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# Toxicological Properties of *O,S,S*-Trialkyl Phosphorodithioates

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A limited series of *O,S,S*-trialkyl phosphorodithioates was prepared. Rat oral toxicities varied widely with very minor changes in alkyl substitutents, ranging from moderately to highly toxic. The order of toxicity to the housefly was different from that of rats. All the compounds were poor in vitro inhibitors at rat erythrocyte cholinesterase, with $k_i$ values ranging from 2 to 177 $M^{-1}$ $min^{-1}$. The poor anti-AChE activity of these compounds when contrasted with the cholinergic symptoms of poisoning elicited in the rat and housefly suggests that bioactivation may play a significant role in their toxicity. *O,S,S*-Trimethyl phosphorodithioate was several-fold more reactive, both chemically and biochemically, than its corresponding phosphorothioate analogue *O,O,S*-trimethyl phosphorothioate.

## INTRODUCTION

Previous reports from this and other laboratories have described the delayed toxic action of *O,O,S*-trimethyl phosphorothioate (OOS-Me) and *O,S,S*-trimethyl phosphorodithioate (OSS-Me). These compounds are present as impurities in such "safe" organophosphorus insecticides as malathion, phenthoate, and acephate (Umetsu et al., 1977; Umetsu et al., 1981; Hammond et al., 1982; Aldridge et al., 1979). Examination of a series of *O,O,S*-trialkyl phosphorothioates related to OOS-Me revealed several other esters with delayed toxic activity, with highest activity found with compounds containing small alkyl groups (Ali and Fukuto, 1982). Some of these compounds were acutely highly toxic to the rat, with oral $LD_{50}$ values ranging from 11 to about 50 mg/kg. Typical signs of poisoning were weight loss, diarrhea, incontinence, and bleeding from the nose or mouth (Gray and Fukuto, 1984; Mallipudi et al., 1979; Umetsu et al., 1981).

The phosphorodithioate ester OSS-Me also displayed toxicological properties similar to OOS-Me at low doses (near the $LD_{50}$ dose of 26 mg/kg), but at high doses ($10LD_{50}$) the signs of poisoning indicated a cholinergic mechanism of intoxication (Aldridge et al., 1979). In an effort to develop additional information on the toxicological properties of compounds of this type, a small series of lower alkyl *O,S,S*-trialkyl phosphorodithioates was synthesized and evaluated for toxicity to rats, anticholinesterase activity, and chemical reactivity. This report presents the results of this study.

## MATERIALS AND METHODS

**Chemicals.** *O,S,S*-Trimethyl phosphorodithioate (**1**) was prepared according to Aldridge et al. (1979). The remaining *O,S,S*-trialkyl phosphorodithioates (**2–6**) were synthesized by reaction between the appropriate alkyl phosphorodichloridate and the sodium alkanethiolate. Typically, the sodium alkanethiolate was added as a powder to a chilled solution of alkyl phosphorodichloridate in anhydrous benzene, followed by heating of the mixture to reflux. After filtration through filter aid, the product was distilled under reduced pressure and subsequently purified by preparative thin-layer chromatography (TLC), using benzene–ethyl acetate (1:1 by volume) as the developing solvent and silica gel GHLF (Analtech, Inc., Newark, DE) as the stationary phase. Structures were verified by NMR and elemental analyses (see Table I). Product purity was determined first by analytical TLC using 1:1 benzene–ethyl acetate. Spots on TLC plates were detected by ultraviolet light and by spraying with 0.75% 2,6-dibromoquinone-4-chloroimide (DBQ) in ether (Menn et al., 1957). Purity was verified further by GLC as described under analysis.

*O,O,S*-Trimethyl phosphorothioate (**7**) was available from an earlier study (Mallipudi et al., 1979).

**Toxicological Evaluation.** Acute and delayed rat toxicity was determined with 200–250-g female albino rats (Sprague-Dawley derived) obtained from Simonsen Laboratories, Gilroy, CA. Solutions of the toxicants in corn oil were administered orally at 0.1 mL/100 g to animals fasted for about 12 h before treatment. The rats were kept under observation for 20 days. At least five different doses with a minimum of four rats per dose were used to determine $LD_{50}$ values.

Insecticidal activity was determined with the susceptible $S_{NAIDM}$ strain of houseflies, *Musca domestica*, at 72 °F according to March and Metcalf (1949). $LD_{50}$ values were based on 24-h mortality by using five dosages per compound.

