BIOORGANIC CHEMISTRY 13, 344–352 (1985)

Phosphinyliminodithiolane Insecticides: Oxidative Bioactivation of Phosfolan and Mephosfolan

GREG W. GORDER, IAN HOLDEN, LUIS O. RUZO, AND JOHN E. CASIDA

Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, California 94720

Received March 20, 1985

2-(Diethoxyphosphinylimino)-1,3-dithiolane (phosfolan) and its 4-methyl analog (mephosfolan) are proinsecticides as determined by microsomal mixed-function oxidase (MFO) activation to potent acetylcholinesterase (AChE) inhibitors. They are similarly activated by peracid oxidation which yields the sulfoxide and sulfone derivatives. The hydrolytically unstable *S*-oxides are irreversible AChE inhibitors that are 160- to 47,000-fold more potent than phosfolan and mephosfolan. MFO *S*-oxidation is indicated for both proinsecticides by (a) NADPH-dependent increases in potency as AChE inhibitors to an extent expected of sulfoxides, and (b) formation of the *S*-oxide hydrolysis product diethyl phosphoramidate.

INTRODUCTION

The commercial systemic insecticides phosfolan (**1**) and mephosfolan (**2**) (Fig. 1) appear to poison as acetylcholinesterase (AChE) inhibitors yet they lack a "classical" acyl moiety or leaving group for enzyme phosphorylation (*1*). This anomaly suggests that they may be proinsecticides. Metabolism of phosphinyliminodithiolanes **1** and **2** is reported to result primarily in cleavage of the P—N bond to give diethyl phosphate and the corresponding iminodithiolane (*2–6*), a detoxification process. In addition, **2** is detoxified by oxidation to its hydroxymethyl and hydroxymethylvinylene derivatives (*3, 4*). It was therefore surprising to discover in enzymatic studies that **1** and **2** undergo metabolic activation (*7*). We have examined this bioactivation by procedures recently used in studying the *S*-alkyl phosphorothiolates (*8, 9*). These are (a) metabolism of the proinsecticide in a microsomal mixed-function oxidase (MFO) system alone and on coincubation with AChE, and (b) peracid oxidation as a possible chemical model for biooxidation reactions.

RESULTS

Oxidative Activation (Fig. 2)

1 and **2** are poor AChE inhibitors but incubation with microsomes and NADPH increases their anti-AChE potency by more than 100-fold as compared to incuba-

0045-2068/85 $3.00

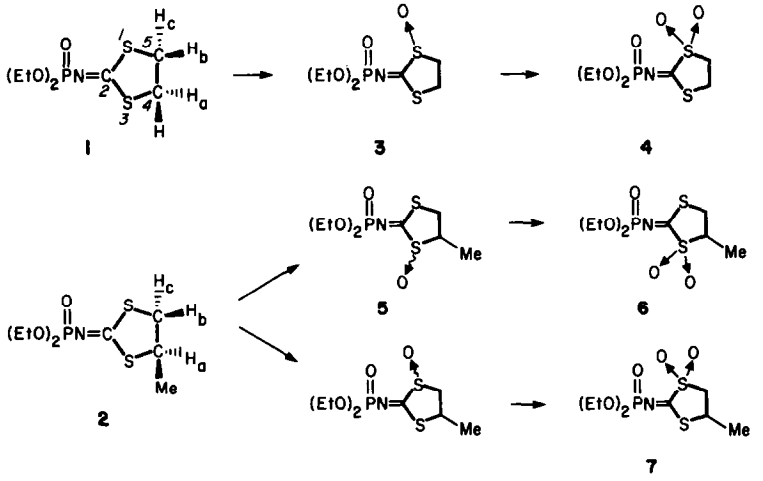

FIG. 1. Phosfolan (1) and mephosfolan (2) and their sulfoxide and sulfone derivatives (3–7).

