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Single and Joint Acute Toxicity of Isocarbophos Enantiomers to *Daphnia magna*

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Although enantioselectivity in the toxicity of chiral pesticides has received considerable attention over recent years, how coexisting enantiomers interact with each other during their toxic action remains unknown. In this study, we attempted to resolve the enantiomers of a chiral organophosphate insecticide, isocarbophos, and investigated the acute toxicity of individual enantiomers and various enantiomer mixtures. Baseline enantiomeric separation of isocarbophos was achieved on a Chiralcel OD column with the mobile phase of *n*-hexane/isopropanol (90/10, v/v) at a flow rate of 0.8 mL/min. The resolved enantiomers were differentiated by their responses on a circular dichroism detector. The median lethal concentrations (LC_{50}) of racemate, (+)-enantiomer, and (−)-enantiomer of isocarbophos toward *Daphnia magna* were 13.9, 7.08, and 353 μg/L, respectively, after 48 h of static exposure, displaying a 50-fold difference between the enantiomers. Toxic unit (TU) analysis was employed to evaluate the joint toxicity of isocarbophos enantiomer mixtures. The calculated TU_{mix} for the acute toxicity (48 h test) of various binary mixtures ranged from 0.83 to 1.04, suggesting a mode of additive effect. Further evaluation of available literature data for chiral organophosphorus insecticides showed that the joint toxicity of enantiomers may be additive, synergistic, and antagonistic. Therefore, when significant enantioselectivity exists for a chiral pesticide, it is important to also evaluate the interaction of enantiomers in the joint toxicity effect when enantiomers are present in a mixture.

KEYWORDS: Chiral; pesticide isocarbophos; enantiomeric separation; joint toxicity; enantioselective toxicity

INTRODUCTION

Organophosphorus pesticides (OPs) are one of the most important classes of currently used pesticides. OPs are also a pesticide class containing many chiral compounds. A survey showed that 30 compounds of 70 OP standards available from a popular commercial source for pesticide standards are chiral compounds (*1*). In OPs, both the phosphorus and the carbon atoms may be the chiral center. Isocarbophos [(*RS*)-(*O*-2-isopropoxycarbonylphenyl *O*-methyl phosphoramidothioate] with an asymmetric center at the phosphorus atom contains one pair of enantiomers (**Figure 1**). Isocarbophos was introduced into agricultural use in 1981 in China. Isocarbophos is a potent acetylcholinesterase inhibitor and is used to control chewing and sucking insects and spider mites on a variety of crops such cotton, tobacco, and rice. Because of its high toxicity and high residue, isocarbophos was banned from use on vegetables in China. Other chiral OPs such as methamidophos (*2*), chloramidophos (*3*), fosthiazate (*4*), trichloronate (*5*), leptophos (*6*), and fenamiphos (*7*) have been found to display enantiomer-specific acute toxicity toward *Daphnia magna*. However, enantioselectivity in the ecotoxicity of isocarbophos has not been studied.

Studies increasingly show that the toxicity of chiral pesticides is enantiomer-specific, suggesting that the environmental risk of chiral pesticides should be evaluated using individual enantiomers (*2*–*10*). However, most chiral OPs are currently applied and released into the environment in their racemic forms, that is, an equimolar mixture of enantiomers. Because microorganisms and the associated enzymes constitute a chiral environment, biodegradation of chiral pesticides is often enantioselective (*11, 12*). As a result, the enantiomeric ratio (ER), defined as the ratio of the concentration of the (+)-enantiomer and the (−)-enantiomer, will often deviate from the racemic composition (ER = 1.0) (*13, 14*). When enantioselective

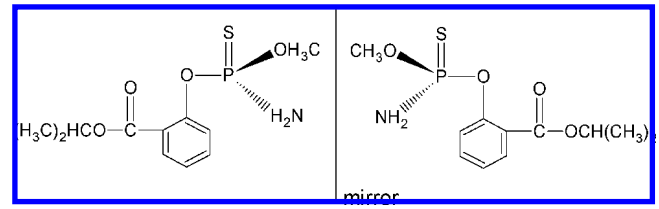

Figure 1. Enantiomers of isocarbophos (the asterisk indicates the chiral center).

