cations. A similar absorbance band, also shown here, was observed earlier in the absorbance spectra of glyphosate solutions equilibrated with CuO, which was interpreted as belonging to a Cu(II)–glyphosate complex (Glass, 1984). The mechanisms in which the complexes of glyphosate are formed from CuO- and Cu^{2+}-saturated clays are uncertain, but one speculation is that a surface interaction between glyphosate and the metal at the liquid/solid interface plays a vital role in the process.

In a similar adsorption study with several amino acids, Bodenheimer and Heller (1967) suggested that the extraction of copper ions was the first stage of the reaction that occurred when amino acids were brought in contact with Cu^{2+}-montmorillonite. The amount of copper extracted from the clay increased with increasing concentrations of glycine and glutamic acids. Cu^{2+} ions that were released into solution after equilibrating Cu^{2+}-montmorillonite with glyphosate solutions in the present study increased with higher glyphosate concentrations (unpublished results) as was found in the earlier investigation with CuO. It is proposed that Cu^{2+} ions are brought into solution from the Cu^{2+}-montmorillonite via a cation-exchange action with solution protons and that the increased adsorption of glyphosate is attributed to the formation of complexes in solution between glyphosate and cations. Motekaitis and Martell (1985) reported that glyphosate formed 1:1 and 1:2 chelates with 13 selected divalent and trivalent metal ions. The equilibrium stability constants for these chelates ranged from 3.29 for Ca(II)–glyphosate to 16.09 for Fe(III)–glyphosate.

In summary, the results of this investigation demonstrate that the order of adsorption for glyphosate on soils is Houston > Muskingum > Sassafras and on the clay minerals is montmorillonite > illite > kaolinite. The results are consistent with reported evidence that suggests that glyphosate adsorbs within the interlayer spaces of the clay minerals. However, the increased glyphosate adsorption on cation-saturated clay minerals suggests that glyphosate is complexed by cations released from the clays via a cation-exchange reaction with solution protons. The new absorbance band (λ_{max} = 226 nm) in the UV spectra of glyphosate solutions equilibrated with Cu^{2+}-montmorillonite provides supporting evidence for the formation of glyphosate complexes.

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Synthesis, Resolution, and Toxicological Properties of the Chiral Isomers of *O,S*-Dimethyl and -Diethyl Ethylphosphonothioate

David J. Armstrong[1] and T. Roy Fukuto*

The effect of chirality on the toxicological properties of *O,S*-dimethyl (1) and *O,S*-diethyl ethylphosphonothioate (2) was determined. The resolved esters were prepared from the respective *O*-methyl and *O*-ethyl ethylphosphonothioic acids, and absolute configurations of the esters were assigned by relating them to the known configuration of the resolved acids. The S_P (−) enantiomers of both 1 and 2 were more acutely toxic and were stronger anticholinesterases than the R_P (+) enantiomers. No delayed deaths were observed with the resolved compounds, but all treated animals lost weight for 3-4 days following dosing.

The work of Mallipudi et al. (1979) and Aldridge et al. (1979) on the toxicity of the malathion impurities *O,O,S*-trimethyl phosphorothioate and *O,S,S*-trimethyl phosphorodithioate has prompted investigation of low molec-

Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, California 92521.
[1] Present address: S-CUBED, La Jolla, CA.

ular weight organophosphates that may be formed as by-products in the synthesis of technical insecticides. Previous papers from this laboratory on phosphonothioate analogues of some malathion impurities, which may also be found as impurities in technical phosphonothioate insecticides, described the unusually high acute toxicity of the *O,S*-dialkyl alkylphosphonothioate esters (Armstrong and Fukuto, 1984) and the insidious delayed toxic effect attributable to *O,S*-diethyl ethylphosphonothioate (Hollingshaus et al., 1981). Since these phosphonothioate esters

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all possess the common feature of a chiral phosphorus center, the question was raised as to the effect of chirality on the toxicity of these esters. Although a number of reports have been published on the synthesis of resolved *O,S*-dialkyl alkylphosphonothioates (Omelanczuk and Mikolajczyk, 1971; Cooper et al., 1975; Hall and Inch, 1979), information is not available on the toxicological properties of the esters. This paper describes results following the resolution and determination of the toxicity and anticholinesterase activity of the chiral isomers of two delayed toxic compounds, *O,S*-dimethyl (1) and *O,S*-diethyl ethylphosphonothioate (2).