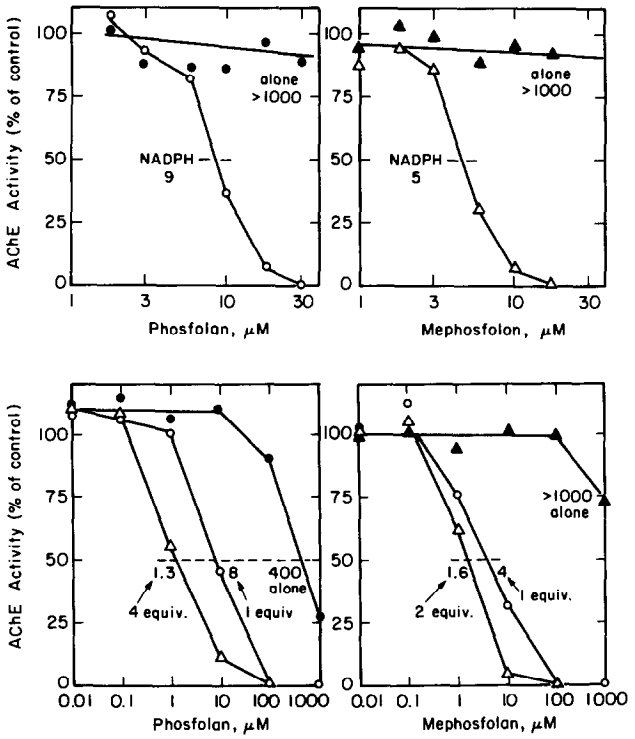

FIG. 2. Microsomal oxidase (top) and peracid (MCPBA, bottom) activation of phosfolan (1) and mephosfolan (2). The oxidase is not active without NADPH. Data points are means of duplicate samples. Comparable data for 50% inhibition (I_{50}'s) with standard compounds under the same conditions (30-min preincubation of inhibitor and enzyme at 37°C prior to substrate addition) are as follows (μM values and 95% confidence limits calculated by linear regression): **1** 270 (230–310), **2** 520 (440–630), **3** 2.6 (2.1–3.1), **4** 0.050 (0.038–0.066), **5** 5.3 (3.3–8.7), **6** 0.92 (0.83–1.02), **7** 0.034 (0.022–0.054), $(EtO)_2P(O)NHC(O)SCH_2CH_2SO_2H$ 95 (61–147), and $(EtO)_2P(O)NH_2$ > 3300. The inhibition values vary somewhat in the presence of microsomes and of the chlorofom–DMSO mixture used with MCPBA oxidation.

tion with microsomes alone. The AChE inhibitory potency of **1** and **2** is also increased by treatment with *m*-chloroperbenzoic acid (MCPBA) but not with ozone, hydrogen peroxide, bis(tributyltin) oxide/bromine, aqueous potassium permanganate, sodium dichromate, sodium hypochlorite, or Fenton's reagent or on photooxidation in organic solvents. The extent of activation varies with the molar ratio of MCPBA and with equimolar oxidant is similar to the microsomal system whereas with 2–4 equivalents of MCPBA there is an additional potency increase of 2- to 6-fold.

Preparation of Phosfolan and Mephosfolan Sulfoxides and Sulfones (Fig. 1)

Treatment of **1** with equimolar MCPBA or monoperphthalic acid in chloroform quickly yields **3** as a single major product that decomposes in the presence of phthalic or *m*-chlorobenzoic acid, necessitating rapid isolation of **3** from the reaction mixture. A similar process occurs with 2 equivalents of MCPBA, perhaps including further oxidation of the decomposition products but without accompanying change in the ^{31}P NMR spectrum. Treatment of **2** with equimolar perphthalic acid gives **5**, which was isolated, plus products having two other similar

TABLE 1

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND MASS SPECTROMETRY DATA FOR PHOSFOLAN, MEPHOSFOLAN, AND THEIR *S*-OXIDES

