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# Enantiomeric Separation and Toxicity of an Organophosporus Insecticide, Pyraclofos

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**ABSTRACT:** Despite the fact that the biological processes of chiral pesticides are enantioselective, knowledge of the toxicities of pyraclofos due to enantiospecificity is scarce. In this study, the optical isomers of pyraclofos were separated and their toxicities to butyrylcholinesterase (BChE) and *Daphnia magna* were assessed. Baseline resolution of the enantiomers was obtained on both Chiralcel OD and Chiralpak AD columns. The effect of the mobile phase composition and column temperature were then discussed. The resolved enantiomers were characterized by their optical rotation and circular dichroism signs. The anti-BChE tests demonstrated that (−)-pyraclofos was about 15 times more potent than its (+)-form. However, acute aquatic assays suggested that (+)-pyraclofos was about 6 times more toxic than its antipode. Moreover, the joint toxicity of pyraclofos enantiomers to *D. magna* was found to be an additive effect. These results demonstrated that the overall toxicity of pyraclofos should be assessed using the individual enantiomers.

**KEYWORDS:** *butyrylcholinesterase, chiral pesticides, enantioselectivity, Daphnia magna*

## INTRODUCTION

Chirality is an important concept in many fields of chemistry, biology, and physics. Recently, significant attention has been paid to the chiral pesticides in relation to their marked differences in biological activity for individual enantiopure isomers.[1] Organophosphorus pesticides (OPs) are used to control insect vectors in fruits, vegetables, and other crops. It has been reported that >30% of the OPs sold are chiral.[2] An increasing number of studies have found that many biological activities, such as acute toxicity,[3−12] delayed neurotoxicity,[11,13] and fate in the environment,[4,14,15] of chiral OPs are enantioselective. Sometimes, only one enantiomer has the desired biological effects on target or nontarget organisms, with other enantiomers being less effective or even completely inactive. For example, the acute toxicity of (+)-isocarbophos to *Daphina magna* was identified to be about 50 times that for (−)-isocarbophos after either 24 or 48 h of exposure.[9] This significant enantioselectivity strongly suggested that the environmental risk of chiral OPs should be evaluated on the basis of their enantiomers.

Pyraclofos [(±)-*O*-1-(4-chlorophenyl)pyrazol-4-yl-*O*-ethyl-*S*-propyl phosphorothioate] is an organophosphorus pesticide (OP) registered in many countries and commonly used to control *Lepidoptera*, *Coleoptera*, *Acarina*, and *Nematode* pests.[16] In recent years, due to its high efficacy, moderate toxicity to mammals,[16] and capacity to control multi-OP-resistant pests,[17] pyraclofos has been considered to be an effective alternative to the highly toxic OPs in China. It is an *O*-ethyl *O*-phenyl *S*-*n*-propyl phosphorothioate and has an optically active phosphorus atom (Figure 1). Profenofos, prothiofos, and sulprofos also belong to this group (Figure 1). Leader and Casida[18] observed that (−)-profenofos was 3.8−22.7-fold more toxic than (+)-profenofos to housefly adults, cabbage looper larvae, mosquito, and mice. In contrast, the (+)-isomer was 2−48-fold more potent than its antipode in inhibiting bovine erythrocyte and housefly head acetylcholinesterase (AChE). This enantioselectivity has also been observed in AChE inhibition assays for nontarget aquatic species.[19] For example, the (−)-enantiomer of profenofos was 4.3−8.5-fold more inhibitory than the (+)-form to AChE in vivo in *D. magna* and Japanese medaka, whereas the (+)-enantiomer had 2.6−71.8-fold more inhibitory potential than the (−)-form in in vitro tests. Moreover, a report indicated that (−)-profenofos underwent preferential metabolic activation and the metabolite formed was 34 times more inhibitory to AChE than the (+)-form.[20] The same study also showed the preferential detoxification of (+)-profenofos. However, despite the universal existence of enantiospecificity in biological processes of these phosphorothioates with similar structures, no corresponding information with respect to pyraclofos has been reported. Moreover, some studies have found that pyraclofos is moderately to highly toxic to many species, such as birds,[16,21] fish,[16] honeybees,[16] and zooplankton,[22] implying its possible adverse effect on the environment. As a result, more studies about the toxicities of pyraclofos, at the level of both racemate and individual enantiomers, should be carried out to sufficiently describe its actual ecological risks.

