# Stereoselectivity in Cholinesterase Inhibition, Toxicity, and Plant Systemic Activity by the Optical Isomers of *O*-2-Butyl *S*-2-(Ethylthio)ethyl Ethylphosphonothioate

David A. Wustner* and T. Roy Fukuto

The synthesis and resolution of *O*-2-butyl *S*-2-(ethylthio)ethyl ethylphosphonothioate (demeton analog), optically active at both the 2-butyl carbon and at phosphorus, are described. Also synthesized were the racemic *O*-2-butyl *S*-2-(ethylsulfinyl)-ethyl ethylphosphonothioate (sulfoxide), *O*-2-butyl *S*-2-(ethylsulfonyl)ethyl ethylphosphonothioate (sulfone), and *O*-2-butyl *S*-2-(dimethylamino)ethyl ethylphosphonothioate (tetram analog). The levorotatory phosphorus isomers gave bimolecular inhibition rate constants 1250-, 105-, and 36-fold larger than corresponding dextrorotatory phosphorus enantiomers against housefly head acetylcholinesterase, bovine erythrocyte acetylcholinesterase, and horse serum cholinesterase, respectively. The levorotatory phosphorus isomers were much more active as toxicants to susceptible and resistant houseflies, *Culex* mosquito larvae, and mice than the dextrorotatory compounds. The dextrorotatory enantiomers were completely inactive as systemic insecticides in cotton plants, while the levorotatory isomers were highly active both as stem and soil treatments. It is apparent that toxicity and plant systemic insecticidal effectiveness are directly related to anticholinesterase activity.

Stereoselectivity in the interaction of molecules containing asymmetric centers with biological systems is a well known phenomenon, and the interaction of asymmetric substrates and inhibitors with cholinesterase is no exception. A short review by Beckett *et al.* (1968) describes the behavior of cholinesterases from different sources toward a variety of resolved chiral substrates. The first observation of rate differences in cholinesterase inhibition by an asymmetric organophosphorus ester was reported by Michel (1955), who observed biphasic kinetics for the inhibition of electric eel acetylcholinesterase by racemic sarin (*O*-isopropyl methylphosphonofluoridate). Aaron *et al.* (1958a,b) described the synthesis of the enantiomers of *O*-ethyl *S*-2-(ethylthio)ethyl ethylphosphonothioate and reported that the levorotatory isomer was 10- to 20-fold more effective as an inhibitor of a variety of cholinesterases than the dextrorotatory enantiomer. Subsequently, a number of papers have appeared concerned with the inhibition of acetylcholinesterase by resolved organophosphorus compounds, *i.e.*, Ooms and Boter (1965), Boter (1970), and De Jong and Van Dijk (1972).

Relatively little has been published on the toxicity of the resolved isomers of organophosphorus esters to insects and mammals. Hilgetag and Lehman (1959) reported that the levorotatory isomer of *O*-methyl *S*-methyl *O*-4-nitrophenyl phosphorothioate was fivefold more toxic to rats than its dextrorotatory enantiomer. Fukuto and Metcalf (1959) reported that the levorotatory isomer of *O*-ethyl *S*-2-(ethylthio)ethyl ethylphosphonothioate was six- to tenfold more toxic to houseflies, mosquito larvae, and honeybees than its enantiomer. Hassan and Dauterman (1968) reported that the dextrorotatory isomers of both malathion and malaoxon ($\alpha$-carbon) were slightly more toxic to mice and twofold more toxic to houseflies than their enantiomers.

This report is concerned with the synthesis, resolution, and examination of the toxicological properties of organophosphorus esters containing two asymmetric centers. In particular, work was conducted to determine the effect of simultaneous chirality at both the phosphorus atom and the $\alpha$-carbon atom of the alkoxy moiety in phosphonothioate esters on the inhibition of cholinesterase from three different sources. Additional experiments were performed to determine whether these effects were reflected in the toxicity of the chiral organophosphorus esters to insects and mice and in their plant systemic insecticidal effectiveness.

Department of Entomology, Division of Toxicology and Physiology, University of California, Riverside, California 92502.

## MATERIALS AND METHODS

**Chemical Synthesis.** The racemic (**1**), and the two *O*-2-butyl ethylphosphonochloridothioates (**1b, 1e**) resolved at the 2-butyl carbon and racemic at the phosphorus atom were synthesized by treating ethylphosphonodichloridothioate (Hoffman *et al.*, 1958; Perry *et al.*, 1963) with racemic or resolved lithium 2-butoxide in ether. The *d*- and *l*-isomers of 2-butanol were obtained from Norse Laboratories, Santa Barbara, Calif., with optical purities of (+)94% and (−)96% (% excess of the resolved over the racemic). Physical constants for these and other compounds synthesized in this study are listed in Table I, and optical rotations are listed in Table II.

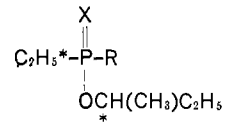
Compounds **1, 1b,** and **1e** were hydrolyzed in *p*-dioxane–water (1:1) with 2 equiv of sodium hydroxide to yield the corresponding *O*-2-butyl hydrogen ethylphosphonothioates (**2, 2b, 2e**) in 90% yield. Further resolution of these phosphonothioic acids (**2b, 2e**), *i.e.*, at the phosphorus atom, was accomplished with (+) and (−)-$\alpha$-phenylethylamine (Theilacker and Winkler, 1954) giving, after four recrystallizations from hexane–ethanol (10:1), the diastereomeric salts (**3a, 3c, 3d, 3f**). Treatment of these salts with acid gave the four fully resolved *O*-2-butyl hydrogen ethylphosphonothioates (**2a, 2c, 2d, 2e**).