Compound	^1H NMR[a]	^{13}C NMR[a] of C=N	^{31}P NMR[a]		
			$CDCl_3$	D_2O	$[MH]^+$[b]
1	3.65 (s)(H, Ha, Hb, Hc)	190.6	−1.5	−1.2	256
2	1.56 (d), J = 6.7 (CH_3); 4.15 (m), J = obs (Ha); 3.31 (dd), J = 11.7, 8.1 (Hb); 3.63 (ddd), J = 11.7, 5.1, 1.0 (Hc)	190.7	−1.5	−1.0	270
3[c]	3.44–3.47 (m), J = obs (H, Ha); 3.61 (ddd), J = 12.4, 5.2, 6.8 (Hb); 3.95 (ddd), J = 12.4, 5.3, 7.2 (Hc)	189.0	−1.5	−1.1	272
4[c]	3.42 (t), J = 6.2 (H, Ha); 3.52 (t), J = 6.2 (Hb, Hc)	174.6	0.5	1.1	288
5	1.60 (d), J = 7.0 (CH_3); 3.25–3.62 (m)(Ha, Hb, Hc)	188.1	−1.6	−1.0	286
6	1.60 (d), J = 7.0 (CH_3); 3.31 (m), J = 8, 7, 5.5 (Ha); 3.21 (dd), J = 12, 8 (Hb); 3.51 (dd), J = 12, 5.5 (Hc)	175.0	0.4	1.0	302
7	1.64 (d), J = 7.0 (CH_3); 3.94 (m), J = 10, 7, 5.2 (Ha); 2.97 (dd), J = 14, 10 (Hb); 3.65 (ddd), J = 14, 5.2, 1 (Hc)	176.2	0.3		302

[a] NMR chemical shifts (δ ppm) and coupling constants [J (in Hz)] taken in $CDCl_3$: ^1H, 300 MHz ($CHCl_3$ δ 7.25); ^{13}C, 75 MHz ($CDCl_3$ δ 77); ^{31}P, 121.5 MHz (external 1% trimethyl phosphate in $CDCl_3$ or D_2O δ 0.0); ^{13}C and ^{31}P spectra with broad band decoupling.

[b] Quasimolecular ion determined by chemical ionization (methane).

[c] ^1H NMR assignments based on oxidation of sulfur at the 1 position and both the sulfoxide oxygen and H appearing on the α face.

^{31}P NMR signals which were not isolated but undoubtedly include an epimer of **5** and the two other stereoisomers. With 3 to 5 equivalents of MCPBA, sulfones **4**, **6**, and **7** become the major products, reaching 40–50% yield.

Structural assignments for sulfoxides **3** and **5** and sulfones **4**, **6**, and **7** are based on NMR and MS (Table 1). In addition, ir indicates the retention of C=N (**1** 1560 cm^{-1}, neat; **3** 1600 cm^{-1}, $CDCl_3$; **4** 1610, neat, 1620 cm^{-1}, KBr) and the presence of a sulfone (**4** 1335 and 1140 cm^{-1}, neat). Identifications of **6** and **7** are based on ^{13}C NMR in chloroform-d (*10*) together with DEPT (*11*) ($\theta = 3\pi/4$) to determine carbon multiplicities [**6** 51.5 (C-4), 30.9 (C-5), 11.2 (CH_3); **7** 34.6 (C-4), 52.8 (C-5), 20.0 (CH_3)]. Similarly **5** is a 3-sulfoxide oxidized on the α face (methyl on β face) [59.3 (C-4), 31.9 (C-5), 14.0 (CH_3)]. ^{13}C NMR of **1** and **2** in acetone-d_6 (TMS = 0 ppm) shows rapid thermal interconversion between the *syn* and *anti* isomers at 20°C but not at −90°C [20°C **1** 38.3 (C-4 and 5); **2** 49.8 (C-4), 44.3 (C-5), 19.7 (CH_3); −90°C **1** 40.5 and 36.1 (C-4 and 5); **2** 52.0 and 47.6 (C-4), 46.5 and 42.1 (C-5), 19.8 and 19.6 (CH_3)]. The oxidized products (**3–7**) apparently occur as only one geometrical isomer as shown by a similar ^{13}C NMR experiment on **4** and the ^1H NMR shows 16-line multiplets (resolution enhanced) for diastereotopic ethoxy methylene protons in **3–7** (δ 4.15 to 4.20) versus "quintets" for equivalent ethoxy methylene protons in **1** and **2** (δ 4.10 and 4.15). In addition, there is a doubling of resonances at −90°C for **1** and **2** in their ^1H NMR spectra and for **2** in its ^{31}P NMR spectrum.