One of the biggest challenges in understanding the chiral selectivity in the biological actions of chiral pesticides is the preparation of enantiomer standards. In the work by Huang,[23] complete enantiomeric separation of pyraclofos was obtained on nonaqueous capillary electrophoresis. However, we consider that this method may not be suitable for preparing optical

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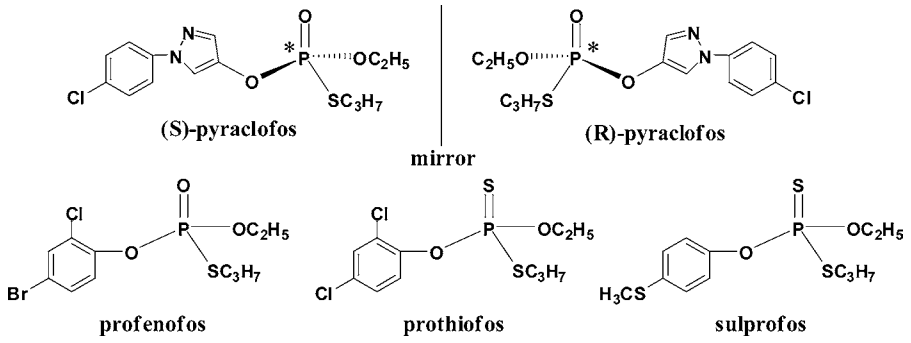


**Figure 1.** Molecular structures of *S*-*n*-propyl phosphorothioate insecticide. * denotes chiral center.

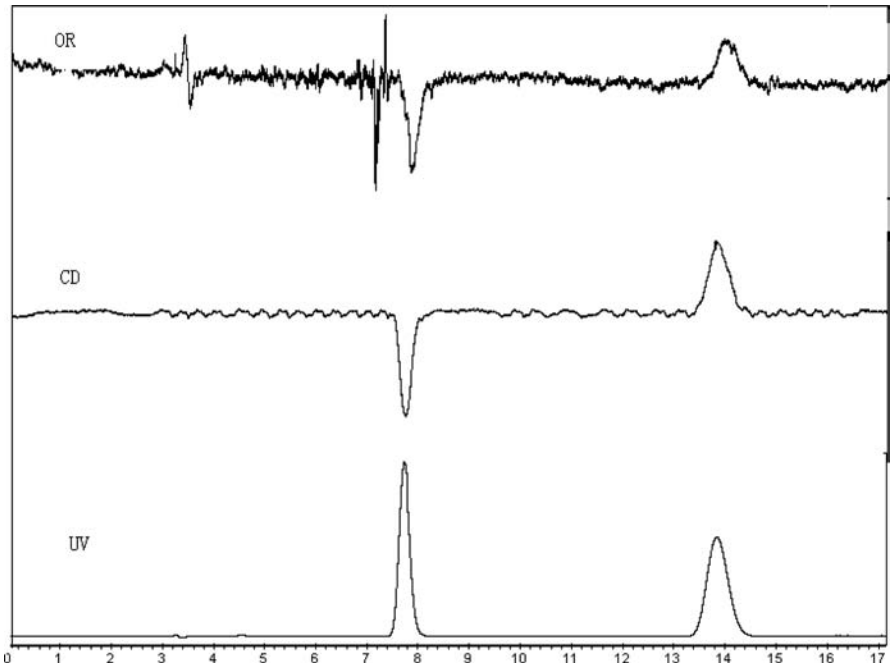


**Figure 2.** HPLC chromatograms for enantiomeric separation of pyraclofos on the Chiralcel OD column. Chromatographic conditions: mobile phase, hexane/isopropanol (85:15, v/v); flow rate, 1.00 mL $min^{-1}$; UV detection wavelength, 254 nm; column temperature, 25 °C.