**Acetylcholinesterase Inhibition.** Rat red blood cell acetylcholinesterase (RBAChE) was prepared by taking 50 μL of blood from a cut in a rat tail and washing the erythrocytes three times with one 10-mL and two 4-mL portions of 0.1 M pH 7.4 sodium phosphate buffer using a Clay Adams desk top centrifuge set at 2000g, for 10, 5, and 5 min, respectively.

The washed erythrocyte pellets were resuspended in 2 mL of buffer, and 0.6 mL of this suspension was diluted

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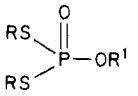
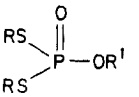
**Table I. Physical Properties and Elemental Analysis of *O,S,S*-Trialkyl Phosphorodithioates**



| | compd | bp, °C (mmHg) | $n_D^{23}$ | $R_f$[a] | % anal. calcd | % anal. found |
|---|---|---|---|---|---|---|
| 1 | R = $CH_3$, $R^1$ = $CH_3$ | 72–74 (0.3) | 1.5311 | 0.53 | C, 20.92; H, 5.27 | C, 21.24; H, 5.55 |
| 2 | R = Et, $R^1$ = $CH_3$ | 82–84 (0.15) | 1.5185 | 0.56 | C, 29.99; H, 6.54 | C, 30.23; H, 6.55 |
| 3 | R = *n*-Pr, $R^1$ = $CH_3$ | 88–90 (0.15) | 1.5092 | 0.63 | C, 36.83; H, 7.51 | C, 36.54; H, 7.33 |
| 4 | R = *i*-Pr, $R^1$ = $CH_3$ | 86–88 (0.15) | 1.5031 | 0.61 | C, 36.83; H, 7.51 | C, 36.52; H, 7.55 |
| 5 | R = *n*-Pr, $R^1$ = Et | 98–100 (0.20) | 1.5031 | 0.63 | C, 39.65; H, 7.90 | C, 39.36; H, 8.06 |
| 6 | R = *n*-Pr, $R^1$ = *n*-Pr | 90–92 (0.10) | 1.4996 | 0.68 | C, 42.17; H, 8.26 | C, 42.26; H, 8.34 |

[a] Plastic thin-layer chromatography, solvent system benzene–ethyl acetate (1:1).

**Table II. Toxicity of *O,S,S*-Trialkyl Phosphorodithioates to Rats and Houseflies**



| | compd | toxicity to rats: time frame for death, days after treatment | toxicity to rats: $LD_{50}$, mg/kg | houseflies $LD_{50}$, μg/g |
|---|---|---|---|---|
| 1 | R = $CH_3$, $R^1$ = $CH_3$ | 3–8 | 42.7[a] (34.4–47.8)[b] | 103.6 (74.6–136.4) |
| 2 | R = Et, $R^1$ = $CH_3$ | 1–5 | 158.5 (140.2–176.0) | 348.6 (281.5–408.7) |
| 3 | R = *n*-Pr, $R^1$ = $CH_3$ | 1 | 6.8 (2.5–11.3) | 15.4 (13.7–17.3) |
| 4 | R = *i*-Pr, $R^1$ = $CH_3$ | 1 | 119.3 (104.5–132.3) | 48.1 (44.5–52.0) |
| 5 | R = *n*-Pr, $R^1$ = Et | 1–5 | 61.8 (55.6–75.6) | 30.8 (28.0–33.4) |
| 6 | R = *n*-Pr, $R^1$ = *n*-Pr | 1–2 | 128.8 (113.7–150.2) | 42.0 (28.8–58.7) |
| 7 | OOS-Me | 2 | ~200 (48 h)[c] | ~95[d] |
| | | 25 | 15–20 (25 days)[c] | |

[a] Data from Hammond et al. (1982). [b] Parenthetical values are 95% confidence limits. [c] Mallipudi et al. (1979). [d] Ali and Fukuto (1982).

to 10 mL with buffer and hemolyzed by adding about 0.9 mg of saponin powder. The lysed cell suspension was diluted 10 times further with phosphate buffer and used immediately after.

Enzyme activity was determined by a modification of the procedure of Ellman et al., 1961.

