The Effect of Chiral Debromoleptophos Oxon Isomers on Acute and Delayed Neurotoxicity and Their Inhibitory Activity against Acetylcholinesterases and Neurotoxic Esterase

Bryan K. Eya and T. Roy Fukuto*

The chiral isomers of 2,5-dichlorophenyl methyl phenylphosphonate (debromoleptophos oxon) were prepared and their toxicological properties were evaluated. Assignment of the absolute configurations of the isomers was made by relating them to the oxon obtained by stereospecific oxidation of $(S)_p$-(−)-leptophos with *m*-chloroperbenzoic acid. The $(R)_p$(+) isomer of debromoleptophos oxon was more acutely toxic to houseflies and mice and was a more potent inhibitor of bovine erythrocyte and housefly head acetylcholinesterase than the $(S)_p$(−) isomer. In contrast, the $(S)_p$(−) isomer was more effective in inhibiting hen brain neurotoxic esterase than its enantiomer. The $(S)_p$(−) isomer was also more potent in inducing delayed neuropathy in hens than the $(R)_p$(+) isomer but racemic debromoleptophos oxon appeared to be more delayed neurotoxic than either enantiomer.

INTRODUCTION

A previous paper (Allahyari et al., 1980) described the resolution, determination of the absolute configuration, and toxicological properties of the chiral isomers of *O*-(4-bromo-2,5-dichlorophenyl) and *O*-(2,5-dichlorophenyl) *O*-methyl phenylphosphonothioate (leptophos and debromoleptophos, respectively). The results revealed that the $(R)_p$(+) isomers of leptophos and debromoleptophos were more acutely toxic to the housefly and white mouse, while the $(S)_p$(−) isomers were more delayed neurotoxic when administered intraperitoneally to the hen. In continuation of this investigation, the chiral isomers of debromoleptophos oxon, the expected metabolic activation product of debromoleptophos (Lee and Fukuto, 1976), were prepared and their toxicological properties were evaluated, including acute toxicity to the mouse and housefly, delayed neurotoxicity to the hen, and inhibition of acetylcholinesterase (AChE) and neurotoxic esterase (NTE). The configurations of the debromoleptophos oxon enantiomers were assigned by relating them to the oxon obtained by stereospecific oxidation of $(S)_p$(−)-leptophos with *m*-chloroperbenzoic acid. This paper presents the results of this study.

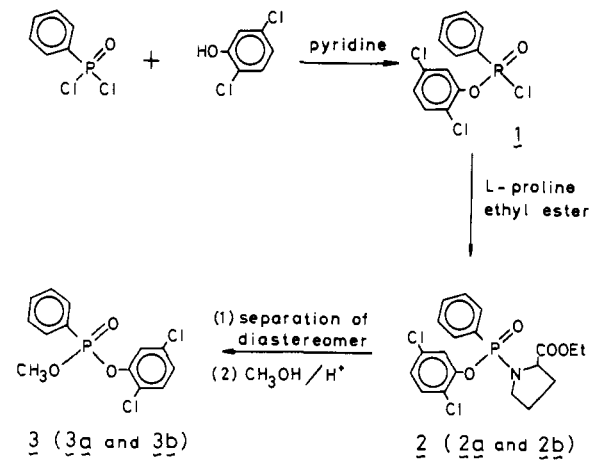
MATERIALS AND METHODS

General Methods. Optical rotations of isomers were determined with a Perkin-Elmer Model 241 polarimeter at the sodium D line (589 nm). Proton magnetic resonance spectra were recorded on a Varian EM-390 spectrometer in either chloroform-*d* or carbon tetrachloride with tetramethylsilane as the internal standard.

Thin-layer chromatography (TLC) was carried out on silica gel 60 F_{254} precoated sheets (EM Reagent) and SilicAR CC-7 Special was used for column chromatography. High pressure liquid chromatography (HPLC) was carried out on a Waters Associate system equipped with M-6000 A solvent delivery and RCM-100 radial compression module with Radial-PAK normal and KC_{18} reverse-phase cartridges. The M-450 variable wavelength UV/visible and M-401 RI detectors were used. A Hewlett-Packard Model 402 chromatograph, fitted with AFID and 5 ft × 2 mm i.d. silanized glass U-tube, was used for gas chromatography (GC). The column packing was 6% EGSP (Applied Science) on HCl-extracted 80/100 mesh chromosorb W, vacuum-coated and fluidized, conditioned for 24 h at 215 °C, and exhaustively extracted with chloroform for 7 days. All melting and boiling points are uncorrected. Elemental analyses were carried out by C. F. Geiger, Ontario, CA.

