# Synthesis and Acute Toxicity of Simple O,S-Dialkyl Alkylphosphonothioate Esters to the Rat and House Fly

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A series of O,S-dialkyl alkylphosphonothioate esters were synthesized and their acute toxic effects evaluated on rats and house flies. All compounds were toxic to rats, and animals initially showed symptoms of cholinergic poisoning. Strong synergism of house fly toxicity was observed with piperonyl butoxide for compounds having an S-methyl moiety; synergism was less for S-ethyl esters and slight for S-n-propyl esters. Bimolecular rate constants for the inhibition of bovine erythrocyte, house fly head, and rat red blood cell acetylcholinesterase were small (ca. $10^2$ $M^{-1}$ $min^{-1}$), indicating poor in vitro anticholinesterase activity. However, sampling of blood 60 min following treatment of rats with four different O,S-dialkyl alkylphosphonothioates at the $LD_{50}$ dose showed essentially 100% inhibition of whole blood acetylcholinesterase. The blood concentration of O,S-dimethyl ethylphosphonothioate reached the level of ca. $3 \times 10^{-5}$ M 45 min following treatment. Administration of atropine and 2-PAM protected rats from the acute symptomology. Overall, the data suggest acetylcholinesterase inhibition to be the mode of action.

The recent discovery of the inherent toxicity of impurities present in many organophosphorus insecticides has initiated reevaluation of the toxicological properties of these compounds. Examination of some of these impurities has led to the discovery of the phenomenon of delayed toxicity (Mallipudi et al., 1979; Hammond et al., 1982). Moreover, a number of impurities proved to be potentiators of the acute mammalian toxicity of such organophosphorus insecticides as malathion and phenthoate (Umetsu et al., 1977, 1979; Pellegrini and Santi, 1972). Hollingshaus et al. (1981) showed that in constrast to many of the trialkyl phosphorothioate impurities, some of the structurally similar O,S-dialkyl alkylphosphonothioates were acutely highly toxic to rats. Since compounds of this type may be present as impurities in phosphonothioate insecticides, e.g., fonofos, a study was initiated to determine the toxicological properties of a broader range of these compounds with the rat and house fly as test animals.

## MATERIALS AND METHODS

**General.** Precoated silica gel 60 $F_{254}$ plastic sheets (0.2 mm, EM Reagents) were used for analytical thin-layer chromatography (TLC). 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 Finnegan 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). Crude house fly head acetylcholinesterase was obtained according to Moorefield (1957).

Determination of acetylcholinesterase activity was conducted spectrophotometrically at 37 °C with a Varian Carey 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).

Rat whole blood assays to determine in vivo inhibition of blood cholinesterases were carried out following an oral $LD_{50}$ dose of toxicant in corn oil. At 10-, 30-, 45-, and 60-min intervals 50 μL of blood was taken from a cut in the rat's tail and diluted with 100 μL of a 2% aqueous solution of Triton X-100 and then 0.1 M, pH 7.6, phosphate buffer to a final volume of 1.5 mL. Aliquots of 50 μL were assayed, and percent inhibition vs. time was plotted to show inhibition profiles for each compound examined. A total of three rats were used for each compound.

**Antidotal Treatment with Atropine and 2-PAM.** Ten rats were poisoned with an oral $LD_{90}$ dose of O,S-dimethyl ethylphosphonothioate (9.2 mg/kg) in corn oil. At the onset of symptoms of cholinergic poisoning, a mixed solution of equal volumes of atropine sulfate (17.4 mg/kg) in saline and 2-PAM (50 mg/kg) in $H_2O$ was administered in a single volume of 2 mL/kg by subcutaneous injection in the flank (Natoff and Reiff, 1970). Injections were readministered as symptoms returned until the animal recovered.

