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ARTICLE

Stereoisomeric Separation and Bioassay of a New Organophosphorus Compound, *O,S*-Dimethyl-*N*-(2,2,2-trichloro-1-methoxyethyl)phosphoramidothioate: Some Implications for Chiral Switch

Shanshan Zhou,[†,‡] Lumei Wang,[§] Ling Li,[†] and Weiping Liu*[,†,‡]

[†]Research Center of Green Chirality, College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou, 310032, People's Republic of China, [‡]MOE Key Lab of Environmental Remediation and Ecosystem Health, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310027, People's Republic of China, and [§]School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 200240, People's Republic of China

The manufacture and use of single- or enriched-enantiomer pesticides are green-chemistry developments advocated in the 21st century, but predictive work for chiral switch of newly produced chiral active ingredients is limited. In the present study, the stereoselective separation, target activity, and nontarget toxicity of *O,S*-dimethyl-*N*-(2,2,2-trichloro-1-methoxyethyl)phosphoramidothioate (MCP), a new organophosphorus compound, were investigated. Because being highly active and safe is a prerequisite for marketing single-isomer products, the above studies were used to offer some implications for the chiral switch of racemic MCP. The results showed that all four stereoisomers of MCP were successfully separated with a Chiralpak AD column on HPLC. The resolved isomers and the pairs of enantiomers were further distinguished using a circular dichroism detector, designating the first and third eluted peaks as one pair of enantiomers and the second and fourth peaks as the other pair. Then, the insecticidal activities and acute and delayed toxicities of the resolved isomers of MCP were evaluated by their acute lethal efficacy against *Daphina magna*, their inhibitory potentials to acetylcholinesterase (AChE), and axon-like outgrowth of the SH-SY5Y cells, respectively. The inhibition potencies of the isomers against AChE in SH-SY5Y cells were low and slightly stereoselective. On the other hand, a significant difference was observed among the isomers in their activities and delayed neurotoxicities. The 48 h acute toxicities of isomers to *D. magna* followed the order peak 1 ≈ pair 1 (i.e., equimolar mixture of peaks 1 and 3) > peak 4 ≈ racemate > pair 2 (i.e., equimolar mixture of peaks 2 and 4) > peak 2 > peak 3, with 1.0−6.3-fold differences among them. In comparison, the inhibitory potentials of the isomers toward axon growth of SH-SY5Y cells decreased in the order peak 2 > pair 2 > peak 4 > racemate > peak 3 > pair 1 ≈ peak 1, with at least a 60-fold difference between the strongest and weakest inhibitors. Those results imply that peak 1 has the optimal target selectivity and ecological profile among the four stereoisomers. It was calculated that two-thirds of the usual pesticide usage can be saved concomitantly with a substantial decrease in neuropathic risk if MCP is present only as peak 1 rather than the racemate. When considering the absence of the economically feasible synthetic methods and techniques to produce optically pure isomers of organophosphorus pesticides, pair 1 of MCP shows considerable worth for future applications on the basis of its biological predominance and cost effectiveness.

KEYWORDS: Stereoselectivity; green pesticide; insecticidal activity; human safety; acute toxicity; organophosphate-induced delayed neuropathy

INTRODUCTION

Organic agrochemical pesticides were first introduced in the 1940s and have been widely used to control, destroy, repel, or attract pests (*1*). Pesticides save up to 40% of crop losses (*2*), greatly minimizing hunger and poverty. They are also useful in protecting humans from many infectious diseases that are spread by harmful pests, such as malaria. However, nonrestricted use of pesticides for several decades has brought about adverse effects to both the environment and nontarget organisms. As a result, the design of novel compounds and technologies for green pesticides, which have a high selectivity on targeted organisms and a low toxicity to humans and the environment, has become a main goal in pesticide research. The advocated approaches may be the use of

*Author to whom correspondence should be addressed (telephone +86 571 8832 0666; fax +86 571 8832 0884; e-mail wliu@zjut.edu.cn).

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biopesticides as alternatives to the traditional synthetic agrochemicals (*3*−*5*) and the development of enantiomerically pure or enriched products instead of the original racemic ones (*6*−*11*).