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degradation occurs, it is inaccurate to assess the toxicity of enantiomeric mixtures with enantiomeric excesses using the data derived from the racemate of chiral pesticides.

Studies considering the enantioselectivity of chiral xenobiotics in toxicity effects so far have neglected the evaluation of interaction of enantiomers. The toxicity of compound mixtures is usually determined by the mode of concentration addition or response addition. Response addition is based on the prerequisite that toxicants have different physiological systems in the organism and is commonly used in assessing the joint effect of pharmaceuticals (*15, 16*). Concentration addition is employed to predict the toxicity of organic compounds that usually pose a nonspecific mode of action, for example, narcosis (*17–19*). In the mode of concentration addition, toxicity of mixtures can be expressed as toxic units of the mixtures (TU_{mix}), which is a ratio of the measured concentration of a chemical in a mixture to the corresponding effect concentration of the single compound in the same medium (*20, 21*). Assuming concentration addition, the TUs for individual components in a mixture can be added to estimate the total toxicity (*21*).

The main objectives of this study were to develop an analytical method for the separation of isocarbophos enantiomers and to evaluate the acute aquatic toxicity of the resolved enantiomers and the mode of toxicity interaction for the coexisting enantiomers.

MATERIALS AND METHODS

Chemicals. An analytical standard of racemic isocarbophos (99.6%) was obtained from Kefa New Technology Development (Shenyang, China). Other chemicals and solvents were of analytical or high-performance liquid chromatography (HPLC) grade. For the enantiomeric separation, isocarbophos was dissolved in the mobile phase at 1000 mg/L.

Chromatographic Conditions and Resolution of Enantiomers. Enantiomer separation and preparation were carried out 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 loop, a CO-2060 column temperature control compartment, a variable-wavelength CD-2095 circular dichroism (CD) detector, and an LC-Net II/ADC data collector. Separation was achieved at 25 °C on a Chiralcel OD column (250 mm × 4.6 mm) with the enantioselective phase [cellulose tris(3,5-dimethylphenyl carbamate)] coated onto a 5 μm silica-gel substrate. The injection volume was 20 μL. The flow rate of the mobile phase was 0.8 mL/min. The detection wavelength of CD was set at 230 nm. 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. Identification of the resolved enantiomers was performed by measuring their CD spectra and by qualitative analysis with an Agilent 6890 gas chromatograph coupled with an Agilent 5975 mass spectrum detector (gas chromatography−mass spectrometry, GC-MS) (Agilent, Wilmington, DE).

The preparation of individual enantiomers was achieved by manually collecting the eluent corresponding to the resolved peaks at the HPLC outlet of the CD detector while observing the UV absorbance. The collected individual enantiomer solutions were evaporated to dryness, redissolved in acetone, and used as the stock solutions for bioassays (the final amount of acetone in assay solution was <0.04%). The concentrations of enantiomers in the stock solutions were determined by analyzing an aliquot on an Agilent 6890 GC coupled with a nitrogen phosphorus detector (Agilent, Wilmington, DE) assuming the same response factor for both enantiomers as for the racemate.

Bioassays. The acute toxicity was measured for individual enantiomers and racemate and enantiomer mixtures using *D. magna* as the test organism. The test organisms were obtained from a continuous culture maintained at 22 ± 1 °C in M4 culture medium (*22*) with a photoperiod of 12 h/day and a density of <50 animals per liter. Stock organisms were originally obtained from the Chinese Academy of Protection and Medical Science (Beijing, China). The medium was renewed three times a week, and daphnids were fed daily with the alga *Scenedesmas obliquus*, which were cultured in the laboratory using a nutrient medium. The test animals used in this experiment were juveniles aged between 6 and 24 h. Prior to the test, a sensitive test for daphnids to potassium dichromate was performed as a positive control and the LC_{50} (24 h) value was in the range of 0.6−1.7 mg/L. The overall acute toxicity test was conducted according to the standard protocol (*23*). Briefly, five neonates were transferred into glass beakers filled with 20 mL of blank or test solutions of known enantiomer concentrations. The test solutions with the highest concentrations were prepared by adding a known amount of enantiomer to the dilution water. Subsequent dilution was made from the highest concentration to derive the lower concentration solutions. The nominal concentrations were 1.0, 5.0, 10.0, 20.0, and 40.0 μg/L for (+)-isocarbophos, 50, 125, 250, 500, and 1000 μg/L for (−)-isocarbophos, and 1.0, 5.0, 10.0, 20.0, and 40.0 μg/L for the racemate. For enantiomer mixtures, the nominal concentrations were chosen according to the ER. For example, the nominal concentrations for the mixture with an ER of 1:5 were 1.0/5.0, 2.5/12.5, 5.0/25.0, 10.0/50.0, and 20.0/100.0 as μg/L (+)-isocarbophos to (−)-isocarbophos. Four replicates were prepared for each treatment. The test animals were not further fed and were incubated at 22 ± 1 °C for 48 h. Mortality of daphnids was observed after incubation for 24 and 48 h. The LC_{50} values were determined from the survival data with ToxCalc (Version 5.0) (Tidepool Scientific Software, McKinleyville, CA).

