Bioactivation of Isofenphos and Analogues by Oxidative N-Dealkylation and Desulfuration

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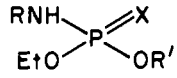
Isofenphos [*O*-ethyl *O*-[2-(isopropoxycarbonyl)phenyl] isopropylphosphoramidothionate] is bioactivated by mixed-function oxidases (MFOs) in two steps that ultimately give *N*-desisopropylisofenphos oxon, a product with 2300-fold greater inhibitory potency than isofenphos oxon toward house fly head acetylcholinesterase (AChE). Bioactivation of *N*-alkyl analogues of isofenphos oxon depends on an *N*-alkyl α-carbon proton and the steric bulk of the largest α-carbon substituent as described by Hansch regression analysis. These reactions are largely mediated by cytochrome P-450 and probably involve N-dealkylation via α-carbon oxidation without formation of AChE-inhibitory intermediates. Thiono oxidation is also required for the phosphorothionates, but MFO inhibition accompanying desulfuration slows the overall bioactivation. Findings on house fly toxicities are generally consistent with the in vitro bioactivation models. The isofenphos isomers, resolved on a chiral HPLC column, have the same stereospecificity for MFO-activated anti-AChE activity and toxicity to house flies, possibly due to enantiomeric differences in AChE inhibitory potency.

INTRODUCTION

Isofenphos (1) is a proinsecticide that is metabolically activated to a more potent acetylcholinesterase (AChE) inhibitor. The activation mechanism is proposed to involve either (a) oxidation to **2** or **4** (Hsin and Coats, 1986) or (b) oxidation at a site close to the nitrogen atom as mimicked by *m*-chloroperbenzoic acid, possibly in a fashion paralleling that of schradan (Ueji and Tomizawa, 1984). Biological oxidation may be catalyzed by flavin monooxygenase at the nitrogen, or cytochrome P-450 at the nitrogen or the *N*-alkyl α-carbon atom, giving an unstable carbinolamine that decomposes with N-dealkylation (Lindeke and Cho, 1982). N-Dealkylation could represent a bioactivation, with **4** having enhanced *P-O*-aryl reactivity toward AChE due to altered polarity or steric bulk with possible analogy in the N-deacetylation of acephate to methamidophos enhancing anti-AChE activity (Magee, 1982). N-Oxidation and rearrangement to P–O–N derivatives, a reaction established with *N,N*-dialkylphosphoramidates (Holden et al., 1982) and an alternative activation mechanism, is examined here with **5**. Chirality can additionally affect the bioactivation of organophosphorus toxicants (Ohkawa, 1982) or the bond reacting with AChE (Wing et al., 1983).



1 (isofenphos): R = *i*-Pr, R′ = Ph-2-C(O)O-*i*-Pr, X = S
2 (isofenphos oxon): R = *i*-Pr, R′ = Ph-2-C(O)O-*i*-Pr, X = O
3 (*N*-desisopropylisofenphos): R = H, R′ = Ph-2-C(O)O-*i*-Pr, X = S
4 (*N*-desisopropylisofenphos oxon): R = H, R′ = Ph-2-C(O)O-*i*-Pr, X = O
5 (diethyl *N*-isopropylphosphoramidate): R = *i*-Pr, R′ = Et, X = O
6 (diethyl phosphoramidate): R = H, R′ = Et, X = O

This report describes structural effects of isofenphos analogues and chiral isomers on the bioactivation mechanism. The key steps of metabolism, studied with mixed-function oxidase (MFO) and esterase inhibitors, and AChE inhibition are evaluated in vitro and in house flies.

MATERIALS AND METHODS

Chemicals. Abbreviations used are as follows: Me = methyl, Et = ethyl, Pr = propyl, Bu = butyl, Pe = pentyl, Ph = phenyl, *i* = iso, *n* = normal, *s* = secondary, and *t* = tertiary.

1–4 and [*phenyl*-^{14}C]-1 (31.4 mCi/mmol) were provided by Mobay Chemical Co. (Kansas City, MO). Additional ethyl 2-(isopropoxycarbonyl)phenyl phosphoramidothionates were prepared by adding ethyl phosphorodichloridothionate (180 mg, 1 mmol) in dichloromethane (2 mL) to isopropyl salicylate (180 mg, 1 mmol) and a catalytic amount of benzyltriethylammonium chloride in 10% aqueous sodium hydroxide (2 mL) at 25 °C. After the mixture was stirred for 3 h, ammonia, methylamine, or ethylamine (1 mmol) in aqueous solution or a higher *N*-alkylamine (1 mmol) in dichloromethane (2 mL) was added, and stirring was continued overnight at 25 °C. The organic layer was washed with saturated sodium chloride solution and then dried over magnesium sulfate. The oil obtained on removal of the solvent was purified by preparative thin-layer chromatography (TLC), developing with hexane–ethyl acetate (2:1) to give the desired compound in essentially quantitative yield. ^{31}P NMR ($CDCl_3$) shifts for RNHP(S)(OEt)OPh-2-C(O)O-*i*-Pr are R = H (**3**) δ 67.3, Me 68.7, Et 67.2, *i*-Pr (**1**) 65.8, *t*-Bu 62.9 [for additional spectral data see Ueji and Tomizawa (1984)]. Ethyl 2-(isopropoxycarbonyl)phenylphosphorochloridate [obtained from the reaction of ethyl phosphorodichloridate (1 mmol) with sodium isopropyl salicylate (1 mmol) in tetrahydrofuran (2 mL)] and diethyl phosphorochloridate were reacted with excess of appropriate amines (≥3 mmol) with stirring. After 4 h, ether was added to the reaction mixture and the organic layer was washed with water and dilute hydrochloric acid and then dried over anhydrous sodium sulfate. After evaporation of the solvent, crude product was purified by preparative TLC on silica gel developed with chloroform. ^{31}P NMR ($CDCl_3$) shifts (mp, °C) [n^{22}_D] for RNHP(O)(OEt)OPh-2-C(O)O-*i*-Pr are R = H (**4**) δ 2.9 (67–68), Me 3.6 [1.4948], Et 2.6 [1.4914], *n*-Pr 2.7 [1.4868], *i*-Pr (**2**) 1.5 (28–29), *n*-Bu 2.7 [1.4882], *i*-Bu 2.8 [1.4801], *s*-Bu 1.8 [1.4837], *t*-Bu 0.0 (39–40), *i*-Pe 2.7 [1.4796], *t*-Pe −0.1 [1.4807], 2-MeBu 3.0 [1.4813], neo-Pe 3.6 [1.4798], 1,2-Me_2Pr 2.3 [1.4816], 1-EtPr 2.1 [1.4810].