MATERIALS AND METHODS

General Methods. Precoated silica gel 60 F_{254} plastic sheets (0.2 mm; EM Reagents) were used for analytical thin-layer chromatography. Compounds were located on TLC plates by use of 2,6-dibromoquinone 4-chloroimide (DBQ) spray reagent (Menn et al., 1957) and ultraviolet detection. Silicic acid (CC-7 Special; Mallinckrodt, St. Louis, MO) was used for column chromatography.

Analytical gas chromatography (GC) was carried out with a Hewlett-Packard Model 402 high-efficiency gas chromatograph fitted with a 6 ft × 2 mm (i.d.) glass U-tube column and an alkali (KCl) flame ionization detector (AFID). The column packing was prepared after the surface-modified support methodology of Aue et al. (1973) using 6% EGSP-Z (Applied Science Laboratories, State College, PA). Gas flows for hydrogen, helium, and air were 40, 36, and 320 mL/min, respectively.

Proton magnetic resonance (¹H NMR) spectra were recorded on a Varian EM-390 spectrometer using carbon tetrachloride as the solvent and tetramethylsilane as the internal standard.

Electron impact mass spectrometry of each compound was conducted by direct injection into a Finnigan Model 3500 mass spectrometer. The ionization energy of the electron source was 75 eV. Compounds were injected in 1.0-μL quantities as neat liquids.

Acetylcholinesterase Inhibition. Purified bovine erythrocyte acetylcholinesterase (BAChE) (Sigma Chemical Co., St. Louis, MO) was diluted with physiological saline to approximately 10 units/250 mL. One unit hydrolyzes 1 μmol of acetylcholine/min at pH 8.0 at 37 °C. Rat red blood cell acetylcholinesterase (RBAChE) was prepared by hemolyzing blood taken from the tail of a rat (Gray et al., 1982).

Determination of acetylcholinesterase activity was conducted spectrophotometrically at 37 °C with a Varian Cary 219 or Beckman Model 25 spectrophotometer equipped with a thermostated cell chamber according to the procedure described by Ellman et al. (1961). Bimolecular inhibition rate constants were determined according to Aldridge and Davison (1952).

Chemicals. Each compound examined was >99% pure by GC analysis. Racemic *O,S*-dimethyl ethylphosphonothioate (1) and *O,S*-diethyl ethylphosphonothioate (2) were available from previous studies (Hollingshaus et al., 1981; Armstrong and Fukuto, 1984). The resolved enantiomeric esters were synthesized from their corresponding resolved chiral acids and dimethyl or diethyl sulfate (Hilgetag and Teichmann, 1959). Resolved 1 or 2 was then purified on silica with hexane–ethyl acetate (1:1) as the elution solvents. Proton chemical shifts and mass spectral data were consistent with assigned structures.

Resolution. The partial resolution of the *O*-alkyl ethylphosphonothioic acids was accomplished by the method described by Hall and Inch (1979). The reaction sequence employs (-)- or (+)-ephedrine as chiral templates

on which to build the partially resolved (-)- or (+)-*O*-methyl or *O*-ethyl ethylphosphonothioic acids.

To a stirred benzene solution of (-)- or (+)-ephedrine and triethylamine was added a benzene solution of ethylphosphonothioic dichloride. The reaction was allowed to sit overnight and poured onto excess water. The water was extracted four times with benzene, the extracts were combined and dried, and the benzene was removed by rotoevaporation to yield a yellow-brown viscous oil. The oil was chromatographed on silica with dichloromethane–hexanes (1:1). Upon concentration of the eluate factions, white needlelike crystals of the isomeric 1,3,2-oxazaphospholane derivatives were obtained in a ratio of approximately 9:1. The crystals were recrystallized four times from cyclohexane–dichloromethane (10:1) or until the NMR showed only one doublet of doublets for the benzyl hydrogen signal. The predominant (-)-ephedrine adduct was obtained in 20–28% yield; mp 109–110 °C. The predominant (+)-ephedrine adduct was obtained in 19–26% yield; mp 107–108 °C.