Joint Toxicity Assay. The extent of toxic interaction was evaluated by converting the LC_{50} estimates associated with the mixture to TUs based on the LC_{50} estimates associated with the individual enantiomers when tested alone. The TU_{mix} could be determined by the following equation:

$$TU_{mix} = \frac{LC_{50\ (+)\text{-isocarbophos (mix)}}}{LC_{50\ (+)\text{-isocarbophos (alone)}}} + \frac{LC_{50\ (-)\text{-isocarbophos (mix)}}}{LC_{50\ (-)\text{-isocarbophos (alone)}}} \quad (1)$$

Accordingly, 1 TU is considered equivalent to the LC_{50} of an individual enantiomer when tested alone. Assuming a strictly additive effect, the LC_{50} concentration of a mixture should equal unity when expressed as TUs (*24*). For example, if the (+)-isocarbophos concentration associated with the LC_{50} of a mixture is 12.2 μg/L, 0.87 TU of (+)-isocarbophos would be present at this concentration, assuming the LC_{50} for (+)-isocarbophos alone is 14.1 μg/L. A TU_{mix} value equal to 1.0 ± 0.2 indicates concentration addition. A TU_{mix} less than 0.8 indicates a synergistic effect (more than additive effect), while a TU_{mix} greater than 1.2 indicates an antagonistic effect (less than additive effect) (*25*).

RESULTS AND DISCUSSION

Enantiomer Separation and Preparation. Successful separation of isocarbophos enantiomers was obtained on the Chiralcel OD column with a mobile phase of *n*-hexane/isopropanol (90/10, v/v) at the flow rate of 0.8 mL/min. A typical chromatogram is shown in **Figure 2**. The separated enantiomers were easily differentiated from the response in the CD chromatograms. According to their CD responses at 230 nm, the first and second eluted enantiomers were designated as (+)-isocarbophos and (−)-isocarbophos, respectively. To confirm that the resolved two peaks corresponded to a pair of enantiomers, their CD spectra were scanned by the online CD detector. The CD spectra of the two peaks (**Figure 3**) were mirror images of each other when taking the axis of CD = 0 as a mirror, indicating that the resolved chemicals were a pair of enantiomers. The resolved enantiomers were separately collected, and an aliquot of the solutions was analyzed with GC-MS. Their mass spectra were found to be consistent with that of the racemic isocarbophos (data not shown).

The developed method was employed to prepare the pure enantiomers of isocarbophos for the subsequent bioassays. A

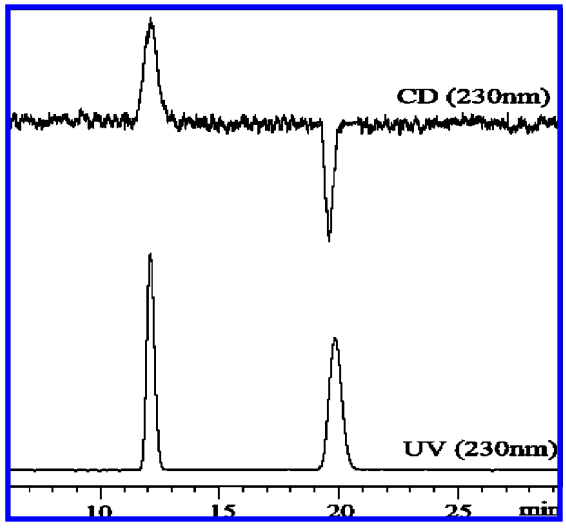

Figure 2. Typical HPLC chromatogram for the enantiomeric separation of isocarbophos on the Chiralcel OD column. Other chromatographic conditions: mobile phase, *n*-hexane/isopropanol (90/10, v/v) at the flow rate of 0.8 mL/min (25 °C).

