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ENANTIOSELECTIVE ACETYLCHOLINESTERASE INHIBITION OF THE ORGANOPHOSPHOROUS INSECTICIDES PROFENOFOS, FONOFOS, AND CROTOXYPHOS

MAE GRACE NILLOS,*† GABRIELA RODRIGUEZ-FUENTES,‡ JAY GAN,† and DANIEL SCHLENK†
†Environmental Toxicology Program, ‡Department of Environmental Sciences, University of California, Riverside, California 92521, USA

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Abstract—A large number of organophosphorous insecticides (OPs) are chiral compounds, and yet enantioselectivity in their environmental fate and effects is rarely addressed. In the present study, we isolated individual enantiomers of three OPs, profenofos, fonofos, and crotoxyphos, and evaluated enantioselectivity in their inhibition of acetylcholinesterase (AChE). Acetylcholinesterase inhibition by the enantiomers and racemates was determined in vivo in the aquatic invertebrate *Daphnia magna* and in Japanese medaka (*Oryzias latipes*) as well as in vitro with electric eel (*Electrophorus electricus*) and human recombinant AChEs. The overall results showed variable sensitivity between AChE enzymes from different species as well as variable magnitude of enantioselectivity in enzyme inhibition. The (−)-enantiomer of profenofos was 4.3- to 8.5-fold more inhibitory to AChE in vivo, whereas (−)-fonofos was 2.3- to 29-fold more potent than the corresponding (+)-enantiomer. The (+)-enantiomer of crotoxyphos was 1.1- to 11-fold more inhibitory to AChE than the (−)-enantiomer. In contrast, the in vitro results showed (+)-profenofos to be 2.6- to 71.8-fold more inhibitory than the (−)-enantiomer and (−)-crotoxyphos to be 1.6- to 1.9-fold more active than the (+)-enantiomer. The reversed direction of enantioselectivity observed between the in vivo and in vitro assays suggests enantioselectivity within toxicodynamic processes such as uptake, biotransformation, or elimination. Findings from the present study provide evidence of enantioselectivity in the AChE inhibition of chiral OPs in nontarget organisms and indicate the need to consider enantiomers individually when assessing environmental risk of these chiral pesticides.

Keywords—Acetylcholinesterase Enantioselectivity Chiral pesticides Organophosphorus Chirality

INTRODUCTION

The importance of stereoselectivity in the bioactivity of natural and synthetic pharmaceutical products has long been recognized [1,2]. More recently, pesticides consisting of active ingredients with chiral centers have increasingly attracted attention [3]. Because biochemical receptors and enzymes generally are stereoselective, enantiomers of the same pesticide are expected to behave as completely different compounds in biologically mediated environmental processes, such as biotransformation and toxicity [4]. Because chiral pesticides are physically and chemically identical in an achiral environment, they often are considered as single compounds, and risk assessment generally is not carried out for individual enantiomers [5]. Their widespread use, however, calls for a better understanding about the importance of enantioselectivity in the environmental fate and effects of these pesticides.

Organophosphorous (OP) insecticides continue to be an important chemical protection against agricultural and household pests in many countries [6,7]. They are potent acetylcholinesterase (AChE) inhibitors and are less persistent in the environment than their chlorinated predecessors. More than 30% of the modern-use OP insecticides are chiral, with a stereogenic center on a carbon, sulfur, pentavalent phosphorus, or other substituent atom of phosphorus [4,8]. Chiral OPs are mostly marketed and used as racemic mixtures (equimolar mixture of its enantiomers).

The number of investigations regarding the occurrence of enantioselectivity in the aquatic toxicity of chiral OPs has been steadily increasing in recent years [5,9–12]. Chiral OP compounds also have been shown to stereoselectively inhibit cholinesterase enzymes in a variety of species [9,13]. However, the magnitude of stereoselectivity in enzyme inhibition may vary and the preferred configuration could reverse between animal species or between in vitro and in vivo determinations [1,14–16].