The racemic and partially resolved (resolved at the 2-butyl carbon atom) *O*-2-butyl *S*-2-(ethylthio)ethyl ethylphosphonothioates (**4, 4b, 4e**) were prepared according to the method of Aaron *et al.* (1958a) from the sodium salts of the phosphonothioic acids and 2-(ethylthio)chloroethane in methanol. The fully resolved esters (**4a, 4c, 4d, 4f**) were prepared directly by treating the $\alpha$-phenylethylamine salts of the phosphonothioic acids (**3a, 3c, 3d, 3f**) with 2-(ethylthio)chloroethane in benzene.

Compound **4** was treated with *m*-chloroperbenzoic acid in dichloromethane to form racemic *O*-2-butyl *S*-2-(ethylsulfinyl)ethyl ethylphosphonothioate (**5**), the sulfoxide. The product was purified on a falling film molecular still to yield 49% of **5**, wall temperature 80° (0.03 mm), $n^{25}$D 1.5084. Similarly, compound **4** was treated with potassium permanganate in acetic acid–water to yield racemic *O*-2-butyl *S*-2-(ethylsulfonyl)ethyl ethylphosphonothioate (**6**), the sulfone. Similar purification gave 54% of **6**, wall temperature 125° (0.05 mm), $n^{25}$D 1.4979.

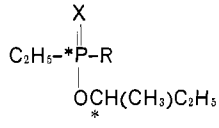
The sodium salt of the racemic phosphonothioic acid (**2**) was treated with 2-(dimethylamino)chloroethane in ethanol, followed by the addition of 1 equiv of oxalic acid to yield, after three recrystallizations from acetone, the race-

**Table I. Physical Constants for the Isomers of Compounds 1 through 4**



| | 1, R = Cl, X = S | | | 2, R = OH, X = S | | 3, R = S⁻H₃N⁺CH*(CH₃)Ph, X = O | | 4, R = -SC₂H₄SC₂H₅, X = O | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | % | Bp (mm) | $n^{25}D$ | Bp (mm) | $n^{25}D$ | % | Mp | % | Bp (mm) | $n^{25}D$ |
| Racemic | 88 | 77–79 (5.2) | 1.4848 | 89–90 (0.17) | 1.4789 | | | 93 | 105–107 (0.05) | 1.5010 |
| **a** | | | | 81–82 (0.05) | 1.4785 | 47 | 131.5–132.5 | 84 | 111.5 (0.08) | 1.5006 |
| **b** | 84 | 76–80 (5.9) | 1.4847 | 85–86 (0.08) | 1.4785 | | | 61 | 108 (0.05) | 1.5012 |
| **c** | | | | 83.5–84 (0.05) | 1.4783 | 55 | 148.5–149 | 54 | 104 (0.05) | 1.5000 |
| **d** | | | | 80–81 (0.03) | 1.4784 | 55 | 149–149.5 | 83 | 108 (0.05) | 1.5000 |
| **e** | 80 | 77–79 (5.2) | 1.4834 | 78–80 (0.05) | 1.4788 | | | 91 | 109 (0.05) | 1.5022 |
| **f** | | | | 76–77 (0.05) | 1.4787 | 37 | 134–134.5 | 80 | 107 (0.1) | 1.5008 |

**Table II. Optical Rotations in Degrees of Arc of the Isomers of Compounds 1 through 4. The Standard Deviations of the Measurements Were Approximately 0.01° Irrespective of the Magnitude of the Measured Rotation**



| | 1, R = Cl, X = S | 2, R = OH, X = S | | 3, R = S⁻H₃N⁺-*CH(CH₃)Ph, X = O | 4, R = $SC_2H_4SC_2H_5$, S = O |
|---|---|---|---|---|---|
| | $\alpha^{25}D$ | $\alpha^{26}D$ | $[\alpha]^{26}D$[c] | $[\alpha]^{26}D$[e] | $\alpha^{26}D$ |
| **a** | | −2.268[a] | +19.731 | −4.306 | −33.317 |
| **b** | +19.861 | +15.239[b] | +12.615[d] | | +11.072[f] |
| **c** | | +33.133 | +8.661 | +13.499 | +60.098 |
| **d** | | −33.967 | −9.128 | −13.718 | −60.276 |
| **e** | −21.408 | −16.187 | −13.233 | | −14.698 |
| **f** | | +2.363 | −20.708 | +4.284 | +33.780 |

[a] t = 25°. [b] t = 22°. [c] c ≈ 0.02 g/ml in absolute ethanol. [d] t = 24°. [e] c ≈ 0.1 g/ml in absolute ethanol. [f] t = 25°.

mic *O*-2-butyl *S*-2-(dimethylamino)ethyl ethylphosphonothioate hydrogen oxalate (**7**), a tetram analog. The yield of **7** was 47%, mp 97–99°.

Elemental analyses were carried out by C. F. Geiger, Ontario, Calif., and are presented in Table III for representative compounds.

**Enzyme Inhibition.** The enzymes used were bovine erythrocyte acetylcholinesterase (BAChE, EC 3.1.1.7), horse serum cholinesterase (HSChE, EC 3.1.1.8), and housefly head acetylcholinesterase (HFAChE). Both the bovine and horse enzymes were obtained from the Sigma Chemical Co., St. Louis, Mo., and were prepared as stock solutions of 1 mg/ml in 0.1 *M* phosphate buffer, pH 7.55. Housefly heads were obtained from susceptible (NAIDM) strain houseflies by the modified method of Moorefield (1957). The heads were ground by hand in an all-glass Duall homogenizer in 0.1 *M* sodium phosphate buffer, pH 7.55. The concentration was adjusted to 50 heads/ml and the crude brei was then spun down in a Sorvall refrigerated centrifuge at 25,000 × *g* for 1 hr. The supernatant was used without further treatment. One large batch, about 7000 heads, sufficed for all of the inhibition experiments. Small samples of brei were stored frozen in small vials and were thawed for use as needed. No significant loss in activity or change in kinetic performance was seen in samples stored in this manner for as long as 8 months.