Up to 25% of the currently used pesticides contained chiral structures in 1996 (*11*), and this ratio is increasing as compounds with more complex structures are being registered for use. Although the pure enantiomers of chiral compounds have identical physicochemical properties, they are known to selectively interact with biological systems and, thus, may have different ecotoxicities. Numerous studies have reported concerns with respect to the chiral switch of racemic pesticides as a green-chemistry development originating from the enantiospecial biological effects toward target organisms. In most cases, the desired activity is preferentially attributable to only one enantiomer, whereas others are less or not active (*12*, *13*). Therefore, it can be simply deduced that if a high difference of activity exists among the stereoisomers, 50% or more (in the case of compounds with more than one asymmetric center) additional pollution is being added to the environment when the racemic pesticides are applied. Furthermore, the unnecessary "isomeric ballast" may cause various adverse side effects, such as toxicity (*14*−*16*), neural disturbance (*17*, *18*), and endocrine-disrupting activity (*19*−*21*). As a result, exploiting single- or enriched-enantiomer pesticides instead of the racemic ones can reduce pesticide use and protect the environment and human health from unintended effects. In the past two decades, some new formulations with enantiopure or enantioenriched active ingredients have been available and sold, accounting for about 7% of the total market value of pesticides (*11*). With the increased complexity of new pesticide structures (which increases the possibility of chiral centers), more routes for the production of single-enantiomer compounds, and an increase in green-chemistry consciousness, registration of single- or enriched-enantiomer pesticides has become a great trend in the 21st century. It is conceivable that if an enantiopure or enantioenriched active ingredient is requested to market instead of the original racemic product, it should be superior in pesticidal activity and/or environmental safety than the latter. Therefore, studies about enantioselective effects against both target and nontarget organisms become a predictive and prerequisite work to assess the possibility of the racemic pesticides' switch to enriched forms. However, despite many new chiral structures being introduced into the agricultural field, few discussions have been presented on their stereochemical properties in terms of biological activity and toxicological problems.

Chloramidophos (CP, *O*,*S*-dimethyl-*N*-(2,2,2-trichloro-1-hydroxyethyl)phosphoramidothioate) is an organophosphorus (OP) insecticide provisionally registered in China in 2005 and has been proven as an effective alternative to the highly toxic organophosphorus pesticides (OPs). However, our previous studies found that CP in either solid state or formulation was unstable during storage, implying its continuous usage doubtful (*22*, *23*). In our subsequent work, a new organophosphorus compound, *O*,*S*-dimethyl-*N*-(2,2,2-trichloro-1-methoxyethyl)phosphoramidothioate (MCP, **Figure 1**) was isolated from the stored commercial formulation of CP (*23*). We fortunately found that MCP was more stable than CP(*23*), and the lethal activities of a 30% emulsion in water of MCP against two lepidopterous pests, *Leucania separate* and *Pluteua xylostella*, are comparable with those of a 30% emulsifiable concentrate of CP (*24*). Therefore, a new challenge is to examine whether MCP could be developed for agricultural use on the basis of its toxicity to nontarget species, especially to humans. Because racemic MCP contains two asymmetric centers, one at the phosphorus atom and the other at the carbon atom, yielding four stereoisomers, the activity and toxicity of each stereoisomer should inclusively be taken into account.

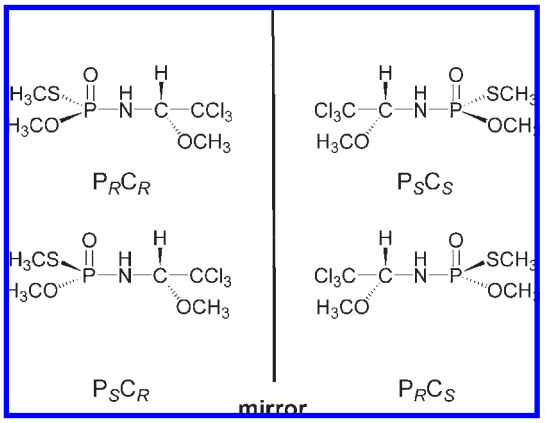

Figure 1. Stereoisomers of MCP.

In the present study, an HPLC method was developed to resolve and prepare all four stereoisomers of MCP. The pure stereoisomers were then used to measure their respective activities by a 48 h acute test against *Daphnia magna*. Meanwhile, because OPs mainly bring on two clinical syndromes, that is, acute cholinergic crisis and organophosphate-induced delayed neuropathy (OPIDN) (*25*, *26*), both the acute and delayed neurotoxicities were chosen to determine the stereospecificity of MCP in toxicities. The acute and delayed neurotoxicities of the stereoisomers of MCP were evaluated by their inhibitory potentials to acetylcholinesterase (AChE) and axon-like outgrowth in SH-SY5Y human neuroblastoma cells, respectively. Our goal for these stereoselective studies was to assess the possibility of using enantiomerically pure or enriched products of MCP in agriculture.

MATERIALS AND METHODS

Chemicals. Racemic methamidophos (Me, *O*,*S*-dimethylphosphoramidothioate) with a purity of 99.0% was purchased from Kefa New Technology Development Co. (Shenyang, China). Racemic MCP with a purity of > 98% was synthesized in our laboratory according to a previous study (*23*). Acetylthiocholine iodide (ATCh-I), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and retinoic acid (RA) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals and solvents were of analytical or HPLC grade.