isomers of pyraclofos due to the complicated removal of the chiral and achiral surfactants used (sodium cholate and sodium dodecyl sulfate, respectively). In recent years, high-performance liquid chromatography (HPLC) with a chiral stationary phase (CSP) column has been employed to prepare individual enantiomers from racemic chiral OPs on a small scale.[3−12] As a result, in this study, chiral separation of pyraclofos was carried out on four commercial chiral HPLC columns, that is, Chiralpak AD, Chiralpak AS, Chiralpak OD, and Chiralcel OJ. The effects of mobile phase composition and column temperature on chiral discrimination were also evaluated. Toxicities of the resolved enantiomers and the racemate of pyraclofos were then tested by in vitro inhibition of human butylcholinesterase (BChE) and in vivo by assessing acute toxicity to *D. magna*. Because there have been no studies on the enantioselective environmental behavior of pyraclofos, data from this study would provide helpful information for a more comprehensive assessment of the environmental risks of pyraclofos.

## ■ MATERIALS AND METHODS

**Chemicals.** The analytical standard of racemic pyraclofos (purity > 99.9%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BChE from human serum, butyrylthiocholine iodide (BTCh-I), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other solvents and chemicals were all of HPLC grade.

**Apparatus.** Chiral separation was performed on a Jasco LC-2000 series HPLC system (Jasco, Tokyo, Japan). The chromatographic system consisted of a PU-2089 quaternary gradient pump, a mobile phase vacuum degasser, an AS-2055 intelligent sampler with a 100 μL loop, a CO-2060 column thermostat, a variable-wavelength CD-2095 circular dichroism (CD) detector, an OR-2090 optical rotation (OR) detector, and an LC-NetII/ADC data collector. Chromatographic data were acquired and processed with computer-based ChromPass software (version 1.7.403.1, Jasco). The BChE activity was spectrophotometrically determined on a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories).

**Chromatographic Conditions and Identification of Enantiomers.** Four commercial chiral HPLC columns were used for the enantiomeric separation of pyraclofos in this study: Chiralpak AD [amylase tris(3,5-dimethylphenylcarbamate)], Chiralpak AS [amylase tris((*S*)-1-methylphenylcarbamate)], Chiralpak OD [cellulose tris(3,5-dimethylphenylcarbamate)], and Chiralcel OJ [cellulose tris(4-methylbenzoate)]. All of these columns were 250 mm × 4.6 mm i.d. and were purchased from Daicel Chemical Industries, Ltd. (Tokyo, Japan). In the preliminary experiments, the mobile phase for all four columns was *n*-hexane modified with isopropanol or ethanol from 5 to

Table 1. Enantiomeric Separation of Pyraclofos on Different Chiral Columns[a]

| column | mobile phase | content | capacity factor $k'_1$ | capacity factor $k'_2$ | separation factor, $\alpha$ | resolution, $R_s$ | CD[b] pk1/pk2[c] |
|---|---|---|---|---|---|---|---|
| Chiralcel OD | hexane/isopropanol | 85:15 | 1.16 | 2.63 | 2.28 | 8.10 | −/+ |
| | | 90:10 | 1.49 | 3.66 | 2.45 | 9.53 | −/+ |
| | | 95:5 | 2.59 | 7.11 | 2.74 | 11.54 | −/+ |
| | hexane/ethanol | 85:15 | 0.80 | 1.26 | 1.58 | 4.15 | −/+ |
| | | 90:10 | 1.01 | 1.70 | 1.68 | 5.33 | −/+ |
| | | 95:5 | 1.85 | 3.26 | 1.77 | 7.08 | −/+ |
| Chiralpak AD | hexane/isopropanol | 85:15 | 3.60 | 4.34 | 1.21 | 2.17 | −/+ |
| | | 90:10 | 5.24 | 6.38 | 1.22 | 2.40 | −/+ |
| | | 95:5 | 9.53 | 11.68 | 1.23 | 2.60 | −/+ |
| | hexane/ethanol | 85:15 | 5.22 | 11.84 | 2.27 | 6.23 | −/+ |
| | | 90:10 | 9.09 | 21.03 | 2.31 | 6.52 | −/+ |
| | | 95:5 | | | | | |
| Chiralpak AS | hexane/isopropanol | 85:15 | 3.50 | 3.50 | 1.0 | 0 | nr[d] |
| | | 90:10 | 4.74 | 5.67 | 1.20 | 0.84 | +/− |
| | | 95:5 | 8.42 | 10.30 | 1.22 | 1.14 | +/− |