^{31}P NMR ($CDCl_3$) shift [n^{22}_D] for Me_2NP(O)(OEt)-OPh-2-C(O)O-*i*-Pr is δ 3.5 [1.4879]. ^{31}P NMR ($CDCl_3$)

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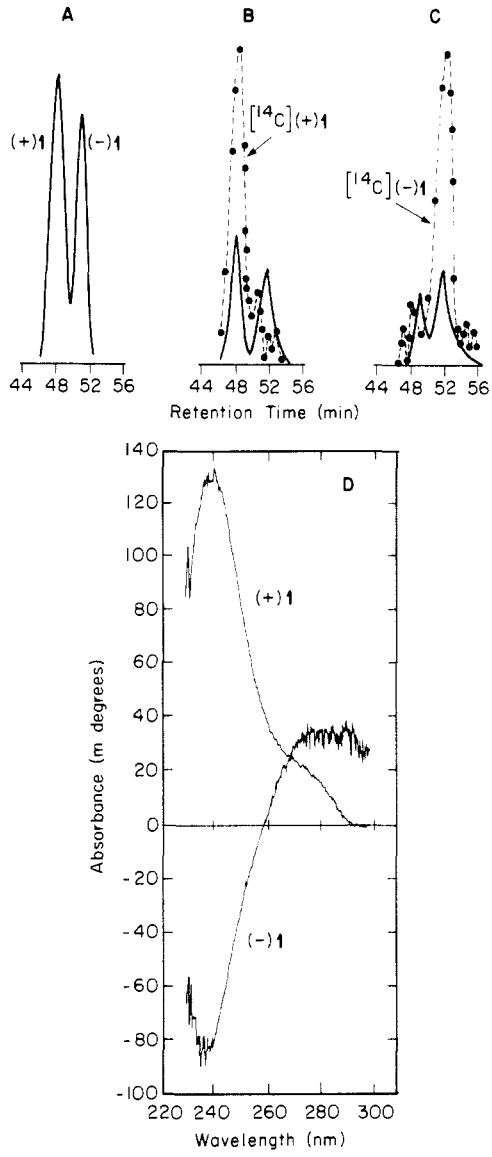

Figure 1. HPLC resolution of isofenphos isomers on a Sumipax 0A-4000 column. (A) Unlabeled racemate (5 μg) with UV detection. (B and C) Radiochemical purity determination of resolved ^{14}C enantiomers detected by radiocarbon analysis for cochromatography with unlabeled racemate analyzed by UV absorbance. (D) CD spectra of the resolved isomers each at 0.28 mM with four determinations and background subtraction (see Materials and Methods for consideration of purity).

shifts (mp, °C) [n^{22}_D] for RNHP(O)(OEt)$_2$ are R = H (6) δ 6.8 (42–49), *i*-Pr (5) 5.8 [1.4222], *s*-Bu 6.1 [1.4268], *t*-Bu 4.6 [1.4255]. ^{31}P NMR ($CDCl_3$) shift [n^{22}_D] for $Me_2NP(O)(OEt)_2$ is δ 8.5 [1.4217].

The enantiomers of 1 and [^{14}C]-1 were resolved on a Sumipax OA-4000 optically active high-performance liquid chromatography (HPLC) column (8 mm × 30 cm; Sumitomo Chemical Co., Osaka, Japan) with hexane (6 mL/min) using 254 nm UV or radiocarbon detection in amounts up to 10 μg/injection (Figure 1). The stereochemical purity achieved in a single passage through the column is >95%, based on rechromatography of the resolved enantiomers of [^{14}C]-1. Circular dichroism (CD) showed pure (+) enantiomer ($\Delta\epsilon$ at 239 nm, +1.37) and contamination of the (−) enantiomer with an optically active compound absorbing at ~280 nm also observed by UV. Concentrations of the (+) and (−) enantiomers were standardized on the basis of UV absorbance at 225 nm. No resolution is obtained by HPLC under comparable conditions with 2–4.

In Vitro Assays. Electric eel AChE and horse serum butyrylcholinesterase (BuChE) assays involved 4 units of enzyme (Sigma Chemical Co., St. Louis, MO) in 2.5 mL of 50 mM, pH 7.4, phosphate buffer with addition of the inhibitor in 10 μL of ethanol, incubation at 37 °C for 30 min, and removal of 0.08 unit for analysis of residual activity by its rate of acetylthiocholine or butrylthiocholine hydrolysis, respectively (Ellman et al., 1961). House fly head AChE was prepared by homogenizing 10 fly heads/mL of phosphate buffer and centrifuging 20 min at 15000 *g*. The assays with 0.25 mL of supernatant diluted to 2.5 mL with buffer were incubated with inhibitor at 30 °C for 30 min, and 1-mL aliquots were tested for residual AChE activity.

MFO incubations consisted of 2.5 mL (except as noted) of 50 mM, pH 7.4, phosphate buffer with 1 mg of microsomal protein (Lowry et al., 1951) (rat liver except the [^{14}C]-1 experiment that was done with mouse liver; no induction) and 2 μmol of NADPH with controls lacking NADPH and the oxidation initiated by substrate or inhibitor addition in 10 μL of ethanol. Oxidase inhibitors, when used, were added 5 min prior to substrate addition. Microsomal incubations were at 37 °C, and analyses were by (a) electric eel AChE inhibition following 5- or 10-min incubation that was stopped by dilution of a 0.05- or 0.20-mL aliquot to obtain the 2.5-mL assay sample, (b) ^{31}P NMR spectroscopy of extracts in $CDCl_3$ or CD_3CN following 30-min incubations (2.0-mL samples extracted with chloroform, 2× v/v; 1.0-mL samples extracted with acetonitrile, 1× v/v after saturation with NaCl), or (c) TLC and radiocarbon analysis of ether–ethanol extracts (3:1; 2× v/v after addition of 0.5 g of ammonium sulfate) following 60-min incubations with [^{14}C]-1. Gas chromatography–mass spectrometry (GC–MS) analysis of extracts was not successful due to inadequate resolution of substrates and metabolites on a 10-m high-performance methylsilicone capillary column.