Both enantioselective and nonselective degradation of several chiral OPs have been reported recently [3,4,12,17]; however, research concerning enantioselectivity in the toxicity of chiral OPs to nontarget and nonmammalian species, particularly aquatic organisms, is still inadequate. The limited research may be attributed to the challenges in chiral separation and analysis and to unavailability of enantiomer standards. To date, studies of the differential AChE activities of OP enantiomers have been conducted mostly in mammalian systems and target insects, and have been contingent with the synthesis and availability of enantiomer standards. Recent advances in chiral separation using high-performance liquid chromatography (HPLC) offer an opportunity to prepare enantiopure samples that could be used in bioassays to improve our understanding of the ecotoxicological risk associated with chiral pesticides [18]. The purpose of the present study was to evaluate the enantioselective inhibition of AChE by individual enantiomers of three chiral OPs (profenofos, fonofos, and crotoxyphos) in an aquatic invertebrate (*Daphnia magna*) and a juvenile fish, Japanese medaka (*Oryzias latipes*). In addition, in vitro studies using *Electrophorus electricus* AChE (EE-AChE) and human recombinant AChE (HR-AChE) were carried out to compare in vitro and in vivo biological activities.

* To whom correspondence may be addressed (mae.nillos@email.ucr.edu).

MATERIALS AND METHODS

Chemicals and enzymes

Analytical standards of racemic profenofos (purity, 94.9%; *O*-4-bromo-2-chlorophenyl-*O*-ethyl *S*-[*R,S*]-propyl phosphorothioate), fonofos (purity, 98.5%; *O*-ethyl *S*-phenyl [*R,S*]-ethylphosphonodithioate), and crotoxyphos (α-methylbenzyl-3-hydroxycrotonate-dimethylphosphate) were purchased from Chem Service (West Chester, PA, USA). All other solvents or chemicals used in the present study were of HPLC or analytical grade and were purchased from Fisher (Fair Lawn, NJ, USA). Acetylthiocholine iodide (ATC), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), EE-AChE (type VI-S), HR-AChE (expressed in Human Embryonic Kidney 293 cells), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Juvenile Japanese medaka (*O. latipes*; age, four to five weeks posthatch; length, 10–12 mm; wt, 11.2–12.8 mg) were obtained from the culture at the University of California–Riverside (CA, USA). The fish were kept in glass aquaria in a chemical-free room with the temperature maintained at 25 ± 2°C and a 16:8-h light:dark photoperiod. Dechlorinated water was used for all stock cultures and experiments. Water-quality parameters were constantly monitored, and fish were fed live brine shrimp twice daily. Fish were not fed during exposure. *Daphnia magna* were purchased from Aquatic Biosystems (Fort Collins, CO, USA), and cultures were maintained in a chemical-free room at 22 ± 2°C with 16:8-h light:dark photoperiod. Reconstituted moderately hard water was used for all stock cultures and experiments. *Daphnia magna* was fed with yeast-cerophylla-trout chow and *Selenastrum capricornutum*. Test organisms were not fed during exposure.

Preparation of enantiomers

Enantiomers were resolved on an 1100 Series HPLC (Agilent, Wilmington, DE, USA) equipped with an in-line laser polarimeter detector (PDR-Chiral, Lake Park, FL, USA). Columns with different chiral stationary phases (CSPs), including a Sumichiral OA-2500-I column (Sumika Chemical Service, Tokyo, Japan) as well as Chiralcel OD-R and Chiralcel OJ columns (Daicel Chemical Industries, Tokyo, Japan), were previously tested in our laboratory [5,10]. Optimal resolution and isolation of enantiomers were achieved for the test OPs on a Chiralcel OJ column. Each OP sample (20 μl) was injected into the HPLC and eluted with hexane (100%), hexane/ethanol (90:10, v/v), and hexane/2-propanol (95:5, v/v) as the mobile phase for the separation of profenofos, crotoxyphos, and fonofos enantiomers, respectively. All analyses were carried out at room temperature (25°C). The flow rate of the mobile phase was fixed at 0.8 ml/min. The ultraviolet detection wavelength was set at 230 nm for all analyses. The specific rotation of the resolved enantiomers was determined at 675 nm, and the cell path was 50 mm. The rotation sign (+ or −) was directly given by a positive or negative peak on the polarimeter. Enantiopure samples for AChE assays were manually collected at the HPLC outlet, evaporated to dryness under a stream of pure nitrogen, and then redissolved in acetone (carrier solvent). Stock solution concentrations were determined on HPLC assuming the same response factor for enantiomers originating from the same compound. The purity of the derived enantiomers was checked with reanalysis on HPLC and found to be greater than 99% in all cases.