Spectrophotometric analyses for the in vitro enzyme assays were carried out with a Varian Cary 219 or Beckman Model 25 UV/visible spectrophotometer equipped with automatic sample changer.

Scheme I



Synthesis and Resolution. The synthesis and resolution of 2,5-dichlorophenyl methyl phenylphosphonate (debromoleptophos oxon) was achieved by application of the method described by Koizumi et al. (1978) (Scheme I) via the 2,5-dichlorophenyl phenylphosphonochloridate (1) and L-proline ethyl ester to give the phenylphosphonoamidate (2). The diastereomers of **2** (**2a** and **2b**) were separated by column chromatography using 95:5 benzene–ethyl acetate as the solvent and the purity of each diastereomer was determined by HPLC at 280 nm with 8:2 isooctane–ethyl acetate solvent. The HPLC retention times of **2a** and **2b** were 6.1 and 8.9 min (flow rate 4 mL/min), respectively, and each diastereomer was ≥97% resolved. The diastereomers **2a**, $[\alpha]^{24}_D$ +8.03 (*c* 121.0, CCl_4), and **2b**, $[\alpha]^{24}_D$ −51.2 (*c* 121.0, CCl_4), were colorless oils and were used directly in the next step. ^{1}H NMR of **2**: δ (Me_4Si, chloroform-*d*) 7–8.3 (m, 8 H, aromatic), 4.4–4.6 (m, 1 H, C*H*COOEt), 4–4.3 (q, 2 H, $COOCH_2CH_3$), 3–3.5 (m, 2 H, NCH_2), 1.5–2.2 (m, 4 H, $NCH_2CH_2CH_2$), 1.1–1.3 (t, 3 H, $COOCH_2CH_3$).

Acid-catalyzed methanolysis of **2a** and **2b** afforded the enantiomers of 2,5-dichlorophenyl methyl phenyl-

Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, California 92521.

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phosphonate (**3a** and **3b**) as colorless oils which were purified by Kugelrohr distillation. **3a**: bp 165–170 °C (0.15 mm); n^{25}_D 1.5690; $[\alpha]^{24}_D$ +58.1 (*c* 25.0, CCl_4). **3b**: bp 165–170 °C (0.15 mm); n^{25}_D 1.5685; $[\alpha]^{24}_D$ −59.5 (*c* 26.0, CCl_4). Racemic **3** was prepared by methanolysis of unresolved **2** and by the reaction of **1** and methanol in the presence of pyridine. The product obtained by methanolysis of **2** showed negative rotation, $[\alpha]^{24}_D$ −12.6 (*c* 25.0, CCl_4), and therefore **3** was also prepared from **1**. Purity of products were verified by GC and HPLC, including both normal (8:2 isooctane–ethyl acetate) and reversed-phase (6:4 and 8:2, methanol–water) HPLC. Racemic **3**, n^{25}_D 1.5697. Anal. Calcd for $C_{13}H_{11}Cl_2O_3P$: C, 49.24; H, 3.50. Found: C, 48.87; H, 3.55. ^1H NMR of **3**: δ (Me_4Si, chloroform-*d*) 6.8–8.2 (m, 8 H, aromatic) 3.8–4 (d, 3 H, OCH_3).

Assessment of the optical purity of **3a** and **3b** was accomplished by NMR with the aid of the pseudocontact shift reagent tris-[3-[(heptafluoropropyl)hydroxymethylene]-*d*-camphorato]europium (III) (Aldrich). Increments of shift reagent in chloroform-*d* (20 μL, 52 mg/0.1 mL) were added to a sample of **3** in chloroform-*d* containing Me_4Si as the internal standard. Addition of the shift reagent (80 μL) to racemic **3** resulted in two doublets (separated by 6 Hz) for the OCH_3 doublet (J = 12 Hz) at δ 3.7–4. The same analysis with **3a** and **3b** revealed no separation of doublets.