The fly-head brei was used directly and the stock solutions of the commercial enzymes were diluted twofold to give an uninhibited substrate (acetylthiocholine) velocity of approximately $2.7 \times 10^{-2}$ $\mu$m ml$^{-1}$, min$^{-1}$ at 30°, pH 7.55.

**Table III. Analytical Data for Representative Compounds**

| Compd | Formula | Carbon | Hydrogen |
|---|---|---|---|
| **3d** | $C_{14}H_{26}NO_2PS$ | Theory, 55.42 | 8.64 |
| | | Found, 55.25 | 8.48 |
| **3f** | $C_{14}H_{26}NO_2PS$ | Found, 55.56 | 8.74 |
| **4a** | $C_{10}H_{23}O_2PS$ | Theory, 44.42 | 8.57 |
| | | Found, 44.49 | 8.90 |
| **4d** | $C_{10}H_{23}O_2PS$ | Found, 44.44 | 8.98 |
| **4e** | $C_{10}H_{23}O_2PS$ | Found, 44.61 | 8.36 |
| **5** | $C_{10}H_{23}O_3PS_2$ | Theory, 41.94 | 8.10 |
| | | Found, 42.09 | 8.28 |
| **6** | $C_{10}H_{23}O_4PS_2$ | Theory, 39.72 | 7.76 |
| | | Found, 40.20 | 7.73 |
| **7** | $C_{12}H_{26}NO_6PS$ | Theory, 41.98 | 7.65 |
| | | Found, 42.42 | 8.01 |

Bimolecular inhibition constants, $k_i$ (Aldridge, 1950), were obtained by incubating 1 ml of the enzyme solutions with 10 $\mu$l of an appropriate concentration of inhibitor in acetone at 37.5°. The inhibitor concentrations used approximated the $I_{50}$, *i.e.*, the molar concentration required to inhibit 50% of the enzyme in 15 min. At zero time and at 3-min intervals thereafter, 50-$\mu$l aliquots of the incubation mixture were diluted with 3 ml of substrate solution and the residual substrate velocities were measured spectrophotometrically at 30° in a Unicam SP-800A ultraviolet spectrophotometer (Pye Unicam Instruments, Ltd., Cambridge, U.K.) at 412 nm. The estimation of substrate hydrolysis was essentially that of Ellman *et al.* (1961). Final concentrations in the cuvette were $4.4 \times 10^{-3}$ *M* of acetylthiocholine and $4.4 \times 10^{-4}$ *M* of DTNB in 0.1 *M* sodium phosphate buffer, pH 7.55.

The rate constant $k_i$ was calculated in the usual manner from plots of natural log $[A_0]/[A_t]$ against inhibition time, where $A_t$ is the enzymatic activity at time t and $A_0$ is the activity at time zero. The plots were analyzed by linear regression analysis using an Olivetti Programma 101 (Figure 1). The slope divided by the inhibitor concentration gave the bimolecular inhibition rate constants ($k_i$) in units of $M^{-1}$ min$^{-1}$.

**Toxicity.** Susceptible (NAIDM) and resistant (SC) strains of houseflies were treated topically by the methods of March and Metcalf (1949). Larvae of the southern house mosquito, *Culex pipiens quinquefasciatus* Say, were tested according to the methods of Mulla *et al.* (1966). Two to five replications of 20 insects each were used at each dose level, with five to seven doses used for each dosage mortality plot. Most of the insect toxicities were determined in acetone solutions. The oxalate (**7**) was dissolved in water. Mouse toxicity was determined on 3- to 6-month-old female Swiss white mice. The test compounds were dissolved in olive oil (**7** in water) and 0.1 ml was introduced orally using a syringe equipped with a

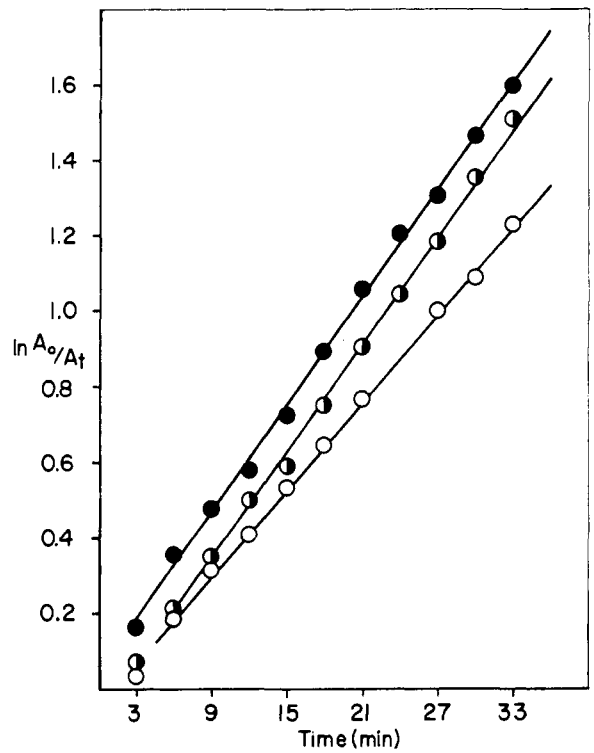



**Figure 1.** Pseudo-first-order plots of the inhibition of horse serum (●), bovine erythrocyte (◑), and housefly head (○), cholinesterases by racemic **4**.