**Blood Concentration of O,S-Dimethyl Ethylphosphonothioate.** The level of O,S-dimethyl ethylphosphonothioate in the blood of a rat treated with an oral dose of 8.3 mg/kg was determined according to Bailey et al. (1981). One-microliter aliquots of ethyl acetate extracts of blood taken from four different animals were analyzed in triplicate by GC at each time point. The means of each set of samples were averaged and used to calculate the blood concentration and standard error. The data were

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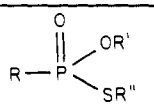
**Table I. Acute Toxicity of O,S-Dialkyl Alkylphosphonothioates to Rats and House Flies**



| no. | R | OR′ | SR″ | $n^{23}_D$ | rat oral $LD_{50}$, mg/kg | HF topical $LD_{50}$, μg/g |
|---|---|---|---|---|---|---|
| 1 | Me | Me | Me | 1.4716[a] | 3.3 (2.8–4.0)[a] | 24 (18–28) |
| 2 | Me | Et | Et | 1.4730 (1.4760)[b] | 6.0 (3.3–9.2) | 45 (40–50) |
| 3 | Me | *n*-Pr | *n*-Pr | 1.4789 (1.4820)[c] | 1.2 (0.6–2.2) | 57 (49–64) |
| 4 | Et | Me | Me | 1.4695 | 8.3 (7.4–9.0) | 24 (18–28) |
| 5 | Et | Et | Et | 1.4697[a] (1.4721)[d] | 7.7 (7.0–8.5)[a] | 31 (27–38) |
| 6 | Et | *n*-Pr | *n*-Pr | 1.4669 | 1.8 (0.9–3.3) | 35 (28–40) |
| 7 | Et | *n*-Bu | *n*-Bu | 1.4671 (1.4660)[e] | 6.1 (4.0–7.9) | 39 (33–44) |
| 8 | Et | Me | Et | 1.4752 | 9.9 (7.6–12.0) | 37 (32–42) |
| 9 | Et | Me | *n*-Pr | 1.4763 | 2.5 (0.7–4.8) | 14 (7–27) |
| 10 | Et | Me | *n*-Bu | 1.4747 | 7.0 (3.9–11.2) | 61 (54–67) |
| 11 | Et | Et | Me | 1.4692 | 4.6 (2.4–6.9) | 14 (12–17) |
| 12 | Et | *n*-Pr | Me | 1.4696 | 3.9 (2.0–5.9) | 11 (9–13) |
| 13 | Et | *n*-Bu | Me | 1.4695 | 3.4 (2.4–4.5) | 13 (11–16) |
| 14 | *n*-Pr | Me | Me | 1.4735 | 25 (20–37) | 86 (68–102) |
| 15 | *n*-Bu | Me | Me | 1.4741 | 52 (37–67) | 108 (102–114) |

[a] Data from Hollingshaus et al. (1981). [b] Data from Rozengart et al. (1965). [c] Data from Godovikov et al. (1963). [d] Data from Kabachnik et al. (1956). [e] Data from Kabachnik et al. (1955).

plotted as micrograms of toxicant per milliliter of blood vs. time (minutes) after dosing.

**Chemicals.** Each compound examined was >99% pure by GC analysis. *O,S*-Dimethyl methylphosphonothioate (1) and *O,S*-diethyl ethylphosphonothioate (5) were available from a previous study (Hollingshaus et al., 1981). The other compounds (2–4, 6–15) were synthesized as follows. *O*-Alkyl alkylphosphonothioic acids were obtained by alkaline hydrolysis of the corresponding *O,O*-dialkyl alkylphosphonothioates, followed by acidification (Kabachnik et al., 1955). The resulting acids were distilled and alkylated with the appropriate alkyl iodide (Kabachnik et al., 1956) or dialkyl sulfate (Hilgetag and Teichmann, 1959). The *O,S*-dialkyl alkylphosphonothioates were then purified on silica with hexane–ethyl acetate (1:1) as the elution solvents. Proton chemical shifts and mass spectral data were consistent with the assigned structures and are available as supplemental materials.

**Toxicological Evaluation.** Acute rat oral toxicity (48 h) was determined with 120–180-g femal albino rats (Sprague-Dawley derived, Simonsens Laboratories, Gilroy, CA). Solutions of the 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 $LD_{50}$ values.

House fly toxicity was determined with the susceptible $S_{NAIDM}$ strain of *Musca domestica* at 60 °F according to March and Metcalf (1949). $LD_{50}$ values were based on 24-h mortaility by using two replicates of five to six dosages per compound.

## RESULTS

**Rat Toxicity.** The acute signs of poisoning of rats treated with the *O,S*-dialkyl alkylphosphonothioates indicated a cholinergic mechanism of intoxication. In general, poisoned animals exhibited salivation, uncontrolled urination, difficulty in breathing, and eventual tetany.