Chromatographic Conditions and Identification of Stereoisomers. Chiral HPLC analyses were carried out on a Jasco LC-2000 series HPLC system (Jasco, Tokyo, Japan). Enantiomeric identification was operated by using an online variable-wavelength CD-2095 circular dichroism (CD) detector connected with HPLC. Various Daicel chiral columns (Tokyo, Japan), including Chiralpak AD [amylose tris(3,5-dimethylphenyl carbamate)], Chiralcel OC [cellulose tris(phenyl carbamate)], Chiralcel OD [cellulose tris(3,5-dimethylphenyl carbamate)], and Chiralcel OJ [cellulose tris(4-methylbenzoate)], were chosen for the stereoselective separation. All columns were 250 mm × 4.6 mm in dimensions (i.d.) with different enantioselective phases coated onto 5 μm silica gel beads. For all of the separation experiments, the injection volume, temperature, flow rate, and detection wavelength of CD were fixed at 20 μL, 25 °C, 1.0 mL min^{-1}, and 220 nm, respectively. When all four stereoisomers were separated, they were identified with CD spectra using the online CD detector with scanning wavelengths from 220 to 400 nm.

The solvents containing the resolved stereoisomers for bioassays were manually collected at the column outlet, evaporated to dryness under a nitrogen stream, and redissolved in ethanol. The purity and concentration of the obtained stereoisomers were determined by HPLC analysis under the conditions described above. The purity was calculated to be > 99% for all of the stereoisomers prepared for the subsequent bioassays.

Activity Assay in Vivo. The concentration causing 50% mortality (LC_{50}) of *D. magna* was used to evaluate the activities of organophosphorus compounds. Stock organisms were originally obtained from the Chinese Academy of Protection and Medical Science (Beijing, China).

Detailed processes for organism culture and test performance were described previously (*15*). The mortality of daphnids was monitored at 24 h intervals for the 48 h exposure period. Four replicates for each treatment were performed. The LC_{50} value of the test population was determined by probit analysis.

Acute and Delayed Neurotoxic Tests in SH-SY5Y Human Neuroblastoma Cells. *Cell Culture Conditions*. SH-SY5Y human neuroblastoma cells were originally purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). They were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C in a medium consisting of equal parts of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum. The cells were maintained in the logarithmic phase of growth and were subcultured at intervals of 4−6 days.

Inhibition of AChE. Cells for measurements of AChE activities were pretreated with 20 μM RA for 2−3 days until reaching 80% confluency to induce differentiation and maximize basal AChE activity. Then the cells were harvested, centrifuged, and resuspended in 0.01 M phosphate-buffered saline (PBS, pH 7.4) at a concentration of 1×10^7 cells mL^{-1}. Three hundred microliters of cells (1×10^7 mL^{-1}) were incubated (5% CO_2 in ambient air) in a 24-well microtiter plate for 30 min at 37 °C with 1.5 μL of the test compounds at various concentrations. Meanwhile, control samples were also prepared by use of 1.5 μL of ethanol in place of the test compounds. At the end of the exposure period, 50 μL of the suspended cells was taken to measure the activity of AChE according to a modified Ellman method (*27*). Briefly, 150 μL of DTNB solution (0.01 M PBS (pH 8.0)) and 50 μL of ATCh-I solution (0.01 M PBS (pH 8.0)) were added to the wells of a 96-well microtiter plate. Cell-control or cell-inhibitor (50 μL) solution was subsequently added to make the final concentrations of DTNB and ATCh-I at 0.33 and 0.5 mM, respectively. The enzymatic activities of the mixtures in the 96-well microtiter plate were determined at 405 nm for 7 min at intervals of 1 min from the addition of the suspended cells. Each organophosphorus compound was incubated with cells on two separate occasions, with four wells for each esterase determination run each day. The anti-AChE potentials of OPs were determined by their concentrations resulting in half-inhibition of enzyme activity (IC_{50}), which was calculated with the method of Zhou et al. (*22*).

Outgrowth of Axon-like Processes. Cells for the measurement of axon outgrowth were harvested and seeded in 35 mm × 10 mm culture dishes at an initial density of 8×10^4 cells mL^{-1} in 1.0 mL of growth medium. After 24 h, the medium was removed and replaced with 1.0 mL of new medium containing 20 μM RA. Samples with final concentrations at 6.25, 25, 100, and 400 μM were synchronously added to the medium. Control cells were exposed to solvent (ethanol) instead of the tested compounds, and negative control cells were refreshed by the medium without either nerve growth factor or inhibitor. The final ethanol concentration in the media of the sample-treated and untreated cells was 0.5% (v/v). The first day of adding RA was designated day 1. Then all of the conditioned cultures from each dish were refreshed once on day 3. At the end of the fourth day of exposure, the cells were washed with PBS (pH 7.4) and fixed for 15 min at −20 °C in a solution containing 90% methanol in PBS. After washing, the cells were stained for 1 min at room temperature (20 ± 3 °C) with Coomassie brilliant blue (1.25% (w/v) in 40% (v/v) methanol and 20% (v/v) glacial acetic acid in distilled water) and washed with PBS and then with water. Twelve to 20 randomly chosen fields, with a minimum of 300 cells per dish and three dishes per concentrations, were viewed with the aid of a Leica DM IRB inverted light microscope (Wetzlar, Germany) at 200× magnification. The total number of cells and axons (considered to be a neurite with a length of greater than two cell body diameters) in each field was counted, and the number of axons per 100 cells was determined. The concentrations that caused a 50% reduction in the number of axon (ND_{50}) in the differentiated SH-SY5Y cells were used to evaluate the neuropathic toxicity of OP compounds; the values of ND_{50} were calculated according to the same method as the IC_{50} values. All of the doses of the samples chosen were noncytotoxic toward the differentiating cells under the conditions employed, as determined by trypan blue exclusion (*28*).