[a]Chromatographic conditions: flow rate, 1.0 mL min$^{-1}$; column temperature, 25 °C. [b]Based on the signs of circular dichroism detected at 254 nm. [c]Pk1 and pk2 represent the first and second eluted enantiomers, respectively. [d]nr, no resolution.

15% with a 5% increment. These reagents were filtered through a 0.45 μm filter and degassed in vacuum before use. The column temperature, flow rate, and injection volume were 25 °C, 1 mL/min, and 20 μL, respectively. Under the determined optimal mobile phase conditions, the column temperature was varied from 15 to 35 °C with an increment of 5 °C to investigate the effect of temperature on chiral separation. The signals of the CD detectors were recorded at 254 nm. Specific rotation of the enantiomers was determined by an online OR detector. The light source for the chiral detector was a 150 W Hg−Xe lamp, and the tapered cell path was 25 mm with a volume of 44 μL. The rotation sign (+ or −) was indicated by a positive or negative peak on the chromatogram.

The enantiopure isomers of pyraclofos used for subsequent bioassays were manually collected at the HPLC outlet on the basis of the retention time under a proper HPLC separation conditions (Figure 2). They were then evaporated to dryness under a nitrogen stream and redissolved in ethanol. The concentrations of the enantiomers were determined by assuming the same response factor for enantiomers as for the racemate on the gas chromatograph coupled with a nitrogen−phosphorus detector. The purities of the prepared enantiomers were confirmed to be >99%.

**Assay of BChE Inhibition.** The inhibitory potentials of the pyraclofos enantiomers and its racemate against BChE were evaluated by calculating their respective concentrations leading to half-inhibition of BChE activity ($IC_{50}$). The BChE inhibition tests were carried out according to a previous study.[12] Briefly, test solutions (20 μL) at various concentrations of each enantiomer or racemate that inhibited enzyme activity by 10−80% were added into 500 μL centrifuge tubes, followed by the addition of 180 μL of BChE solution. Control samples were also prepared using 20 μL of phosphate buffer (pH 8.0) instead of the test solutions. Final concentrations of the solvent (ethanol) in both the BChE-inhibitor solution and the BChE-control solution were fixed at 0.5% (v/v). The mixture was incubated at 37 °C for 30 min. Then, 80 μL of the BChE-inhibitor solution (or BChE-control solution) was taken to measure the residual activity of BChE. BChE activity was spectrophotometrically determined at 37 °C using a modified Ellman method.[12] $IC_{50}$ was calculated by the logit transition model.[24]

**Aquatic Toxicity Assays.** The enantioselectivity of pyraclofos on aquatic toxicity was evaluated using a 48 h acute toxicity assay against *D. magna*. Stock organisms were originally obtained from the Chinese Academy of Protection and Medical Science (Beijing, China). The overall cultivation and testing procedures followed a published study.[7] The concentration that caused 50% mortality of the test population ($LC_{50}$) was determined by probit analysis (ToxCalc v5.0, Tidepool Scientific Software, McKinleyville, CA, USA). [25]

**Statistical Analysis.** All of the above tests and measurements were performed in four replicates. Student's *t* test at a significance level of 0.05 was used to compare the differences between groups.