Toxicological data presented in Table I reveal the high acute rat toxicity of these compounds with $LD_{50}$ values ranging from 1.2 mg/kg for compound 3 to 52 mg/kg for 15. Increasing the size of the P-alkyl moiety (R in Table I) from one to four carbons resulted in a general decrease in rat toxicity, e.g., compare 1, 4, 14, and 15 where an overall 16-fold decrease was observed. In contrast, increasing the size of the alkoxy moiety (OR′ in Table I) to four carbons, the rest of the molecule remaining constant, resulted in a small increase in rat toxicity, e.g., compare 4, 11, 12, and 13. A change in the alkylthio moiety (SR″ in Table I) had relatively little effect on rat toxicity except in those cases where R″ was *n*-propyl. For reasons that are not immediately apparent, phosphonothioates containing the *n*-propylthio moiety were unusually toxic (cf. 3, 6, and 9).

**Table II. Effect of Piperonyl Butoxide on the House Fly Toxicity of Selected Compounds[a]**

| no. | $LD_{50}$, μg/g | SR[b] |
|---|---|---|
| 1 | 0.9 (0.7–1.1) | 27 |
| 2 | 4.5 (3.5–5.0) | 10 |
| 3 | 33 (29–37) | 1.7 |
| 4 | 0.8 (0.2–1.5) | 29 |
| 8 | 5.4 (4.4–6.8) | 6.9 |
| 9 | 9.3 (5.7–15.8) | 1.5 |
| 11 | 0.8 (0.7–1.1) | 16.7 |
| 12 | 0.9 (0.7–1.1) | 12.9 |

[a] Piperonyl butoxide was administered in 5-fold excess simultaneously with toxicant. [b] Ratio of $LD_{50}$ without PB to $LD_{50}$ with PB.

**House Fly Toxicity.** All of the compounds produced signs of cholinergic poisoning in house flies following topical application. The data in Table I reveal moderate house fly toxicity for the *O,S*-dialkyl alkylphosphonothioates with $LD_{50}$ values ranging from 11 μg/g for compound 12 to 108 μg/g for 15. As in the case of rat toxicity, an increase in size of the P-alkyl moiety from one to four carbons resulted in a decrease in toxicity although the decrease was less sensitive to change in the number of carbons; e.g., only a 4-fold overall decrease was observed for the series 1, 4, 14, and 15. Examination of the series where the alkoxy moiety was enlarged systematically by one carbon, i.e., compounds 4, 11, 12, and 13, revealed a significant increase in toxicity in progressing from one to two carbons but essentially no change thereafter. The effect of change in the alkylthio moiety was variable, and no general trend was observed.

**Synergism.** Data for the effect of the synergist piperonyl butoxide (PB) on the house fly toxicity of a selected number of *O,S*-dialkyl alkylphosphonothioates are presented in Table II. The results reveal wide variability in the synergistic activity of PB with synergistic ratios ($LD_{50}$ of the phosphonothioate alone/$LD_{50}$ phosphono-

**Table III. Bimolecular Inhibition Rate Constants for Various Acetylcholinesterases**

| | $k_i$, $M^{-1}$ $min^{-1}$ | | |
|---|---|---|---|
| no. | BAChE | RBAChE | HFAChE |
| 1 | 267 | 290 | 350 |
| 2 | 157 | 308 | 710 |
| 3 | 352 | 369 | 364 |
| 4 | 164 | 258 | 399 |
| 5 | 203 | 292 | |
| 6 | 343 | 353 | |
| 7 | 246 | | |
| 8 | 127 | | 368 |
| 9 | 327 | | 388 |
| 10 | 167 | | |
| 11 | 303 | | 623 |
| 12 | 325 | | 774 |
| 13 | 316 | | |
| 14 | 83 | | |
| 15 | 72 | | |

thioate + PB) ranging from 1.5 (9) to 29 (4). The degree of synergism was primarily dependent on the size of the alkylthio moeity although synergistic activity also appeared to be affected by the size of the alkoxy moiety. Phosphonothioates where R′ = R″ = Me (1 and 4) were synergized to the greatest extent (and those containing the *n*-propylthio (R″ = *n*-PrS) (3 and 9) were synergized the least.