Statistic Analysis. Unless otherwise stated, all data were expressed as mean ± SD. Student's *t* test, at a significance level of 0.05, was used to compare the differences between the groups.

Table 1. Capacity Factors (k), Separation Factors (α), Resolutions (R_s), and CD Signals Using the Chiralpak AD Column[a]

capacity factor				separation factor			resolution			CD signal[d]			
k_1[b]	k_2[b]	k_3[b]	k_4[b]	α_{12}[c]	α_{23}[c]	α_{34}[c]	R_{s12}[c]	R_{s23}[c]	R_{s34}[c]	peak 1	peak 2	peak 3	peak 4
3.78	4.46	7.25	10.54	1.19	1.63	1.45	2.92	8.25	6.32	+	+	−	−

[a] The operating chromatographic conditions were as follows: detection wavelength, 220 nm; *n*-hexane/ethanol (85:15, v/v), 1.00 mL min^{-1}, 25 °C. [b] Subscripts 1−4 indicate the effluent order of the four isomers. [c] Subscripts 12, 23, and 34 indicate the effluent order of the two adjacent peaks. [d] The detection wavelength of CD was set at 230 nm.

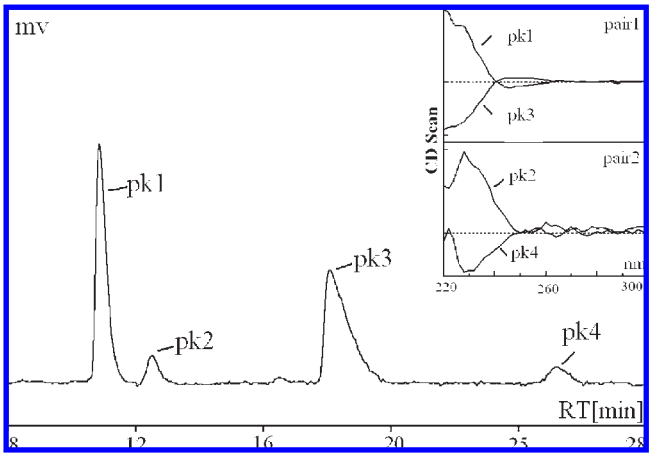

Figure 2. Representative chromatogram of MCP and a CD spectrum of the separated stereoisomers. The chromatographic conditions are described in **Table 1**. Peaks (pk) 1, 2, 3, and 4 represent the first, second, third, and fourth eluted stereoisomers, respectively. Pair 1 represents the equimolar mixture of peaks 1 and 3, and pair 2 represents the equimolar mixture of peaks 2 and 4.

RESULTS AND DISCUSSION

Stereoisomeric Separation and Identification. Enantiomeric separation is highly chiral stationary phase-specific in HPLC analyses. In our study, satisfactory resolution was achieved only on the Chiralpak AD, with a baseline resolution obtained for all four stereoisomers of MCP when a mixture of 85% *n*-hexane and 15% ethanol was used as mobile phase. The corresponding capacity factors (k), separation factors (α), and resolutions (R_s) are listed in **Table 1**, and a representative chromatogram is shown in **Figure 2**. In previous studies, stereoisomers of three similar chiral OPs, isomalathion (*29*), chloamidophos (*15*), and fosthiazate (*30*), with two asymmetric centers at the phosphorus and carbon atoms, were successfully resolved by HPLC. It was surprising that the separation of the stereoisomers of the above four OPs was coincidentally accomplished on the Chiralpak AD column. These results suggest that Chiralpak AD columns are likely to have the ability to separate stereoisomers of chiral OP compounds with two chiral centers on the phosphorus and carbon atoms. However, because of the complex mechanisms of chiral recognition as well as the lack of information on the absolute configurations of the resolved MCP stereoisomers, it is difficult to elucidate the exact reasons why they can be separated more easily on the Chiralpak AD column.