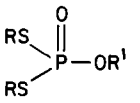
Forty-eight milliliters of the enzyme preparation, spiked with 400 μL of 1% w/v quinidine sulfate and 400 μL of each test inhibitor ($10^{-2}$–$10^{-1}$ M) was incubated at 30 °C in a thermostated water bath. At appropriate intervals, 3-mL aliquots were taken in a glass cuvette and rapidly mixed with 50 μL each of 0.075 M acetylthiocholine iodide and 0.1 M 5,5′-dithiobis(2-nitrobenzoic acid). The change in absorbance was followed at 412 nm in a Beckman Model 25 spectrophotometer at 30 °C. Controls were spiked with 50 μL of phosphate buffer in place of the substrate. Initial rate determinations ($A_0$) were carried out just prior to each assay. Data were plotted as activity vs. time using a Hewlett-Packard 11C calculator. Correlation coefficients ranged from 0.9 to 1.0.

**Hydrolysis.** Rates of hydrolysis of compounds **1–7** were determined at three temperatures (30, 37, 44 °C) in a Brinkman Lauda thermostated water bath. Aliquots of an ethyl acetate solution containing weighed amounts of each compound were concentrated to dryness under a gentle stream of nitrogen. The residue was then dissolved in 0.1 M phosphate buffer (pH 11.1) containing 2.5% (v/v) methanol. At appropriate intervals, 1-mL aliquots were removed and added to 3–30 mL of ethyl acetate. Anhydrous sodium sulfate was added to the mixture to take up the water, and samples were taken for the quantification of the parent compound by GLC.

**Analyses.** A Varian 1400 or a Hewlett-Packard 402 gas chromatograph equipped with an alkaline flame ionization detector (AFID) was used to quantify parent materials. For the Varian 1400, the column packing was 6% Carbowax 20M TPA (Applied Science Division, Milton Roy Co. Laboratory Group, State College, PA) on acid-washed Chromosorb W, 80/100 mesh, HCl extracted, vacuum coated, and fluidized, conditioned for 24 h at 280 °C, and exhaustively extracted with chloroform. For the HP-402, the column packing was 6% EGSP-Z (Applied Science Laboratories, State College, PA) on acid-washed Chromosorb W, 80/100 mesh, vacuum coated and fluidized, conditioned for 24 h at 230 °C, and exhaustively extracted with chloroform. Column temperatures ranged from 120 to 180 °C for compounds **1–7** with detector and injection port temperatures of 260 and 230 °C, respectively. Hydrogen, air, and carrier gas flow rates were set as appropriate for each compound.

Quantification was carried out by extrapolating the experimental sample peak heights to that of a standard

**Table III. Hydrolysis Rate Constants and Bimolecular Inhibition Rate Constants toward Rat Red Blood Cell Acetylcholinesterase ($k_i$)**



| | compd | $k_{HYD}$, $\times 10^{-2}$ min | | | $t_{1/2}$(30 °C), min | $k_i$(30 °C), $M^{-1}$ $min^{-1}$ |
|---|---|---|---|---|---|---|
| | | 30 °C | 37 °C | 44 °C | | |
| 1 | R = $CH_3$<br>$R^1$ = $CH_3$ | 1.84 ± 0.10 | 5.64 ± 0.28 | 12.72 ± 0.16 | 38 | 177 ± 90 |
| 2 | R = Et<br>$R^1$ = $CH_3$ | 0.77 ± 0.09 | 2.63 ± 0.13 | 5.47 ± 0.21 | 90 | 33 ± 13 |
| 3 | R = *n*-Pr<br>$R^1$ = $CH_3$ | 0.69 ± 0.03 | 2.22 ± 0.01 | 2.82 ± 0.01 | 100 | 31 ± 3 |
| 4 | R = *i*-Pr<br>$R^1$ = $CH_3$ | 0.56 ± 0.06 | 1.11 ± 0.31 | 1.51 ± 0.15 | 124 | 22 ± 12 |
| 5 | R = *n*-Pr<br>$R^1$ = Et | 0.55 ± 0.00 | 1.16 ± 0.16 | 2.39 ± 0.72 | 126 | 4 ± 3 |
| 6 | R = *n*-Pr<br>$R^1$ = *n*-Pr | 0.57 ± 0.15 | 1.18 ± 0.09 | 1.63 ± 0.30 | 122 | 2 ± 1 |
| 7 | OOS-Me | 0.59 ± 0.05 | 2.57 ± 0.24 | 3.68 ± 0.10 | 117 | 1 ± 0.5 |

concentration curve of authentic standards, using a Hewlett-Packard 11C calculator. Correlation coefficients ranged from 0.97 to 1.0.