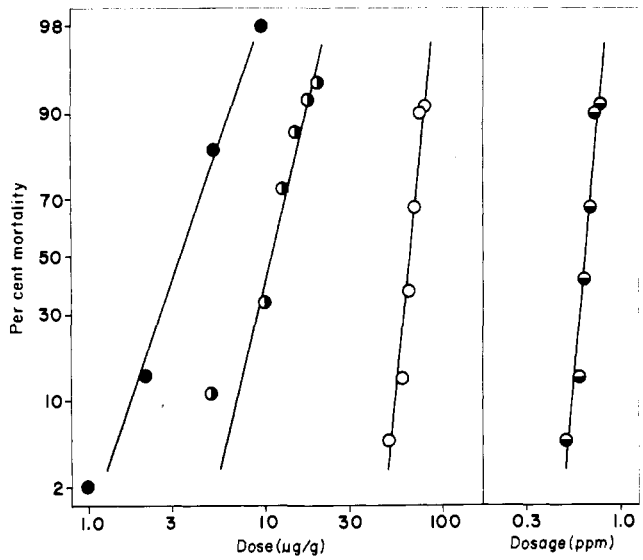



**Figure 2.** Log dose probit mortality plot of the toxicity of racemic **4** against white mice (●), susceptible (◑), and resistant (○) houseflies, and *Culex* mosquito larvae (◒).

small animal feeding needle. In all cases, mortality was determined 24 hr after treatment. The average per cent mortality of the replicates within each dose was plotted on logarithm probability paper and a line was eye-fitted to the points (Figure 2).

**Plant Systemic Activity.** Tests for systemic activity were run under greenhouse conditions on eight-leaf stage Delta Pine Smooth Leaf cotton seedlings. Two modes of application were used, topical and soil. For the topical treatment, 5 μl (10 mg in acetone if solid) of the pure material was applied directly to the stem between the soil level and the origin of the cotyledons. The compound was formulated as 5% (by weight) Attaclay granules for the soil treatment. The granules (400 or 800 mg) were uniformly applied to the soil in four equally spaced holes in the soil about 2 in. from the stem. The test organisms were *Tetranychus cinnabarinus* (adult females), *Aphis gossypii* Glover (cotton aphid, adult apterous females), *Estigmene acrea* Drury (salt marsh caterpillar, first-instar larvae), and *Bucculatrix thurberiella* Busck (cotton leaf perforator, fourth-instar larvae). Ten individuals were confined on a leaf for 48 hr in small "clip-on" cages and then mortality was evaluated. Separate tests were made 3 and 10 days after treatment and at 1-week intervals thereafter, until mortality was no longer observed relative to untreated controls.

The systemic effectiveness against pink bollworm (*Pectinophora gossypiella* Saunders) in the boll was also determined. Larvae were placed on a young boll (1 to 2 weeks) on a soil-treated plant 1 week after treatment and mortality was evaluated, relative to untreated controls, 10 to 12 days later.

**Table IV. Anticholinesterase Properties of 4, Its Resolved Isomers, 5, and 6 Against Housefly Head Acetylcholinesterase (HFAChE), Bovine Erythrocyte Acetylcholinesterase (BAChE), and Horse Serum Cholinesterase (HSChE)**

| No. | Configuration α-C | Configuration P | HFAChE, $k_i$ (95% CI) $M^{-1}$ min$^{-1}$ × $10^{-3}$ | BAChE, $k_i$ (95% CI) $M^{-1}$ min$^{-1}$ × $10^{-3}$ | HSChE, $k_i$ (95% CI) $M^{-1}$ min$^{-1}$ × $10^{-3}$ |
|---|---|---|---|---|---|
| **4** | Racemic | | 969 (29) | 31.8 (0.9) | 5.95 (0.21) |
| **4a** | + | − | 1710 (50) | 65.3 (2.1) | 17.3 (0.9) |
| **4c** | + | + | 1.35 (0.02) | 0.629 (0.020) | 0.483 (0.042) |
| **4d** | − | − | 1690 (110) | 54.5 (2.2) | 6.85 (0.30) |
| **4f** | − | + | 6.62 (0.30) | 1.45 (0.07) | 1.01 (0.18) |
| **5** | Racemic | | 556 (21) | 43.9 (1.4) | 3.67 (0.17) |
| **6** | Racemic | | 696 (39) | 126 (8) | 15.1 (0.6) |

## RESULTS AND DISCUSSION

**Cholinesterase Inhibition.** Anticholinesterase data ($k_i$) for the various isomers of **4** against housefly head (HFAChE), bovine erythrocyte (BAChE), and horse serum (HSChE) cholinesterases are presented in Table IV. Wide variability was observed in the inhibitory behavior of the various isomers to each enzyme and in the behavior of each isomer to the different enzymes. For example, the enantiomers **4c** and **4d** gave inhibition constants of $1.35 \times 10^3$ $M^{-1}$ min$^{-1}$ and $1.69 \times 10^6$ $M^{-1}$ min$^{-1}$, respectively, against HFAChE, a rate difference of about 1250-fold. Similar and consistent, though smaller, rate differences were observed between the enantiomers of **4** in their inhibition of the other two cholinesterases. The largest rate differences were caused by chirality at the phosphorus atom. The (−)-phosphorus isomers consistently gave larger inhibition constants than the corresponding (+)-phosphorus isomers; *i.e.*, compare **4a** with **4c** and **4d** with **4f**.