Coupled MFO/AChE assays (Wing et al., 1983) were done with 1 mg of rat liver microsomal protein (no induction) and 4 units of electric eel AChE and 0 or 2 μmol of NADPH (for control and active samples, respectively) in 2.5 mL of 50 mM, pH 7.4, phosphate buffer with the substrate added in 10 μL of ethanol. Incubations were at 37 °C for 30 min prior to analysis of residual AChE activity. Means and standard deviations (SD) in AChE inhibition experiments are based on duplicate samples, and concentrations giving 50% inhibition (I_{50} values) are based on linear regression of log dose and percent AChE inhibition for 4–12 samples at four concentrations.

In Vivo Assays. Insecticidal activity was determined with adult female house flies (*Musca domestica* L., SCR strain, 3–5 days after emergence, ~20 mg each) with the test compounds applied topically in acetone (0.5 μL) to the ventral abdominal surface or injected in methoxytriglycol (0.2 μL) into the mesothorax. Piperonyl butoxide (PB) and phenyl saligenin cyclic phosphonate (PSCP) were used as selective oxidase and esterase inhibitors, respectively, topically applying these candidate synergists at 250 μg/g 1 h before administering the insecticide either by topical means or by injection. Mortality determinations for the treated house flies held in batches of 10 with sugar and water at 25 °C, were made after 24 h. LD_{50} values, estimated by log dose–probit mortality analysis, were reproducible within 2-fold in repeated tests.

Insecticide metabolism was examined by injecting [^{14}C]-1 (2 μg/g) into normal house flies or those pretreated as above with PB or PSCP. Some of the treated flies showed poisoning signs during the 2-h holding period prior to

Table I. Thin-Layer Chromatographic Properties of 1 and Some of Its Metabolites

compd	R_f[a] chloroform	R_f[a] hexane–ethyl acetate
RNHP(S/O)(OEt)OPh-2-C(O)O-*i*-Pr		
i-Pr/P=S (1)	0.52	0.66
i-Pr/P=O (2)	0.06	0.21
H/P=S (3)	0.35	0.55
H/P=O (4)	0.02	0.11
HOPh-2-C(O)OR		
i-Pr	0.66	0.74
H	0.32	0.36

[a] Two-dimensional development, first with chloroform and then with hexane–ethyl acetate (2:1).

Table II. Bioactivation of 1–3 as Inhibitors of Electric Eel AChE Assayed Directly in MFO-Coupled Systems and as Chloroform Extracts of MFO Reaction Mixtures

	inhibn, % (indicated assay)			
	MFO-coupled syst		chloroform extr	
compd[a]	no NADPH	NADPH	no NADPH	NADPH
i-Pr/P=S (1)	0 ± 4	47 ± 2	−3 ± 3	60 ± 1
i-Pr/P=O (2)	−3 ± 9[b]	74 ± 3[b]	−1 ± 6	87 ± 1
H/P=S (3)	10 ± 4	91 ± 1	−3 ± 1	96 ± 1

[a] RNHP(S/O)(OEt)OPh-2-C(O)O-*i*-Pr at 1 μM. [b] House fly microsomes assayed under the same conditions with 100 μM 2 gave −7% AChE inhibition without NADPH and 41% inhibition with NADPH.

extracting with acetone (1 mL × 2 per group of 10 flies). The acetone extract was subjected directly to TLC with preliminary development with benzene (Shrivastava et al., 1969) to remove lipids from the origin, minimizing their interference in metabolite separation. Data for individual metabolites are reported as means and SD for three experiments.

Analyses. NMR spectra (^{1}H at 300 MHz, ^{31}P at 121.5 MHz with broad-band decoupling) were taken in $CDCl_3$ or CD_3CN with a Bruker WM-300 spectrometer with internal ^{1}H reference to $CHCl_3$ (δ 7.25) or tetramethylsilane (δ 0.00) and external ^{31}P reference to trimethyl phosphate (δ 0.00). Melting points determined on a micro hot stage are uncorrected. TLC (Table I) utilized silica gel 60 F-254 chromatoplates with detection by UV and radioautography and quantitation by liquid scintillation counting.

Structure–activity relationships were analyzed by the method of Hansch and Fujita (1964) using physicochemical substituent parameters (Hansch and Leo, 1979) and regression techniques.

RESULTS

MFO-Catalyzed Bioactivation. Each of 1–3 is activated to a more potent AChE inhibitor by rat liver NADPH-dependent MFOs. House fly microsomes also activate 2. The AChE-inhibitory metabolite in each case is extracted from the MFO incubations with chloroform as shown by similar percent AChE inhibition for the MFO-coupled system directly and the residue of the evaporated chloroform extract (Table II).

^{31}P NMR analyses of the chloroform extracts of microsomal incubations (100 μM substrate) establish that the major products from 1 are 2 and 3 and both of these products (100 μM substrate) are oxidized to 4 (Figure 2). At a lower substrate concentration (0.8 μM), 6–9% conversion to 4 is detected with [^{14}C]-1. In tests against electric eel AChE (for consistency with the bioactivation studies) only 4 shows inhibitory activity (Figure 3). House fly toxicity data by injection establish that 4 is the most

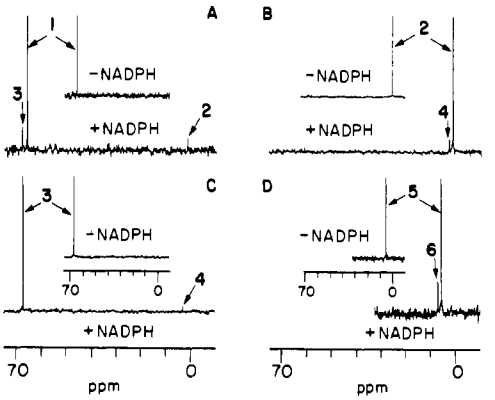

Figure 2. ^{31}P NMR spectra (pulse angle 30°, recycle delay 2 s) showing the following NADPH-dependent conversions: (A) 1 (δ 65.8) → 2 (δ 1.5) + 3 (δ 67.3); (B) 2 → 4 (δ 2.9); (C) 3 → 4; (D) 5 [*i*-PrNHP(O)(OEt)$_2$, δ 5.8] → 6 [$H_2NP(O)(OEt)_2$, δ 7.3]. Results are based on pooled and concentrated chloroform (A–C, 10 000 pulses) or acetonitrile (D, 15 000 pulses) extracts of four microsomal incubations each for 30 min with 0.25 μmol of substrate alone (inset plots) or with NADPH.