## RESULTS

**Toxicity.** Data for the toxicity of the different *O,S,S*-trialkyl phosphorodithioates (1–6) to the rat and housefly are presented in Table II; data for OOS-Me are included for comparison (Mallipudi et al., 1979). The results indicate wide variability in the rat oral toxicities of the phosphorodithioates, despite relatively minor changes in either the SR or $OR^1$ alkyl moieties. For example, **3** with an *S*-*n*-propyl moiety is more than 23-fold more toxic to the rat than **2**, the analogue containing the *S*-ethyl moiety. Further, a 9-fold drop in rat toxicity was observed when $OR^1$ in **3** was changed from methyl to ethyl (**5**).

Delayed toxic activity was observed with compounds **2** and **5**, with mortality occurring as late as 5 days following treatment. At doses near the $LD_{50}$ level, typical signs of delayed poisoning were apparent, including weight loss, uncontrolled urination, and staining around the mouth, nose, or eyes (Hammond et al., 1982). Mild to moderate signs of cholinergic poisoning were also observed with these compounds at or near the $LD_{50}$ level. However, animals poisoned with compounds **3**, **4**, and **6** appeared to die mainly of cholinergic poisoning, and in these cases the signs of cholinergic poisoning were severe at the $LD_{50}$ level.

The order of toxicity of compounds 1–6 to the housefly was different from that to the rat, although **3**, which was most toxic to the rat, was also most toxic to the housefly. In the limited series of compounds examined, those with larger alkyl groups appeared to be more effective against houseflies.

**Anticholinesterase Activity.** Compounds 1–6 were examined for inhibition of RBAChE (Table III). All of the compounds were poor inhibitors of this enzyme with bimolecular inhibition constants ($k_i$, $M^{-1}$ $min^{-1}$) ranging from 2 to 177.

The magnitude of $k_i$ decreased with increasing alkyl chain length of the SR moiety, dropping by more than 88-fold from **1** to **6**. In the *S*-*n*-propyl series, increasing the alkyl chain length for $OR^1$ also decreased the $k_i$ values by more than 15-fold from compounds **3** to **6**. These $k_i$ values appear to parallel the pseudo-first-order hydrolysis rate constants, indicating that a bimolecular reaction between RBAChE and a nucleophile was taking place.

These $k_i$ values, however, did not have any obvious correlation with the rat oral $LD_{50}$ values of these compounds. For example, compound **3**, in spite of its high acute oral toxicity and cholinergic symptoms of poisoning, showed a $k_i$ value more than 5-fold smaller than that of **1**. Moreoever, the cholinergic compound **6** showed the smallest $k_i$ value of the limited series of phosphorodithioates examined.

The $k_i$ value of OOS-Me, the monothioate analogue of **1**, was 177-fold smaller than that of **1**.

**Hydrolysis.** Rate constants for the hydrolysis of compounds 1–7 in pH 11.1 phosphate buffer are presented in Table III. For the phosphorodithioates 1–6, the pseudo-first-order constants, as in the case of $k_i$, generally decreased with increase in the size of SR and $OR^1$ although the decrease in rate constants was relatively small. The rate constants for the trimethyl phoshorodithioate ester (**1**) was significantly larger than the constants for the remaining phosphorodithioate esters, and little difference was observed between these compounds. Further, the rate constants for compounds 2–5 were close to that of OOS-Me, the phosphorothioate analogue of **1**.

## DISCUSSION

Examination of this limited series of *O,S,S*-trialkyl phosphorodithioates provided interesting information on the toxicological properties of these compounds. As in the case of the *O,O,S*-trialkyl phosphothioates examined previously (Ali and Fukuto, 1982), the toxicity of the *O,S,S*-trialkyl phosphorodithioates was highly sensitive to change in structure and minor changes had a strong effect on rat toxicity. The *S,S*-di-*n*-propyl analogue (**3**), with an $LD_{50}$ value of 6.8 mg/kg, was highly toxic to the rat and also showed the highest toxicity to the housefly. The unusually high rat toxicity of **3** is difficult to explain since it, along with the other compounds, is a poor antichloinesterase, and rats treated with **3** died with typical cholinergic signs of poisoning within 24 h after treatment. Although it may be purely fortuitous, highest rat toxicity was also observed with the *S*-*n*-propyl analogues of *O,O,S*-trialkyl phosphorothioates (Ali and Fukuto, 1982) and *O,S*-dialkyl alkylphosphonothioates (Armstrong and Fukuto, 1984).