Enzyme Assays. Neurotoxic esterase (NTE) activity was determined according to Johnson (1977) by using the brain homogenate from adult white leghorn hens 1.3–1.8 kg in weight and 55–57 weeks in age. Acetylcholinesterase (AChE) activity was determined at pH 7.6 according to Wustner and Fukuto (1973) by using acetylthiocholine as the substrate. Bovine erythrocyte AChE (BAChE) was obtained from the Sigma Chemical Co. and housefly head acetylcholinesterase (HFAChE) was prepared according to Wustner and Fukuto (1973). BAChE assays were at 37 °C and HFAChE assays were at 30 °C.

Delayed Neurotoxicity. Delayed neurotoxic activity was determined with white leghorn hens of the same weight and age used for the NTE assay. **3**, **3a**, and **3b** were each administered intraperitoneally in dimethyl sulfoxide (Me_2SO) with both untreated and Me_2SO-treated controls. Three to nine hens were treated per dose at doses ranging from 3 to 30 mg/kg. Hens receiving doses higher than 3 mg/kg were given 14 mg/kg atropine sulfate in 0.9% saline intramuscularly 15 min before treatment and 9 mg/kg at 1, 8, and 16 h posttreatment as needed for survival. Hens were observed every other day for signs of ataxia over a 4-week period. The severity of ataxia was graded as described by Davies and Holland (1972).

Acute Toxicity. Insecticidal activity was determined by using the susceptible S_{NAIDM} strain, as described by March and Metcalf (1949). Mammalian toxicity was determined with 20–32-g female ICR white mice obtained from Harlan Sprague-Dawley, Inc., Indianapolis, IN. Test compounds dissolved in corn oil were administered intraperitoneally at 0.01–0.08 mL/mouse, with untreated and corn oil treated controls. LD_{50} values were based on 24 h mortality by using the moving average method described by Weil (1952) where n = 4 and K = 3.

Oxidation of $(S)_p$(−)-Leptophos with *m*-Chloroperbenzoic Acid. The *m*-chloroperbenzoic acid (MCPBA) oxidation of $(S)_p$(−)-leptophos (Allahyari et al., 1980) to leptophos oxon was carried out according to Herriott (1971). MCPBA (46.5 mg) in 2.7 mL of dichloromethane was gradually added to a stirring ice-chilled solution of 100 mg of $(S)_p$(−)-leptophos ($[\alpha]^{24}_D$ −26.55 (*c* 85.0, CCl_4)) in 15 mL of dichloromethane. The mixture was stirred at room temperature for 2 h. The oxon was isolated in 84% yield by preparative TLC (Analtech) with benzene–ethyl acetate (95:5) as the solvent. The leptophos oxon ($[\alpha]^{24}_D$ −35.20 (*c* 83.6, CCl_4)) gave the following ^1H NMR signals: δ (Me_4Si, chloroform-*d*) 7.2–8.1 (m, 7 H, aromatic), 3.7–4.0 (d, 3 H, OCH_3).

Table I. Acute Toxicity of (+), (±), and (−) Isomers of Debromoleptophos Oxon to Houseflies and Mice

isomer	LD_{50} housefly (topical), μg/g	LD_{50} mouse (ip), mg/kg
(+)	0.0321[a] (0.0243–0.0400)[b]	28.5 (21.8–35.2)[b]
(±)	0.0460[a] (0.0378–0.0541)[b]	61.1 (56.0–66.2)[b]
(−)	0.187 (0.162–0.213)[b]	363.0 (340.9–385.1)[b]
(−)/(+)	5.8-fold	12.7-fold

[a] $t > 1.96$ are different data sets, t = 2.5. [b] 95% confidence interval. The data sets are different when confidence intervals do not overlap.