To a solution of (-)-ephedrine or (+)-ephedrine adduct in methanol was added a solution of anhydrous hydrochloric acid in methanol. The solution was allowed to stir for 1 h after the addition was completed, after which enough potassium hydroxide in water was added to make the solution basic. The solution was then filtered, washed with ether, and acidified with dilute hydrochloric acid. The acidic solution was extracted three times with chloroform, the extracts were combined and dried, and chloroform was removed to give the partially resolved (+)- or (-)-*O*-methyl ethylphosphonothioic acid in 83–85% yield. The same procedure with ethanolic HCl resulted in the partially resolved (+)- or (-)-*O*-ethyl ethylphosphonothioic acid. Further resolution of the acids by repeated recrystallization of their (-)- or (+)-α-methylbenzylamine salts (Theilacker and Winkler, 1954) afforded a high degree of enantiomeric purity.

The optical purities of (-)-1, (+)-1, (-)-2, and (+)-2 were assessed by the addition of the chiral shift reagent, tris-[3-[(trifluoromethyl)hydroxymethylene]-*d*-camphorato]-europium(III) (Aldrich), in small increments directly to samples of the compounds in chloroform-*d*, the NMR spectrum being recorded after each addition.

Toxicological Evaluation. Rat oral toxicity was determined with 120–180-g female albino rats (Sprague–Dawley derived; Simonsens Laboratories, Gilroy, CA). Solutions of toxicant in corn oil were administered orally at 0.15 mL/150-g rat to animals fasted 16–18 h before treatment. At least four different doses with a minimum of five rats per dose were used to determine 48-h LD_{50} values.

Therapeutic Treatment. The effectiveness of the cholinergic poisoning antidotes atropine and 2-PAM against acute poisoning by an LD_{90} oral dose of 1 in corn oil was determined. Rats (10) were treated with 1 and given a simultaneous subcutaneous injection of atropine sulfate (17.4 mg/kg) and 2-PAM (50 mg/kg) at the onset of the first sign of cholinergic poisoning (Natoff and Reiff, 1970). The antidotal treatment was repeated as needed as poisoning signs reappeared for 24 h. No injections were required after 24 h.

RESULTS AND DISCUSSION

Resolution and Optical Purity. The values for the observed (neat) rotations of the resolved acids and their alkylated esters (1, 2) are given in Table I. The values provide evidence for the high degree of resolution obtained by employing the ephedrine method of Hall and Inch (1979) combined with recrystallization of the partially

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Table I. Observed Rotations (Degrees Arc) for Resolved Acids and Esters

$$Et-\underset{\underset{SR}{|}}{\overset{\overset{O}{\|}}{P}}-OR'$$

compound	R	R'	config	obsd	lit.[a]
(−)-1 acid	H	Me	S_P	−11.71	−11.72
(+)-1 acid	H	Me	R_P	+11.54	+11.45
(−)-2 acid	H	Et	S_P	−15.33	−15.41
(+)-2 acid	H	Et	R_P	+15.16	+14.82
(−)-1	Me	Me	S_P	−65.08	
(+)-1	Me	Me	R_P	+64.51	
(−)-2	Et	Et	S_P	−74.92	−70.09[b]
(+)-2	Et	Et	R_P	+74.09	

[a] Data from Allahyari et al. (1977). [b] Data from Omelanczuk and Mikolajczyk (1971).