**Enzyme Inhibition.** Results of the determination of the anticholinesterase activity of the *O,S*-dialkyl alkylphosphonothioates against bovine erythrocyte (BAChE), hemolyzed rat red blood cell (RBAChE), and house fly head (HFAChE) acetylcholinesterase (Table III) show that all of the compounds are relatively poor inhibitors of these enzymes. Very little difference in inhibitory potency between the different compounds and in the susceptibility of the three enzymes to inhibition was observed. HFAChE appeared to be slightly more sensitive to inhibition than the other two enzymes, but the significance of the difference in inhibition was not determined. The values for $k_i$, the bimolecular inhibition constant, are similar to those reported by Kabachnik et al. (1970) for the inhibition of BAChE by another series of *O,S*-dialkyl alkylphosphonothioates.

Despite their poor in vitro anticholinesterase activity, several of the compounds in Table III were effective in inhibiting rat whole blood cholinesterase following an oral $LD_{50}$ dose. The results are summarized graphically in Figure 1 as a plot of the percent inhibition of whole red blood cholinesterase vs. time after treatment with compound 1, 2, 4, or 5. Significant inhibition (35–60%) was observed with each compound at the earliest time interval of 10 min following treatment, and virtually 100% inhibition was observed after 60 min.

**Antidotal Treatment.** The effectiveness of the cholinergic antidotes atropine and 2-PAM in protecting rats against acute poisoning by *O,S*-dimethyl ethylphosphonothioate (4) was determined. Rats were treated orally with 9.2 mg/kg 4 ($LD_{90}$ dose) 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. The antidotal treatment was repeated as poisoning signs reappeared. Of the 10 rats treated, most required two to three injections during the first 12 h following oral dosing and a few required one or two additional injections during the next 12 h. No injections were given after 24 h, and all animals survived the acute symptoms of the $LD_{90}$ dose of 4.

**Blood Concentration of *O,S*-Dimethyl Ethylphosphonothioate.** The concentrations of *O,S*-dimethyl ethylphosphonothioate (4) present in the blood of rats at different time intervals following oral administration of an $LD_{50}$ dose (8.3 mg/kg) were determined. The results are presented graphically in Figure 2 where concentration (μg/mL) of 4 is plotted against time. Peak concentrations of 4 were observed 40–50 min after treatment, followed by about a 4-fold drop in concentration during the next 4 h. Measurable amounts of 4 were still present in the blood after 12 h. The amount of 4 in the blood at the peak concentration level (~45 min after treatment) corresponds to approximately 5% of the applied dose.

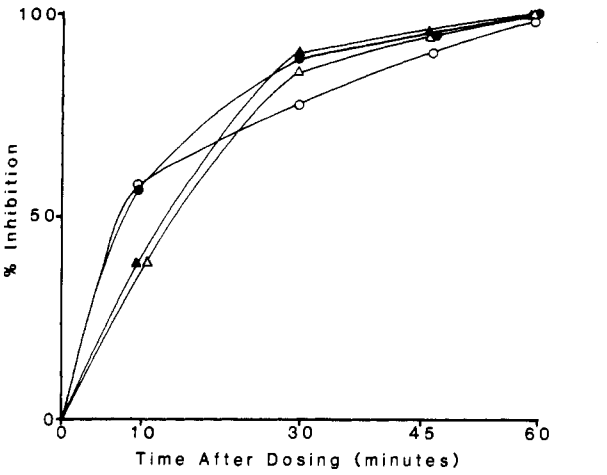



**Figure 1.** Percent inhibition of rat blood acetylcholinesterase after an oral $LD_{50}$ dose: (○) 1; (●) 2; (△) 4; (▲) 5. $n$ = 3.