Statistical Analysis. To test whether the plots from the enzyme assays were significantly different from each other, the slope of each plot was calculated by linear regression analysis and the test for parallelism was used to compare the slopes. The parallelism to error ratio of the slopes, F, was computed and tested for significance at P = 0.05. Also, the pooled error was calculated to compute the standard score, t, for pairwise comparison of slopes at P = 0.05 (Goldstein, 1964). ED_{50} and LD_{50} values and their 95% confidence intervals were determined by logit transformation (Ashton, 1972). A Data General Eclipse S/140 computer was used for statistical analyses.

RESULTS AND DISCUSSION

The (+)-rotating isomer of debromoleptophos oxon (**3a**) was more toxic to houseflies and mice than was its (−)-rotating enantiomer **3b** (Table I). A larger difference in toxicity between the enantiomers was observed with mice (12.7-fold) than with houseflies (5.8-fold). The toxicity of the racemic mixture fell in between the enantiomers in both cases. In general, the acute toxicity data were similar to those reported previously for the (+) and (−) enantiomers of phenylphosphonothioates such as leptophos and debromoleptophos (Allahyari et al., 1980), *O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate (EPN) (Ohkawa et al., 1977b) and *O*-(4-cyanophenyl) *O*-ethyl phenylphosphonothioate (cyanofenphos) (Ohkawa et al., 1977a). In all cases the (+)-rotating isomers were higher in activity than the (−) enantiomers although the differences in toxicities between the phenylphosphonothioate enantiomers were substantially smaller than between the enantiomers of debromoleptophos oxon. For example, the (+) isomers of EPN and cyanofenphos were only 2- to 3-fold more toxic to mice and houseflies than the (−) isomers. This may be attributable to racemization which may take place during the metabolic activation of the phosphonothioate to the respective phosphonate.

Results from the inhibition of bovine erythrocyte (BAChE) and housefly head acetylcholinesterase (HFAChE) were qualitatively consistent with the housefly and mouse toxicity data (Table II). Quantitatively, however, the differences in anticholinesterase activities were much greater than the respective differences in toxicities to houseflies and mice, e.g., **3a** was 107- and 18.4-fold more effective in inhibiting HFAChE and BAChE than **3b**. Differences of this nature have been observed previously with enantiomers of other organophosphorus esters (Lee et al., 1978).

In contrast to their anticholesterase potencies, the (−)-rotating isomer **3b** was more effective than **3a** in inhibiting hen brain neurotoxic esterase (NTE), although the

Table II. Inhibitory Activity of (+), (±), and (-) Isomers of Debromoleptophos Oxon against Housefly Head (HFAChE) and Bovine Erythrocyte Acetylcholinesterase (BAChE)

	HFAChE				BAChE			
isomer	[I],[a] M	k_i[b]	M[c]	r[d]	[I],[a] M	k_i[b]	M[c]	r[d]
(+)	1×10^{-7}	4.4×10^5	0.135	0.997	1×10^{-6}	1.93×10^5	0.193	0.997
(±)	3×10^{-7}	3.0×10^5	0.0909	0.996	1×10^{-6}	1.04×10^5	0.104	0.995
(-)	2×10^{-5}	0.041×10^5	0.00124	0.995	1×10^{-6}	0.105×10^5	0.0105	0.972
(+)/(-)		107-fold				18.4-fold		

[a] [I] = molar concentration of inhibitor used. [b] k_i = bimolecular inhibition constant. [c] M = slope of regression line. [d] r = linear correlation of regression line.

Table III. Inhibitory Activity of (+), (±), and (-) Isomers of Debromoleptophos Oxon against Hen Brain Neurotoxic Esterase (NTE)

comparison among four regression lines				pairwise comparison of slopes		
isomer	I_{50}, μM[a]	M[b]	r[c]	isomers	t[g]	DF
(+)	0.35	-0.859	0.995	(+) vs. (-)	12.7[h]	24
(±)	0.26	-1.13	0.994	(+) vs. (±)	6.42[h]	24
(-)	0.21	-1.46	0.995	(-) vs. (±)	6.16[h]	24
F[d] = 78.8[h] with 2[e] and 36[f] DF						
(+)/(-)	1.7-fold					

[a] I_{50} (μM) = molar concentration of inhibitor which inhibits 50% of enzyme activity. [b] M = slope of regression line. [c] r = linear correlation of regression line. [d] F = variance ratio = parallelism/error [Goldstein, 1964, p 171]. [e] 2 = degree of freedom (DF) of parallelism. [f] 36 = degree of freedom (DF) of error. [g] t = t-test for significance of a difference between two slopes [Goldstein, 1964, p 144]. [h] Slopes are significantly different at $P = 0.05$.