Table II. Bimolecular Rate Constants for the Inhibition of Bovine and Rat Erythrocyte Acetylcholinesterase by Racemic and Resolved O,S-Dimethyl Ethylphosphonothiolate (1) and O,S-Diethyl Ethylphosphonothioate (2)

	k_i,[a] M^{-1} min^{-1}	
compound	RAChE	BAChE
(S_P)-(−)-1	212	273
1	164	192
(R_P)-(+)-1	117	137
(S_P)-(−)-2	289	205
2	203	158
(R_P)-(+)-2	150	104

[a] BAChE = bovine erythrocyte acetylcholinesterase; RAChE = rat erythrocyte acetylcholinesterase.

resolved acids with a chiral amine (Theilacker and Winkler, 1954). The rotations of the acids compare favorably with those reported by Allahyari et al. (1977), which were obtained by the classical method of repeated recrystallization of alkaloid–acid salts. The observed roation of (−)-2 is somewhat higher than that reported by Omelanczuk and Mikolajczyk (1971) and is probably attributable to differences in the degree of resolution of the O-ethyl ethylphosphonothioic acid.

The NMR spectra of racemic 1 and 2 showed a symmetrical doublet for the SCH$_3$ protons of 1 and an overlapping doublet of quartets for the SCH$_2$ signal of 2. Addition of the pseudo contact shift reagent, tris[3-[(trifluoromethyl)hydroxymethylene]-d-camphorato]europium(III), to the racemic materials resulted in two doublets for the SCH$_3$ proton signal of 1 and two overlappping double quartets for the SCH$_2$ proton signal of 2. The separation of these signals was proportional to the amount of shift reagent added. A similar change in spectra was not observed with either enantiomer of 1 or 2, indicating enantiomeric purity greater than the limit of detection of the instrument (98%).

Since alkylation of O-methyl or O-ethyl ethylphosphonothioic acid with dimethyl or diethyl sulfate does not involve any bond directly attached to the phosphorus atom, the absolute configurations of the enantiomers and 1 and 2 are identical with those of the corresponding acids (Allahyari et al., 1977). Absolute configurations of the acids and esters are indicated in Table I.

Acetylcholinesterase Inhibition. Bimolecular rate constants for the inhibition of rat erythrocyte acetylcholinesterase (RAChE) and bovine erythrocyte acetylcholinesterase (BAChE) by resolved and racemic 1 and 2 are given in Table II. The S_P enantiomers of 1 and 2

Table III. Acute Toxicity[a] of Racemic and Resolved O,S-Dimethyl Ethylphosphonothioate (1) and O,S-Diethyl Ethylphosphonothioate (2) to Rats

compound	rat oral LD$_{50}$, mg/kg
(R_P)-(+)-1	9.0 (5.3–12.2)
1	8.3 (7.4–9.0)
(S_P)-(−)-1	5.2 (3.1–6.6)
(R_P)-(+)-2	13.3 (10.3–16.0)
2	7.7 (7.0–8.5)[b]
(S_P)-(−)-2	3.9 (2.3–6.2)

[a] Defined as 48-h toxicity. [b] Data from Hollingshause et al. (1981).

Table IV. Delayed Toxicity of O,S-Dimethyl Ethylphosphonothioate (Me) Administered Orally to Rats

dose, mg/kg	no. treated	time of occurrence of death, days						total dead	% mortality
		1	2	3	4	5	6	7–25		
10	6	6							6	100
9	6	2	3						5	83
8	6	1	1	3					5	83
7	6		1		1		1		3	50
6	6			1					1	17
5	6								0	0

appeared to be stronger inhibitors of either enzyme although all of the compounds were relatively poor inhibitors of acetylcholinesterase. The small difference in anticholinesterase activity of the enantiomers of 1 and 2 is in strong contrast to the 9- and 57-fold greater anticholinesterase potency of (S_P)-fonofos oxon (O-ethyl S-phenyl ethylphosphonothioate) compared to (R_P)-fonofos oxon for the inhibition of BAChE and RAChE, respectively (Lee et al., 1978). The larger difference in AChE inhibition between the enantiomers of fonofos oxon may be attributed to the greater bulk of S-phenyl compared to S-ethyl or S-methyl, thus providing greater asymmetry to the fonofos oxon isomers.