Potency and Kinetics of AChE Inhibition by Phosfolan and Mephosfolan Sulfoxides and Sulfones (Figs. 2 and 3, Table 2)

The sulfoxides formed with 1 equivalent of peracid have similar AChE-inhibitory potencies to the 1-equivalent MCPBA mixture and the activated microsomal mixture. The higher AChE inhibitory activity observed with additional peracid is consistent with the greater potency of the sulfones. The sulfoxides and sulfones

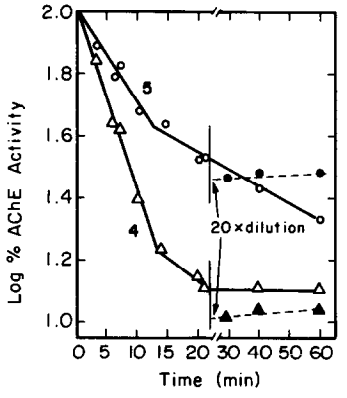

FIG. 3. Reaction rate of electric eel AChE with phosfolan sulfone (**4**, 1.8×10^{-7} M) and mephosfolan sulfoxide (**5**, 8.4×10^{-6} M) and lack of reactivation following 20-fold dilution of the partially inhibited enzyme at 22 min. Points from 0 to 21 min are composites from two experiments including the dilution study.

TABLE 2

OXIDATIVE ACTIVATION OF PHOSFOLAN AND MEPHOSFOLAN AS ACETYLCHOLINESTERASE INHIBITORS

Compound	k_i (M^{-1} min^{-1})[a]	Activation factor
1	$8.5 \pm 0.6 \times 10^1$	—
3	$1.4 \pm 0.3 \times 10^4$	160
4	$8.1 \pm 0.4 \times 10^5$	9,500
2	$3.6 \pm 0.3 \times 10^1$	—
5	$7.7 \pm 1.1 \times 10^3$	210
6	$4.5 \pm 0.3 \times 10^4$	1,250
7	$1.7 \pm 0.3 \times 10^6$	47,000

[a] k_i values are derived from the slopes of the least-squares regression lines of log % residual activity versus time for three inhibitor concentrations giving first-order kinetics for 3-, 6-, and 10-min readings (**1** and **2** also 20 min). They are reported as means ± standard errors.

hydrolyze to thiolcarbamates [e.g., $(EtO)_2P(O)NHC(O)SCH_2CH_2SO_2H$] and eventually diethyl phosphoramidate (*12*). Both hydrolyses represent detoxifications with the thiolcarbamate being much less potent than **3–7** but more potent than **1** or **2** and diethyl phosphoramidate being inactive.

The reactions of **4** and **5** with AChE follow first-order kinetics for 10–15 min. The partially inhibited enzyme does not reactivate following dilution after 22 to 60 min. Inhibitor reactivities (k_i's) based on the initial linear rates of AChE inhibition

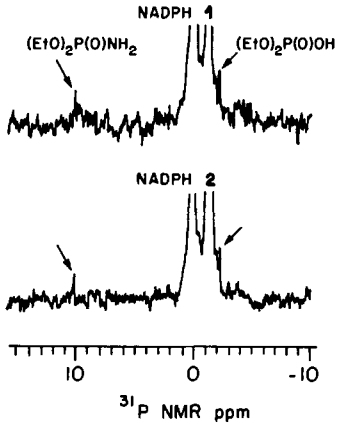

FIG. 4. ^{31}P NMR spectra (pulse angle 30°, recycle delay 3 s) of microsomal reaction mixtures of phosfolan (**1**) (4000 pulses) and mephosfolan (**2**) (2700 pulses) with microsomes and NADPH. Diethyl phosphate and diethyl phosphoramidate (δ -2.7 and 10.0, respectively) are detected only when both microsomes and NADPH are present (-NADPH spectrum, 12,000 pulses, not shown). Sulfoxides and sulfones **3–7**, if present, would be obscured by the NADPH or substrate signals.