Using resolved inhibitors similar to compound **4**, *i.e.*, *O*-isopropyl *S*-2-(ethylthio)ethyl (+)- and (−)-methylphosphonothioate, rate differences of only 5.4-fold were observed between the enantiomers against BAChE (Boter, 1970) compared to about a 100-fold difference for **4a** and **4c** against the same enzyme. The actual bimolecular inhibition constants observed by Boter, *e.g.*, $2.2 \times 10^4$ $M^{-1}$ min$^{-1}$ for the (−)-phosphorus enantiomer, were intermediate to those of **4a** and **4c**. Against HSChE, a pseudocholinesterase, only a 1.7-fold difference in inhibition rate was observed between the enantiomers studied by Boter (1970), compared to 36-fold for **4a** and **4c**. The increased size of the groups around phosphorus, *i.e.*, *sec*-butyl compared to isopropyl and ethyl compared to methyl, possibly accounts for the larger rate differences. However, De Jong and Van Dijk (1972), using the enantiomers of *O*-isopropyl *S*-(2-trimethylammonium)ethyl (+)- and (−)-methylphosphonothioate iodide, observed a 3000-fold difference

in the inhibition rates against BAChE. The (−)-phosphorus enantiomer was the more potent inhibitor.

The effect of chirality at the α-carbon (2-butyl) on cholinesterase inhibition was dependent upon chirality at the phosphorus atom. In the two isomers with the (−)-phosphorus atom, the (+)-α-carbon compound (**4a**) gave a larger $k_i$ than the (−)-α-carbon compound (**4d**). However, in the other two isomers with the (+)-phosphorus atom, the (−)-α-carbon compound (**4f**) gave a larger $k_i$ than the (+)-α-carbon compound (**4c**). Thus, in all three cholinesterases, the order of rate constants ($k_i$) for the four fully resolved isomers of **4** is **4a** > **4d** ≫ **4f** > **4c**. The consistency of these results indicates that these asymmetric inhibitors are interacting with the active site of each enzyme in a similar manner and, therefore, the stereochemistry involved at the active site of the three enzymes is probably similar.

For *O*-2-hexyl methylphosphonofluoridate resolved at both the 2-carbon and phosphorus, a greater than 300-fold difference in inhibition rate against BAChE was observed between the enantiomers involving the phosphorus atom, but less than a 1.2-fold rate difference was observed between the enantiomers involving the 2-carbon atom (Boter, 1970). The ($R_C$) configuration was slightly more effective as an anticholinesterase than the ($S_C$). Similarly, for the fully resolved isomers of soman (*O*-3,3-dimethyl-2-butyl methylphosphonofluoridate), a 12,000-fold difference in inhibition rate was observed between the phosphorus isomers, but only a fivefold difference between the carbon isomers was observed (Keijer and Wolring, 1969). For the isomers of **4**, a 1.3- to 7-fold difference in inhibition rates was attributable to asymmetry at the carbon atom.

Several additional points of interest emerge from the $k_i$ data in Table IV. It can be seen that among the compounds listed, HFAChE is more sensitive to inhibition than BAChE, which in turn is more sensitive than HSChE. Comparing, for example, the relative rates of inhibition of the three enzymes by **4a**, HFAChE is 26-fold more sensitive to inhibition than BAChE which, in turn, is 3.8-fold more sensitive than HSChE. It can also be seen that the largest rate differences are produced by the more effective levorotatory (−)-phosphorus inhibitors (**4a, 4d**). The dextrorotatory (+)-phosphorus compounds (**4c, 4f**) showed rather low and approximately equal activity against the three enzymes, although **4f** was moderately active against HFAChE. In contrast, anticholinesterase activity increased dramatically for the (−)-phosphorus isomers (**4a, 4d**) in proceeding from HSChE to BAChE to HFAChE. Similar results have been reported by Boter (1970) and Ooms (1971).

As expected, $k_i$ for racemic **4** against each of the three cholinesterases closely equalled the weighted sum of $k_i$ values of the individual resolved inhibitors (**4a, 4c, 4d, 4f**) after adjustment for concentration. Against BAChE, the weighted sum of the $k_i$ values for the four resolved isomers is $3.06 \times 10^4\ M^{-1}\ min^{-1}$, compared to an observed $k_i$ of $3.18 \times 10^4\ M^{-1}\ min^{-1}$ for **4**. These results indicate that the individual isomers in the racemic mixture are acting independently of each other.

Against all of the enzymes used, $k_i$ for the sulfone (**6**) was larger than that for the sulfoxide (**5**), probably because of the greater reactivity of **6**. Against BAChE and HSChE, $k_i$ of **5** was larger than that for **4**; however, against HFAChE, $k_i$ for both **5** and **6** was smaller than that for **4**.

Compounds **4c** and **4f** against HSChE gave nonlinear kinetics with the inhibition rate decelerating at longer incubation times and, therefore, the $k_i$ values that are given are approximate. The nonlinearity is reflected in depressed values of $r^2$ and the large 95% confidence intervals (CI). This curving may be attributed to multiple kinetic forms of the enzyme (Main, 1969); however, the forms were not resolved. The regression lines for the other $k_i$ determinations all had $r^2$ values >0.99 and 95% CI of from 1 to 6%.