(S_P)-fonofos oxon (S_P)-2
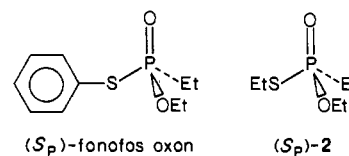
Toxicity. Data for 48-h toxicity of racemic and resolved 1 and 2 to rats are presented in Table III. Enantiomers having the S_P configuration appeared to be more toxic than the R_P enantiomer, although the difference was small. In fact, R_P, S_P, and racemic 1 were statistically equally toxic to rats. All animals treated with 1 or 2 exhibited signs of cholinergic poisoning, including diarrhea and incontinence. The small differences in the acute toxicity of resolved and racemic esters of 1 or 2 are in general agreement with the small differences in the anticholinesterase activities of these compounds. However, the significance of this relationship is not clear owing to the very poor anticholinesterase potencies of 1 or 2 compared to high rat toxicities. The descrepancy between high toxicity and poor anticholinesterase activity of compounds of this type has prompted speculation on the possibility of the metabolic activation to more potent anticholinesterases, e.g. the corresponding S-oxides (Armstrong and Fukuto, 1984).

Poisoning by 1 or 2 appeared to involve two different modes of action. Initial signs of poisoning after dosing with either compound were typically cholinergic. However, delayed toxic effects previously reported for 2, e.g. weight loss and red staining around the nose and mouth (Hollingshaus et al., 1981), were also observed with 1, and animals died as late as 6 days after dosing (Table IV). All

of the rats treated with 1 lost weight for 2–3 days after treatment. Rats that survived the first 48 h and lost less than 30% of their initial body weight usually survived the treatment. These rats rapidly regained their lost weight. However, rats that survived the first 48 h and thereafter lost more than 30% of their initial body weight usually died within a few days. Weight loss for animals that died beyond the initial 48-h holding period varied from 29 to 38% at the time of death.

Diarrhea and incontinence are common signs of acute cholinergic poisoning, usually subsiding within 48 h after treatment (Koelle, 1980). A general loss in weight is also common to cholinergic poisoning owing to cessation of food intake and increased elimination of body wastes. As the signs of poisoning subside, normal rates of food consumption resume and weight gain resumes. However, rats treated with 1 that died after 48 h continued to refuse food and lose weight after the cholinergic signs of poisoning had ceased. The delayed toxic effects observed for 1 and previously with 2 were similar to those reported earlier for poisoning by *O,O,S*-trimethyl phosphorothioate (Mallipudi et al., 1979) and *O,S,S*-trimethyl phosphorodithioate (Aldridge et al., 1979).

Antidotal treatment with atropine and 2-PAM of rats treated with the oral LD_{90} dose of 1 (9.2 mg/kg) resulted in the protection of all animals from acute cholinergic poisoning. Administration of atropine and 2-PAM was terminated after 24 h, and signs of cholinergic poisoning did not appear. However, all 10 animals in this experiment lost weight for 2–3 days, and four of these animals continued to lose weight and eventually died 4–6 days after dosing with 1 and the antidotes. The remaining six animals recovered. These results provide additional support for a dual mechanism of poisoning for 1, i.e. cholinergic and noncholinergic (delayed toxicity).

The enantiomers of 1 and 2 were also examined for delayed toxic effects. Although treated animals lost weight for the first 2–3 days following treatment and some for 4–6 days, those animals that did not die within 48 h after dosing were able to survive the treatment. Thus, except for weight loss and red staining around the nose and mouth, delayed toxic effects were less severe with the re-solved isomers of 1 and 2 compared to the racemates. An explanation for this observation is not available.

The cholinergic aspect of the toxicity of these compounds can be therapeutically treated with the typical antidotes atropine and 2-PAM. However, the delayed toxicity expressed with the racemic esters is not affected by therapeutic treatment with these antidotes, and no antidote for the delayed toxic symptomology is known.

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