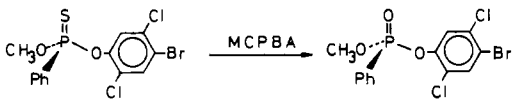
difference in inhibitory potencies between the enantiomers was small (Table III), i.e., less than 2-fold. This observation is in good agreement with data obtained previously by Ohkawa et al. (1980) on the inhibition of hen brain NTE by the resolved isomers of EPN oxon and cyanofenphos oxon. With each of these compounds, the enantiomers and the racemate were all potent inhibitors of NTE with inhibitory activity in the order (-) > (±) > (+) but large differences between enantiomers were not observed as observed with the inhibition of AChE.

Determination of the delayed neurotoxicity of the enantiomers and racemic mixture of debromoleptophos oxon provided results which were difficult to interpret (Table IV). As expected from the antineurotoxic esterase data, the (-)-enantiomer with a MED_{50} value of 7.7 mg/kg was more potent in inducing delayed neuropathy in hens than the (+)-enantiomer (MED_{50} 15.3 mg/kg). However, in contrast to antineurotoxic esterase activity, racemic debromoleptophos oxon with a MED_{50} value of 4.9 mg/kg appeared to be more delayed neurotoxic than either enantiomer. In all other cases where neurotoxic activity is enantiomers and reacemates have been compared, e.g., debromoleptophos (Allahyari et al., 1980), EPN (Ohkawa et al., 1977b), and EPN oxon (Abou-Donia et al., 1978, 1980), the activity of the racemate was in between that of the enantiomers.

The period required for the onset of ataxia for the racemate and the (-) enantiomer was the same, i.e., paralysis occurred 10–12 days following treatment with 20 mg/kg while 14 days were required before symptoms of neuropathy were observed following dosing with the same amount of (+) enantiomer. In contrast, ataxia was observed 2–4 days later upon treatment of hens with racemic and (+)-rotating debromoleptophos than with the same amount of (-) enantiomer (Allahyari et al., 1980).

Owing to the unexpectedly high neurotoxic activity of the racemic material, assay for neurotoxicity was repeated on three separate occasions with no significant change in results. Further, the enantiomers and racemate were checked for both chemical and optical purity prior to each assay and no change in purity of the chemicals was noted. Therefore, the results obtained cannot be repudiated on the basis of variability in the purity of the samples nor on inadequacy in the number of bioassays. At the present time, no explanation may be offered for the higher neurotoxic activity of the racemic debromoleptophos oxon.

A sample of (-)-rotating $(S)_p$-leptophos ($[\alpha]^{24}_D$ -26.55), available from a previous study (Allahyari et al., 1980), was treated with *m*-chloroperbenzoic acid (MCPBA), resulting in (-)-rotating leptophos oxon ($[\alpha]^{24}_D$ -35.20). Since



$(S)_p$(-)-leptophos $(S)_p$(-)-leptophos oxon

previous results by Herriott (1971) and Allahyari et al. (1977) revealed that MCPBA oxidation of P=S to P=O esters occurs with retention of configuration, the absolute configuration of (-)-leptophos oxon is most likely also $(S)_p$ and (+)-leptophos oxon is $(R)_p$. Since it is unlikely that removal of a bromine atom from leptophos oxon will have

Table IV. Delayed Neurotoxicity of (+), (±) and (-) Debromoleptophos Oxon in Atropinized Hen after 31 Days

	(+)				(±)				(-)			
dose,[a] mg/kg	no. of hens	acute toxicity[b]	no. ataxic	degree of ataxia[c]	no. of hens	acute toxicity[b]	no. ataxic	degree of ataxia[c]	no. of hens	acute toxicity[b]	no. ataxic	degree of ataxia[c]
3	3	0	0	0	6	0	0	0	3	0	0	0
5	6	0	0	0	6	0	4	6–8	6	0	0	0
7	6	0	1	2	6	0	5	8	9	0	4	2–4
10	6	0	3	2–8	6	0	6	8	6	0	5	8
20	6	0	4	4–8	3	0	3	8	3	0	3	8
30	3	1	3	8								
MED_{50}, mg/kg	15.3[d] (8.8–21.8)[f]				4.9[e] (3.5–6.4)[f]				7.7[d,e] (5.7–9.7)[f]			