AChE inhibition assay

In vivo AChE inhibition by individual OP enantiomers and racemates was determined in juvenile medaka following a 96-h exposure and in *D. magna* following a 24-h exposure. The organisms were exposed to various aqueous concentrations derived from the literature or determined from range-finding experiments with the OP racemates. All exposures were performed in triplicate. The exposure procedure was based on established protocols [19]. Following aqueous exposure, homogenates were prepared from pooled whole animals in sodium phosphate buffer with 3% Triton-X 100 (Sigma; 1 ml homogenization buffer/g tissue) using a tissue tearer, followed by centrifugation of the homogenate at 12,500 rpm for 15 min at 4°C. The supernatant was then used to assay for AChE activity using a modified Ellman method adapted to the microplate technique [20,21]. The total reaction volume was 225 μl, consisting of 5 μl of homogenate and 1.5 mM and 0.22 mM final concentrations of ATC and DTNB, respectively. In all cases the homogenate was preincubated at pH 7.5 and 20°C for 10 min with DTNB to allow completion of the background binding of DTNB to the sulfhydryl groups in the sample. All assays were performed in 96-well microtiter plates and read in a SOFTmax Pro microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm for 2 min. Residual AChE activity was normalized to protein content by the Bradford method, using Coomasie Plus-200 Protein Assay reagent and bovine serum albumin standard (Pierce, Rockford, IL, USA) [21].

The in vitro inhibition of AChE was evaluated using EE-AChE and also with HR-AChE for comparison. Briefly, test solutions (5 μl) at various concentrations of each enantiomer were added to microplate wells, followed by the addition of AChE-DTNB solution (205 μl). The mixture was incubated at room temperature (25°C) for 30 min, and then the residual AChE activity was determined with the microplate reader at 405 nm for 2 min following the addition of ATC (20 μl). The final concentrations of AChE, ATC, and DTNB were 0.005 U/ml, 1.5 mM, and 0.22 mM, respectively. All assays were conducted with at least three replicates. The median inhibitory concentration (IC50) based on nominal aqueous concentrations was determined by linear interpolation analysis using ToxCalc™ Version 5.0 (Tidepool Scientific Software, McKinleyville, CA, USA).

RESULTS AND DISCUSSION

Enantiomer resolution

The structures of the chiral OP compounds investigated in the present study are shown in Figure 1. Fonofos, a phosphonodithioate insecticide also known as dyfonate, is primarily applied to control insects in a number of crops and in turfs [4,17]. Profenofos is a phosphorothiolate OP compound that has been widely used in cotton-growing areas in most countries worldwide [22]. Crotoxyphos is a phosphate used as contact and stomach poison, primarily for pests on lactating dairy and beef cattle [4]. Both fonofos and profenofos have a pentavalent phosphorus stereogenic center (denoted by an asterisk in Fig. 1), whereas crotoxyphos has a chiral carbon substituent of phosphorus.

Resolution of enantiomers was highly column-specific [5]. Successful separation of enantiomers of profenofos, fonofos, and crotoxyphos was achieved only on the Chiralcel OJ column (Fig. 2). The corresponding retention (k), separation (α), and resolution (R_S) factors are given in Table 1. The separated

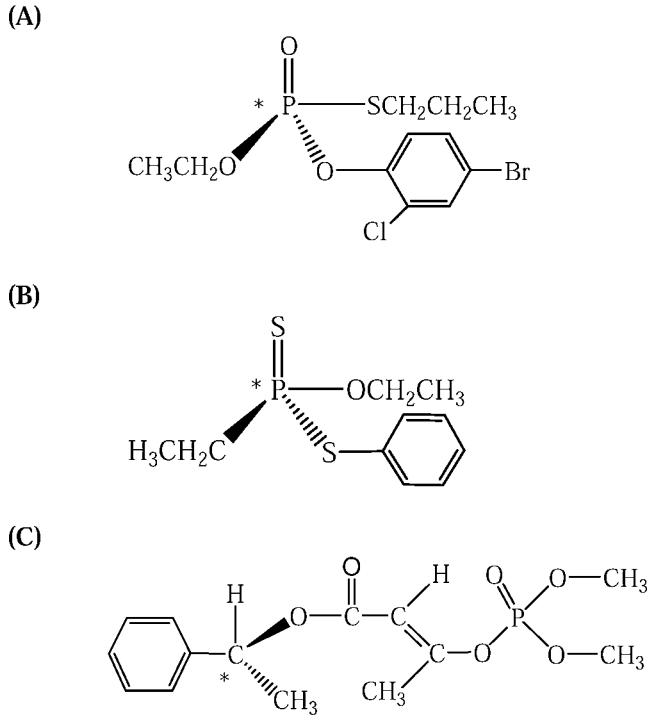

Fig. 1. Structures of test compounds (the asterisk indicates the chiral position). (**A**) Profenofos. (**B**) Fonofos. (**C**) Crotoxyphos.