**Table V. Toxicity of 4, Its Isomers, 5, 6, and 7, to Houseflies (Topical, Adult, Female), Mosquito Larvae, and White Mice (Oral)**

| No. | Configuration α-C | Configuration P | *Musca domestica* NAIDM, $LD_{50}$ μg/g | *Musca domestica* SC, $LD_{50}$ μg/g | *Culex* larvae, $LC_{50}$ ppm | Mouse ♀, $LD_{50}$ mg/kg |
|---|---|---|---|---|---|---|
| **4** | Racemic | | 10.8 | 78 | 0.65 | 3.1 |
| **4a** | + | − | 6.7 | 33 | 0.25 | 3.2 |
| **4b** | + | ± | 9.4 | 84 | 0.66 | |
| **4c** | + | + | >500 | >500 | >1 | 110 |
| **4d** | − | − | 6.9 | 42 | 0.15 | 2.8 |
| **4e** | − | ± | 12.1 | 122 | 0.57 | |
| **4f** | − | + | >500 | >500 | >1 | 125 |
| **5** | Racemic | | 39 | 310 | >1 | |
| **6** | Racemic | | 27 | >500 | >1 | |
| **7** | Racemic | | >500 | >500 | >1 | 1.0 |

**Toxicity.** In general, toxicity to either insects or to mice paralleled anticholinesterase activity, *i.e.*, the stronger inhibitors proved to be more effective toxicants to each test animal (Table V). The (+)-phosphorus isomers (**4c, 4f**) were nontoxic at the maximum dosages used to all of the insects tested and only moderately toxic to mice. The (−)-phosphorus isomers (**4a, 4d**) were highly toxic to susceptible houseflies and mice and moderately toxic to resistant houseflies and mosquito larvae. Small differences in toxicity owing to chirality at the 2-butyl carbon atom also were noted.

Because the (+)-phosphorus isomers, (**4c, 4f**) were nontoxic to insects at the maximum dosages used, it is not possible to determine whether a direct correlation exists between *in vitro* anticholinesterase activity of the four isomers of **4** and insecticidal activity. For example, against HFAChE, $k_i$ ($M^{-1}\ min^{-1}$) for **4a** and **4c** are $1.71 \times 10^6$ and $1.35 \times 10^3$, respectively, a difference of about 1270-fold. In comparison, the $LD_{50}$ values (μg/g) of **4a** and **4c** to houseflies are 6.7 and >500, respectively, a difference of >75-fold. It should be pointed out that none of the houseflies treated with **4c** at 500 μg/g were affected and, therefore, the ratio of toxicity of **4a** and **4c** is probably significantly greater than 75.

A similar comparison may be made between inhibition of BAChE and mouse toxicity. Owing to the appreciable toxicity of the (+)-phosphorus enantiomers, calculation of finite values for the ratio of toxicities was possible. Against BAChE, **4a** ($k_i$ $6.53 \times 10^4\ M^{-1}\ min^{-1}$) was 104-fold more effective as an inhibitor than **4c** ($k_i$ $6.29 \times 10^2\ M^{-1}\ min^{-1}$) and 34-fold more toxic to the white mouse (3.2 and 110 mg/kg, respectively, for **4a** and **4c**). Similarly, **4d** was 45-fold more effective as an anticholinesterase than **4f** and 37-fold more toxic to the white mouse. Considering that biological systems are involved, the relationship between inhibition of a mammalian acetylcholinesterase, BAChE, and toxicity to the white mouse is quite good and suggests that other factors involved in intoxication (metabolism, translocation, etc.) are similar for the different isomers in the mouse.

Although good correlation is observed between BAChE inhibition and mouse toxicity for the isomers of **4**, the actual toxicities of these isomers to the mouse are greater than that expected from their anticholinesterase activities. On an equal weight basis all of the isomers were substantially more toxic to mice than to houseflies, even though they were much more potent inhibitors of HFAChE compared to BAChE. Any explanation of this observation would be pure conjecture at this time.

The toxicities of the racemic mixtures (**4, 4b, 4e**) to the insects tested are approximately equal to the weighted

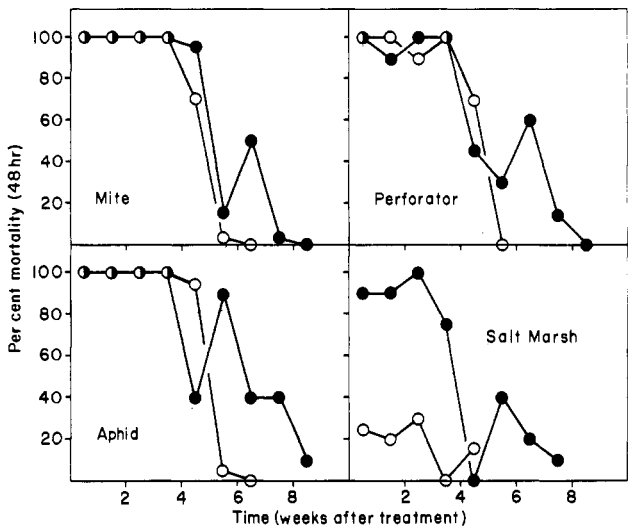



**Figure 3.** Cotton plant systemic insecticidal activity of racemic **4** as a soil treatment of 400 (○) and 800 (●) mg of 5% Attaclay granules per pot.

sums of the toxicities of the component isomers. These data indicate that the portion of the observed toxicity exerted by each of the isomers in a mixture is independent of the toxicity exerted by each of the other component isomers.

Racemic **7**, an amine salt, was nontoxic to all of the insects tested, but was very toxic to mice. It is possible that this charged organophosphorus ester was not able to penetrate the insect cuticle.

**Plant Systemic Activity.** The results obtained from the plant systemic screen following granular treatment of the soil with **4** are shown in Figure 3. The data shown are typical of most of the data obtained, *i.e.*, early consistent high mortality was observed followed by a rather abrupt loss in activity to less than 50% kill. With this type of data, the number of weeks with 50% or greater mortality of the test arthropods is a reasonable measure of plant systemic activity. The longer the mortality is sustained at a high level, the better the toxicant is as a plant systemic insecticide. This information is summarized in Table VI for four test arthropods after stem and soil application of **4**, its four optical isomers, and **7**. Similar data for aldicarb, a well known plant systemic insecticide, are included for comparison.