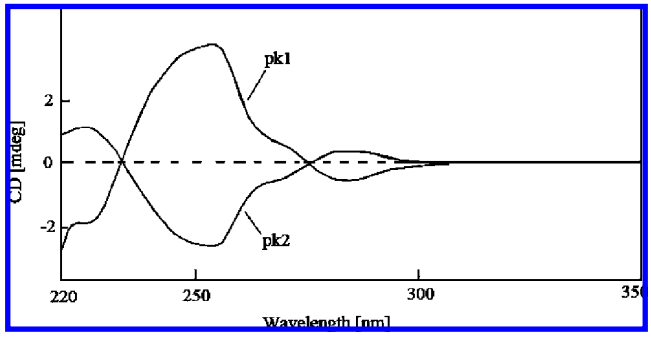

Figure 3. CD spectra of resolved isocarbophos enantiomers. Chromatographic conditions were the same as given for **Figure 2**.

Table 1. Acute Median Lethal Concentrations (LC_{50}, μg/L) of Racemate and Enantiomers of Isocarbophos for *Daphnia magna*

	LC_{50} and 95% confident interval	
compound	24 h	48 h
(±)-isocarbophos	23.5 (18.9−28.1)	13.9 (11.1−17.4)
(+)-isocarbophos	14.1 (13.2−15.0)	7.08 (6.45−7.71)
(−)-isocarbophos	709 (648−776)	353 (302−413)

resolution factor (R_s) of 1.5 can theoretically provide 99.7% separation. With $R_s = 9.9$, the pair of isocarbophos enantiomers was completely resolved on the Chiralcel OD column (with $R_s = 9.9$) under the experimental conditions and warranted the sufficient purity of the enantiomers prepared for bioassays.

Acute Toxicity of Enantiomers and Racemate. The LC_{50} values obtained from the tests are summarized in **Table 1**. The order of toxicity potency at both 24 and 48 h was (+)-isocarbophos > racemate > (−)-isocarbophos. The (+)-isocarbophos enantiomer exhibited acute toxicity to *D. magna* was about 50 times of that for (−)-isocarbophos after either 24 or 48 h of exposure (**Table 1**). It is common for the acute aquatic toxicity of a chiral OP to be associated primarily with only one of the enantiomers. For example, the toxicity differences against *D. magna* for methamidophos (*2*), fenamiphos (*7*), leptophos (*6*), fonofos (*8*), profenofos (*8*), and trichloronate (*5*), were 7.0-, 2.6-, 20.0-, 15.0-, 6.6-, and 8.2-fold, respectively. In addition, the enantioselectivity among the stereoisomers of chlorami-

Table 2. Acute Median Lethal Concentrations (LC_{50}, μg/L) of Isocarbophos Enantiomers in Their Binary Mixtures for *Daphnia magna* and the Calculated TUs

	24 h test			48 h test		
ER[a]	(+)	(−)	TU	(+)	(−)	TU
1:200	2.22	444	0.78	1.29	258	0.91
1:100	3.49	349	0.74	1.91	191	0.81
1:50	6.31	316	0.89	3.31	166	0.93
1:25	8.30	158	0.81	3.92	98.0	0.83
1:5	10.5	50.3	0.81	6.53	32.6	1.03
1:2	11.8	23.6	0.87	7.08	14.2	1.04
1:1	11.8	11.8	0.85	6.95	6.95	1.00
2:1	12.2	6.10	0.87	7.07	3.54	1.01
5:1	12.2	2.40	0.87	7.07	1.41	1.00

[a] The concentration ratio of (+)-isocarbophos and (−)-isocarbophos.

dophos (*3*) and fosthiazate (*4*), which have two chiral centers, were 13.0- (pk2/pk4) and 3.1-fold (pk1/pk3), respectively. However, the 50-fold difference between isocarbophos enantiomers in the acute toxicity toward *D. magna* is the largest between enantiomers for chiral OPs that have been considered thus far.