## RESULTS AND DISCUSSION

**Chiral Separation on Different Chiral Stationary Phases (CSPs).** Polysaccharide-based CSPs have been used for the enantiomeric separation of chiral OPs,[26] especially for the four columns we chose, that is, the Chiralcel OD, Chiralcel OJ, Chiralpak AD, and Chiralpak AS columns.[27] Relative selectivity of the enantiomers of pyraclofos was first examined on these columns. The chromatographic separation results are shown in Table 1 (results of the Chiralcel OJ column are not shown). As listed in Table 1, the Chiralcel OD column showed the best enantiomeric recognition ability for pyraclofos among the tested columns. Moreover, the selectivity factor ($\alpha$) and resolution factor ($R_s$) values were substantially greater than those obtained by capillary electrophoresis methods, and the elution times were significantly shorter.[23] A typical chromatogram of the enantiomeric separation of pyraclofos on the Chiralcel OD column is shown in Figure 2. Baseline resolution of the enantiomers of pyraclofos was also achieved when Chiralpak AD was employed. However, little or no separation was obtained for the analyte on the Chiralpak AS and Chiralcel OJ columns with all of the *n*-hexane/alcohol combinations used (hexane/ethanol or hexane/isopropanol from 95:5 to 85:15 v/v).

The chiral discrimination mechanisms of the polymeric CSPs at the molecular level remain unclear. However, the highly specific enantiomeic resolution by the different CSPs may give us some indication. First, as mentioned above, the Chiralcel OD column provided the highest resolutions and retentions, followed by the Chiralpak AD and Chiralpak AS columns, whereas separation of the enantiomers was not obtained using the Chiralcel OJ column. It should be noted that the structures of the CSPs which exhibited rather high enantiomer-resolving

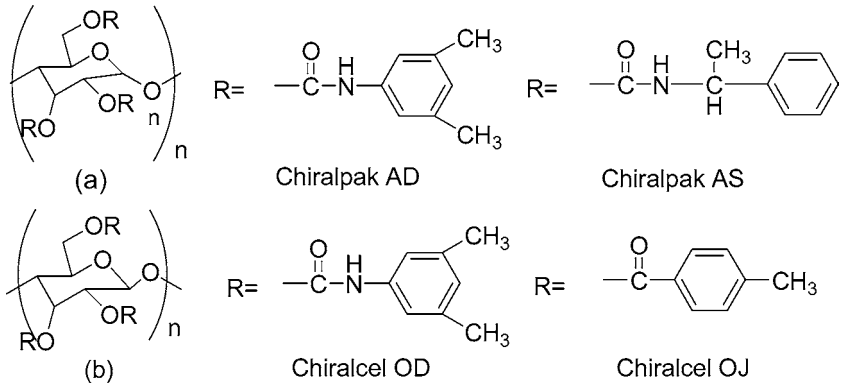


**Figure 3.** Chemical structures of derivatized polysaccharide CSPs: (a) Amylose-O-R, ChiralpakAD, and Chiralpak AS; (b) Cellulose-O-R, Chiralcel OD, and Chiralcel OJ.

abilities all had NH groups (Figure 3), which may interact via hydrogen bonding with the electronegative atoms (nitrogen, chlorine, oxygen, or sulfur) of pyraclofos and contribute to chiral recognition. Second, the Chiralcel OD column showed higher enantiomeric separation ability for this compound than the Chiralpak AD column, which could be due to the differences in the higher order structure between the amylase and cellulose derivatives (Figure 3). Moreover, the analyte contained a P═O group and phenyl and imidazole rings, which may lead to different preferences in their interaction with the polysaccharide CSPs through dipole−dipole and $\pi-\pi$ interactions. In addition, the degree of steric into the chiral cavities of the CSPs may also play a role in chiral recognition, and the chiral cavities of the different CSPs may have different accessibilities for pyraclofos. Given its better performance, the Chiralcel OD column was chosen for more specific tests.