The dextrorotatory (+)-phosphorus isomers (**4c, 4f**) were completely inactive as plant systemic insecticides. It is not certain whether the absence of activity is attributable to decreased rates of absorption, translocation, and perhaps metabolism in the plant owing to stereoisomerism or whether the translocated isomers (or their metabolites) themselves are nontoxic owing to their poor anticholinesterase properties. In contrast, the levorotatory (−)-phosphorus isomers (**4a, 4d**) were highly active, a finding which is consistent with their high anticholinesterase activity toward HFAChe. Since it is unlikely that the physical properties of the individual isomers which affect movement in plants vary to any large extent, the poor systemic activity of **4c** and **4f** probably is attributable to poor anticholinesterase activity.

Little difference in the duration of activity between the two different dosages of 800 and 400 mg was found for any of the compounds, although the activity of the 800-mg treatment occasionally lasted an additional week. The activities of 4a and **4d**, the (−)-phosphorus isomers, are similar, with 4a slightly more effective against salt marsh caterpillars and **4d** more effective against mites. The striking observation here is that persistence of systemic activity following treatment with **4a** or **4c** was approximately three times longer than after treatment with racemic **4** at 800 mg, even though the combined amount of **4a** and **4c** in **4** at the higher dosage is equal to 400 mg. As soil insecticides, **4a** and **4d** are superior to aldicarb against mites and aphids in these tests, but slightly inferior to aldicarb against perforators and salt marsh caterpillars.

When applied to the stem, racemic **4** was as effective as **4a** and **4d**, but equal or inferior to aldicarb. An interesting

**Table VI. Plant Systemic Activity, the Number of Weeks with a >50% Mortality of the Test Arthropods. The Arthropods are *Tetranychus cinnabarinus* (Mite), *Aphis gossypii* (Aphid), *Bucculatrix thurberiella* (Perforator), and *Estigmene acrea* (Salt Marsh Caterpillar) (4 = 400 mg Soil Treatment, 8 = 800 mg Soil Treatment, S = Stem Treatment)**

| Compd | Treatment | Mite | Aphid | Perforator | Salt marsh caterpillar |
|---|---|---|---|---|---|
| **4** | 4 | 4 | 4 | 4 | 0 |
| | 8 | 4 | 5 | 4 | 3 |
| | S | 4 | 4 | 4 | 1 |
| **4a** | 4 | 12[a] | 15 | 12 | 2[b] |
| | 8 | 12 | 16 | 12 | 4[a] |
| | S[c] | 3 | 3 | 3 | 2 |
| **4c** | 4, 8, S | All completely inactive | | | |
| **4d** | 4 | 14[a] | 15 | 12[a] | 1 |
| | 8 | 14 | 15 | 12 | 1 |
| | S | 3 | 5[a] | 2 | 1 |
| **4f** | 4, 8, S | All completely inactive | | | |
| **7** | 8[d] | 7 | 0 | 7 | 0 |
| | S | 36 | 0[e] | 36+ | 26–33[f] |
| Aldicarb | 4 | 10 | 14 | 15 | 3 |
| | 8 | 10 | 15 | 16 | 4 |
| | S | 3 | 7 | 14 | 0 |

[a] Variable, but with at least half of the points 90% +. [b] Maximum to only 60% mortality. [c] Phytotoxic, plant dead in 3 weeks. [d] No mortality for first 3 weeks. [e] Average mortality about 40% for some 14 weeks. [f] Variable for the last 7 weeks; mortality averaging at least 50%.

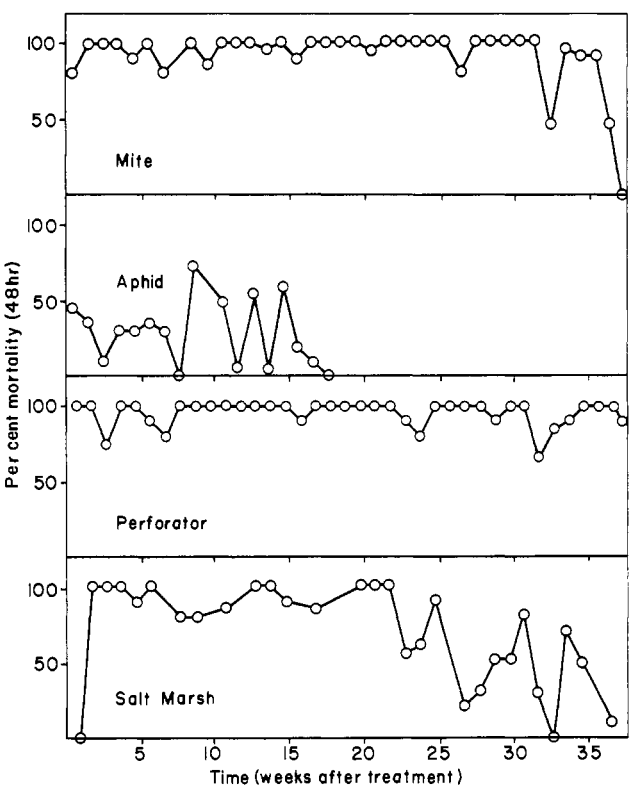



**Figure 4.** Cotton plant systemic insecticidal activity of **7** after stem treatment (10 mg in acetone) against the four arthropods tested.