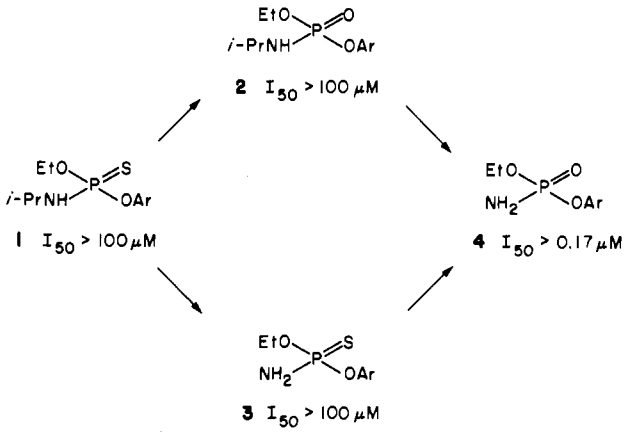

Figure 3. Pathways for oxidative metabolism of 1 with the bioactivities of 1–4 shown as I_{50} values for electric eel AChE after 30-min preincubation. Ar = Ph-2-C(O)O-*i*-Pr.

Table III. Toxicity and Synergism of Injected 1 and Some of Its Metabolites in House Flies

	LD_{50}, μg/g (following indicated pretreatment)		
compd[a]	none	PB	PSCP[b]
i-Pr/P=S (1)	1.3	1.3	0.26
i-Pr/P=O (2)	0.45	0.49	0.04
H/P=S (3)	0.33	0.36	0.03
H/P=O (4)	0.07	0.07	0.02

[a] RNHP(S/O)(OEt)OPh-2-C(O)O-*i*-Pr. [b] The large factor of synergism by PSCP does not extend to topical treatment (Table VI).

toxic compound and that its activity is further enhanced by PSCP (Table III).

Effect of *N*-Alkyl Substituents on Anti-AChE Activity (Table IV). House fly head AChE is more sensitive than electric eel AChE and horse serum BuChE to derivatives of RNHP(O)(OEt)OPh-2-C(O)O-*i*-Pr. With each esterase, inhibitory activity decreases with increasing numbers of carbon atoms in the *N*-alkyl group; 4 is the most potent inhibitor.

Effect of *N*-Alkyl Substituents on MFO-Catalyzed Bioactivation (Table V; Figure 4). In the series RNHP(O)(OEt)OPh-2-C(O)O-*i*-Pr R = H (4) is the only potent AChE inhibitor with microsomes minus NADPH,

Table IV. Potency of 2 and Analogues as Inhibitors of Three Cholinesterases

	AChE				BuChE	
	electric eel		house fly head		horse serum	
compd[a]	I_{50}[b]	pot.[c]	I_{50}[b]	pot.[c]	I_{50}[b]	pot.[c]
H (4)	0.17[d] (0.14–0.21)	100	0.0056 (0.0045–0.0071)	100	0.066 (0.052–0.083)	100
Me	15 (14–16)	1	0.37 (0.35–0.40)	2	6.5 (5.5–8.6)	1
Et	>100	<0.2	4.6 (3.7–5.6)	0.1	7.6 (6.2–9.3)	1
i-Pr (2)	>100[d]	<0.2	13 (12–14)	0.04	25 (24–32)	0.3
t-Bu	>100	<0.2	>100	<0.006	>100	<0.07

[a] RNHP(O)(OEt)OPh-2-C(O)O-*i*-Pr. [b] Inhibition after 30-min preincubation (95% confidence limits). [c] Relative to H (4) as 100. [d] These values also appear in Figure 3.

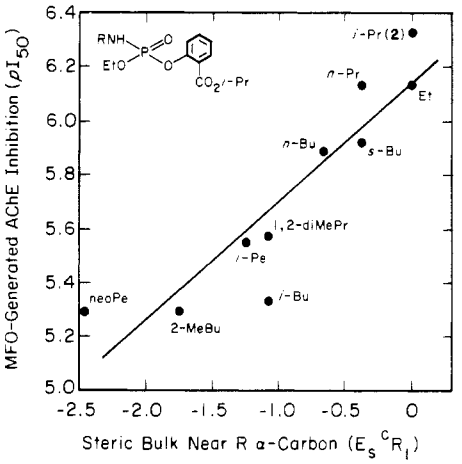

Figure 4. Hansch regression analysis for RNHP(O)(OEt)OPh-2-C(O)O-*i*-Pr analogues (R = CHR_1R_2) of the effect of the steric bulk of the *N*-alkyl α-carbon ($R_1 \neq H$, R_2 = H or Me) on the extent of N-dealkylation measured by MFO-generated AChE inhibition (30 min). Data are derived from Table V and alkyl names refer to R.