enantiomers were identified by determining their optical rotation. For fonofos and profenofos, the (+)-enantiomer were eluted before the corresponding (−)-enantiomer, whereas for crotoxyphos, the (−)-enantiomer was eluted before the (+)-enantiomer under the described conditions (Table 1). Fonofos, profenofos, and crotoxyphos have been previously separated on the same or similar CSP column under slightly different conditions or using different systems [4,5,23]. Because of the complexity of investigating the chiral recognition mechanisms of CSPs, the present study did not attempt to elucidate any structural features of the selected OPs or the CSP that correlated with the resolution. Several researchers, however, have suggested hydrogen bonding, π–π interaction, and dipole–dipole stacking, determined by the degree of steric fit of the enantiomers in the chiral cavity, to be responsible for the chiral discrimination [18]. The adequate resolution of the selected OPs in the present study allowed individual enantiomers to be recovered for use in bioassays.

In vivo inhibition of AChE

Acetylcholinesterase inhibition of profenofos, fonofos, and crotoxyphos enantiomers and racemates were investigated in vivo in the aquatic invertebrate *D. magna* and in juvenile Japanese medaka. The AChE IC50 of the individual enantiomers and racemic mixtures of the selected chiral OPs are given in Table 2. The selectivity ratios of inhibition between enantiomers are given in Table 3. The more active enantiomer is assigned the value of unity. The (−)-enantiomers of profenofos and fonofos were observed to be more potent AChE inhibitors than the (+)-enantiomers in vivo. The (−)-enantiomer of profenofos was an approximately 8.5-fold more potent inhibitor than the corresponding (+)-enantiomer to *D. magna* AChE (Table 3). Similarly, the (−)-enantiomer of profenofos was more inhibitory to the medaka AChE than (+)-profenofos, but the magnitude of enantioselectivity was only 4.3-fold. In both species, the racemic profenofos had near intermediate toxicity between the (+)- and (−)-enantiomers. This observation clearly indicates that most of the AChE activity in both aquatic invertebrate and fish in the racemic mixture can be attributed to the more potent enantiomer of profenofos in vivo.

R-(−)-fonofos was reported to have higher biological activity than *S*-(+)-fonofos to housefly and mosquito fly and also to mice in vivo [16]. In the present study, the (−)-enantiomer of fonofos was approximately twofold as inhibitory to a non-