**Table VII. Screen for Plant Systemic Activity Against the Pink Bollworm, *Pectinophora gossypiella*, in the Boll. Bolls Infested 7 Days After Treatment**

| Compd | Treatment | % larval mortality: Treatment | % larval mortality: Control | Days after infestation |
|---|---|---|---|---|
| **4** | 5λ on stem | 75 | 16 | 10 |
| **4a** | 800 mg gran. | 100 | 12 | 10 |
| **4d** | 800 mg gran. | 100 | 6 | 10 |
| **7** | 10 mg in water on stem | 7 | 21 | 12 |
| **7** | 10 mg in acetone-water on stem | 7 | 6 | 12 |

phenomenon was that **4a**, by stem application, was phytotoxic and killed the plant within 3 weeks, while **4d** was not. However, within the 3-week period, a nearly total kill of the test arthropods was observed after treatment with **4a** in spite of its effect on the plant.

Compound **7** gave very interesting results. As a soil treatment, **7** evidently was slow to absorb and/or translocate, since no mortality was observed for the first 3 weeks, followed by a 4-week period in which mortality was observed only against mites and perforators. As a stem treatment, however, **7** was extremely effective, giving long term control (26–36 weeks) of mites, perforators, and salt marsh caterpillars. However, it was nearly ineffective against aphids. These results are shown graphically in Figure 4. The reason for the low activity of **7** as a soil systemic and as a toxicant against aphids is not known. Ghosh and Newman (1955) reported that amiton (*O,O*-diethyl *S*-2-(diethylamino)ethyl phosphorothioate), an analog of **7**, was active against aphids both as a soil systemic and as a contact poison. Except for the high mammalian toxicity of **7** and related compounds, *e.g.*, amiton, examination of other organophosphorus esters of this type might prove fruitful in the development of new systemic insecticides.

Table VII shows the results of the plant systemic screen against the pink bollworm larvae (*Pectinophora gossypiella*). It can be seen from the data that **4a** and **4d** show promise against this insect, while **7** does not.

## CONCLUSION

Chirality in the organophosphorus ester *O*-2-butyl *S*-2-(ethylthio)ethyl ethylphosphonothioate (**4**) evidently has a profound effect on its toxicological properties. Of the two asymmetric centers in this molecule, the effect of chirality on anticholinesterase activity and toxicity was much more pronounced at the phosphorus atom than at the 2-butyl carbon atom. On an overall basis, toxicological activity, *i.e.*, toxicity to insects and mice, and plant systemic activity of the individual isomers of **4** appeared to be related to their ability to inhibit stereoselectively the target enzyme, acetylcholinesterase, suggesting that other factors involved in intoxication and detoxication of the individual isomers are similar. As plant systemic insecticides, however, the more toxic (−)-phosphorus isomers were far more effective as translocated insect toxicants than predictable from the activity of the racemic mixture alone. This enhancement of systemic activity, produced by resolution of the isomers, may have practical implications and deserves further study.

## LITERATURE CITED


Aaron, H. S., Michel, H. O., Witten, B., Miller, J. I., *J. Amer. Chem. Soc.* **80**, 456 (1958a).
Aaron, H. S., Shryne, T. M., Miller, J. I., *J. Amer. Chem. Soc.* **80**, 107 (1958b).
Aldridge, W. N., *Biochem. J.* **7**, 451 (1950).
Beckett, A. H., Michard, M., Clithrow, J. W., *Biochem. Pharmacol.* **17**, 1601 (1968).
Boter, H. L., Ph.D. Dissertation, University of Leiden, 1970.
De Jong, L. P. A., Van Dijk, C., *Biochem. Biophys. Acta* **268**, 680 (1972).
Ellman, G. L., Courtney, K. D., Andres, V., Jr., Featherstone, R. M., *Biochem. Pharmacol.* **7**, 88 (1961).
Fukuto, T. R., Metcalf, R. L., *J. Econ. Entomol.* **52**, 739 (1959).
Ghosh, R., Newman, J. F., *Chem. Ind.* 118 (1955).
Hassan, A., Dauterman, W. C., *Biochem. Pharmacol.* **17**, 1431 (1968).
Hilgetag, G., Lehmann, G., *J. Prakt. Chem.* **280**, 224 (1959).
Hoffman, F. W., Wadsworth, D. H., Weiss, H. D., *J. Amer. Chem. Soc.* **80**, 3945 (1958).
Keijer, J. H., Wolring, G. Z., *Biochem. Biophys. Acta* **185**, 465 (1969).
Main, A. R., *J. Biol. Chem.* **244**, 525 (1969).
March, R. B., Metcalf, R. L., *Calif. Dept. Agr. Bull.* **38**, 1 (1949).
Michel, H. O., *Fed. Proc.* **14**, 255 (1955).
Moorefield, H. H., *Contrib. Boyce Thompson Inst.* **18**, 463 (1957).
Mulla, M. S., Metcalf, R. L., Geib, A. F., *Mosquito News* **26**, 236 (1966).
Ooms, A. J. J., *Bull. W. H. O.* **44**, 113 (1971).
Ooms, A. J. J., Boter, H. L., *Biochem. Pharmacol.* **14**, 1839 (1965).
Perry, B. J., Reesor, J. B., Ferron, J. L., *Can. J. Chem.* **41**, 2299 (1963).
Theilacker, W., Winkler, H. G., *Chem. Ber.* **87**, 690 (1954).


Received for review April 6, 1973. Accepted July 9, 1973. This investigation was supported in part by Training Grant No. ES00047 from the National Institute of Environmental Health Sciences, Research Triangle Park, N. C., Research Grant No. R801837 from the Environmental Protection Agency, Washington, D. C., and by Research Grant No. 73-261 from Cotton Incorporated, Raleigh, N. C.