show that the sulfoxides and sulfones are 160- to 47,000-fold more potent than **1** or **2**. Phosfolan sulfoxide (**3**) and mephosfolan 3-sulfoxide (**5**) have similar k_i's, as do the more potent sulfones **4** and **7**, but mephosfolan 3-sulfone (**6**) has intermediate potency. Inhibition of AChE by sulfone **4** (10^{-6} M, 60 s, 37°C) was blocked in a concentration-dependent manner by the natural substrate, acetylcholine (ACh), with results as follows (ACh concentration and percent AChE activity): 0 mM, 11 ± 1%; 0.25 mM, 31 ± 4%; 1 mM, 76 ± 4%; and 4 mM, 87 ± 8%.

Microsomal Metabolism (Fig. 4)

Direct examination of the microsomal incubation mixtures by ^{31}P NMR reveals that both **1** and **2** are converted to new products, in the presence but not in the absence of NADPH. The ^{31}P NMR shifts of the NADPH-dependent products, which are formed in small yields, correspond to diethyl phosphate and the *S*-oxide hydrolysis product, diethyl phosphoramidate (confirmed by GC–MS).

DISCUSSION

Phosfolan and mephosfolan are proinsecticides based on their activation to more potent AChE inhibitors by microsomal MFOs. Similar activation occurs on *S*-oxidation by peracids. Reactivities with AChE (k_i's) and 50% AChE inhibition levels (I_{50}'s) show that the *S*-oxides rather than the parent compounds or hydrolysis products are the potent inhibitors in the peracid system. The reactivities of phosfolan *S*-oxides are consistent with comparable mephosfolan *S*-oxides except that **6** is considerably less potent than **4** or **7**. This might result from a less favorable steric interaction for **6** at the AChE active site or a less reactive imino carbon. Detection of diethyl phosphoramidate as a NADPH-dependent MFO metabolite also implicates the *S*-oxides as bioactivation products because the *S*-oxides (obscured in the ^{31}P NMR spectrum by **1** or **2** or NADPH) are rapidly hydrolyzed to the stable phosphoramidate (*12*). Further evidence for MFO *S*-oxidation is provided by the similar potencies of **1** and **2** activated by MFOs or by equivalent peracid to those of the pure sulfoxides.

The deviation from linear inhibition kinetics (*13, 14*) after 10–15 min is attributable to the rapid hydrolysis of **3–7** (*12*) rather than to reactivation of the inhibited enzyme as established by the lack of recovery on dilution. AChE derivatization probably occurs at or near the ACh binding or reaction site since the substrate protects the enzyme from inhibition by **4** (*13, 15*). These findings are consistent with phosphorylation of AChE but do not rule out other addition reactions at the active site (*12*).

Peracid synthesis of the *S*-oxides of **1** and **2** appears to be influenced by their stereochemistry and similar effects are possible during biooxidation. Geometrical isomerization occurs in **1** and **2** but not in **3–7**, suggesting that nitrogen inversion results when the iminodithiolane ring is not oxidized. The rate of nitrogen inversion may also depend on a substituent forming a conjugated system with the iminodithiolane since it occurs in 2-phenyl- and 2-benzoylimino-1,3-dithiolanes

but not in 2-alkylimino-1,3-dithiolanes (*16*). Peracid oxidation is apparently influenced by nitrogen inversions since oxidation occurs at S-1 or S-3 in **2** (e.g., leading to **6** or **7**) but neither **1** nor **2** gives disulfoxides. The sulfoxides may therefore exist as *anti* isomers resulting in steric hindrance to oxidation at the *syn* sulfur atom. The failure to isolate the 1-sulfoxide and the lower yields of **7** than **6** suggest that peracids may preferentially oxidize S-3 of **2**. It is also possible that the 1-sulfoxides are less stable than **5** and its epimer in the reaction mixture.

EXPERIMENTAL

Spectroscopy and Analysis

NMR spectroscopy was carried out with a Bruker WM-300 spectrometer and MS with a Hewlett–Packard 5985 instrument under CI conditions with methane at 230 eV. GC–MS utilized a 10-m high-performance methyl silicone capillary column at 100 to 220°C (20°C/min).