[a] Administration intraperitoneally in Me_2SO. [b] Death occurring within 72 h of treatment. [c] 2 = mild; 4 = moderate; 6 = severe; 8 = total paralysis and death as described by Davies and Holland (1972). [d,e] MED_{50} values compared with t-test when 95% confident intervals overlapped, $t = (MED_{50_1} - MED_{50_2})/\sqrt{SE_1^2 + SE_2^2}$; DF = $n_1 + n_2 - 2$; n = sample size; (+), n = 27, SE = 3.3; (±), n = 24, SE = 0.8; (-), n = 24, SE = 1.0; $t > 2.02$ are different data set at $P = 0.05$; [d] $t = 2.20$, [e] $t = 2.19$. [f] 95% confidence interval.

any effect on the sign of rotation of the enantiomers, the same conclusion is applicable to debromoleptophos oxon. Therefore, the enantiomer of debromoleptophos oxon with greater anticholinesterase activity and acute toxicity is $(R)_p$ while the enantiomer with greater antineurotoxic esterase activity and delayed neuropathy is $(S)_p$.

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Registry No. 1, 97151-12-7; **2a**, 97151-14-9; **2b**, 97151-15-0; **3**, 97151-13-8; **3a**, 97232-57-0; **3b**, 97232-58-1; acetylcholinesterase, 9000-81-1; esterase, 9013-79-0.

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Analysis of Phosphate Ores and Related Raw Materials by X-ray Spectrometry

Wendell D. Wilhide* and Doris H. Ash

Both pressed-powder and fusion sample preparation methods have been compared in the application of an automated X-ray spectrometer to a broad range of phosphate ore types and their products of beneficiation treatments. Emphasis has been on minimum dilution techniques. The methods developed show good precision for six of the more important constituents followed in process control and experimental studies. The X-ray fluorescence (XRF) method is in general agreement with routine chemical methods, although no attempt was made to establish ultimate accuracy. Sample preparation methods are evaluated for the various types of materials studied.

INTRODUCTION

World production of phosphate rock products increased by nearly two-thirds during the past decade to the current production rate of about 150 million metric tons of concentrate product. Domestic U.S. production accounts for about one-third of this total production, mainly from major sedimentary deposits in Florida, North Carolina, and the western U.S. phosphate district (Idaho, Utah, Wyoming) (IFA, ISMA, 1983).

The principal use of phosphate rock products is as raw material for the manufacture of chemical fertilizers and wet-process phosphoric acid (WPA) intermediates. Worldwide market requirements for fertilizers, however, have accelerated rapidly to meet agricultural production needs of food and fiber. This is resulting in increased dependence on lower quality phosphate rock resources as high-grade reserves are being depleted. As a consequence, the progressive slippage in chemical quality of phosphate concentrate grades is adversely affecting the chemical fertilizer processes and product compositions (Lehr, 1984).

Compositional variations among phosphate rock products may result from a combination of factors. Phosphate rocks occur in a broad range of geological settings ranging from igneous intrusives and metamorphic carbonatites to sedimentary deposits of widely differing geochemical origin. These individual deposits differ in the compositional form of the apatitic phosphate minerals, as well as in the variety of accessory mineral impurities (McClellan and Gremillion, 1980). Furthermore, physical metallurgy treatments to upgrade the phosphate values in raw ores to commercial concentrate grade lead to more or less random rejection of accessory mineral impurities. Consequently, phosphate rock products meeting specific P_2O_5 grade specifications may differ markedly in their chemical compositions, especially for key impurities such as Na, K, Mg, Al, Fe, and Si, that are mainly derived from the accessory mineral matter retained in rock products (Lehr,

Division of Chemical Development, National Fertilizer Development Center, Tennessee Valley Authority, Muscle Shoals, Alabama 35660.