and its activity is not affected by the addition of NADPH. The Me derivative also shows some activity without NADPH, but the other derivatives are inactive (I_{50} > 100 μM). All *N*-alkyl analogues potentially N-dealkylated by MFOs to 4 [i.e., having a possible R_1R_2(HO)CNHP(O)(OEt)OPh-2-C(O)O-*i*-Pr intermediate] are strongly bioactivated, suggesting that they undergo N-dealkylation; the *t*-Bu and *t*-Pe derivatives are not bioactivated. The extent of MFO-generated AChE inhibition in 30 min is therefore a measure of the N-dealkylation rate, which is quantitatively represented by eq 1. In this equation, $E_s{}^c$

$$\log (1/I_{50}) = 0.44\ (\pm 0.08)E_s{}^cR_1 + 6.14\ (\pm 0.09)$$
$$n = 10,\ \mathrm{SD} = 0.18,\ r = 0.90,\ F = 35 \qquad (1)$$

is the Hancock's corrected steric substituent parameter (Hansch and Leo, 1979), R_1 is the largest substituent on the α-carbon atom ($R_1 > R_2 > R_3$) excluding the phosphoramidyl group, *n* is the number of data points, SD is the standard deviation, *r* is the correlation coefficient, and *F* is the *F* ratio. The figures in parentheses are the standard errors, and all terms are justified more than 99% by a *t*-test. According to eq 1, the activity is decreased with increasing steric bulk of R_1. Use of other parameters (e.g., $E_s{}^c$ for whole alkyl groups, electronic σ*, hydrophobic π, van der Waals volume r_v, molar refractivity MR, or STERIMOl L and B_5) instead of $E_s{}^cR_1$ or addition of the other parameters to eq 1 does not improve the correlation. The regression applies only to derivatives with one or two α-carbon protons and only one α-carbon substituent larger than Me and thus excludes the Me, *t*-Bu, *t*-Pe, and 1-EtPr derivatives.

In partial agreement with the in vitro system, R = H is most potent against house flies and R = *t*-Bu and *t*-Pe with tertiary α-carbon atoms are relatively inactive; however, there is no overall correlation between house fly toxicity and MFO-activated AChE-inhibitory activity. PB increases the topical toxicity of all compounds, and there is a fairly good correlation (*r* = 0.70) between the toxicity without PB and that with PB.

Table V. Potency of 2 and Analogues as Inhibitors of Electric Eel AChE in a MFO-Coupled Assay and as Topical Toxicants for House Flies

	I_{50}		LD_{50}, μg/g (following indicated pretreatment)	
compd[a]	μM[b]	activn factor[c]	alone	PB
H (4)	0.30 (0.26–0.35)	none	0.38	0.22
Me	5.9 (5.2–6.6)	10	5.3	1.1
Et	0.74 (0.68–0.81)	>140	10	1.8
i-Pr (2)	0.47 (0.40–0.55)	>210	10	1.9
n-Pr	0.74 (0.55–1.0)	>140	6.3	0.78
n-Bu	1.3 (1.1–1.5)	>77	12	1.5
i-Bu	4.7 (3.7–5.9)	>21	13	1.4
s-Bu	1.2 (1.1–1.3)	>83	14	1.7
i-Pe	2.8 (2.6–3.0)	>36		
neo-Pe	5.1 (4.3–6.2)	>20	16	4.3
2-MeBu	5.1 (4.1–6.5)	>20		
1,2-Me_2Pr	2.7 (2.3–3.2)	>37	60	8.1
1-EtPr	16 (12–20)	>6	60	7.0
t-Bu	>100	none	130	63
t-Pe	>10	none	>500	140

[a] RNHP(O)(OEt)OPh-2-C(O)O-*i*-Pr. [b] Inhibition after 30-min preincubation in the presence of microsomes and NADPH. Values in minus NADPH samples were >100 μM for all compounds except H [0.31 (0.29–0.33)] and Me [60 (50–72)]. Numbers in parentheses are 95% confidence limits. [c] I_{50} with microsomes minus NADPH divided by I_{50} with microsomes plus NADPH.

Effects of Potential Leaving Groups on Toxicity and Anti-AChE Activity. $RNHP(O)(OEt)_2$ analogues, lacking the isopropylsalicyl leaving group, are not bioactive in the test systems. Assays with R = H (**6**), *i*-Pr (**5**), *s*-Bu, and *t*-Bu gave topical house fly LD_{50}s over 300 μg/g in the presence and absence of PB. In vitro tests showed no electric eel AChE inhibition at 100 μM with or without MFO activation. These phosphoramidates however are N-dealkylated by MFOs in similar fashion to **2** as shown by **5** → **6** (Figure 2).

N,N-Dialkyl derivatives gave somewhat similar results to their *N*-alkyl counterparts. $Me_2NP(O)(OEt)_2$ also was not bioactivated but had slight biological activity, possibly due to a minor impurity not easily removed by TLC (67 ± 2 and 69 ± 2% inhibition at 100 μM in the electric eel AChE–microsome system for minus and plus NADPH, respectively; >300 and 130 μg/g house fly LD_{50}s for alone and plus PB, respectively). $Me_2NP(O)(OEt)$OPh-2-C(O)O-*i*-Pr was bioactivated to a slight extent (2 ± 5 and 26 ± 3% inhibition at 100 μM in the electric eel AChE–microsome system for minus and plus NADPH, respectively; 260 and 88 μg/g house fly LD_{50}s for alone and plus PB, respectively).

Table VI. Potency of 1 and Analogues as Topical Toxicants for House Flies Alone and with Synergists and as Inhibitors of Electric Eel AChE in a MFO-Coupled Assay

	LD_{50}, μg/g following indicated pretreatment			AChE inhibn, % (with compd at indicated concn,[b] μM)	
compd[a]	none	PB	PSCP	1	100
H (3)	0.8	0.5	0.6	88 ± 1	100 ± 0
Me	3.6	3.6	2.1	5 ± 1	8 ± 5
Et	4.7	4.0	2.8	36 ± 9	10 ± 2
i-Pr (1)	6.5	2.8	2.5	60 ± 19	18 ± 1
t-Bu	>50	>50	46	5 ± 4	−4 ± 5

[a] RNHP(S)(OEt)OPh-2-C(O)O-*i*-Pr. [b] Data are means ± SD of duplicate samples with NADPH. Controls (−NADPH) for Me, Et, *i*-Pr, and *t*-Bu, respectively, at 1 μM were 3 ± 7, −2 ± 7, −4 ± 5, and −1 ± 1% and at 100 μM were 3 ± 7, 0 ± 3, 8 ± 0, and 1 ± 11%.