Chemicals

Structures for the compounds are given in Fig. 1. **1** and **2** from Chem Service (Westchester, Pa.) were obtained >99% pure by HPLC (C_{18} column, acetonitrile–water 1 : 1 and 1.3 : 1, respectively). Sulfoxides **3** and **5** were obtained in ~10% yield on treatment of **1** and **2** (0.4 mmol) with equivalent monoperphthalic acid (*17*) in dry chloroform–ether 4 : 1 (4 ml) with reaction for 5 min at 0°C and 25 min at 20°C followed by solvent evaporation, filtration, and HPLC (μPorasil, −25°C, acetonitrile–chloroform 3 : 2). The same procedure but in acetone with 4 equivalents of MCPBA gives sulfones **4** and **6** in 20% yield and **7** in 10% yield following isolation by transfer to chloroform, extraction with 5% $NaHCO_3$ and water (both at 5°C, 3 ×, v/v) and HPLC (μPorasil, 20°C, ethyl acetate to ethyl acetate–acetonitrile 2 : 3). An improved procedure to obtain **3** in 40% yield involved treatment of 0.4 mmol of **1** in 1 ml of dry chloroform with 0.4 mmol of purified MCPBA (*18*) for 10 min at 20°C followed by addition of 1.2 mmol of dry dimethyl sulfoxide (DMSO), and rapid purification by HPLC (ethyl acetate–acetonitrile as above and then acetonitrile–chloroform 3 : 2). In each HPLC system the sulfoxides elute after **1** and **2** and sulfone **7** before **6**. The *S*-oxides were all >99% pure immediately after HPLC isolation.

$(EtO)_2P(O)NHC(O)SCH_2CH_2SO_2H$ is obtained in quantitative yield by allowing **4** (10 mM) to stand in water (~pH 2 due to product formation) for 10 h at 20°C followed by lyophilization to dryness. Diethyl phosphorochloridate was treated with ammonia to prepare diethyl phosphoramidate (*19*) or with bicarbonate to obtain diethyl phosphate.

Biochemical Studies

AChE inhibition was studied with 4 units of electric eel enzyme (Sigma Chemical Co., St. Louis, Mo.) in 2.5 ml of buffer by introducing the inhibitor in 10 μl of

acetone, incubating at 37°C, removing 0.08-unit aliquots of enzyme at appropriate times, and measuring residual AChE activity by its rate of acetylthiocholine (ATCh) hydrolysis (*20*). The incubation times for enzyme and inhibitor before substrate addition were: 30 min for I_{50}'s; 3, 6, 10, 20, 40, and 60 min for k_i's; 7, 14, and 21 min with 20-fold dilution at 22 min and again at 30, 40, and 60 min to determine possible reactivation. ACh protection of AChE was studied by adding the inhibitor in 2 μl of acetone to 2.5 ml of buffer containing ACh (0, 0.25, 1, or 4 mM) and immediately introducing 2.5 units of electric eel enzyme in 12.5 μl of buffer, incubating 60 s at 37°C, stopping the reaction by 100-fold dilution with 37°C buffer, and measuring the rate of ATCh hydrolysis relative to controls containing ACh but lacking inhibitor. Similar results were obtained in these direct inhibition studies with pH 7.4, 50 mM Hepes and phosphate buffers.

The peracid-activation system involved treating **1** or **2** (25 μmol) in acetone (0.2 ml) with MCPBA (0, 1, 2, or 4 equivalents) for 30 min at 20°C and then addition of 100 equivalents of DMSO (20 min, 20°C) to quench any unreacted MCPBA before diluting in acetone for assay of aliquots. Percentage inhibition was calculated based on controls in which the MCPBA was quenched and the same acetone–DMSO solvent ratios were used.