The CD spectra with wavelengths ranging from 220 to 400 nm were obtained for distinguishing the stereoisomers of MCP using the online CD detector (**Figure 2**). Theoretically, the spectra of the enantiomers of a pair are mirror images of each other. It is tentatively concluded that the first (peak 1) and third (peak 3) eluted isomers are one pair of enantiomers, whereas the second

Table 2. LC_{50}[a] of CP and MCP against Three Target Organisms

	LC_{50} (mg L^{-1})		
	Daphnia magna	*Leucania separate*	*Pluteua xylostella*
CP	1.31[b]	329.87[c]	52.38[c]
MCP	1.41	372.21[d]	53.10[d]

[a] LC_{50}, the concentration that caused 50% mortality of target organisms in 48 h. [b] Reference *17*. [c] 30% emulsifiable concentrates (*27*). [d] 30% emulsion in water (*27*).

(peak 2) and fourth (peak 4) isomers are the other pair (**Figure 2**). We refer to the equimolar mixture of peaks 1 and 3 as pair 1 and that of peaks 2 and 4 as pair 2. Judging from the peak area of each stereoisomer in ultraviolet absorbance, the molar ratio for the four stereoisomers in racemic MCP was about 6.5:1.0:6.5:1.0 (peak 1/peak 2/peak 3/peak 4) (**Figure 2**). Because the amount of peaks 2 and 4 in racemic MCP was low, the sample for CD scan was a synthetic MCP mixed with concentrated peaks 2 and 4 that were collected at the HPLC outlet. The final concentration of pair 2 in the sample for CD scan was about half that of pair 1.

Stereoselectivity in Activity. It is well-known that the capability of OPs to control insects is due to their inhibition of the type B esterases by binding to the active site and phosphorylating the enzyme. *D. magna*, an arthropod, offers an excellent model system to investigate the B esterase inhibition patterns (*31*). It is very sensitive to OPs (*32*) and widely inhabits agricultural fields receiving OPs treatments (*33*). Therefore, we used *D. magna* as the target organism, and the activity of MCP was evaluated by the LC_{50} values of *D. magna*.

As shown in **Table 2**, the LC_{50} value of racemic MCP toward *D. magna* was a little higher than that of racemic CP. A subsequent *t* test suggested that these two values were not significantly different from each other, suggesting that the insecticidal activity of MCP was nearly the same as that of the original compound CP. As mentioned above, some similar phenomena have been observed when the LC_{50} values of formulated MCP are compared with those of the commercial formulation of CP toward *Leucania separate* and *Pluteua xylostella* (**Table 2**) (*24*). These observations suggest that many biological properties affecting the toxicity in vivo, especially the anti-AChE activity, the penetration, and detoxication rate, are similar for these two chemicals against the insects. Meanwhile, the comparable results obtained from *D. magna* and the two target insects support the use of *D. magna* as a target organism for MCP, and therefore it was further used in the activity measurement of the stereoisomers.

The *t* test indicated significant differences in the LC_{50} values among the stereoisomers of MCP. On the basis of the values of LC_{50}, the acute insecticidal activities of the isomers to *D. magna* followed the order peak 1 > peak 4 > peak 2 > peak 3, with 1.2−6.3-fold differences among them (**Table 3**). In fact, studies in the past five years have revealed that toxic selectivity was very common among the stereoisomers of OPs toward *D. magna*. For example, the toxicity differences against *D. magna* for leptophos (*34*), fenamiphos (*35*), fonofos (*14*), profenofos (*14*), trichloronate (*36*), and isocarbophos (*37*) were 20.0-, 2.6-, 15.0-, 6.6-, 8.2-, and 50.0-fold, respectively. In addition, a maximum difference of 3.1-fold in the toxicity to *D. magna* was observed among the stereoisomers of fosthiazate (*30*), an OP that has two chiral centers at the phosphorus and carbon atoms. In comparison, the discrimination increased to 13.0-fold in the case of another analogous OP compound, CP (*15*).

On the basis of the listed LC_{50}, the calculated relative efficacies toward *D. magna* between the racemate and the stereoisomers of MCP were 3.1-, 0.6-, 0.5-, and 1.0-fold for peaks 1, 2, 3, and 4, respectively (**Table 4**), demonstrating peak 1 as predominating in activity. Traditionally, the pesticidal activity of a chiral compound is based on its racemate. If MCP is only presented in peak 1, only one-third of the conventionally used amount is enough to provide the desired insecticidal efficacy.

Table 3. Data for Activity and Acute and Delayed Toxicities of Individual Stereoisomers and Equimolar Mixtures of Enantiomers of MCP

	LC_{50}[a] (mg L^{-1})	IC_{50}[b,d] (μM)	ND_{50}[c] (μM)
peak 1	0.45 ± 0.07	>2000.00	>2338.13[f]
peak 3	2.83 ± 0.26	>2000.00	2338.13 ± 605.59[e]
pair1	0.48 ± 0.11	>2000.00	>2338.13[f]
peak 2	2.46 ± 0.37	>2000.00	38.91 ± 17.82
peak 4	1.36 ± 0.32	>2000.00	175.09 ± 40.66
pair 2	2.12 ± 0. 32	>2000.00	51.82 ± 10.67

[a] Definition of LC_{50} was given in **Table 1**. [b] IC_{50}, the concentration of inhibitor leading to half-inhibition of cellular AChE activity in 30 min. [c] ND_{50}, the concentration that caused 50% reduction of the number of axons. [d] The highest tested concentration generated about 20−30% AChE inhibition. [e] About 70% of the developed axons of the control cells were left at 400 μM; 400 μM was the highest concentration used for axon growth tests. [f] No significant inhibition at 400 μM, suggesting that the values of the apparent ND_{50} of peak1 and pair 1 must be larger than that of peak3.