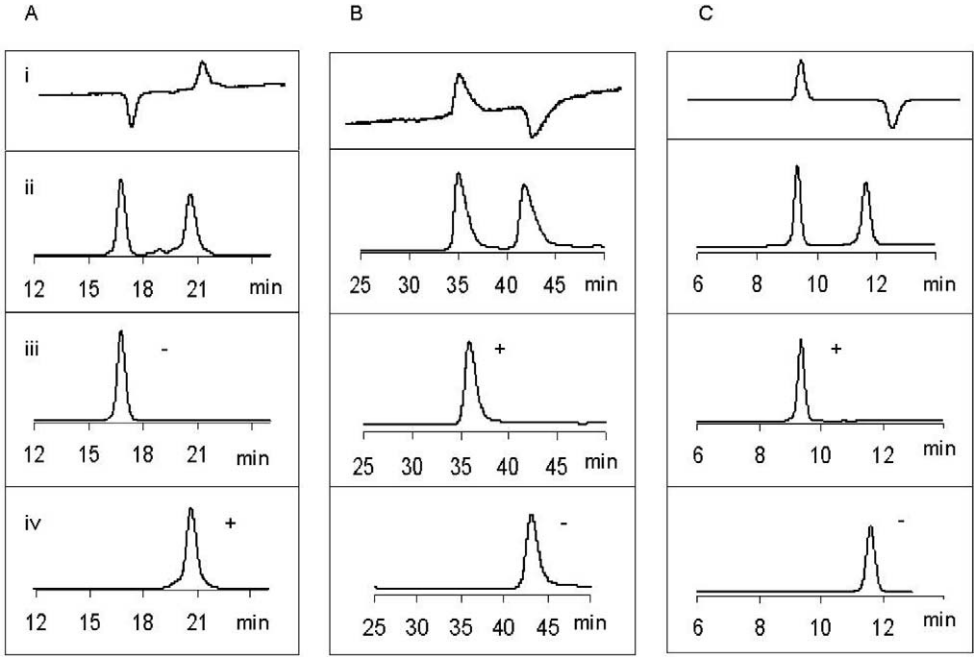

Fig. 2. High-performance liquid chromatogram and optical rotation spectra for the enantiomeric separation of (**A**) crotoxyphos, (**B**) profenofos, and (**C**) fonofos on Chiralcel OJ column (Daicel Chemical Industries, Tokyo, Japan). i = optical rotation; ii = chromatogram of the separated enantiomers; iii = chromatogram of the first-eluting enantiomer; iv = chromatogram of the second-eluting enantiomer.

Table 1. The separation (α) and resolution (R_S) factors of profenofos, fonofos, and crotoxyphos on the Chiralcel OJ column[a]

	Retention time (min)			Peak width (min)		Retention factor				
	T_0	T_+	T_-	w_+	w_-	k'_+	k'_-	α	R_S	Rotation (*pk1/p2*)[b]
Profenofos	4.91	35.06	41.86	1.32	1.60	6.14	7.53	1.23	1.12	+/−
Fonofos	4.94	12.85	16.85	0.33	0.47	1.60	2.41	1.50	2.39	+/−
Crotoxyphos	4.68	24.19	19.37	0.80	0.55	4.17	3.14	1.33	1.81	−/+

[a] Daicel Chemical Industries, Tokyo, Japan. T_0 = solvent retention time; the subscripts + and − indicate the (+)- and (−)-enantiomers, respectively. $k'_+ = (T_+ - T_0)/T_0$; $k'_- = (T_- - T_0)/T_0$; $\alpha = k'(pk2)/k'(pk1)$; $R_S = 1.18[k'(pk2) - k'(pk1)]/0.5(w_- + w_+)$.
[b] *pk1/p2* = peak 1/peak 2.

target aquatic invertebrate (*D. magna*) AChE and more than 29-fold more active to medaka AChE than the (+)-enantiomer (Tables 2 and 3). Interestingly, the racemic fonofos was almost equipotent to the (−)-enantiomer in medaka and approximately fivefold more potent to *D. magna* AChE than the active enantiomer. The results of the in vivo AChE inhibition by profenofos and fonofos in *D. magna* are qualitatively consistent with the previously observed acute toxicity of profenofos and fonofos, in which the (−)-enantiomers were found to be more toxic than the (+)-enantiomers to the aquatic invertebrate [5]. Similar relationships were previously reported for other chiral OPs. In the acute toxicity test with *Daphnia pulex*, no significant difference in the median lethal concentrations was observed between (+)-fenamiphos and its racemate, but the (−)-enantiomer showed significantly lower toxicity [12]. In another study, no significant difference was observed in both AChE IC50 in housefly heads and acute toxicity to *D. pulex* between the more active (+)-enantiomer and racemic leptophos [11]. These findings suggest that for certain chiral OPs, chirality may affect the toxicodynamic processes, so a straightforward relationship between enantiomers and racemates may not always be found [24].

Enantiomers of both malathion and malaoxon exist because of an asymmetric carbon center in the thiosuccinyl substituent [16]. Rodriguez et al. [14] showed significant enantioselectivity in activity of malaoxon against cholinesterase enzymes, where the *R*-enantiomer was up to 22-fold more inhibitory than the *S*-enantiomer to bovine erythrocyte AChE. Similar to malaoxon, crotoxyphos chirality is a result of an asymmetric carbon center in a substituent group (Fig. 1).

In the present study, stereoselectivity also was observed, with (+)-crotoxyphos being the more potent AChE inhibitor in vivo (Tables 2 and 3). Additionally, the (+)-enantiomer of crotoxyphos was more than 11-fold more inhibitory to *D. magna* AChE. Such selectivity, however, was not as clearly defined in medaka, although the IC50 was not reached for the (−)-enantiomer at the highest concentration tested (Tables 2 and 3). A wide range of inhibition selectivity ratios have been reported for the inhibition of different AChE enzymes, suggesting that enantioselectivity in AChE inhibition may be dependent on the species of test organisms [14].