The microsomal oxidase incubation mixtures in 2.5 ml of pH 7.4, 50 mM Hepes buffer (pH 7.4, 50 mM phosphate buffer gave similar results) contained 1 mg of microsomal protein (*21*) (rat liver, no induction), 4 units AChE, and 0 or 2 μmol NADPH (for control and active oxidase incubations, respectively). The substrates were added in 10 μl of acetone and the remaining AChE activity was assayed as previously described after incubation for 30 min at 37°C. Microsomal incubations for NMR and GC–MS analyses were carried out as above but with 1 μmol **1** or **2** and buffer including deuterium oxide but without AChE. Six reaction mixtures without NADPH were pooled for NMR analysis in a 20-mm tube as were six mixtures with NADPH. Similar samples were extracted with ether, dried, concentrated, and analyzed by GC–MS with retention time and ion profile comparisons to standards.

ACKNOWLEDGMENTS

Helpful advice was provided by Marian Saeman Bleeke, formerly of this laboratory. This research was supported in part by Grant P01 ES00049 from the National Institutes of Health.

REFERENCES

1. FEST, C., AND SCHMIDT, K.-J. (1982) The Chemistry of Organophosphorus Pesticides, 2nd ed., pp. 120–121, Springer-Verlag, New York.
2. KAPOOR, I. P., AND BLINN, R. C. (1977) *J. Agr. Food Chem.* **25,** 413–417.
3. ZULALIAN, J., AND BLINN, R. C. (1977) *J. Agr. Food Chem.* **25,** 1033–1039.
4. KU, C. C., AND KAPOOR, I. P. (1981) *in* Sulfur in Pesticide Action and Metabolism (Rosen, J. D., Magee, P. S., and Casida, J. E., eds.), pp. 97–109, *ACS Symp. Ser.* **158,** Amer. Chem. Soc., Washington, D.C.

5. BAHIG, M. E., AND WAFA, D. M. (1982) *Isotope Rad. Res.* **14,** 57–62.
6. HOLLINGSHAUS, J. G., SANDER, G., AND LITTLE, R. J. (1984) *J. Econ. Entomol.* **77,** 1393–1399.
7. GORDER, G. W., HOLDEN, I., AND CASIDA, J. E. (1984) American Chemical Society, Division of Pesticide Chemistry, 187th National Meeting, St. Louis, Mo., Abstract 35.
8. WING, K. D., GLICKMAN, A. H., AND CASIDA, J. E. (1983) *Science (Washington, D.C.)* **219,** 63–65.
9. SEGALL, Y., AND CASIDA, J. E. (1982) *Tetrahedron Lett.* **23,** 139–142.
10. BLOCK, E., BAZZI, A. A., LAMBERT, J. B., WHARRY, S. M., ANDERSEN, K. K., DITTMER, D. C., PATWARDHAN, B. H., AND SMITH, D. J. H. (1980) *J. Org. Chem.* **45,** 4807–4810.
11. DODDRELL, D. M., PEGG, D. T., AND BENDALL, M. R. (1982) *J. Magn. Reson.* **48,** 323–327.
12. GORDER, G. W., HOLDEN, I., RUZO, L. O., AND CASIDA, J. E. (1985) *Bioorg. Chem.* **13,** 353–361.
13. ALDRIDGE, W. N., AND REINER, E. (1972) Enzyme Inhibitors as Substrates. Interactions of Esterases with Esters of Organophosphorus and Carbamic Acids, Amer. Elsevier, New York.
14. O'BRIEN, R. D. (1976) *in* Insecticide Biochemistry and Physiology (Wilkinson, C.F., ed.), pp. 271–296, Plenum, New York.
15. O'BRIEN, R. D. (1968) *Mol. Pharmacol.* **4,** 121–130.
16. UENO, Y., NAKAI, T., AND OKAWARA, M. (1970) *Bull. Chem. Soc. Japan* **43,** 162–167.
17. ROYALS, E. E., AND HARRELL, L. L., JR. (1955) *J. Amer. Chem. Soc.* **77,** 3405–3408.
18. FIESER, L. F., AND FIESER, M. (1968) *in* Reagents for Organic Synthesis, Vol. 1, p. 135, Wiley, New York.
19. WEIR, W. D., AND KILBOURN, E. E. (1978) U.S. Patent 4 076 809.
20. ELLMAN, G. L., COURTNEY, K. D., ANDRES, V., JR., AND FEATHERSTONE, R. M. (1961) *Biochem. Pharmacol.* **7,** 88–95.
21. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.