538.
(32) Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M, and Gustafsson, J. A. (2001) Mechanisms of esreogen action. *Physiol. Rev. 81*, 1535–1565.
(33) Billsson, K., Westerlund, L., Tysklind, M., and Olsson, P. (1998) Developmental disturbances caused by polychlorinated biphenyls in zebrafish (*Brachydanio rerio*). *Mar. Environ. Res. 46*, 461–464.

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