In vitro inhibition of AChE

In contrast to the in vivo results, the (+)-enantiomer of profenofos and the (−)-enantiomer of crotoxyphos were found to have higher activity against both EE-AChE and HR-AChE in the in vitro assays (Table 4). As observed in vivo, however, the magnitude of difference in activity between the two enantiomers of crotoxyphos was relatively small in both EE-AChE and HR-AChE in the in vitro assay (Table 3). The (−)-enantiomer of crotoxyphos was only 1.6- and 1.9-times more inhibitory than the (+)-enantiomer to EE-AChE and HR-AChE, respectively. Larger differences in toxicity were observed between the enantiomers of profenofos (Table 3). The differences in the magnitude of selectivity may be related to the differences in the stereogenic centers between the two chiral OPs [16,25].

Comparison of in vivo and in vitro inhibition

Regardless of the magnitude of enantioselectivity, the apparent reversal in potency of enantiomers between in vivo and in vitro assays may be explained by the possible occurrence of stereoselectivity in metabolic processes in vivo [16]. It is likely that one enantiomer is preferentially bioactivated to a

Table 2. In vivo acetylcholinesterase median inhibitory concentration (IC50; μmol/L) of the enantiomers and racemic profenofos, fonofos, and crotoxyphos[a]

	(+)	(−)	Racemate
Profenofos			
Daphnia magna[bc]	0.102 ± 0.007	0.012 ± 0.002	0.020 ± 0.0004
Japanese medaka[de]	0.226 ± 0.019	0.053 ± 0.010	0.094 ± 0.003
Fonofos			
Daphnia magna[be]	>0.406	0.176 ± 0.025	0.037 ± 0.006
Japanese medaka[de]	>0.812	0.028 ± 0.0004	0.029 ± 0.0004
Crotoxyphos			
Daphnia magna[bc]	0.029 ± 0.007	>0.318	0.0727 ± 0.009
Japanese medaka[de]	0.894 ± 0.010	>0.954	0.781 ± 0.017

[a] All values are presented as the mean ± standard error.
[b] Assayed following 24-h exposure.
[c] $n = 6$.
[d] Assayed following 96-h exposure of juvenile medaka (age, four to five weeks posthatch).
[e] $n = 3$.

Table 3. Comparison of in vivo and in vitro inhibition of acetylcholinesterase between enantiomers of the chiral organophosphorous (OP) compounds profenofos, fonofos, and crotoxyphos[a]

OP	In vivo[b]		In vitro[c]	
	Daphnia magna	Japanese medaka	EE-AChE	HR-AChE
Profenofos				
Racemic	1.7	1.8	0.88	2.03
(+)	8.5	4.3	1.0	1.0
(−)	1.0	1.0	>2.6	71.8
Fonofos				
Racemic	0.21	1.03		
(+)	>2.3	>29	NA[d]	NA
(−)	1.0	1.0		
Crotoxyphos				
Racemic	2.5	0.87	0.83	2.06
(+)	1.0	1.0	1.6	1.9
(−)	>11.0	>1.1	1.0	1.0

[a] Selectivity = IC50 of less active enantiomer (or racemate)/IC50 of the more active enantiomer, where IC50 is the median inhibitory concentration.
[b] Calculated from the in vivo IC50.
[c] Calculated from the in vitro IC50. EE-AChE = *Electrophorus electricus* acetylcholinesterase; HR-AChE = human recombinant acetylcholinesterase.
[d] NA = Not assayed in vitro.

metabolite more inhibitory to AChE, whereas the other is preferentially detoxified and its capacity to inhibit AChE reduced. An earlier study with rat liver microsomes coupled with AChE inhibition quantification [26] indicated that the individual enantiomers reacted in an opposite manner. In that study, stereospecific metabolic activation resulted in the (−)-enantiomer of profenofos becoming 34-fold more inhibitory to AChE, whereas (+)-profenofos was preferentially detoxified and its capacity to inhibit the enzyme reduced by twofold. Such a shift in enantioselectivity in AChE inhibition following metabolism was attributed to either differences in rates of oxidation or the intrinsic potency of metabolite enantiomers. Results similar to that observed in the present study have been reported in housefly and mice (in vivo) and in bovine erythrocyte, housefly head, and EE-AChE (in vitro) for profenofos [15,16,26]. In addition, differential metabolism also was demonstrated for the chiral OP, isofenfos, in rat liver microsomes, in which (−)-isofenfos produced nearly fourfold more oxon than the (+)-enantiomer, although AChE inhibition and mixed-function oxidases-dependent metabolism of the oxon was higher for the (+)-enantiomer [27].