Table 4. Relative Activities and Delayed Toxicities of Individual Stereoisomers and Equimolar Mixtures of Enantiomers of MCP with Those of Its Racemate

	peak 1/ racemate	peak 2/ racemate	peak 3/ racemate	peak 4/ racemate	pair 1/ racemate	pair 2/ racemate
D. magna[a]	0.3	1.7	2.0	1.0	0.3	1.5
axon growth[b]	>1.4	0.02	1.4	0.1	>1.4	0.03

[a] LC_{50} in the 48 h static tests toward *D. magna*. [b] ND_{50} in 96 h static tests in SH-SY5Y cells.

Stereoselectivity in Toxicity. The current EPA guidelines for hazard identification and risk assessment of OP insecticides are based on toxicity tests using in vivo animal models (*38*). However, for financial, scientific, and ethical concerns, the use of cell cultures in neurotoxicology has been duly emphasized (*39*, *40*). Many cell lines, such as SH-SY5Y (human, neuroblastoma) (*41*−*44*), NB41A3 (mouse, neuroblastoma) (*42*, *45*), N2a (mouse, neuroblastoma) (*46*, *47*), N-18 (mouse-brain neuroblastoma) (*48*), PC-12 (rat, pheochromocytoma) (*45*, *47*), and C-6 (rat, glioma) (*48*), recently have been proven to be useful for differentiating the neuropathic OPs (i.e., those causing OPIDN) from acutely toxic OPs (i.e., those highly capable of inhibiting AChE). For the prediction of human toxicity from in vitro studies, human cells have an advantage compared to animal cells. Consequently, we chose the SH-SY5Y cell line as the biological model for toxicological evaluation. The cholinergic crisis of the OP compound was measured by the IC_{50} values of AChE, the generally acknowledged target enzyme of acute poisoning of OPs. However, the precise molecular mechanisms of OPIDN remain to be established. Some known information is that OPIDN is initiated by the inhibition and aging of neuropathy target esterase (NTE) and is characterized by distally located swelling in the large axon of central and peripheral nerves, with subsequent axonal degeneration (*25*, *26*, *49*). In this case, two methods are commonly used to screen the neuropathic OPs. One is based on the inhibition and/or aging of NTE by organophosphates (*41*, *42*, *45*). The other one focuses on the morphological target, that is, assessing the effects of OPs in decreasing the number of large-diameter axons (*43*, *46*−*48*). Because the roles of NTE inhibition and aging in OPIDN are not clearly understood (*49*), we chose the morphological biomarker to assess the neuropathic toxicity of OP compounds.

The acute and delayed neurotoxicities of racemic MCP were characterized by comparison to Me, because both the cholinergic crisis and OPIDN risks of the latter have been well studied (**Table 5**) (*50*). Certain structural requirements have been elucidated

Table 5. Acute and Delayed Toxicities to Human Cells of Racemic MCP and Racemic Me

	acute (IC_{50},[a] μM)	delayed (ND_{50},[a] μM)	ND_{50}/IC_{50}
MCP	>2000.00[b]	1669.57 ± 631.04[c]	<0.8
Me	3.91 ± 0.38	>50.00[d]	>12.8

[a] Definitions of IC_{50} and ND_{50} have been given in **Table 3**. [b] The highest tested concentration generated 22.90% AChE inhibition. [c] 75% of the developed axons of the control cells were left at 400 μM. [d] The highest tested concentration generated 4.74% neurite degeneration.