The significant enantioselectivity strongly suggests that the aquatic toxicity of isocarbophos enantiomers should be considered individually. Currently, the environmental risk of most chiral pesticides is evaluated based on their racemates. Thus, overestimation or underestimation may result because of the enantioselective toxicity to nontarget organisms. After a racemate of a chiral pesticide is applied, its ER may change due to enantioselective biodegradation in the environment, leading to an enantiomeric excess (*11, 26*). Taking the toxicity of isocarbophos to *D. magna* as an example, the predicted acute toxicity (24 h) based on the racemate might be overestimated by 30 times if only the (−)-enantiomer were present in the residue. However, it might cause a 1.7-fold underestimation if only the (+)-enantiomer were present in the residue. It is unknown if enantioselective attenuation occurs to isocarbophos; however, studies on other chiral pesticides suggest a frequent occurrence of enantioselective degradation in soil (*9*). For example, deforestation in Brazil caused soils to shift from preferentially removing the less toxic (−)-ruelene to exclusively transforming the (+)-enantiomer (*9*). Soil warming in Norway, on the other hand, caused soils to shift from preferentially removing the (+)-ruelene to preferentially removing the less toxic enantiomer (*9*).

Joint Acute Toxicity of Isocarbophos Enantiomer Mixtures. To understand the interaction of enantiomers in the joint acute toxicity of isocarbophos enantiomer, the LC_{50} values at 24 and 48 h for binary enantiomer mixtures with ERs ranging from 1:200 to 5:1 were determined. On the basis of the LC_{50} values from the tests on the individual enantiomers and binary mixtures, the results for the mixtures were converted to TUs of the individual enantiomer and summed (**Table 2**). In 24 h test, the values of calculated TU_{mix} ranged between 0.74 and 0.89, with an average of 0.83 ($n = 9$). Similarly, the values of calculated TU_{mix} in the 48 h test ranged between 0.83 and 1.04, with an average of 0.95 ($n = 9$). Collectively, these values were very close to the hypothetical value of 1.0, suggesting that isocarbophos enantiomers exhibited additive toxicity to *D. magna* when both were present in the solution. The additive effect between isocarbophos enantiomers may be reasonably explained by the fact that both enantiomers have a similar mode of toxic action by binding with acetylcholinesterase. This observation implies that the toxicity to *D. magna* for isocarbo-

Table 3. Calculated TUs for Chiral Organophosphorus Insecticides with One Chiral Center Using Reported Data

compound	exposure time (h)	LC_{50}(μg/L) racemate	(+)-enantiomer	(−)-enantiomer	TU[a]	ref
methamidophos	24	1.09×10^2	70.7	2.77×10^2	0.97	*2*
	48	34.0	33.8	2.38×10^2	0.57	*2*
fenamiphos	24	1.90	1.60	6.10	0.75	*7*
leptophos	24	4.10×10^{-2}	3.90×10^{-2}	0.80	0.55	*6*
fonofos	96	0.58	3.45	0.23	1.35	*8*
profenofos	96	0.69	2.32	0.35	1.13	*8*
trichloronate	96	0.51	1.39	0.17	1.68	*5*

[a] TU was calculated as $TU = [LC_{50\,(+)\text{-enantiomer in racemate}}/LC_{50\,(+)\text{-enantiomer alone}}] + [LC_{50\,(-)\text{-enantiomer in racemate}}/LC_{50\,(-)\text{-enantiomer alone}}]$.