The separated enantiomers were identified by measuring their OR and CD signs. As shown in Figure 2, (−)-pyraclofos (peak I) was eluted prior to its (+)-form (peak II) on the Chiralcel OD column. However, the elution orders of (±)-pyraclofos were reversed when using the Chiralpak AS column (Table 1), indicating a change in chiral recognition interaction between the two columns.

**Effect of Polar Modifiers.** The type and percentage of organic modifier are the factors that most influence the chiral separation. According to the reports by Wainer et al.,[28,29] alcohol in the mobile phase not only competes for chiral bonding sites with chiral solutes but can also alter the three-dimensional structure of the stationary phase. On the basis of our previous experiences,[7,12,30] the effects of two organic modifiers, ethanol and isopropanol, were investigated. As illustrated in Table 1, with an increased percentage of alcohol in the mobile phase, the values of retention factor ($k'$) and $R_s$ decreased; this effect was more pronounced on the Chiralpak AD column, which showed no elution peaks within 2 h for pyraclofos using 95:5 hexane/isopropanol as the mobile phase. This indicated that hydrogen bonding was the dominant interaction for retention and enantioselectivity.[31] When the polarity of the mobile phase increased, the hydrogen bonds between the analyte and CSP decreased, which was consistent with the increased ability of the solvent to displace the solute from the CSP.[29,32] Nevertheless, the values of $\alpha$ changed very little over the entire alcohol concentration range (5−15%), suggesting that there was no competition between the analyte and the organic modifier for the active sites of CSPs.[33] In addition to the polarity of the mobile phase, the resolution was also affected by the type of mobile phase. In this study, the solvent performed differently on different columns. As seen from Table 1, better resolution was obtained in hexane/isopropanol than in hexane/ethanol on the Chiralcel OD column and the Chiralpak AS column. By comparison, ethanol was a better organic modifier for the Chiralpak AD column. These results indicated that the selection of the polar additive to obtain satisfactory enantioselectivity for OPs sometimes depended on the column used.

**Effect of Column Temperature.** Column temperature is another important factor in enantioseparation. In this study, the effect of temperature was investigated on the Chiralcel OD column in the temperature range from 15 to 35 °C using the mobile phase composition of hexane/isopropanol (85:15, v/v) at a flow rate of 1 mL min$^{-1}$. The calculated parameters including $k'$, $\alpha$, and $R_s$ are summarized in Table 2. The results showed that higher temperature led to decreases in retention, selectivity, and resolution.

**Table 2. Effect of Temperature on Enantiomer Separation of Pyraclofos on the Chiralcel OD Column**[a]

| $T$ (°C) | $k'_1$ | $k'_2$ | $\alpha$ | $R_s$ |
|---|---|---|---|---|
| 15 | 1.32 | 3.28 | 2.48 | 8.45 |
| 20 | 1.19 | 2.84 | 2.38 | 8.32 |
| 25 | 1.16 | 2.63 | 2.28 | 8.10 |
| 30 | 0.97 | 2.14 | 2.21 | 7.66 |
| 35 | 0.90 | 1.87 | 2.09 | 6.95 |

[a]Chromatographic conditions: mobile phase, hexane/isopropanol (85:15, v/v); flow rate, 1.0 mL min$^{-1}$.

According to Péter et al.,[34] temperature can affect chiral separation. For example, it can change the separation factor ($\alpha$), which is investigated according to the van't Hoff equation

$$\ln k' = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \phi \quad (1)$$

where $\Delta H^\circ$ and $\Delta S^\circ$ are the standard molar enthalpy and molar entropy of the transferring solute from the mobile phase to the stationary phase, respectively, $R$ is the gas constant, $T$ is the absolute temperature, and $\phi$ is the phase ratio. Then, ln $\alpha$ can be expressed as

$$\ln \alpha = -\frac{\Delta\Delta H^\circ}{RT} + \frac{\Delta\Delta S^\circ}{R} \quad (2)$$


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where $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ values are the differences $(\Delta H^\circ_2 - \Delta H^\circ_1)$ and $(\Delta S^\circ_2 - \Delta S^\circ_1)$, respectively. Provided that ln $\phi$ is independent of temperature, a plot of ln $k'$ versus $1/T$ will be linear with a slope of $-(\Delta H/R)$ and an intercept of $(\Delta S/R + \ln \phi)$. For the linear plot of ln $\alpha$ versus $1/T$, the slope and intercept are $-\Delta\Delta H^\circ/R$ and $\Delta\Delta S^\circ/R$, respectively.