for OP inhibitors of AChE. For example, the anti-AChE potentials of N-unsubstituted phosphoramidates in vitro are preferred over those of N-substituted analogues (*51*). In the present study, we obtained a similar result as for the anti-AChE potentials of the samples dropped by a factor of over 500 in going from Me (IC_{50} = 3.91 μM) to MCP (IC_{50} > 2000 μM), manifesting that MCP is an extremely more friendly structure than Me based on the acute toxicity (**Table 5**). In contrast to the cholinergic impairment, the observation of Coomassie-stained cells by light microscopy indicated a more distinct reduction in the number of axon-like processes in MCP-treated cells compared with Me-treated cells (**Figure 3c**,**d**). Further quantitative analysis revealed an ND_{50}/IC_{50} ratio of < 0.8 (**Table 4**) for racemic MCP, indicating that the dose of MCP required for producing OPIDN might be lower than that inducing acute poisoning. In comparison, the ND_{50}/IC_{50} ratio for Me was > 12.8, meaning that Me might be more potent at inducing cholinergic crisis than at causing OPIDN (**Table 4**). These results closely agreed with previous in vivo tests indicating that Me caused a mild OPIDP associated with very high inhibition of NTE at doses estimated to be > 8 times the unprotected LD_{50} of adult hens (*52*). In the poisoning events caused by OPs, a very high dose will be ingested by humans, especially after incorrect use or sometimes for suicidal reasons. Under this extremely high exposure, MCP seems to more easily induce delayed neurotoxicity than Me. That is probably because people who are exposed to Me may die before they suffer OPIDN, whereas with MCP they may not. We further suggest that the potential of MCP to inhibit axon outgrowth is closely related with the derivative group at the amino nitrogen. The reason is that trichlorphon, a pesticide with a similar 2,2,2-trichloro-1-substitiuted-ethyl group, also can greatly inhibit the axon outgrowth of N2a cells at a low concentration (1 μg mL^{-1}) (*46*). In summary, racemic MCP has high potency to induce OPIDN, and therefore its use in agricultural is doubtful. These results underscore the requirement for stereoselective bioassays. Even though only specific stereoisomers possess the significant toxicities, the remaining ones may also deserve to be examined.

All of the stereoisomers of MCP exhibited comparative sustained levels of inhibitory potentials in the cell anti-AChE assays, with a 20−30% decrease of AChE activity after exposure at the highest concentration (2000 μM, **Table 3**). Considering that none of the stereoisomers or the racemate proved to be effective inhibitors of AChE, it should be anticipated that the structure of MCP, either in individual isomeric forms or in full isomeric mixture, poses no practical hazard of causing cholinergic effects to humans. However, a marked stereospecificity appeared in the inhibitions toward axon growth. As shown in **Figure 3e**,**f**, the number of axon-like processes was obviously reduced for peak 2-treated cells, but not for peak 1-treated cells. According to the values of ND_{50}, the order of the potentials for neurodegeneration was peak 2 > peak 4 > peak 3 > peak 1, with at least a 60-fold difference between the strongest and weakest inhibitors (**Table 3**). When the ND_{50} of each individual stereoisomer was divided by that of the racemate (**Table 4**), the delayed neurotoxicities of the two enantiomeric pairs were separated into two groups. On the one hand, values of ND_{50} of peaks 2 and 4 were only a 40th and 10th of that of the racemate, respectively, indicating that these two isomers were on average 25 times more potent at inducing OPIDN than the racemate. On the other hand, the values of ND_{50} of peaks 1 and 3 were greater than that of the racemate, suggesting that their contributions to the delayed neurotoxicity of the racemate were negligible. In other words, the neurite outgrowth inhibitory potency recorded for the racemic MCP was fundamentally attributable to the effects of peaks 2 and 4, the two isomers that accounted for only about 13% of the mass percent of racemic MCP (**Figure 2**). This type of neuropathic risk of a chiral compound associated primarily with part of the stereoisomers has been observed for other OPs. For example, the administration of high doses of (+)-Me at 5−7 times the LD_{50} to hens (protected for cholinergic toxicity by antidotal therapy with atropine and oxime) induced OPIDP, but no evidence of OPIDP was observed in similar studies using (−)-Me (*52*). Johnson and Read demonstrated that only the (−)-enantiomer of EPN was responsible for the OPIDP seen with the racemic EPN (*53*). In the case of soman, an OP posessing two optical centers, the pure P(+)-isomers were predicted to cause the delayed neuropathy in hens dosed with about the unprotected LD_{50}, whereas the corresponding dose would have to be raised to 1000-fold the LD_{50} for the P(−)-isomers (*54*). When it comes to isomalathion, another stereoisomeric organophosphorus compound, the NTE IC_{50}/AChE IC_{50} values of P_S-isomers were about $(1.5-2.5)\times10^3$, whereas those of the P_R-isomers increased to $(4.9-15)\times10^4$ (*55*).

The contributions of each asymmetric center to the overall delayed toxic potencies between MCP and the other two stereoisomeric compounds, soman and isomalathion, were very different.