Table VII. Effects of MFO Inhibitors on Bioactivation of 2 and 3 Based on Electric Eel AChE Inhibition

	% AChE inhibn with indicated substr[b]		
MFO inhibitor[a]	none	2	3
none	control	80 ± 3	85 ± 3
PB	−3 ± 12	36 ± 1	79 ± 2
n-octylamine	−2 ± 12	49 ± 6	77 ± 2
$(EtO)_2P(S)OPh$	*c*	7 ± 4	73 ± 2
t-BuNHP(S)(OEt)OPh-2-C(O)O-*i*-Pr	1 ± 7	12 ± 1	72 ± 3
methimazole	−2 ± 4	57 ± 3	75 ± 2

[a] PB and *n*-octylamine are reversible inhibitors of cytochrome P-450 isozymes involved in methylene and amine oxidations, respectively, whereas thiono compounds $(EtO)_2P(S)OPh$ and presumably *t*-BuNHP(S)(OEt)OPh-2-C(O)O-*i*-Pr are irreversible nonspecific cytochrome P-450 inhibitors (Testa and Jenner, 1981) as is methimazole, but to a lesser extent (Wislocki et al., 1980). *n*-Octylamine activates and methimazole is a competitive substrate for flavin monooxygenase (Ziegler, 1980). [b] 5-min preincubation of microsomes alone or with an MFO inhibitor (1 mM) and then 10-min incubation alone or with saturating substrate (1 mM) before dilution of 0.2 mL (for none and 3) or 0.05 mL (for 2) to 2.5 mL and determination of 30-min AChE inhibition values (means ± SD of duplicate samples). All values are for microsomes plus NADPH incubations. Controls minus NADPH were −10 ± 3 (2) and 7 ± 0% (3) AChE inhibition. [c] The control for 2 was 3 ± 10% and for 3 was 33 ± 9% AChE inhibition.

Effect of Phosphorothionate Group on MFO-Catalyzed Bioactivation (Tables VI and VII; Figure 5). In in vitro tests with RNHP(S)(OEt)OPh-2-C(O)O-*i*-Pr derivatives, R = *i*-Pr (1) and Et show far greater MFO activation to AChE inhibitors at a low than at a high concentration. This is not the case for the Me and *t*-Bu derivatives. All of these phosphorothionate derivatives are very toxic to house flies, except for the *t*-Bu compound. The bioactivation steps 2 → 4 and 3 → 4 each show positive dose-response relationships with enzyme saturation at lower substrate levels for 3 (0.04 μmol) than for 2 (0.32–2.56 μmol). Similar yields of 4 are obtained with both substrates at low concentration, but 2 → 4 is more efficient at high concentrations. Each of the MFO inhibitors reduces activation by N-dealkylation to a much greater degree than that by thiono oxidation. The nonselective cytochrome P-450 inhibitors [$(EtO)_2P(S)OPh$, *t*-BuNHP(S)(OEt)OPh-2-C(O)O-*i*-Pr] blocked N-dealkylation more effectively than the more selective cytochrome P-450 inhibitors (PB and octylamine). The flavin monooxygenase inhibitor (methimazole) that also causes some nonselective cytochrome P-450 inhibition was least effective.

Effects of Chirality on Bioactivity, Bioactivation, and Detoxification (Tables VIII and IX). The (+) isomer of 1 is much more potent than the (-) isomer in the MFO-activated anti-AChE assay. Interestingly, MFO

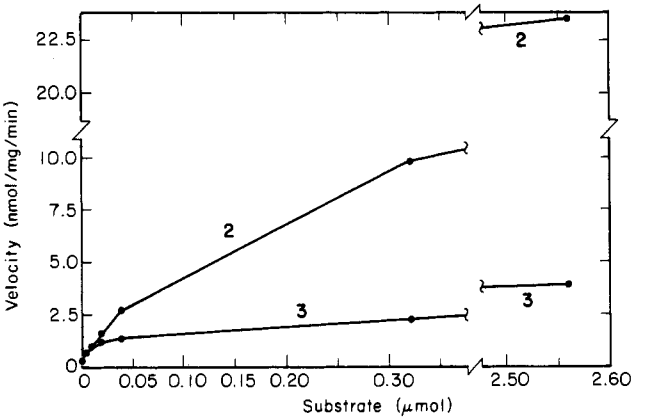

Figure 5. Kinetics of oxidative N-dealkylation (2 → 4) and desulfuration (3 → 4) reactions involved in bioactivation of 1. Results are means of duplicate MFO incubations for 5 min with 1 mg of microsomal protein in 2.5 mL. Levels of 4 are from 30-min AChE inhibitions for 0.2 mL of microsomal incubation mixtures with 2 or 3 and NADPH in comparison with 0.2 mL of microsomal incubation mixtures with 4 and no NADPH. As an exception, AChE inhibition assays with 2 at 0.32 and 2.56 μmol are for 0.05 mL of incubation mixtures with substrate and 0.15 mL without substrate. Controls with 2 or 3 at 2.56 μmol and no NADPH showed no 4. Formation of 4 occurs at the same rate from 2 and 3 at substrate levels up to 0.01 μmol.

Table VIII. Potency of 1 and Its Resolved Enantiomers as Injected Toxicants for House Flies and as Inhibitors of Electric Eel AChE in a MFO-Coupled Assay

synergist or cofactor	isomer(s) (+)	(−)	racemate
LD_{50} (μg/g) following Indicated Pretreatment			
none	0.47	8.4	1.3[a]
PB	0.36	8.8	1.3[a]
PSCP	0.12	3.7	0.26[a]
AChE Inhibition (%) with Indicated Coupled System[b]			
microsomes alone	3 ± 6	0 ± 3	−4 ± 5
mirosomes plus NADPH	72 ± 13	5 ± 1	60 ± 19

[a] These data also appear in Table III. [b] Isofenphos at 1 μM.