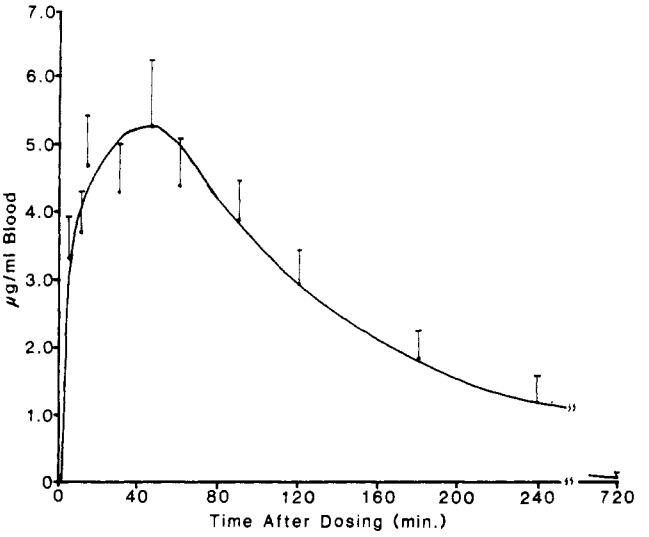



**Figure 2.** Blood concentration of *O,S*-dimethyl ethylphosphonothioate (4) after an oral $LD_{50}$ dose (8.3 mg/kg). $n$ = 4.

## DISCUSSION

The *O,S*-dialkyl alkylphosphonothioates were acutely highly toxic to the rat and moderately toxic to the house fly with each exhibiting signs of cholinergic poisoning. Antidotal treatment of rats with atropine and 2-PAM after pretreatment with an oral $LD_{90}$ dose of *O,S*-dimethyl ethylphosphonothioate protected the animals from the acute poisoning effects of the compound. Moreover, examination of rat red blood cell acetylcholinesterase following oral treatment with $LD_{50}$ doses of several different *O,S*-dialkyl alkylphosphonothioates revealed virtually complete inhibition of the enzyme 1 h after dosing. These results all point to a cholinergic mechanism of poisoning for these compounds.

Despite evidence pointing to a cholinergic mechanism of intoxication, the relatively poor in vitro anticholinesterase activity of the O,S-dialkyl alkylphosphonothioates is difficult to reconcile with their unusually high rat toxicities. Organophosphorus esters of comparable toxicity, e.g., paraoxon and tetraethyl pyrophosphate, are generally $10^4$–$10^5$-fold more potent as anticholinesterases (Aldridge and Davison, 1952). The rat blood concentration of 4 after an oral $LD_{50}$ dose rose to a level corresponding to 5% of the applied dose in 45 min. Calculations based on the in vitro anticholinesterase activity of 4 ($k_i$ = 258 $M^{-1}$ $min^{-1}$) indicate that this concentration is not high enough to cause the approximately 90% inhibition observed in vivo 45 min after an oral $LD_{50}$ dose. The contradiction between poor anticholinesterase activity and high toxicity therefore raises the possiblity of the metabolic activation of these compounds to more potent anticholinesterases. In vivo activation of O,S-dimethyl phosphoramidothioate (methamidophos), a poor anticholinesterase, to the corresponding S-oxide has been suggested to explain its high toxicity to insects and mammals (Eto et al., 1977), and spectroscopic evidence for its formation by peracid oxidation has been obtained (Thompson, 1982). Evidence has also been obtained for peracid oxidation of phosphorothioates to phosphinyloxysulfonates believed to be formed by rearrangement of an S-oxide intermediate (Segall and Casida, 1982).

The house fly synergism data with piperonyl butoxide, an inhibitor of mixed-function oxidase (Nakatsugawa et al., 1968), suggest oxidative metabolism to be important in the detoxication of O,S-dialkyl alkylphosphonothioates in this insect. However, since piperonyl butoxide should also inhibit the oxidative activation, the role of activation in the mode of action of these compounds in the house fly is not clear. It is noteworthy that the O,S-dialkyl alkylphosphonothioates were all substantially less toxic to house fly (without piperonyl butoxide) than to the rat, and this may be due in part to the ability of the house fly to detoxify these compounds. However, in the case of the rat, the observed low in vitro inhibition rates and high anticholinesterase activity against rat blood enzyme in vivo are suggestive of bioactivation of the parent compound to a more potent enzyme inhibitor.

**Registry No.** 1, 58259-60-2; 2, 2511-10-6; 3, 90220-14-7; 4, 84044-17-7; 5, 2511-11-7; 6, 90220-15-8; 7, 2511-15-1; 8, 90245-33-3; 9, 90220-16-9; 10, 40618-52-8; 11, 2511-12-8; 12, 90220-17-0; 13, 90220-18-1; 14, 90220-19-2; 15, 90220-20-5; acetylcholinesterase, 9000-81-1.

**Supplementary Material Available:** Mass spectral and $^1H$ NMR data for compounds in Table I (8 pages). Ordering information is given on any current masthead page.

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