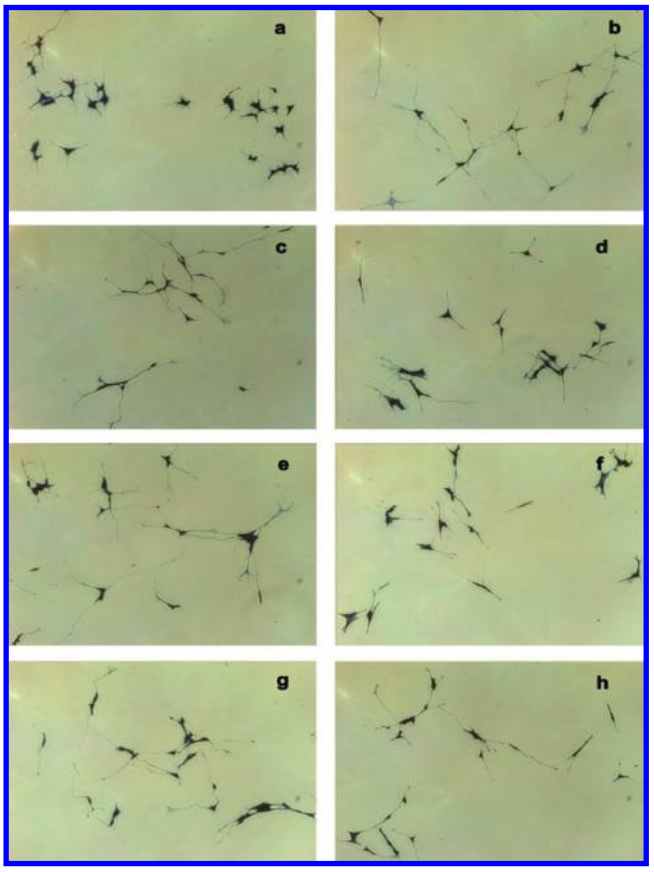

Figure 3. Light microscopy of SH-SY5Y cells: (**a**) blank; (**b**) control; (**c**) 50 μM racemate of Me; (**d**) 400 μM racemate of MCP; (**e**) 400 μM peak1 of MCP; (**f**) 400 μM peak 2 of MCP; (**g**) 400 μM peak 3 of MCP; (**h**) 400 μM peak 4 of MCP.

In the case of soman and isomalathion, the effect of changing the configuration at phosphorus alone was greater than that by changing the configuration at carbon alone, with at least 10-fold discrimination occurring in the toxic potentials between the P_S- and P_R-isomers. In comparison, the most significant selectivity in the delayed toxicity among the isomers of MCP was displayed between the two enantiomeric pairs (pairs 1 and 2). However, as both of the studies of soman and isomalathion were based on the NTE inhibition against hen brain homogenate and the mechanisms with respect to how the primary inhibition and aging of NTE lead to the resulting axonal degeneration are unknown, we could not elucidate the exact mechanisms for this discrepancy in the relative contribution of the chiral centers. Moreover, except for the actual molecular and cellular events with respect to the development of OPIDN, some other factors also will act in the toxic selectivity among the isomers in cells, making the problem more complex. First, many biological activities affecting the toxicity, especially metabolism, transfer, and accumulation, are stereoselective. Second, the chemical stability of the diastereoisomers under the cell culture conditions employed may be different.

An exciting result we obtained is that peak 1, the least potent compound at inducing OPIDN, is contrarily the most active stereoisomer toward the target organism (**Table 3**). Therefore, peak 1 must be the optimal substitute for the racemate among the four stereoisomers. It can be determined that if racemic MCP is replaced by peak 1, two-thirds of the usual pesticide usage can be saved in addition to having better prevention for humans from delayed neurotoxic risks.

Because of the polarization of the OPIDN potentials of the stereoisomers of MCP, the activity and toxicity of pairs mixed with equimolar enantiomers (pairs 1 and 2) were also measured. As shown in **Tables 3** and **5**, the inhibitions of both pairs 1 and 2 to cell AChE were not significantly different from that of the racemate. Additionally, pair 1, which was 4.4-fold more active than the other, was also > 45 times less potent at causing OPIDN. Furthermore, the insecticidal activity and the potencies of cholinergic toxicity and neuropathic hazard of pair 1 were all comparable with those of peak 1. With regard to the absence of the economically feasible synthetic methods and techniques for producing single optically pure isomers of OPs, pair 1 of MCP shows considerable worth for future applications.

In conclusion, racemic MCP was shown to be highly active against insects and scarcely toxic toward humans. However, its threat of OPIDN makes its pesticidal use doubtful. Fortunately, pair 1, that is, an equimolar mixture of peaks 1 and 3, was found to be an improved substitute on the basis of its biological predominance and cost effectiveness. At present, no enantiopure phosphorus agrochemicals have been marketed, because no appropriate economic reactions for producing a chiral phosphorus center are available (*11*). The results obtained in this study indicate that when a carbon asymmetry is introduced into the structure of OPs, there may be new potential.

ABBREVIATIONS USED

AChE, acetylcholinesterase; ATCh-I, acetylthiocholine iodide; CD, circular dichroism; CP, chloramidophos; *D. magna*, *Daphnia magna*; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); IC_{50}, concentration resulting in half-inhibition of enzyme activity; LC_{50}, concentration causing 50% mortality; MCP, (*O*,*S*-dimethyl-[(2,2,2)-trichloro-1-methoxyethyl]phosphoramidothioate); Me, methamidophos; ND_{50}, concentration causing a 50% reduction of numbers of axon; NTE, neuropathy target esterase; OP, organophosphorus; OPs, organophosphorus pesticides; OPIDN, organophosphate-induced delayed neuropathy; RA, retinoic acid.

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