Of considerable interest is the poor in vitro anticholinesterase activity of the trialkyl phosphorodithioates. Evidently, these compounds are not reactive enough to phosphorylate the enzyme at a rate that would cause rapid inhibition. Previous studies with the *O,O,S*-trialkyl phosphorothioates (Ali and Fukuto, 1982) and *O,S*-dialkyl alkylphosphonothioates (Armstrong and Fukuto, 1984) revealed that they also were poor anticholinesterases, al-

though a number of these compounds were highly toxic to rats. Poisoning in a substantial number of these cases was cholinergic. The *O,S,S*-trialkyl phosphorodithioates, therefore, may be included with the *O,O,S*-trialkyl phosphorothioates and *O,S*-dialkyl alkylphosphonothioates as compounds that appear to be toxic by a cholinergic mechanism but are poor in vitro anticholinesterases. These observations raise the possibility of an activation step of some kind in the mode of action of these compounds. The absence of correlation between $k_i$ values and rat oral toxicity also suggests that structural features might play a role in this proposed activation process.

The similarity in hydrolysis rates of the phosphorodithioates and OOS-Me (7) was surprising since the dithioates would be expected to be substantially more susceptible to hydrolysis owing to less $d\pi$–$p\pi$ interaction between the two sulfur atoms and phosphorus (Murdock and Hopkins, 1968). The similarity in hydrolysis rates, however, is consistent with the poor anticholinesterase activity of the trialkyl phosphorothioates and phosphorodithioates.

**Registry No.** 1, 22608-53-3; 2, 22082-34-4; 3, 4104-02-3; 4, 99559-84-9; 5, 13194-48-4; 6, 74125-01-2; 7, 2953-29-9; acetylcholinesterase, 9000-81-1.

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# Phenolic Acid Content of Food Plants and Possible Nutritional Implications[1]

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The *p*-coumaric, ferulic, and caffeic acid contents of alfalfa, cabbage, and spinach were determined by gas chromatography after derivatization with *N,N*-bis(trimethylsilyl)trifluoroacetamide. Total phenolic acids were estimated by extraction with 1% NaOH containing 0.5% $NaBH_4$. Free or nonesterified acids were extracted with 80% methanol. Alfalfa contained the highest concentration of total *p*-coumaric acid (1 mg/g) and of free caffeic acid (0.68 mg/g). Lower levels of free caffeic acid were found in cabbage and spinach but none in wheat bran. Wheat bran had the highest content of ferulic acid (4.4 mg/g). Only the free form of caffeic acid was found in alfalfa and cabbage, probably because the alkaline treatment destroyed all that was present.

Fiber-rich plant foods stimulate the growth rate of immature guinea pigs fed purified diets (Ershoff, 1957; Reid and Mickelson, 1963; Lakhanpal et al., 1966; Singh et al., 1968; Knehans et al., 1979). They also protect against radiation damage (Calloway et al., 1963) and salmonellosis (Nabb and O'Dell 1964). The growth stimulant in alfalfa and other plants studied is water insoluble and associated with cell wall components and holocellulose prepared from the plant. It is unstable to alkaline solutions and loses activity with long-term dry storage, presumably by oxidation (Knehans et al., 1979).

The mechanism by which fibrous plant foods and the water-insoluble residues prepared from them stimulate growth is unknown but fibrous products do change the intestinal microflora of the guinea pig (Knehans and O'Dell, 1980). Recent observations (Johanning et al., 1984) have shown that fibrous residues prepared from alfalfa, cabbage, and spinach impair the growth rate of an intestinal anaerobe, *Bacteroides ovatus*, by removal of hemin from the medium. Alkaline treatment of the fiber-rich residues decreases their antibacterial action (Johanning et al., 1984) as well as the guinea pig growth stimulating activity. The active component may stimulate the growth rate by an antibiotic effect on certain intestinal microflora, but this is unknown. In any case its chemical properties suggest a compound that is easily hydrolyzed by alkaline solution or is prone to oxidation in alkaline solution or both.

Some of the polyphenolic acids are covalently linked to cell wall components and possess chemical properties similar to the growth stimulant found in alfalfa and its water-insoluble components. Caffeic acid, 3,4-dihydroxycinnamic acid, is widely distributed in plants and is highly sensitive to air oxidation, particularly in alkaline solution. Although there is not good evidence that they are esterified to cell wall components, several phenolic acids, including

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