As shown in Figure 4, both the plots of natural logarithms of retention factor (ln $k'$) and separation factor (ln $\alpha$) as a function of the inverse of temperature ($1/T$) could be fitted by straight lines. The $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ values calculated from Figure 4 were −6.13 and −13.73 kJ mol$^{-1}$, respectively. The negative value of $\Delta\Delta H^\circ$ demonstrated that better chiral resolution of pyraclofos on the Chiralcel OD columns may be achieved at a lower temperature. Moreover, the values of $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ for pyraclofos were all negative, implying that enantiomeric separation of pyraclofos is an enthalpy-driven process.[31]

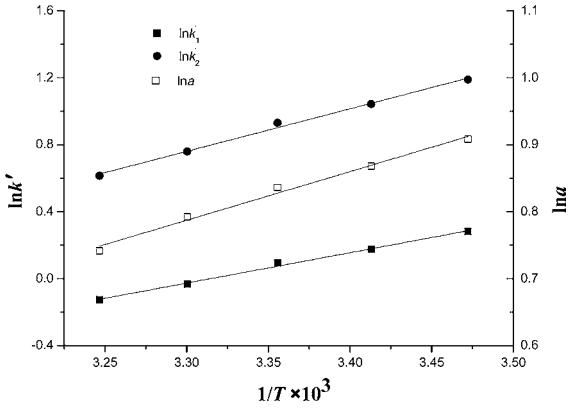


**Figure 4.** van't Hoff plots of $k'$ and $\alpha$ for the enantiomers of pyraclofos. Chromatographic conditions: Chiralcel OD column; *n*-hexane/isopropanol (85:15, v/v); flow rate, 1.0 mL min$^{-1}$.

**Enantioselective Toxicity.** It has been known for many years that the mechanism of the acute toxicity of OPs at both the pharmacological and toxicological levels is due to inhibition of the serine esterase AChE. In this study, the activities of AChE from both bovine erythrocytes and *Electrophorus electricus* were not significantly inhibited by pyraclofos in the in vitro assays (data not shown). However, the in vitro inhibitory potential of pyraclofos against another serine esterase, BChE, was relatively high with an $IC_{50}$ value of 0.21 ± 0.01 mg L$^{-1}$ (Table 3). In fact, this discrepancy of inhibitory potentials against AChE and BChE has been found for many OPs, such as soman,[35,36] FP-biotin,[37] and chlorpyrifios oxon.[38] In the case of dichlorvos, the mean biomolecular rate constants for in vitro inhibition of BChE were about 4 orders of magnitude higher than those of AChE.[39] These examples imply that BChE is more sensitive to OPs than AChE sometimes and is an indicator at lower substrate levels.[40] As a result, it is possible that the dissolved pyraclofos is inactive to AChE, but is enough to inhibit BChE. Moreover, a significant difference was found between the BChE inhibitory activities of the pyraclofos enantiomers. From the values of $IC_{50}$, (−)-pyraclofos was about 15-fold more potent toward BChE than (+)-pyraclofos (Table 3). Similar enantioselectivity in BChE inhibitory potentials was also observed for corresponding studies on leptophos, fenamiphos, and salithion, with the (+)-isomers being about 4.8−18.8-fold more active than the (−)-isomers.[3,4,12] Although BChE has not been demonstrated to play a physiological role, it may have a protective function by sequestering OP compounds, which might inhibit the pivotal functions of AChE.[41,42] According to this hypothesis, increased inhibition of BChE might signify the organism's potential risk for acute cholinergic crisis (inhibition of AChE). In addition, different inhibitory potentials of pyraclofos enantiomers may be more important for unborn humans than humans. This is because BChE appears in the development of the central nervous system before AChE and has been suggested to function as an embryonic acetylcholinesterase.[43] Under this circumstance, (−)-pyraclofos may be more acutely toxic to the fetus than its antipode.