Table IX. Metabolites of Injected [^{14}C]-1 and Its Resolved Enantiomers in House Flies Alone and with Synergists

synergist and enantiomer of 1	radiocarbon recovery as indicated products,[a] % 1	2	4	polar[b]	other[c]
none					
(+)	51 ± 1	17 ± 1	3 ± 1	25 ± 1	3 ± 1
(−)	51 ± 6	26 ± 1	6 ± 2	12 ± 2	5 ± 2
racemate	55 ± 1	24 ± 1	5 ± 1	12 ± 1	4 ± 2
PB					
(+)	69 ± 1	7 ± 1	3 ± 1	17 ± 2	3 ± 2
(−)	72 ± 2	15 ± 3	4 ± 2	5 ± 1	4 ± 2
racemate	77 ± 2	13 ± 2	3 ± 2	4 ± 1	3 ± 2
PSCP					
(+)	62 ± 1	18 ± 1	5 ± 1	11 ± 1	4 ± 1
(−)	48 ± 7	25 ± 1	9 ± 2	10 ± 3	7 ± 4
racemate	59 ± 1	22 ± 0	6 ± 1	9 ± 1	5 ± 2

[a] Means ± SD in three experiments. [b] TLC with hexane–ethyl acetate (2:1), R_f 0.00. [c] TLC with hexane–ethyl acetate (2:1), R_f = 0.38–0.55, possibly including 3.

conversion of [^{14}C]-1 to [^{14}C]-4 occurs to a similar extent with the (+) and (−) isomers. The (+) isomer is also ~20-fold more toxic than the (−) isomer to house flies. PB does not alter the potency, but PSCP increases the toxicity of both enantiomers.

Metabolism of [^{14}C]-1 in house flies gives large amounts of 2 and small levels of 4. Much of the dose is present as

unidentified metabolites. PB retards the transformation of the parent compound while PSCP does not. The overall metabolism is similar for the enantiomers. Differences in the quantity of radiocarbon at the TLC origin suggest more rapid detoxification of the more toxic (+) isomer by PSCP-inhibited esterases. There are no indications of greater bioactivation of the more toxic (+) isomer.

DISCUSSION

The potent AChE inhibitor formed on bioactivation of 1 is stable and extractable into chloroform, and it is therefore not a highly reactive or transient intermediate as suggested by Ueji and Tomizawa (1984). Identification of the chloroform-soluble products from the MFO system shows the metabolic conversions of 1 → 2 and 3 and of 2 and 3 → 4 involving oxidative N-dealkylation and desulfurization. Similarly house flies produce 2 and 4 with 3 as a possible minor product in agreement with results in corn rootworms (Hsin and Coats, 1986). The insecticidal activity to house flies increases in the order 1 < 2 or 3 < 4 on injection as expected for the proposed activation sequences. This is in contrast to similar potencies for 1–4 on topical treatment of corn rootworm larvae and adults (Coats and Hsin, 1983). The bioactivation pathways are confirmed with the coupled AChE/MFO system, which shows marked activation of 1–3 but little or no change in the activity of 4, the most potent AChE inhibitor and terminal bioactive metabolite.

An interesting interplay between bioactivation and detoxification occurs in the toxicity of 1 and its analogues to house flies. In vitro hepatic MFO studies establish a major role for cytochrome P-450 in activation based on the requirements for NADPH and the inhibitory effect of PB. It is therefore surprising to find that PB synergizes the toxicity to house flies, perhaps due to partial cytochrome P-450 inhibition blocking detoxification to a greater extent than bioactivation. Esterases are most important in house fly detoxification as shown by analysis of metabolites from injected 1 and by an unusually high degree of synergism on topical application of PSCP and injection of 1–4. The treatment method affects the relative importance of esterase and oxidase detoxification since topically applied PSCP and PB are equally effective synergists for the topically applied toxicants.

The bioactivation pattern of *N*-alkyl analogues of 4 requiring one α-carbon proton is consistent with 4 being the terminal inhibitor. The absence of bioactivation of compounds that cannot form carbinolamine intermediates (i.e., the *t*-Bu and *t*-Pe derivatives) argues against *N*-oxidation. The extent of bioactivation of derivatives with one or two α-carbon protons decreases with increasing bulk of the largest α-carbon substituent (R_1), possibly due to increasing steric hindrance for approach of the crucial C–H bond to the oxidase active site. The R_2 substituent does not interfere with bioactivation when it is H or Me, but Et (as in 1-EtPr) reduces bioactivation, possibly due to a similar detrimental steric effect. Less bioactivation than expected occurs when R = Me, not for steric but perhaps for electronic reasons; i.e., cytochrome P-450 oxidations probably occur via a radical, and this mechanism disfavors alkyl oxidations at primary carbon atoms (White and Coon, 1980). In contrast to the in vitro system and a previous study with other phosphoramidates (Neely and Whitney, 1968), the house fly toxicity data do not fit a regression equation, suggesting along with the effects of PB and PSCP that in the isofenphos oxon series detoxification and penetration are as important as bioactivation.

The diethyl *N*-alkylphosphoramidates lack insecticidal and anti-AChE activity, indicating that a good leaving group is neither present nor generated metabolically. Metabolism of 5 → 6 is parallel to 2 → 4, showing that the P–N bond is not activated for AChE inhibition. Although P–N bonds are activated by N-oxidation, this is seemingly an unfavorable reaction with *N*-methyl relative to *N,N*-dimethylphosphoramidates (Holden et al., 1982). The slight bioactivation of *O*-ethyl *O*-[2-(isopropoxycarbonyl)phenyl] dimethylphosphoramidate also could result from di-N-demethylation rather than N-oxidation. Schradan is seemingly an unusual compound, with N-oxidation activating P–N bonds (Holden et al., 1982) and additionally P–O–P linkages that are critical relative to esterase inhibition (Casida et al., 1952). The enhanced potency of 4 relative to analogues of 2 probably results from enhanced reactivity (Neely and Whitney, 1968) and improved fit at the AChE active site.

Efficient blockage of N-dealkylation by phosphorothionates is probably due to nonspecific cytochrome P-450 suicide inhibition, and the same mechanism may account for inhibition by methimazole (Neal, 1980). The lesser effect of methimazole suggests that flavin monooxygenase has little or no role in N-dealkylation. The low substrate saturation level and yield of 4 from 3 probably result from the same suicide inhibition reaction. It is interesting that phosphorothionates and other cytochrome P-450 inhibitors are less effective in blocking 3 → 4, perhaps in part due to the reaction kinetics or differences in sensitivities to inhibition of the cytochrome P-450 isozymes. The overall reaction 1 → 4 occurs in the MFO system most efficiently at low substrate concentrations, but in agreement with the PB effect, this is less critical in house flies as evidenced by the insecticidal potency of 1.