The enantioselectivity in AChE inhibition of fonofos enantiomers was not assayed in vitro in the present study because of the inability of the system to form fonofos-oxon. The mode of action of fonofos that contains a P=S moiety requires metabolic activation by oxidative desulfuration to the P=O (oxon) form for enzyme inhibition to take place [14]. Preliminary assays with the fonofos racemate showed no significant inhibition of EE-AChE at the highest concentration assayed (1,000 μM). In an earlier study regarding the acute toxicity of fonofos and fonofos-oxon enantiomers in housefly, mosquito fly, and mice [24], results showed the *R*-enantiomer of fonofos to be 1.8- to 4.0-fold more toxic than the *S*-enantiomer. The same study also reported the *S*-oxon as 2.6- to 12.2-fold more toxic than the corresponding *R*-oxon to the same animals. In addition, in an in vitro metabolic activation study of fonofos in rat liver microsomes [28], it was found that *R*-fonofos was predominantly converted to the *S*-oxon, which explains the greater toxicity of *R*-fonofos over *S*-fonofos.

CONCLUSION

The successful chiral chromatographic separation and isolation of enantiomers of the selected chiral OPs made possible the evaluation of enantioselectivity in the inhibition of AChE in the present study. The in vivo and in vitro results with aquatic organisms suggest that for the selected OPs, enantioselectivity for AChE inhibition does occur. The magnitude of enantioselectivity, however, varied greatly between species, from aquatic invertebrates to fish to humans. In addition, the present results also suggest that in vivo AChE inhibition by chiral OPs is influenced by biological processes other than the compound's mode of action. Findings from the present study provide evidence for enantioselectivity in the AChE inhibition of chiral OPs in nontarget organisms in the aquatic environment. The observed enantioselectivity indicates the need to consider enantiomers individually when assessing environmental risk of chiral pesticides. In addition, the reversed direction of enantioselectivity observed between the in vivo and in vitro AChE inhibition assays suggests significant enantioselective contributions of toxicodynamic processes, such as biotransformation, uptake, or elimination.

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REFERENCES

1. Kuriahara N, Miyamoto J, Paulson GD, Zeeh B, Skidmore MW, Hollingworth RM, Kuiper HA. 1997. Chirality in synthetic agrochemicals: Bioactivity and safety considerations. *Pure Appl Chem* 69:2007–2025.
2. Ladik J, Szekeres Z. 2002. A study on chirality in biomolecules: The effect of the exchange of l-amino acids to d ones in Sso7d ribonuclease. *Journal of Molecular Modeling* 12:462–467.

Table 4. In vitro acetylcholinesterase median inhibitory concentration (μmol/L) of the enantiomers and racemic profenofos and crotoxyphos[a]

	Profenofos[b]			Crotoxyphos[b]		
	(+)	(−)	Racemate	(+)	(−)	Racemate
EE-AChE[c]	193.2 ± 23.4	>500	169.84 ± 2.94	14.06 ± 0.19	8.95 ± 0.57	7.46 ± 0.54
HR-AChE[d]	2.26 ± 0.16	162.3 ± 6.58	4.60 ± 0.27	5.58 ± 0.18	2.92 ± 0.15	6.02 ± 0.40

[a] All values are presented as the mean ± standard error.
[b] $n = 3$.
[c] EE-AChE = *Electrophorus electricus* acetylcholinesterase.
[d] HR-AChE = human recombinant acetylcholinesterase.