**Table 3. Half-Inhibition Concentration ($IC_{50}$) for Butyrylcholinesterase and Median Lethal Concentrations ($LC_{50}$) for *Daphia magna* of Pyraclofos Racemate and Its Enantiomers**

| compound | $IC_{50}$ (mg L$^{-1}$) | $LC_{50}$ ($\mu$g L$^{-1}$) |
|---|---|---|
| *rac*-pyraclofos | 0.21 ± 0.01 | 1.38 ± 0.20 |
| (+)-pyraclofos | 1.19 ± 0.10 | 0.71 ± 0.00 |
| (−)-pyraclofos | 0.08 ± 0.00 | 4.04 ± 0.50 |

To investigate the enantioselective toxicity to nontarget organisms, an aquatic toxic assay with *D. magna* was performed for 48 h. The results showed that the more potent BChE inhibitor, (−)-pyraclofos, was less toxic to *D. magna*, with an $LC_{50}$ value about 6 times higher than that of the antipode (Table 3). This enantioselective discrepancy in the in vitro and in vivo toxicities has also been reported for many other OPs, such as methamidophos,[7] chloramidophs,[10] and salithion.[12] For example, (−)-methamidophos was about 8.0−12.4 times more potent to enzymes than its (+)-form in comparison to the (+)-enantiomer, which was 7.0 times more toxic to *D. magna* during 48 h tests. Moreover, as mentioned in previously published studies, the possible causes include the following factors:[7,10,12] First, the enzymes of different species might have different enantiosensitivities to the chiral OPs. Second, many biological processes, such as metabolism, transformation, and accumulation, also have enantioselectivity, resulting in different amounts of different enantiomers reaching the active sites of target enzymes.[44,45]

The available literature on toxicity data showed that the joint acute toxicity for enantiomer mixtures of chiral OPs has many different modes of interaction.[9] For instance, the joint acute toxicity of the individual enantiomers of salithion, methamidophos, and profenofos clearly showed an additive effect in *D. magna*. However, synergistic and antagonistic effects were also evident for leptophos and chloramidophos, respectively. To understand the interaction of pyraclofos enantiomers in joint acute aquatic toxicity, the toxic unit of the mixture ($TU_{mix}$), which is the ratio of the measured concentration of a chemical in a mixture to the corresponding effective concentration of the single compound in the same medium, was determined.[46] The $TU_{mix}$ could be calculated by the following equation:

$$TU_{mix} = \frac{LC_{50(+)\text{-pyraclofos (mix)}}}{LC_{50(+)\text{-pyraclofos (alone)}}} + \frac{LC_{50(-)\text{-pyraclofos (mix)}}}{LC_{50(-)\text{-pyraclofos (alone)}}} \quad (3)$$

A $TU_{mix}$ value equal to 1.0 ± 0.2 indicates concentration addition. A $TU_{mix}$ value <0.8 indicates a synergistic effect (more than additive effect), whereas a $TU_{mix}$ value >1.2 indicates an


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antagonistic effect (less than additive effect).[47] On the basis of the $LC_{50}$ values listed in Table 3, the toxic units for (+)-pyraclofos ($TU_{(+)\text{-pyraclophos}}$), (−)-pyraclofos ($TU_{(-)\text{-pyraclophos}}$), and the racemic mixture ($TU_{\text{rac-pyraclofos}}$) were 0.97, 0.17, and 1.14, respectively, suggesting that pyraclofos enantiomers exhibited additive toxicity to *D. magna* when both were present in the solution. The additive effect of pyraclofos enantiomers may be reasonably explained by the fact that both enantiomers have a similar mode of toxic action by binding with acetylcholinesterase.

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### Notes

The authors declare no competing financial interest.

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