The enantiomers of 1 show similar specificity toward house flies and in the coupled MFO/AChE system. The chiral specificity is most likely to involve the AChE phosphorylation step since it is not attributable to metabolic activation or detoxification reactions. The optical configuration is retained on metabolic desulfuration (Ohkawa, 1982) or N-dealkylation, so the chirality of 4 is probably the same as 1. The ultimate toxicant 4 on bioactivation of 1 probably has the absolute configuration (*S*) based on the relative potency of the enantiomers in other series where it is assigned (Sasaki, 1985).

ACKNOWLEDGMENT

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Registry No. 1, 25311-71-1; (+)-1, 103982-06-5; (−)-1, 103982-07-6; 2, 31120-85-1; 2 (R = Pr), 96183-10-7; 2 (R = Bu), 96183-11-8; 2 (R = *i*-Bu), 96183-12-9; 2 (R = *s*-Bu), 96183-13-0; 2 (R = *i*-Pe), 103982-00-9; 2 (R = neo-Pe), 103982-01-0; 2 (R = 2-MeBu), 103982-02-1; 2 (R = 1,2-Me_2Pr), 103982-03-2; 2 (R = 1-EtPr), 103982-04-3; 2 (R = *t*-Pe), 103982-05-4; 3, 25205-08-7; 3 (R = Me), 25205-10-1; 3 (R = Et), 96183-03-8; 3 (R = *t*-Bu), 96183-08-3; 4, 31120-83-9; 4 (R = Me), 31120-86-2; 4 (R = Et), 96183-09-4; 4 (R = *t*-Bu), 96183-14-1; 5, 22685-19-4; 6, 1068-21-9; PSCP, 4081-23-6; MFO, 9040-60-2; AChE, 9000-81-1; NADPH, 53-57-6; PB, 51-03-6; BuChE, 9001-08-5; *o*-$HOC_6H_4CO_2Pr$-*i*, 607-85-2; *o*-$HOC_6H_4CO_2H$, 69-72-7; $(EtO)_2P(S)OPh$, 32345-29-2; $CH_3(CH_2)_7NH_2$, 111-86-4; cytochrome P-450, 9035-51-2; methimazole, 60-56-0.

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Formamidine–S–Carbamates: A New Procarbamate Analogue with Improved Ovicidal and Acaricidal Activities

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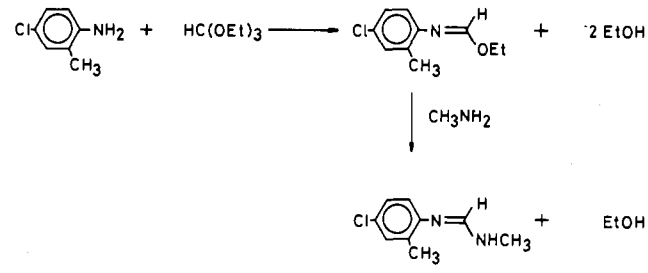
A series of [N^1-(4-chloro-2-methylphenyl)-N^2-methylmethanimidamido]thio and [N^1-(2,4-dimethylphenyl)-N^2-methylmethanimidamido]thio derivatives of methylcarbamate insecticides were prepared and examined for toxicity to houseflies, white mice, and a variety of agricultural pests. These compounds have the combined pesticidal activity of the parent formamidine (i.e., demethylchlordimeform and BTS-27271) and *N*-methylcarbamate, being active against acarines in addition to a wide variety of insects. The compounds also display activity as systemic pesticides.

INTRODUCTION

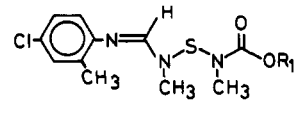
In previous papers from our laboratory, we described the favorable toxicological properties of sulfide derivatives of methylcarbamate insecticides (Black et al., 1973; Fahmy et al., 1974, 1978). Examples of these are carbosulfan, CGA-73,102, and benfuracarb, i.e. derivatives of carbofuran (2,3-dihydro-2,2-dimethylbenzofuranyl-7-yl methylcarbamate) that have insecticidal activity similar to that of carbofuran but are substantially less toxic to mammals (Fukuto, 1984). Also many sulfide derivatives of insecticidal and acaricidal formamidines have been described that usually retain the activity of the parent compound or in some cases are superior. For example, the phenylthio derivative of demethylchlordimeform [DCDM or *N'*-(4-chloro-2-methylphenyl)-*N*-methylmethanimidamide] was superior against the two-spotted spider mite (LC_{50} 6 ppm) compared to DCDM (12 ppm) and chlordimeform (19 ppm) (Knowles, 1982).

Carbamate esters are known to be effective against a broad spectrum of insects. However, they are generally relatively ineffective against mites, ticks, and other acarines. On the other hand, certain formamidines such as CDM, DCDM, and BTS-27271 [*N'*-(2,4-dimethylphenyl)-*N*-methylmethanimidamide] are known to be highly effective against acarines as well as insects (Atkinson and Knowles, 1974; Gemrich et al., 1976a,b; Hollingworth, 1976; Knowles, 1982). It would be desirable to combine the insecticidal activity of the carbamate and the acaricidal activity of the formamidine into a single pesticidal compound for the control of both insects and acarines.

Scheme I

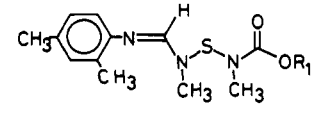


This report is concerned with the synthesis and toxicological properties of a series of DCDM–S–carbamates and (BTS-27271)–S–carbamates of the general structures I and II where R_1 is the phenolic moiety of carbofuran or



DCDM-S-carbamate

I



(BTS 27271)-S-carbamate

II

3-isopropylphenyl methylcarbamate or the oxime of methomyl [methyl *N*-[[(methylamino)carbonyl]oxy]ethan-

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