Table 4. Calculated TUs for Chiral Organophosphorus Insecticides with Two Chiral Centers Using Reported Data

compound	exposure time (h)	LC_{50} (mg/L) racemate	pk1[a]	pk2[a]	pk3[a]	pk4[a]	TU[b]	ref
chloramidophos[c]	48	1.31	0.34	0.04	0.13	0.40	12.9	*3*
	96	1.12	0.16	0.02	0.05	0.26	18.2	*3*
fosthiazate[d]	24	1.10	0.50	0.65	0.87	0.57	1.77	*2*
	48	0.66	0.22	0.32	0.68	0.49	1.85	*2*
	72	0.33	0.16	0.29	0.51	0.34	1.20	*2*
	96	0.26	0.16	0.16	0.40	0.28	1.21	*2*

[a] pk1 to pk4 are the first, second, third, and fourth eluted stereoisomers on a chiral column. More detailed information is available in the original references. [b] TU was calculated as $TU = \sum_{i=1}^{4} [LC_{50\,pk(i)\text{ in racemate}}/LC_{50\,pk(i)\text{ alone}}]$. [c] The molar ratio for the pk1:pk2:pk3:pk4 in racemic chloramidophos was 1:2.7:1:2.7. [d] The molar ratio for the pk1:pk2:pk3:pk4 in racemic fosthiazate was 1:1:1:1.

phos enantiomer mixtures with different ERs can be predicted by the calculation of TU_{mix}.

The joint toxicity of enantiomers of a chiral compound is usually thought to be additive effect, as observed for isocarbophos in the present study. Using the TU analysis approach, we further calculated the values of TU_{mix} for some other chiral OPs with one or two asymmetric centers for which toxicity values were reported (**Tables 3** and **4**). Judging from the values of TU_{mix}, the joint acute toxicity to *D. magna* for methamidophos (24 h test), fenamiphos, profenofos, and fosthiazate (72 and 96 h tests) can also be explained by the mode of additive effect. However, a synergistic effect between the enantiomers is evident for methamidophos (48 h test) and leptophos, with TU_{mix} values being 0.57 and 0.55, respectively. On the other hand, an antagonistic effect between isomers was found for trichloronate, chloramidophos, fonofos, and fosthiazate (24 and 48 h test). The values of TU_{mix} for racemic mixtures of trichloronate, fonofos, and fosthiazate (24 and 48 h test) showed small deviations from 1.0, displaying a limited antagonistic effect. However, a significant antagonistic effect was observed in racemic mixtures of chloramidophos, with TU_{mix} being 12.9 and 18.2 in 48 and 96 h tests, respectively.

No studies so far have attempted to understand such antagonistic or synergistic interactions between enantiomers. It may be attributable to in vivo enantiomerization or other in vivo enantioselective biological reactions. Enantiomers of a chiral compound may undergo chiral inversion or enantiomerization in organisms (*27, 28*), implying that the ER of a mixture binding to active sites may be different from that of the exposure mixture. In this case, TU_{mix} does not accurately reflect the joint toxicity of enantiomer mixtures. For example, enantiomerization from the less toxic enantiomer to the more toxic enantiomer would lead to an underestimated TU_{mix}, while the opposite chiral inversion would lead to an overestimated TU_{mix}. Enantioselectivity in the biological activity may be another important factor. Before the enantiomers bind to the active sites in *D. magna*, they may undergo many enantioselective biological processes such as uptake, bioconcentration, transformation, and metabolism (*11, 26*). Furthermore, the presence of one enantiomer may facilitate or hinder the other enantiomer's binding to the active sites in an organism simply because the enantiomers have the same mode of toxic action.

In conclusion, racemic isocarbophos was enantiomerically separated on a Chiralcel OD column, which allowed the preparation of small quantities of pure enantiomers. About a 50-fold difference was observed between the two enantiomers of isocarbophos in eliciting acute toxicity to *D. magna*, with the (+)-isocarbophos being more active than the (−)-enantiomer. Analysis of the joint toxicity of binary mixtures of isocarbophos enantiomers toward *D. magna* clearly showed an additive effect from the individual enantiomers. Applying the same approach to literature available toxicity data showed that while an additive effect was found also for methamidophos and profenofos, antagonistic and synergistic effects were observed for chloramidophos and leptophos, respectively. These results together imply that the joint acute toxicity for enantiomer mixtures of chiral xenobiotics may show different modes of interactions when present in a mixture. Given that most chiral pesticides are released into the environment as racemates and that enantiomeric excesses may occur from enantioselective degradation, it is important to understand not only the toxicity differences between enantiomers but also the mode of toxicity interaction between enantiomers. More comprehensive studies are needed to further understand the mechanisms involved in the different interactions of enantiomers in causing toxicity to nontarget organisms.

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