3. Garrison AW. 2004. On the issue of enantioselectivity of chiral pesticides: A green chemistry opportunity. *Green Chemistry* 6: G77–G78.
4. Ellington JJ, Evans JJ, Prickett KB, Champion WL. 2001. High-performance liquid chromatographic separation of the enantiomers of organophosphorous pesticides on polysaccharide chiral stationary phases. *J Chromatogr A* 928:145–154.
5. Liu W, Gan J, Schlenk D, Jury W. 2005. Enantioselectivity in environmental safety of current chiral insecticides. *Proc Natl Acad Sci USA* 102:701–706.
6. Scholz N, Hopkins W. 2006. Ecotoxicology of acetylcholinesterase pesticides: Data gaps and research challenges. *Environ Toxicol Chem* 25:1185–1186.
7. Singh B, Walker A. 2006. Microbial degradation of organophosphorous compounds. *FEMS Microbiol Rev* 30:428–471.
8. Garrison AW. 2006. Probing the enantioselectivity of chiral pesticides. *Environ Sci Technol* 40:16–23.
9. Lin K, Zhou S, Xu C, Liu W. 2006. Enantiomeric resolution and biotoxicity of methamidophos. *J Agric Food Chem* 54:8134–8138.
10. Liu W, Lin K, Gan J. 2006. Separation and aquatic toxicity of enantiomers of the organophosphorous insecticide trichloronate. *Chirality* 18:1–4.
11. Yen J, Tsai C, Wang Y. 2003. Separation and toxicity of enantiomers of organophosphorous insecticide leptophos. *Ecotoxicol Environ Saf* 55:236–242.
12. Wang P, Tai K, Yen J. 2004. Separation, bioactivity and dissipation of enantiomers of the organophosphorous insecticide fenamiphos. *Ecotoxicol Environ Saf* 57:346–353.
13. Thompson C, Ryu S, Berkman C. 1992. Consequence of phosphorus stereochemistry upon the postinhibitory reaction kinetics of acetylcholinesterase poisoned by phosphorothiolates. *J Am Chem Soc* 114:10710–10715.
14. Rodriguez OP, Muth GW, Berkman CE, Kim K, Thompson C. 1997. Inhibition of various cholinesterases with the enantiomers of malaoxon. *Bull Environ Contam Toxicol* 58:171–176.
15. Leader H, Casida J. 1982. Resolution and biological activity of the chiral isomers of *O*-(4-bromo-2-chlorophenyl) *O*-ethyl *S*-propyl phosphorothioate (profenofos insecticide). *J Agric Food Chem* 30:546–551.
16. Sasaki M. 1998. Importance of chirality in organophosphorous agrochemicals. In Kurihara N, Miyamoto J, eds, *Chirality in Agrochemicals*. Wiley, Chichester, UK, pp 85–139.
17. Jarman JL, Jones WJ, Howell LA, Garrison AW. 2005. Application of capillary electrophoresis to study the enantioselective transformation of five chiral pesticides in aerobic soil slurries. *J Agric Food Chem* 53:6175–6182.
18. Lao W, Gan J. 2006. High-performance liquid chromatographic separation of imidazolinone herbicide enantiomers and their methyl derivatives on polysaccharide coated chiral stationary phases. *J Chromatogr A* 1117:184–193.
19. U.S. Environmental Protection Agency. 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. EPA-821-R-02-012. Washington, DC.
20. Ellman G, Courtney D, Andres V, Featherstone R. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88–95.
21. Sandahl J, Jenkins J. 2002. Pacific steelhead (*Oncorhynchus mykiss*) exposed to chlorpyrifos: Benchmark concentration estimates for acetylcholinesterase inhibition. *Environ Toxicol Chem* 21: 2452–2458.
22. Kumar A, Chapman JC. 1998. Profenofos toxicity to the eastern rainbow fish (*Melanotaenia duboulayi*). *Environ Toxicol Chem* 17:1799–1806.
23. Wang P, Jiang S, Liu D, Zhang H, Zhou Z. 2006. Enantiomeric resolution of chiral pesticides by high performance liquid chromatography. *J Agric Food Chem* 54:1577–1583.
24. Lee P, Allahyari R, Fukuto TR. 1978. Studies on the chiral isomers of fonofos and fonofos oxon: I. Toxicity and antiesterase activities. *Pestic Biochem Physiol* 8:146–157.
25. Doorn JTT, Thompson C, Richardson R. 2001. Probing the active site of butyrylcholinesterase and cholesterol esterase with isomalathion: Conserved stereoselective inactivation of serine hydrolases structurally related to acetylcholinesterase. *Chem Res Toxicol* 14:807–813.
26. Wing K, Glickman A, Casida J. 1983. Oxidative bioactivation of *S*-alkyl phosphorothiolate pesticides: Stereospecificity of profenofos insecticide activation. *Science* 219:63–65.
27. Ueji M, Tomizawa C. 1987. Metabolism of chiral isomers of isofenphos in the rat liver microsomal system. *J Pestic Sci* 12: 269–272.
28. Lee P, Allahyari R, Fukuto TR. 1978. Studies on the chiral isomers of fonofos and fonofos oxon: II. In vitro metabolism. *Pestic Biochem Physiol* 8:158–169.